Microbiome

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Milk sialyl-oligosaccharides mediate the early colonization of gut commensal microbes in piglets

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Abstract

Background The suckling period in pigs is a key phase in development for shaping the gut microbiota, which is essential for maintaining biological homeostasis in neonates. In piglets fed sow milk, the gut microbiota comprises predominantly lactobacilli, indicating a host–gut microbiota symbiosis that is influenced by sow milk components. In this study, we sought to elucidate the mechanisms underlying the establishment and maintenance of the gut microbiome in suckling piglets, with a specific focus on the metabolism of sialyl-oligosaccharides by lactobacilli.

Results Based on liquid chromatography–mass spectrometry analysis, we identified 3′-sialyl-lactose (3′SL) as the major oligosaccharide in porcine milk, and microbiome profiling revealed the predominance of *Ligilactobacillus salivarius* during the suckling period, with a subsequent transition to *Limosilactobacillus reuteri* dominance post-weaning. Notably, sialic acid metabolism was established to be exclusively attributable to *L. salivarius*, thereby highlighting the pivotal role of 3′SL in determining species-specific bacterial segregation. *L. salivarius* was found to metabolize 3′SL when co-cultured with *Bacteroides thetaiotaomicron*, resulting in a shift in the predominant short-chain fatty acid produced, from lactate to acetate. This metabolic shift, in turn, inhibits the growth of enterotoxigenic *Escherichia coli*. Furthermore, the comparison of the gut microbiota between suckling piglets and those fed a low-3′SL formula revealed distinct diversity profiles. We accordingly speculate that an absence of sialyl-oligosaccharides in the formula-fed piglets may have restricted the growth of sialic acid-utilizing bacteria such as *L. salivarius*, thereby leading to a higher abundance of Enterobacteriaceae.

Conclusions Our findings reveal the influence of sialyl-oligosaccharides in promoting microbial diversity and gut homeostasis, thereby highlighting the importance of sialic acid as a key factor in shaping milk-driven microbial colonization during the early stages of piglet development.

Keywords Lactobacilli, Microbiome, Piglet, Sialyl-oligosaccharide, Sow milk

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Introduction

Milk production and parenting are unique traits of mammals that have profound effects on offspring growth, including gut microbiome development [1–3]. The establishment of gut microbiota immediately after birth is essential for the maturation of the neonatal immune system and has a lasting impact on health [4–6]. The early microbial community also plays a critical role in protecting against pathogenic infections and lowering the risk of various pathologies [7–10]. The neonatal gut is rapidly colonized by bacteria from multiple sources, such as environmental exposure, maternal transmission during delivery, and breastfeeding, which continue to diversify over the subsequent months.

Pigs are highly efficient at converting feed into meat and are an important source of protein in many regions of the world. The expanding pig farming market is fueled by several factors such as increasing food demand, global demand for pork, and population growth. Cognitive market research estimates that the global pig farming market will be valued at US$311.52 million in 2024, with a compound annual growth rate of 2.3% projected from 2024 to 2031 [11]. In the pig farming industry, early microbial colonization in piglets is crucial for immune development and disease resistance, particularly in relation to post-weaning diarrhea susceptibility [12]. During the suckling period in piglets (between 3 and 4 weeks old), the dominant genera are *Lactobacillus* and *Bacteroides*, the latter being gradually replaced by *Clostridium* sensu *stricto* 1 as piglets age [13, 14], with a marked shift in the gut microbiota occurring after weaning when a solid cereal-based diet is introduced. However, individual variability and environmental factors can have a pronounced influence on microbial development in piglets. The gut microbiota of piglets is closely associated with their average daily growth (ADG), with piglets exhibiting the highest ADG relative to their birth weight class and showing a higher abundance of *Lactobacillus*, *Ruminococcaceae* UCG-005, and unclassified *Prevotellaceae* during the suckling period [14, 15]. A common post-weaning phenomenon that has been observed in multiple studies is the marked proliferation of *Prevotella* coinciding with the dietary shift from milk to solid feed. Furthermore, Li et al. [16] demonstrated that low-birthweight piglets harbored a distinct fecal microbiota compared to normal-birthweight piglets during early life, with a notably lower abundance of the genus *Lactobacillus*. Early life formation of the gut microbiome has significant implications for long-term health and productivity outcomes in piglets [12, 17, 18].

The microbial community in the neonatal gut is influenced by milk compounds, including immune components (e.g., secretory IgA, lactoferrin, and antimicrobial peptides) and milk-derived oligosaccharides [19]. Indeed, the composition and dynamics of the neonatal gut microbiota are strongly influenced by the presence of milk-derived oligosaccharides [20]. Mammalian milk oligosaccharides exist in diverse repertoires decorated with fucose and/or sialic acid moieties [21–23]. Among these, 2′-fucosyllactose (2′-FL) is the most abundant fucosylated milk oligosaccharides in humans, selectively promoting the growth of specific *Bifidobacterium* species [24, 25]. These species play an important role in promoting a development trajectory that establishes homeostatic interactions with the mucosal immune system in infants [26, 27]. In addition, sialylated milk oligosaccharides alter the composition of the gut microbiota in gnotobiotic animals transplanted with infant microbiota, and the resulting metabolites promote animal growth [28].

Despite advances in our understanding of the evolutionary development of the gut microbiome in humans, there is still relatively little information available regarding the specific effects of milk-derived oligosaccharides in shaping the composition and function of the microbiota in mammals, particularly in piglets. In this study, using microbiome profiling and bacterial culture assays, we sought to elucidate the mechanisms whereby milk-derived sialyl-lactose (SL) influences the composition and metabolic activity of the gut microbiome in suckling piglets. Specifically, we investigated how lactobacilli utilize sialic acid released from SL and examined the consequent changes in short-chain fatty acid (SCFA) profiles, as well as their effects on the growth of pathogenic *Escherichia coli*. Our findings enabled us to identify the pivotal role played by milk-derived SL in the establishment of a *Lactobacillus*-dominant microbiota and maintenance of gut homeostasis in suckling piglets.

Results

Dynamics of fecal microbiota composition in piglets: weaning-induced species-level shifts within lactobacilli

Fecal samples were collected from six piglets of the same litter born naturally on a local farm in Miyagi Prefecture, Japan, from days 0 to 56. The piglets were forcibly weaned from sow milk and transitioned to solid feed at 21 d of age. Total DNA was extracted and sequenced by targeting the V3–V4 region of the 16S rRNA gene. Microbiota composition was analyzed using the QIIME2 pipeline based on amplicon sequence variants (ASVs). The top of detected genera are shown in a bar plot (Fig. 1A). During the suckling period (days 0–21), *Lactobacillus* species (encompassing all formerly classified *Lactobacillus* species) were the most abundant immediately after birth (days 0–3), followed by *Escherichia-Shigella* and *Bacteroides* spp. In contrast, after weaning (days 28–56), *Prevotella* was predominant (Fig. 1B). These results

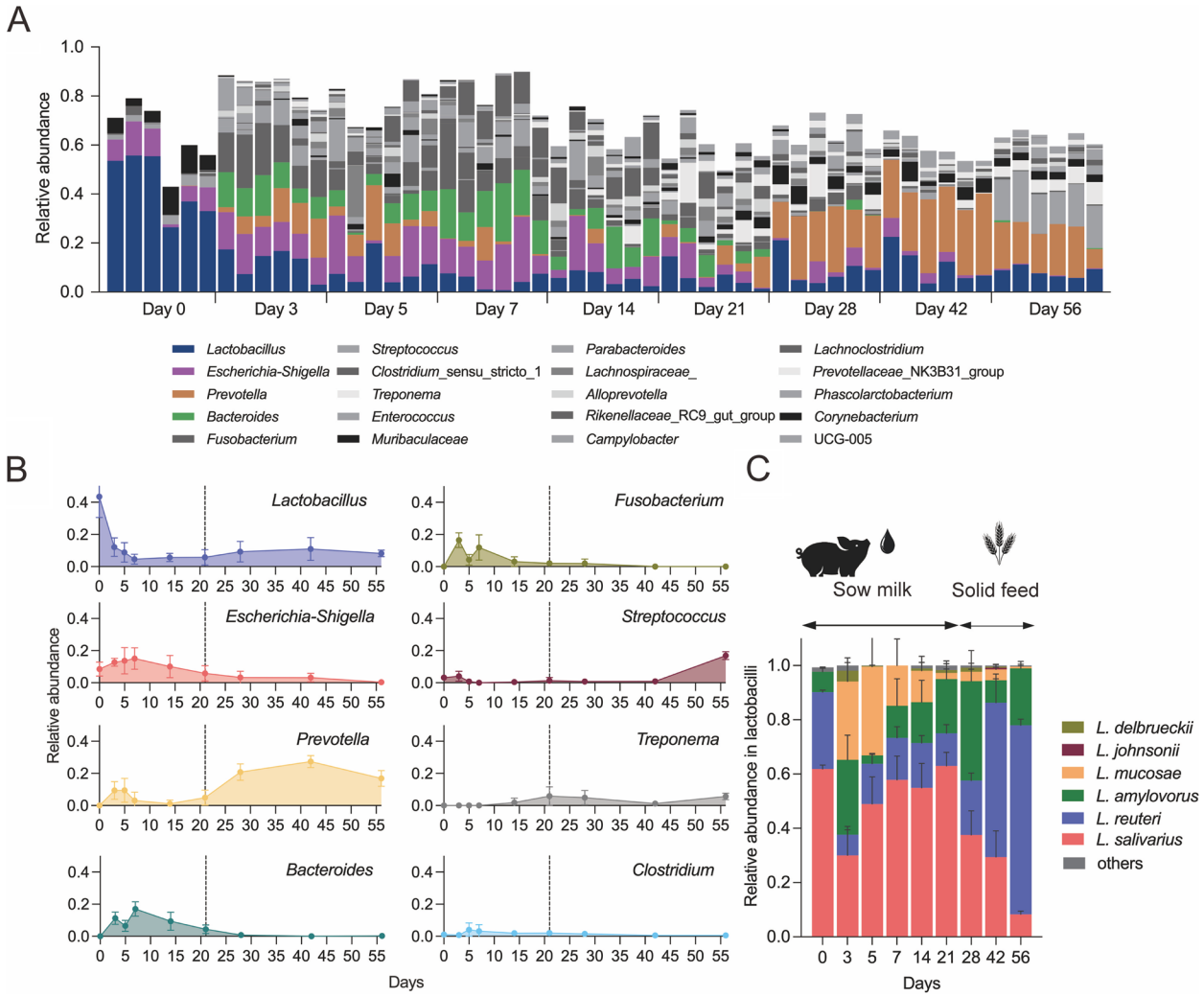

Fig. 1 Fecal microbiota dynamics in piglets from suckling to weaning. **A** Relative abundance of the top 20 bacterial genera is illustrated based on a genus-level analysis of fecal microbiota in piglets born via natural parturition, spanning from 0 to 56 days of age ($n = 6$). Piglets were weaned from sow milk at 21 days of age and transitioned to solid feed. **B** Abundance of major bacterial genera is tracked up to 56 days of age, with vertical, dotted lines indicating the start of the weaning period. **C** Relative abundance of various *Lactobacillus* species detected in the feces of piglets, encompassing all formerly classified *Lactobacillus* species. Species-level classifications were performed by the stand-alone BLAST applications. **B**, **C** Data represent mean ± SEM

confirm the significant changes in piglet microbiota composition before and after weaning, consistent with previous studies [12, 14, 15]. Interestingly, *Lactobacillus* were detected at a constant rate of 10–20% from suckling to weaning. Among *Lactobacillus* species, *Ligilactobacillus salivarius* dominated the suckling period, comprising approximately 60% of the total *Lactobacillus* (Fig. 1C). However, after weaning, the relative abundance of *L. salivarius* gradually declined and *Limosilactobacillus reuteri* became the dominant species, substantially increasing in proportion and effectively replacing *L. salivarius*. These findings highlighted a significant species-level shift in the lactobacilli from pre- to post-weaning (Fig. 1C).

Sialyl-oligosaccharide metabolic adaptations of lactobacilli during the suckling period

We focused on the relationship between sialyl-oligosaccharides in sow milk and the transitions in lactobacilli. The oligosaccharides in sow's milk are enriched in sialyllactose [29]. Sialyl-lactose content in colostrum (within 2 days postpartum) and mature milk (1 week postpartum) from five sows and commercially available pig formulas was quantified using liquid chromatography–mass spectrometry (LC–MS). The representative chromatogram peaks are shown in Fig. 2A (all chromatogram peaks are shown in Fig. S1). In colostrum, 3'-sialyl-lactose (3'SL) was the most abundant at an average of 1879

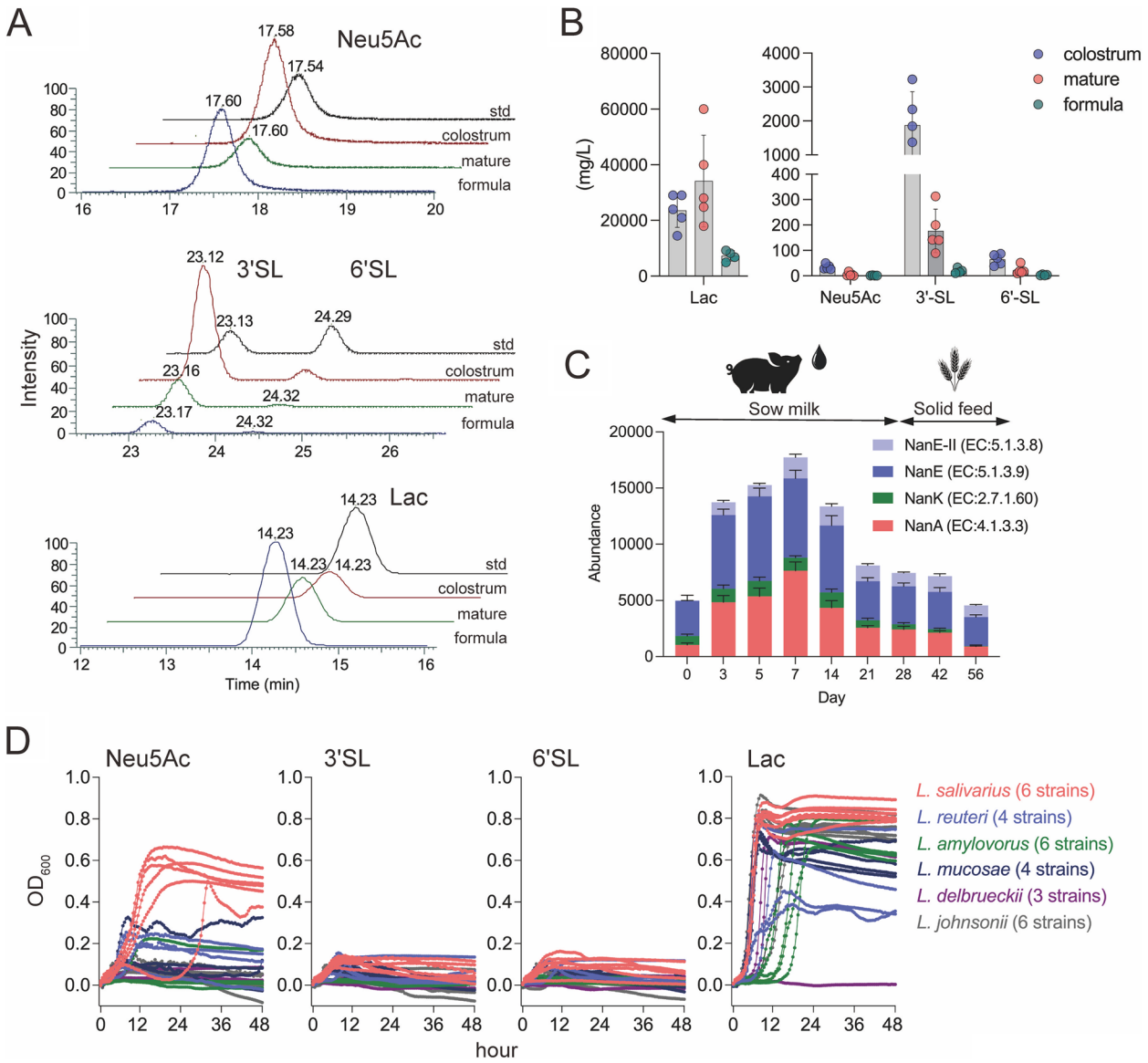

Fig. 2 Determination of sialyl-oligosaccharides in the milk produced by sows and the metabolic capacity of lactobacilli. **A**, **B** Sialyllactose content in colostrum (collected within 2 days after birth) and mature milk (collected 1 week after birth) from five sows, alongside a commercial pig formula, utilizing high-performance liquid chromatography coupled with mass spectrometry. **A** Representative chromatogram peaks are shown; samples were diluted 30-fold for Neu5Ac and 3'SL, 6'SL measurements, and 7500-fold for lactose measurements. **B** The concentration of Neu5Ac, 3'SL, 6'SL, and Lac in each sample is shown. Data represent mean ± SEM. **C** Prediction of the presence of sialic acid metabolic enzymes, namely, *N*-acetylneuraminate lyase (NanA), epimerase (NanE, NanE-II), and kinase (NanK), within the microbiome at various ages, based on the analysis performed using PICRUSt2 (Table S8). **D** Growth assays of various *Lactobacillus* strains (both isolated strains and their corresponding type strains) were identified in piglets utilizing Neu5Ac, lactose, 3'SL, and 6'SL. Growth curves for each strain are displayed in Fig. S2

mg/L, followed by 6'-sialyl-lactose (6'SL) and *N*-acetylneuraminic acid (Neu5Ac) at average 65 mg/L. Although 3'SL was the most abundant substance in colostrum, its concentration in mature milk decreased gradually (Fig. 2B). There was no significant difference in the lactose content between mature milk and colostrum. It has been reported that the sialyl-lactose content in human milk is high in colostrum (approximately 500 mg/L for 6'SL and approximately 200 mg/L for 3'SL) and gradually decreases over time [30, 31]. In contrast, pig milk oligosaccharides are characterized by a high concentration of 3'SL compared to human milk, which has lower concentrations. In addition, commercially available pig formulas contain low levels of sialyl-lactose and Neu5Ac.

Bacteria scavenge sialic acid from the surrounding environment and use it as a carbon and nitrogen source [32]. The sialic acid degradation pathway in bacteria primarily consists of a cluster of genes (the Nan cluster) encoding the enzymes *N*-acetylneuraminate lyase (NanA), kinase (NanK), and epimerase (NanE) required for sialic acid degradation (see Fig. 4A) [33]. Based on the ASVs obtained from the 16S rRNA gene analysis shown in Fig. 1A, PICRUSt2 (https://github.com/picrust/picrust2) was used to analyze the predicted distribution of NanA, NanE, NanE-II, and NanK in the piglet microbiota at different ages (Fig. 2C). The detection frequency of Nan clusters was significantly higher during the suckling period than during the post-weaning period (21–28 days). This supports the hypothesis that during the suckling period, bacteria utilize 3'SL-rich sow milk as a sugar source for gut bacteria.

Among the reference strains corresponding to the six *Lactobacillus* species (*L. delbrueckii, L. johnsonii, L. mucosae, L. amylovorus, L. reuteri,* and *L. salivarius*) detected in the piglet microbiota (see Fig. 1C), we searched for homologs of NanA (P59407) characterized in *Lactiplantibacillus plantarum* WCFS1 [34]. We identified a NanA homolog only in *L. salivarius* JCM1231^T (sialic acid lyase [nanA], EEJ73413.1) and found no homologs in any of the other strains: *L. delbrueckii* subsp. *bulgaricus* JCM 1002 ^{T}, *L. johnsonii* JCM 2012 ^{T}, *L. mucosae* JCM 7177, *L. amylovorus* JCM 1126 ^{T}, and *L. reuteri* JCM 1112 ^{T}. We then assessed the growth of reference strains and those isolated from piglet feces on media with Neu5Ac, lactose, 3'SL, or 6'SL as the sole sugar source, measuring OD_{600} (Fig. 2D, Fig. S2). As expected, all *L. salivarius* strains (six isolates and type strains) grew in the Neu5 Ac-supplemented modified MRS (mMRS) medium, whereas other strains hardly grew in the presence of Neu5Ac. In addition, bacterial growth was observed in most strains grown on the lactose-supplemented medium. Conversely, none of the six *Lactobacillus* species, including *L. salivarius*, exhibited growth in the 3'SL- or 6'SL-supplemented media. These results indicated that among the lactobacilli detected in suckling piglets, only *L. salivarius*, the most predominant species during the sucking period, was able to metabolize Neu5Ac. This supports the hypothesis that *L. salivarius* has a metabolic advantage in preferentially utilizing sialyl-oligosaccharides in sow milk. The comparative genomic analysis identified only four *Lactobacillus* species with the Neu5 Ac metabolic pathway: *Lactobacillus antri*, *L. plantarum*, *Lactobacillus sakei*, and *L. salivarius* [35, 36]. *L. salivarius* and *L. plantarum* were the most frequently detected species in suckling piglets (with the predominance of these species varying by breeding year; data not shown), highlighting the critical role of sialic acid metabolism as a survival strategy for lactobacilli in the piglet gut.

Sialidase-dependent cross-feeding facilitates Neu5Ac utilization by *L. salivarius* in the piglet gut

In pig milk, Neu5 Ac is predominantly found as part of 3'SL rather than as a free sugar. Previous studies have described cross-feeding relationships wherein bacterial species equipped with *exo*-α-sialidase hydrolyze sialyl-oligosaccharides, releasing free Neu5Ac and thus providing sialic acid to other bacteria in the same environment [37–39]. We hypothesized that acquiring Neu5Ac relies on the presence of *exo*-α-sialidase in certain bacterial species. We examined the gut microbiota of suckling piglets for bacteria with *exo*-α-sialidase (EC 3.2.1.18) using PICRUSt2. Our analysis revealed that *Bacteroides*, *Prevotella*, and *Parabacteroides* were the predominant genera (Fig. 3A). Notably, *Bacteroides* were present in the microbiota during the same period when *L. salivarius* was predominant, suggesting a potential nutrient symbiosis between these two species.

To clarify nutrient symbiosis, we focused on the role of the sialidase NanH from *Bacteroides thetaiotaomicron* JCM 5827^T. *B. thetaiotaomicron* JCM 5827^T wild-type and *nanH* mutant were cultured overnight in dextrose-free GAM supplemented with Neu5Ac, 3'SL, 6'SL, and lactose (noting that *B. thetaiotaomicron* JCM 5827^T cannot metabolize sialic acid [37, 40]. *B. thetaiotaomicron* scarcely released free Neu5 Ac in the culture GAM medium (data not shown). Following a previous study by Buzun et al. [41], *B. thetaiotaomicron* was then cultured in PBS supplemented with the same sugars. In the wild-type culture of *B. thetaiotaomicron*, small amounts of free Neu5Ac were detected, which correlated with moderate growth of *L. salivarius* S22 (Fig. 3B, C). Conversely, in the *B. thetaiotaomicron nanH* mutant strain, there was scarcely any free Neu5Ac or growth of *L. salivarius* S22 (Fig. 3B, C). To address this, we tested a strain heterologously expressing *B. thetaiotaomicron nanH* in *E. coli* JW5538-KC (*nanK*-mutant strain), referred to as *E. coli* JW5538-KC p*nanH*. In *E. coli* JW5538-KC p*nanH*, a significant release of sialic acid was observed (Fig. S3A), whereas this release was not detected in *E. coli* JW5538-KC pEmpty (Fig. S3A). The subsequent growth of *L. salivarius* was also confirmed in *E. coli* JW5538-KC p*nanH* (Fig. S3B). These results demonstrated that bacteria with sialidase activity are essential for *L. salivarius* to utilize 3'SL as a nutrient source. In the presence of both lactose and Neu5 Ac, *L. salivarius* preferentially metabolized lactose and then gradually utilized sialic acid (Fig. 3D). To confirm the ability of *L. salivarius* to metabolize sialic acid, a nutrient competition test was performed between *L. salivarius* and *L. reuteri*. In the presence of Neu5Ac,

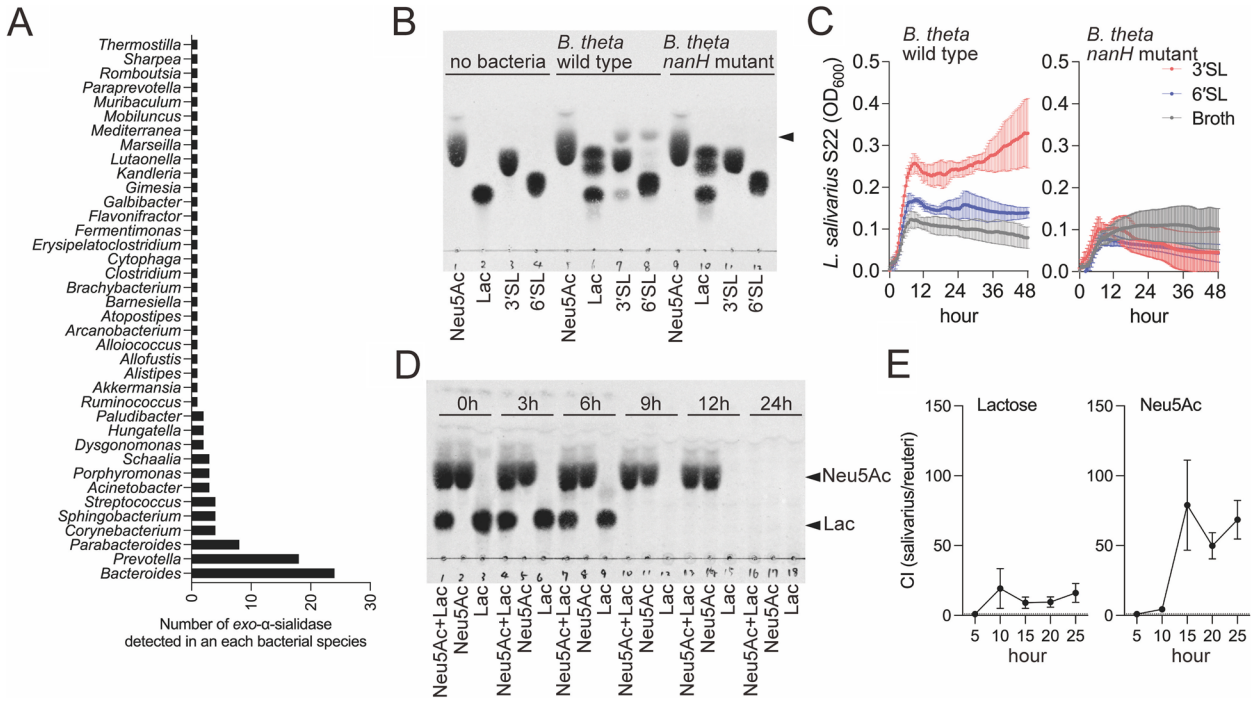

Fig. 3 Sialidase-dependent cross-feeding enables access to Neu5Ac by *Ligilactobacillus salivarius*. **A** Prediction of bacterial species possessing *exo*-α-sialidase (EC 3.2.1.18) detected in the gut microbiota of piglets, based on the analysis performed using PICRUSt2 (Table S9). **B**, **C** *Bacteroides thetaiotaomicron* JCM 5827^{T} wild-type or *nanH*-deficient strains were incubated in PBS supplemented with Neu5Ac, 3′-SL, 6′-SL, or lactose. The sugar components released in the medium after incubating (24 h) were detected using thin-layer chromatography (TLC). The black arrow indicates the position of Neu5Ac. **C** Growth curves of *L. salivarius* strain S22 in mMRS broth mixed with an equal volume of *B. thetaiotaomicron* conditioned buffer ($n = 3$). Data represent mean ± SEM. **D** *L. salivarius* S22 was cultured in a medium supplemented with lactose and Neu5 Ac, with residual sugars in the medium detected using TLC. **E** *L. salivarius* and *L. reuteri* were co-cultured in a medium supplemented with lactose or Neu5Ac. Individual strains were quantified using qPCR ($n = 4$). Data represent mean ± SEM

the proportion of *L. salivarius* significantly increased after 15 h (Fig. 3E). This indicates that the ability of *L. salivarius* to metabolize sialic acid is advantageous in the presence of competitive bacteria.

Sialic acid metabolism by *L. salivarius* enhances acetate production and inhibits pathogenic *E. coli* growth

Sialic acid metabolism in the Nan cluster leads to acetic acid production from *N*-acetylglucosamine-6-phosphate (GlucNAc6P) via *N*-acetylglucosamine-6-phosphate deacetylase (NagA) (Fig. 4A) [33]. Pathogenic *E. coli* infections, including enterohemorrhagic *E. coli* (EHEC) and enterotoxigenic *E. coli* (ETEC), are major causes of diarrhea in piglets [42, 43]. Acetate produced by commensal *Bifidobacteria* protects hosts from EHEC O157 infection [44]. To investigate the biological significance of sialic acid in *L. salivarius*, we assessed the changes in the composition of SCFAs during sialic acid metabolism and their impact on *E. coli* growth. *L. salivarius* (five isolates and one type strain) was cultured in media containing either lactose or Neu5Ac as the sole sugar source to analyze the SCFA profile (Fig. 4B). Lactate was the predominant SCFA in the presence of lactose; however, with Neu5 Ac, acetate accounted for 50–80% of the total SCFAs. Furthermore, the total amount of SCFAs significantly increased in *L. salivarius* S23, S24, and JCM1231^{T} strains.

Among the five assessed *L. salivarius* strains, S22 was selected for further analyses given that it produced comparable amounts of total SCFAs in media supplemented with either lactose or Neu5 Ac, whereas strains S23, S24, and JCM1231^{T} produced significantly higher amounts of acetate when cultured in Neu5Ac-supplemented media (Fig. 4B). The strains were cultured in media supplemented with 0.5% (w/v) lactose or Neu5Ac, and cell-free supernatants were subsequently collected and added in 0–50% volumes to the *E. coli* culture medium. Notably, Neu5Ac-supplemented media exhibited a dose-dependent inhibitory effect on *E. coli* JCM1649^{T} growth, surpassing the effects observed in lactose-supplemented media (Fig. 4C). Further tests showed that acetate exhibited stronger antibacterial activity than did lactate when added individually to the culture medium (Fig. 4D). This is consistent with our observation that

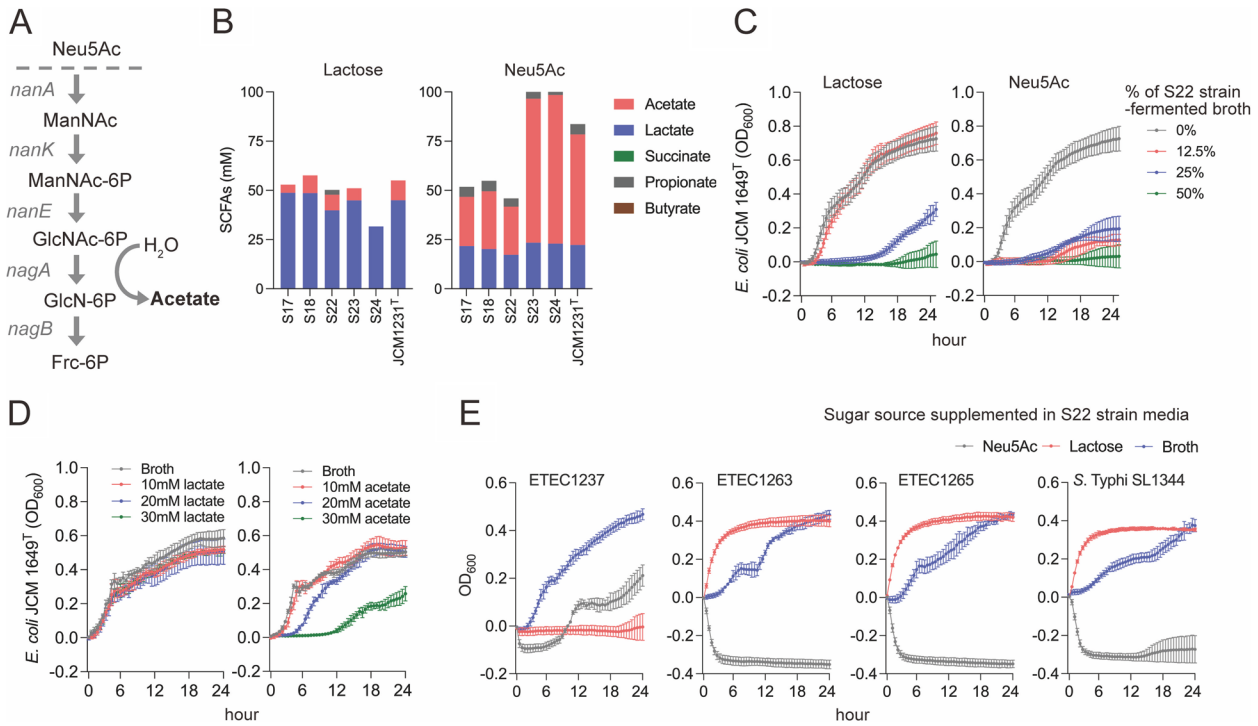

Fig. 4 Sialic acid metabolism by *L. salivarius* promotes acetate production and inhibits pathogenic *E. coli* growth. **A** Factors involved in sialic acid (Neu5Ac) metabolism in bacteria include the transporter NanT, as well as *N*-acetylneuraminate lyase (NanA), *N*-acetylmannosamine-6-P epimerase (NanE), *N*-acetylmannosamine kinase (NanK), *N*-acetylglucosamine-6-phosphate deacetylase (NagA), and glucosamine-6-phosphate deaminase (NagB). **B** Short-chain fatty acid (SCFA) composition in *L. salivarius* culture medium. Lactose or Neu5Ac was used as the sole sugar source, and the SCFA profile was analyzed using HPLC. **C**, **D** Growth curves of *E. coli* JCM1649^T ($n = 3$). **C** *L. salivarius* S22 strain was cultured in media supplemented with 0.5% (w/v) lactose or Neu5Ac, and the cell-free supernatants were added to the *E. coli* culture medium at the indicated concentrations. **D** Growth curves of *E. coli* when acetate or lactate was added. **E** Growth curves of ETEC1237, ETEC1263, ETEC1265, and *Salmonella* Typhimurium SL1344. The culture supernatant from *L. salivarius* S22 cultured in media supplemented with lactose or Neu5Ac was added in equal volume to the ETEC or *Salmonella* culture broth ($n = 3$). All data represent mean ± SEM

Neu5Ac-supplemented media exhibited significant inhibitory activity despite similar total SCFA levels. The effect of Neu5Ac-supplemented medium on ETEC strains isolated from diarrheic piglets was also evaluated. The inhibitory effect varied across strains, with Neu5Ac-supplemented media significantly inhibiting the growth of ETEC1263, ETEC1237, and ETEC1265, as well as *Salmonella* Typhimurium SL1344 (Fig. 4E, Fig. S4). These results indicate that *L. salivarius* not only utilizes sialic acid as a nutrient source but also modifies its metabolite profile, potentially contributing to the suppression of pathogenic *E. coli* colonization.

Influence of sialyl-oligosaccharides on the composition and diversity of the microbiome in suckling piglet

We conducted a comparative analysis of the microbiome in suckling piglets (SP) and formula-fed piglets (FP), which differed in sialo-oligosaccharide content (Fig. 2B). Piglets in the SP group were fed exclusively sow milk from birth, whereas those in the FP group received an artificial formula for 14 d after an initial 5 mL dose of colostrum. Fecal morphology analysis revealed a normal stool consistency in the SP group, whereas the FP group exhibited diarrhea-like stools (Fig. 5A). In addition, fecal pH measurements showed that the FP group exhibited significantly higher pH levels than the SP group (Fig. 5B). Although the total fecal SCFA concentrations were similar between the two groups, acetate levels were significantly higher in the SP group (Fig. 5C). To investigate differences in gut microbiota composition, we assessed β diversity using Bray–Curtis dissimilarity. Principal coordinate analysis (PCoA) revealed a clear distinction in the microbial community structure between the SP and FP groups on day 14 (Bray–Curtis permanova pairwise, q-value = 0.043; Fig. 5D, Table S1).

To further elucidate these differences, we examined the daily shifts in the relative abundance of the gut microbiota in both groups (Fig. 5E). In the SP group, the genus *Escherichia-Shigella* was predominant on day 1, followed by a rapid diversification of other bacterial taxa (Fig. 5E). In contrast, the microbiota of the FP group remained dominated by three genera (*Escherichia-Shigella*,

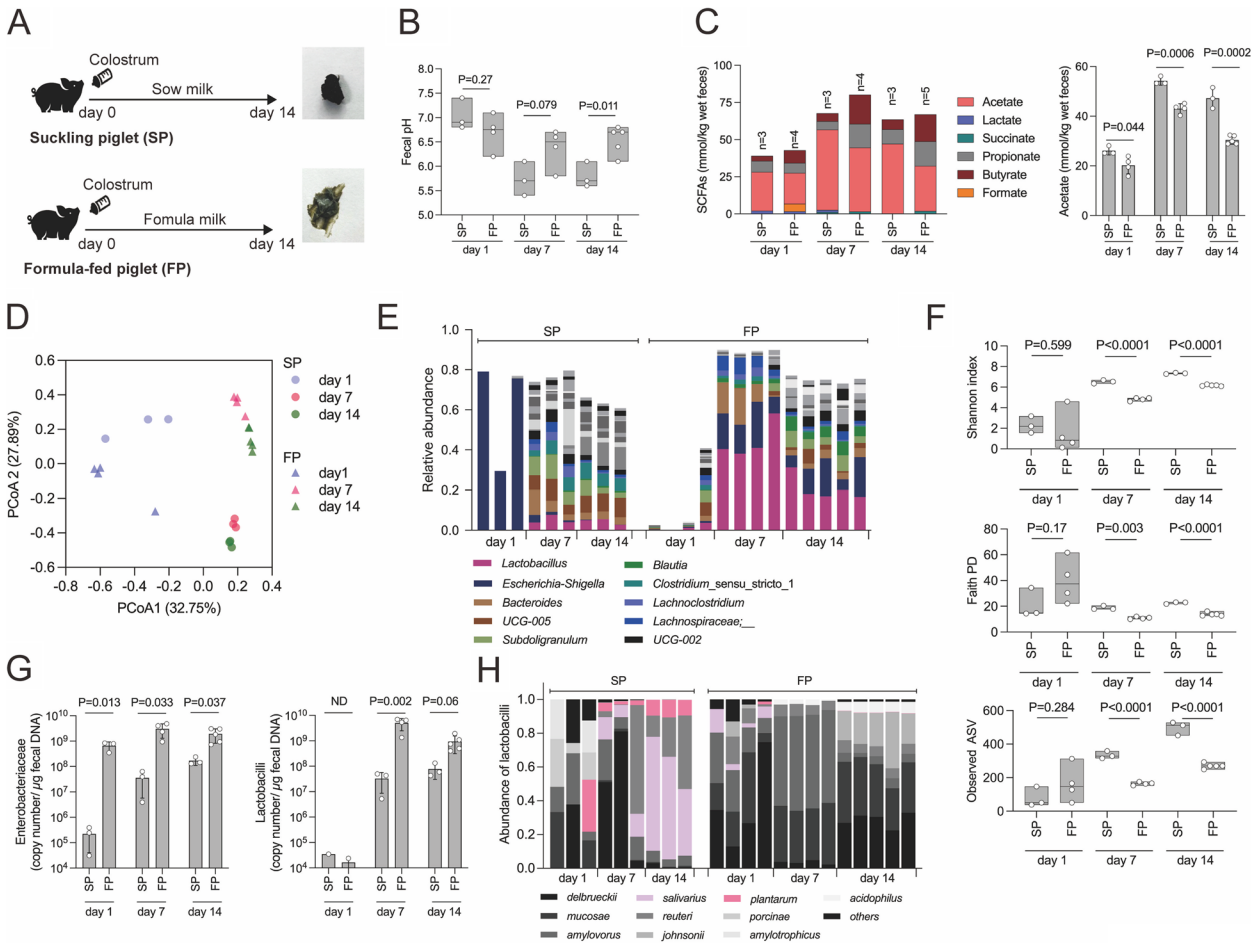

Fig. 5 Influence of sow milk on the composition and diversity of the microbiome in suckling piglets. **A** Comparative analysis of the microbiomes of suckling (SP) and formula-fed (FP) piglets. Piglets in the SP group were exclusively fed sow milk from birth, whereas those in the FP group received an artificial formula for 14 days following an initial administration of colostrum. **B**, **C** Fecal pH (**B**) and short-chain fatty acid (SCFA) composition (**C**) are presented. The number of piglets in each group is indicated by *n*. Data represent median ± SEM (**B**) and mean ± SEM (**C**). **D** Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity for ASV-level microbiome communities in SP and FP piglets, with Bray–Curtis Permanova pairwise comparisons performed between the two groups. All statistical data are shown in Table S1. **E** Relative abundance of the top 20 bacterial genera is illustrated based on a genus-level analysis of fecal microbiota from SP and FP piglets. **F** Shannon index, Faith's PD, and observed ASV of fecal samples are plotted, with comparisons made using the unpaired *t*-test. Data represent median ± SEM. **G** Quantification of Lactobacillaceae and Enterobacteriaceae in the fecal samples as detected by using qPCR. Variations between groups were analyzed using the unpaired *t*-test. Data represent mean ± SEM. **H** Relative abundance of various *Lactobacillus* species detected in SP and FP samples. Species-level classifications were performed by the stand-alone BLAST applications

Bacteroides, and *Lactobacillus*), which was associated with lower alpha diversity (as measured by the Shannon index, Faith's phylogenetic diversity [PD], and observed ASV) (Fig. 5F). Quantitative PCR (qPCR) validation of Enterobacteriaceae confirmed the significantly lower levels in the SP group than in the FP group (Fig. 5G). The reduced diversity in the FP group may be linked to diarrhea-like stools and elevated *Escherichia* levels, underscoring the critical role of sow milk in maintaining intestinal homeostasis. Interestingly, the abundance of Lactobacillaceae was significantly higher in the FP group on days 7 and 14 (Fig. 5G). Focusing on the composition of lactobacilli, *L. salivarius* harboring the Nan cluster genes was the dominant species in the SP group. In contrast, among piglets in the FP group, we were unable to detect *L. salivarius* from day 7 onward, likely owing to the absence of milk-derived sialyl-oligosaccharides, thereby limiting its competitive growth advantage. In the absence of this species, we identified as *L. delbrueckii*, *L. mucosae*, and *L. amylovorus* as predominant lactobacilli (Fig. 5H). Although all six *Lactobacillus* species were capable of metabolizing lactose (Fig. 2D), the detection of

Nan cluster-containing lactobacilli, such as *L. salivarius* and *L. plantarum* in the SP group suggests that sialyl-oligosaccharides play a pivotal role in the habitation of lactobacilli. To elucidate the effect of sialic acid on the microbiome, we used PICRUSt2 to predict the distribution of NanA, NanE/NanE-II, and NanK within the microbiomes of the SP and FP groups (Fig. S5A). Except for the day 1 immature microbiome sample, the frequency of Nan cluster detection was similar in the SP and FP groups. Notably, the microbiome diversity harboring the Nan cluster genes was relatively lower in the FP group than in the SP group (Fig. S5B). In addition, Enterobacteriaceae were the predominant bacteria encoding the *nanA* and *nanE* genes in the FP group. These results provide evidence that the presence of sialyl-oligosaccharides in sow milk promotes greater diversity among bacterial species capable of sialic acid metabolism.

Motivated by these findings, we confirmed whether microbiome diversity and SCFA levels were sialic acid-dependent and investigated changes in microbiota composition in an in vitro culture using piglet feces (Fig. S6 A–D). The addition of Neu5Ac, lactose, 3'SL, and 6'SL as sugar sources revealed a progressive diversification of the bacterial microbiome, in the order of lactose, Neu5Ac, followed by 3'SL and 6'SL (Fig. S6B, C). Interestingly, acetate concentrations were notably higher in cultures with added Neu5Ac, 3'SL, and 6'SL (Fig. S6E). These findings indicate that sialyl-oligosaccharides promote a more diverse bacterial community and exert a pronounced influence on metabolite concentrations, particularly those of acetate.

Discussion

In this study, we investigated the predominance of lactobacilli in the intestinal microbiome of piglets during the suckling period and explored the mechanisms underlying microbiome formation. Given the high concentration of 3'SL in sow milk, we analyzed the distribution of lactobacilli before and after weaning. Our results showed that *L. salivarius*, which can metabolize sialic acid, was dominant in the suckling phase, while *L. reuteri* became prevalent after weaning, indicating habitat segregation. Notably, sialic acid metabolism was exclusive to *L. salivarius*, highlighting the critical role of 3'SL in driving species-specific segregation and symbiosis in suckling piglets.

Interestingly, the metabolic activity of *L. salivarius* led to a shift in the major SCFAs from lactate to acetate during sialic acid metabolism. This metabolic shift was also observed in the fecal contents of suckling piglets, which showed higher levels of acetate and lower pH than those fed formula milk. The substantial production of acetate by *L. salivarius* appears to be sufficient to inhibit the growth of pathogenic *E. coli*. This finding suggests that *L. salivarius*, as a sialic acid-metabolizing symbiont, plays a protective role in the gut of suckling piglets by creating an unfavorable environment for *E. coli* proliferation, thereby highlighting the critical role of sialic acid in maintaining homeostasis through the relationship between milk and gut bacteria in piglets. However, considering that the theoretical maximum acetate yield from the deacetylation of 0.5% (w/v) Neu5Ac is approximately 16 mM, the notably higher acetate concentrations observed indicate a substantial metabolic re-routing in *L. salivarius* grown in the presence of Neu5 Ac (Fig. 4B). Additionally, the pronounced inhibitory effects on *E. coli* growth observed upon addition of as little as 12.5% S22 supernatant (Fig. 4C), corresponding to less than ~3 mM acetate, suggest the presence of other unidentified metabolites that may exert synergistic or additive antibacterial effects.

Moreover, sialyl-oligosaccharides from sow milk are broken down into sialic acid and lactose by enteric bacteria possessing sialidase enzymes, which coexist with *L. salivarius*. Although most lactobacilli primarily metabolize lactose, the unique capacity of *L. salivarius* to utilize sialic acid confers a competitive advantage to the *Lactobacillus* community. Indeed, the findings of our in vivo experiment revealed a near absence of *L. salivarius* among the gut microbiota of FP group piglets, thus indicating the nutritional advantage of the capacity of this bacterium to utilize sow milk-derived sialyl-oligosaccharides (Fig. 5H). Additionally, nutrient competition assay confirmed that in the presence of Neu5Ac, *L. salivarius* significantly outcompeted other lactobacilli, such as *L. reuteri* (Fig. 3E). These findings thus tend to indicate that the selective colonization of *L. salivarius* in sow-fed piglets is directly associated with the availability of sialyl-oligosaccharides, thereby highlighting their important influence in shaping a distinct *Lactobacillus* community and promoting intestinal homeostasis. However, these free sugars may also become accessible to both commensal and pathogenic bacteria, including *E. coli*, which are otherwise unable to directly utilize monosialyl residues from intact oligosaccharides [45]. Thus, the presence of bacteria capable of degrading sialyl-oligosaccharides may inadvertently promote the growth of pathogenic bacteria, thereby potentially posing risks to host health [38, 45]. This emphasizes the essential role played by commensal species, such as *L. salivarius*, which can effectively metabolize free sialic acid, thereby potentially limiting the proliferation of pathogens. These observations are consistent with those reported previously indicating that bacterial species with the capacity to utilize sialyl-oligosaccharides modulate metabolic shifts, such as an increase in acetate production, thereby contributing to

the development of a more complex and dynamic microbial community [28, 46].

Although habitat isolation was evident among lactobacilli, notably with respect to the emergence of *L. reuteri* as a predominant taxon post-weaning (Fig. 1B), a broader examination of the gut microbiome revealed a pronounced increase in the abundance of bacteria in the genus *Prevotella* following the transition to solid feed (days 28–56; Fig. 1B). This marked microbial shift highlights the considerable influence of dietary change (i.e., from sow's milk to solid feed) on the composition and structure of the intestinal microbiota in piglets. Our findings in this regard are consistent with those of previous studies, such as those conducted by Gaukroger et al. [15], who have similarly reported a clear predominance of *Prevotella* in response to dietary shifts during early piglet development. Collectively, these findings highlight the pivotal role played by diet as a primary selective force that has a pronounced modulatory effect on gut microbial communities, shaping their structure and metabolic potential during this important developmental window.

Intriguingly, our findings in this study revealed a significant increase in the overall diversity of the gut microbiome in the presence of sialyl-oligosaccharides compared with exposure to sialic acid or lactose alone (Fig. S6). Diarrhea-associated growth retardation in piglets, which is often linked to pathogenic *E. coli* infections, remains a significant concern for the pig industry [47, 48]. Given that commercial artificial milk formulas for piglets generally contain low levels of sialyl-oligosaccharides, supplementing these formulas with sialyl-oligosaccharides could potentially shift the microbiome composition from an Enterobacteriaceae-dominant community to one enriched with beneficial bacteria such as *L. salivarius*. In addition, our findings are consistent with those previously reported by Young et al. [49], who, using an RNA-based stable isotope probe, revealed that among the cecal microbiota in piglets, *Prevotella* and lactobacilli are the primary utilizers of ^{13}C-labeled sialic acid, resulting in the suppression of *Enterobacteriaceae* populations. These findings collectively emphasize the significant influence of sialyl-oligosaccharides in shaping the composition of the gut microbiota [49]. Accordingly, dietary intervention using sialyl-oligosaccharides could potentially contribute to mitigating the risk of diarrhea. Similarly, in humans, *Bifidobacterium infantis* and *Bifidobacterium breve*, which are both capable of metabolizing sialic acid, establish themselves in the infant gut microbiome and aid in infection prevention [44]. These results underscore the broader significance of sialyl-oligosaccharides in fostering beneficial host-associated microbial communities and suggest their potential applications in both livestock and human health.

Conclusions

In this study, we demonstrated the ability of *L. salivarius* to metabolize 3′SL, a prominent sialyl-oligosaccharide found in the milk produced by sows. We speculate that this metabolic capacity is associated with the predominance of specific lactobacilli, particularly *L. salivarius*, within the intestinal microbiota of suckling piglets. Moreover, by generating an environment unfavorable to the growth of pathogenic bacteria, notably *E. coli*, the utilization of 3′SL by *L. salivarius* may make a substantial contribution to the maintenance of host health. Comparative analyses between SP and FP piglets highlighted the pivotal influence of milk-derived sialyl-oligosaccharides in maintaining gut microbial homeostasis. These findings accordingly provide important insights into milk-promoted microbiota dynamics and could serve as a basis for potential dietary strategies designed to optimize gut health during early development.

Nevertheless, despite our important findings, this study does have certain limitations. Notably, by primarily focusing on *L. salivarius*, we may have arrived at an oversimplified interpretation of the complex metabolic interactions within the diverse gut microbial ecosystem. Other bacterial taxa, such as *Bacteroides*, *Escherichia*, and *Clostridium* [38, 50], similarly have the capacity to metabolize sialic acid, and thus also probably contribute to acetate production and gut homeostasis. Furthermore, in this study, we only compared sow's milk with formula milk, without directly evaluating the physiological impact of artificially supplemented sialyl-oligosaccharides. Moreover, the small number of animals assessed and the practical constraints that prevented consistent fecal sampling from the same individuals may have introduced variability. Differences in housing conditions, including the presence or absence of the maternal sow and environmental exposure, may also have a certain influence on the gut microbiota of piglets. Future studies should accordingly incorporate sialyl-oligosaccharides into artificial diets and use larger more controlled cohorts to confirm the physiological relevance of these findings and clarify the dynamic interactions among diet, microbes, and host development.

Materials and methods

Pigs

Fecal samples were collected from pigs housed at Miyagi University and the Gifu Prefectural Livestock Research Institute. Detailed descriptions of the experimental procedures are presented in sections (ii) and (iii).

(i) Sow milk sample collection

Milk samples were obtained from sows housed at the Gifu Prefectural Livestock Research Institute and Tohoku University. Colostrum was defined as milk collected within 2 days postpartum, whereas mature milk refers to milk collected between day 3 and approximately 1 week postpartum. Both colostrum and mature milk samples were collected from the same five sows. Two of these sows were housed under specific pathogen-free (SPF) conditions at the Gifu Prefectural Livestock Research Institute, whereas the remaining three sows were maintained under conventional conditions at Tohoku University, all of which had previously received vaccinations and antibiotics and were multiparous, with five to ten parities. Milk samples were collected manually using gloved hands and immediately stored at − 30 °C.

(ii) Collection of fecal samples from piglets (0–56 days) at Miyagi University

All eight healthy piglets born to a single sow were individually identified at birth. Piglets were housed with the sow in the same breeding pen and allowed free access to maternal milk, although had no access to the sow's commercial feed. Fecal samples were collected daily from day 0 to day 56 from six piglets; however, owing to practical constraints, it was not always possible to collect samples from the same individuals at the designated time points. From approximately day 14, piglets had access to creep feed (Zennoh Feed Mills Co., Ltd., Japan) in addition to sow milk. On day 21 after weaning, piglets were transitioned to a commercial fattening diet (Zennoh Feed Mills). No weaning-related problems were observed. Fecal samples were stored at − 80 °C until DNA extraction.

(iii) Collection of fecal samples from suckling and formula-fed piglets at the Gifu Prefectural Livestock Research Institute

Piglets born from two different Duroc sows were divided into two groups, namely, a suckling piglet group (SP: $n=4$) and a formula-fed group (FP: $n=5$), all of which were orally administered 5 mL of colostrum at birth. Piglets in the SP group were housed in a pen with their sow and exclusively suckled on maternal milk until day 21, during which time, they had no access to the sow's commercial feed. Piglets in the FP group were separated from the sow immediately after the administration of colostrum and housed independently in a pen without a sow. These piglets were fed a commercial formula (Winny Milk, Nosan Co., Ltd, Japan)-based diet using a milk feeder until day 14 and thereafter received frozen-stored maternal milk orally for health management purposes. Fecal samples were collected from each piglet until day 14. Throughout the study, samples were successfully obtained from three SP piglets and four FP piglets (on day 14, samples were collected from all five FP piglets). Following collection, the fecal samples were frozen for subsequent DNA extraction and SCFA analyses. The piglets in both groups were housed separately in distinct breeding pens under SPF conditions. The milk formula fed to the FP group piglets contains probiotics and oligosaccharides (details of which are available at: https://www.nosan.co.jp/business/fodder/swine.htm).

Milk sialyl-oligosaccharide quantification using LC/MS

(i) Samples and reagents used for LC/MS analysis

Colostrum and mature milk samples were collected from five sows. Four commercially available milk formulae for piglets were obtained from Japanese feed manufacturers (Zennoh Feed Mills, Nosan Co., Ltd., and Scientific Feed Laboratory Co., Ltd.) and were prepared by dissolving in water in accordance with the manufacturers' instructions. Neu5 Ac hydrate (86.6% purity), 3'SL sodium salt (79.2% purity), and 6'SL sodium salt (88.1% purity) were obtained from Tokyo Chemical Industry Co., Ltd., and Kyowa Hakko Bio Co., Ltd., Tokyo, respectively. The purity levels of Lac, Neu5Ac, and the SLs were confirmed using quantitative NMR analysis. High-performance LC (HPLC)-grade water (ADVANTEC) and acetonitrile (Merck) were used as the solution and mobile phases, respectively. Ammonium formate and Lac monohydrate (94.4% purity) were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan).

(ii) Sample preparation

An aqueous solution of ammonium formate (25 mM, diluent A) was used. A 0.10 mL aliquot of milk sample was mixed with 0.10 mL of diluent A, sonicated for 10 s, and transferred to an Amicon Ultra-3 K centrifugal filter unit (Merck Millipore). The mixture was centrifuged at 23,470 ×g for 15 min to remove proteins. The filter and pipette tips were washed twice with 0.20 mL of diluent A; the pooled filtrates (approximately 0.6 mL) were collected. Protein removal was confirmed using laser light scattering. The filtrate was applied to an ODS solid-phase extraction cartridge that was pre-conditioned with 0.60 mL of acetonitrile and diluent A and centrifuged at 2500 ×g for 2 min. Two washes of 0.60 mL of diluent A were also centrifuged; the combined filtrates (approximately 1.8 mL) were collected, yielding a clear, fat-free solution. The filtrate was diluted to 3.0 mL with acetonitrile for Neu5Ac, 3'-SL, and 6'-SL quantification. For Lac, 0.10 mL of this solution was diluted to 25 mL with a 1:1 mixture of diluent A and acetonitrile. Calibration curves for Lac and Neu5Ac ranged from 0.125 to 10 mg/L; for SLs, it ranged from 0.20 to 10 mg/L, with correlation coefficients (r^2)

≥ 0.995. SL standards were prepared separately to avoid contamination by Lac and Neu5Ac impurities.

(iii) LC/MS

LC/MS analysis was performed using a Shimadzu LC-20 series liquid chromatograph coupled to a Thermo Scientific Q-Exactive mass spectrometer. Chromatographic separation was achieved using Shodex HILICPak VG-50 4 A and VN-50 4D columns (Resonac, Tokyo, Japan) connected in series. Solvent A was a 25-mM ammonium formate aqueous solution; solvent B was acetonitrile. The flow rate was 1.00 mL/min, with the column oven and sample rack maintained at 60 °C and 10 °C, respectively. A 1-µL injection volume was used with a linear gradient: 90% B for 2 min, decreasing to 72% B over 24 min, then to 62% B over 6 min, holding at 62% B for 5 min, increasing to 90% B over 2 min, and maintaining at 90% B for 11 min. A 100-µL mixer in the column oven mixed the mobile phases before injection; the needle was flushed with water.

The MS system was operated in negative ion mode using a heated electrospray ionization source. Operating parameters included a spray voltage, sheath gas flow rate, auxiliary gas flow rate, sweep gas flow rate, capillary temperature, auxiliary gas heater temperature, and S-lens RF level of −2.5 kV, 65 units, 20 units, 1 unit, 269 °C, 300 °C, and 50, respectively. Full MS scans were performed over the m/z range of 100 to 1500. Targeted selected-ion monitoring was performed with a mass resolution, an AGC target, a maximum injection time, and an isolation window of 70,000, 1×10^5, 200 ms, and ± 4.0 m/z, respectively. For Neu5Ac, 3'-SL, and 6'-SL, the target ions are listed in Table S2. Mass spectral data were analyzed using Thermo Xcalibur 4.2.47. Quantification was based on extracted ion chromatograms (± 10 ppm tolerance) from two independent measurements, including pretreatment steps. Representative chromatograms are shown in Fig. 2A.

Miseq 16S rRNA gene sequencing

Genomic DNA was extracted from the bacterial isolates using QIAamp PowerFecal Pro DNA Kits (Qiagen, USA) following the manufacturer's protocol. DNA concentration was measured using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The V3-V4 region of the 16S rRNA gene was amplified using primers S-D-Bact-0341-b-S-17 (5'- TCG TCGGCAGCGTCAGATGTGTATAAGAGACAGCCT ACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGGACTACHVGGGTATCTAATCC-3') [51]. A 16S rRNA gene sequencing library was prepared according to the manufacturer's instructions (Illumina). The DNA concentrations of the PCR products were measured using the Qubit dsDNA HS Assay Kit (Thermo Fisher, MA, USA) for normalization. The DNA and internal controls (PhiX control V3; Illumina, Tokyo, Japan) were subjected to paired-end sequencing using a 600-cycle MiSeq reagent kit (Illumina). Raw FASTQ files (33,428–189,692 reads/sample) were analyzed using the QIIME2 platform (version 2023.2) [52]. The DADA2 pipeline was used for denoising, merging paired FASTQ files, obtaining the ASV table, and removing chimeras, resulting in 11,267 to 136,424 high-quality reads per sample. ASV sequences were classified using two methods: (i) the Silva 138 99% reference database on QIIME2 and (ii) BLAST + (version 2.6.0 +) for species-level classification using a 16S rRNA gene database (last modified 2024–08–27). For α diversity, values were calculated at a sampling depth of 10,000 (Observed ASV, Faith's PD, and Shannon index). Bray–Curtis distance–based β-diversity was calculated at rarefaction depths of 72,333 (Fig. 5D) and 49,000 (Fig. S6B), which correspond to the minimum read counts among samples in each dataset. The resulting distance matrices were visualized using PCoA. Functional gene pathway predictions were performed using the QIIME2 plugin for PICRUSt2 (version 2023.2, #17) [53].

Quantitative PCR (qPCR) for microbiome analysis

qPCR quantified the total bacterial load in the fecal samples. Reactions (20 µL) contained DNA template, THUNDERBIRD Next SYBR qPCR Mix (Toyobo, Japan), and 0.3 µM of each primer (sequences listed in Table S3). Amplification was performed using the CFX Opus 96 system (Bio-Rad) with the following thermal conditions: initial denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 10 s, annealing at primer-specific temperatures for 30 s, and extension at 72 °C for 30 s. Standard curves for Enterobacteriaceae and Lactobacillaceae were constructed using tenfold serial dilutions of *E. coli* and *L. salivarius* genomic DNA (10^3–10^8 copies). Standard curve reactions were performed in triplicate, and the amplification efficiency and R^2 were calculated to ensure reliability. These curves were used to calculate the absolute bacterial copy number in fecal samples.

Bacteria and culture conditions

L. salivarius JCM1231^T, *L. reuteri* JCM1112^T, *L. amylovorus* JCM1126^T, 1029, *L. mucosae* JCM7177, *L. delbrueckii* subsp. *bulgaricus* JCM1002^T, *L. johnsonii* JCM2012^T, and *L. plantarum* JCM1149^T were obtained from the Japan Collection of Microorganisms (RIKEN Bioresource Center, Tokyo, Japan). *L. salivarius* S17, S18, S22, S23, and S24; *L. reuteri* R20, R22, and R25; *L. amylovorus* A2, A19, A23, and A30; *L. mucosae* M1, M3, and M16; *L. delbrueckii* D19, D24, and D30; *L. johnsonii* J1,

J2, J3, J4, and J5; and *L. plantarum* P21, P22, P25, P26, and P27 were isolated from fecal samples of piglets bred at Miyagi University farm. The identification of bacterial strains using 16S rRNA gene sequencing has been described [54]. Species identification was performed by PCR amplification of the 16S rRNA gene region with universal primers (27 Fmod 5'-AGRGTTTGATYMTGG CTCAG-3'; 1492R 5'-GGYTACCTTGTTACGACTT-3') for Sanger sequencing and by querying the NCBI genome database. Isolates with >98.0% 16S rRNA gene sequence homology were assigned species names. All lactobacilli were routinely cultured in MRS broth (Becton Dickinson) at 37 °C under anoxic conditions using an AnaeroPack (Mitsubishi Gas Chemical Co., Tokyo, Japan). The pathogenic bacteria were cultured in LB broth with shaking at 180 rpm at 37 °C. *B. thetaiotaomicron* JCM5828^T was purchased from JCM. *B. thetaiotaomicron* was cultured in modified GAM (Shimadzu Diagnostics, Tokyo, Japan) under anaerobic conditions at 37 °C using an anaerobic chamber (COY Laboratory Products).

Generation of *B. thetaiotaomicron ΔnanH* strain

The deletion of *nanH* from *B. thetaiotaomicron* JCM 5827^T was performed using pLBG13 and GAM according to the method described by Garcia-Bayona et al. [55]. pLBG13 was a gift from Laurie Comstock (Addgene, plasmid #126618). *E. coli* JM109 λpir was used as the cloning host. The upstream and downstream 1-kb regions of *nanH* were amplified by PCR using the primer pairs Pr_GYS79/Pr_GYS80 and Pr_GYS81/Pr_GYS82 (Table S4), respectively. Amplified DNA was cloned into pLBG13 using the In-Fusion Snap Assembly Master Mix (TaKaRa Bio) to link the upstream and downstream sequences. The resulting plasmid pGYS175 was introduced into *B. thetaiotaomicron* by bacterial conjugation with *E. coli* S17-1 λpir as the donor. Plasmid insertion mutants were selected on GAM agar plates containing gentamicin (200 μg/mL) and erythromycin (5 μg/mL). A single colony was selected from those appearing on a selective plate, anaerobically cultivated in GAM overnight, and plated on GAM agar plates containing aTc (100 ng/mL) to induce a second crossover event. The deletion of *nanH* was verified by PCR using the primer pair Pr_GYS84 and Pr_GYS85 (Fig. S7). Plasmid curing was confirmed by the loss of erythromycin resistance. No mutations were detected in the deleted region, as confirmed by sequencing analysis.

Construction of a surface expression strain of NanH from *B. thetaiotaomicron* in *E. coli*

For cell surface expression of NanH from *B. thetaiotaomicron* JCM 5827^T, the expression plasmid pSyn15-Blc-NanH (p*nanH*) was constructed as follows: the plasmids and oligonucleotide primers used for plasmid construction are listed in Table S5. *E. coli* TG1 (Zymo Research, Irvine, CA, USA) was used as the cloning host. The replication origin and chloramphenicol-resistance genes were amplified from pACYC184 (Nippon Gene, Tokyo, Japan) and pHLA, respectively [56], using the primer pairs p15 A ori_F/p15 A ori_R and cat_F/cat_R. The amplified fragments were connected by overlap PCR using the primer pair p15 A ori_F/cat_R. The resulting fragment was digested with EcoRV and self-ligated to generate p15 A-cat. The high constitutive expression (HCE) promoter [57] was amplified from pHLA using the primer pair P-HCE_F/P-HCE_R and inserted into the XhoI and SacI sites of p15 A-cat, resulting in pHC15. The gene encoding the Blc anchor protein [58] was amplified from the genome of *E. coli* BW 25113 (National Institute of Genetics, Shizuoka, Japan) using the primer pair Blc_F/Blc_R and inserted into the EcoRV and SacI sites of pHC15, resulting in pHC15-Blc (pEmpty). The codon-optimized gene encoding *nanH*, excluding the signal sequence (Table S6), was synthesized using Integrated DNA Technologies (Skokie, IL, USA). It was amplified using NanH_F/NanH_R and introduced into the SacI and SalI sites of pHC15-Blc (pEmpty), resulting in pHC15-Blc-NanH. Since overexpression of *nanH* was highly toxic to *E. coli* growth, the HCE promoter of pHC15-Blc-NanH was replaced with a synthetic promoter showing low expression levels. The entire sequence of pHC15-Blc-NanH, except the HCE promoter, was amplified by inverse PCR using the SynP_F/cat_R primer pair. The amplified fragment was digested with XhoI and self-ligated to generate p*nanH*. pHC15-Blc (pEmpty) and p*nanH* were introduced into *E. coli* JW5538-KC (NBRC Keio Collection, Mishima, Japan), a *nanK*-mutant strain.

Thin-layer chromatography (TLC)

The bacterial culture medium was centrifuged at 6000 ×g for 5 min; the supernatant obtained was spotted on silica gel (TLC-silica gel 60, Merck), dried, and developed using a developing solvent (water/1-propanol/ammonia water = 37.5:15:2.5). When the solvent reached the top of the plate, the plate was collected, dried, sprayed with Orcinol sulfate reagent, and heat-treated at 110 °C until spots appeared.

Bacterial growth assay

(i) Growth of lactobacilli in Neu5Ac, 3'SL, 6'SL, and Lac-supplemented media

To evaluate carbohydrate utilization, modified MRS (mMRS) without glucose (the composition of the media is presented in Table S7) was supplemented with 0.5% Neu5 Ac, 3'SL, 6'SL, Lac, or mixtures of Neu5Ac and Lac in a 96-well plate (200 μL/well). Overnight cultures

were inoculated (1% v/v) and incubated at 37 °C for 48 h under anaerobic conditions. Growth was monitored by OD_{600} measurement every 0.5–1 h using a Stratus microplate reader (Cerillo Inc.); sugar release was confirmed by TLC.

(ii) Growth of L. salivarius in B. thetaiotaomicron-fermented media

B. thetaiotaomicron wild-type or *nanH*-deficient strains were cultured anaerobically on modified GAM agar, suspended in PBS with 0.5% 3'SL or 6'SL to $OD_{600} = 1$, and incubated at 37 °C for 24 h. Sugar liberation was confirmed by TLC. The culture was centrifuged at 6000 ×g for 5 min, filtered through a 0.22 μm filter, and mixed 1:1 with mMRS medium. The mixture was inoculated with 1% *L. salivarius* and incubated at 37 °C for 24 h. Growth was monitored using OD_{600}, as aforementioned.

(iii) Growth of L. salivarius in E. coli pnanH-fermented media

E. coli p*nanH* or *E. coli* pEmpty strains were cultured in LB medium containing 0.5% 3'SL or 6'SL. Sugar release was confirmed by TLC. The culture was centrifuged at 6000 ×g for 5 min, filtered through a 0.22 μm filter, and mixed 1:1 with mMRS medium. The mixture was inoculated with 1% *L. salivarius* and incubated at 37 °C for 24 h. Growth was monitored using OD_{600}, as aforementioned.

Competition assay for carbohydrate utilization by *L. salivarius*

A competition assay evaluated the carbohydrate utilization efficiency of *L. salivarius*. Five milliliters of mMRS broth supplemented with 0.5% (w/v) Neu5Ac or Lac were prepared. The medium was inoculated with 0.5% (v/v) of precultured bacterial broths of *L. salivarius* S22 and *L. reuteri* R21, respectively, which were grown anaerobically at 37 °C for 24 h. Equal volumes of *L. salivarius* and *L. reuteri* inocula were added to the medium; the cultures were incubated under anaerobic conditions at 37 °C.

Bacterial growth was quantified using qPCR with species-specific primers. Genomic DNA was extracted using a GenCheck DNA Extraction Kit [Type S/F] (FASMAC, Atsugi, Japan). qPCR was performed with the following thermal cycling conditions: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 5 s and annealing/extension at 65 °C for 1 min, using Platinum™ SYBR™ Green qPCR SuperMix-UDG (Thermo Fisher Scientific, USA). The primer sequences are listed in Table S3. Standard curves were constructed using tenfold serial dilutions of genomic DNA from *L. salivarius* and *L. reuteri* (50 ng to 5×10^{-2} ng per reaction). Standard reactions were performed in triplicate; the amplification efficiency and R^2 were calculated for reliability.

Bacterial growth inhibition assays

Enterotoxigenic *E. coli* strains E1237, E1263, and E1265 (isolated from piglet diarrhea samples) and *Salmonella enterica* serovar Typhimurium SL1344 (gift from Dr. Nobuhiko Okada, Kitasato University) were tested in inhibition assays. *L. salivarius* was cultured anaerobically in a non-sugar MRS medium supplemented with 0.5% (w/v) Neu5 Ac at 37 °C for 24 h. The culture medium was centrifuged at 6000 ×g for 5 min and filtered through a 0.22 μm filter. The resulting culture supernatant was used for the experiment. The bacteria were cultured overnight in an LB medium; a suspension was prepared in a fresh LB medium at an OD_{600} of approximately 0.01. The *L. salivarius* culture medium was then mixed with this suspension at ratios of 0%, 12.5%, 25%, and 50% to a total volume of 200 μL. Bacterial growth was monitored every 0.5 to 1 h by measuring OD_{600} using a microplate reader set at 37 °C.

SCFAs and pH measurements

SCFAs (acetic acid, propionic acid, butyric acid, succinic acid, and lactic acid) in the culture medium and fecal samples were analyzed using HPLC (Shimadzu, Kyoto, Japan), as aforementioned [59] with some modifications. For fecal samples, 200–300 mg of feces were homogenized with 1 mL of 0.15 mM sulfuric acid and subsequently centrifuged (22,000 ×g, 4 °C, for 20 min). The supernatant was extracted and filtered through a 0.45-μm PTFE filter into a 0.4-mL vial (GE Healthcare Technologies, Inc., Chicago, IL, #UN503 NPUORG), sealed, and placed at 4 °C for subsequent HPLC analysis. For the culture medium, samples were centrifuged (22,000 ×g, 4 °C, for 20 min); 1 mL of the supernatant was used for sulfuric acid treatment, as aforementioned. The injection volume for HPLC analysis was 10 μL. Separation was performed on an Aminex HPX-87H column (300 mm ×7.8 mm, Bio-Rad Laboratories, Hercules, CA) with a Cation H Cartridge (Bio-Rad Laboratories) as a guard column at a temperature of 40 °C, using a 5-mM sulfuric acid mobile phase at a flow rate of 0.6 mL/min. A refractive index detector (RID-20 A; Shimadzu) was operated for a total analysis time of 70 min. Fecal pH was measured from the supernatant of a 0.1 g/mL fecal suspension in distilled water using a pH meter (Horiba).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software (GraphPad Software). The statistical analysis of β diversity was conducted using the Pairwise

PERMANOVA on the QIIME2 platform; multiple comparisons were performed by calculating q values ($q < 0.05$). Data were visualized using the GraphPad Prism software.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-025-02129-3.

Supplementary Material 1: Fig. S1. Typical extracted ion chromatograms obtained from HPLC/MS analysis of sow milk and formula milk. Fig. S2. Growth assays of Neu5Ac, lactose, 3'SL, and 6'SL by various lactobacilli (both isolated strains and their corresponding type strains) identified in piglets ($n = 3$). All data represent mean ± SEM. OD_{600} values (vertical axis) were measured over time (horizontal axis, hours). Fig. S3. (A) Heterologously expressing *B. thetaiotaomicron nanH* in *Escherichia coli* JW5538-KC (*nanK*-mutant strain), referred to as *E. coli* p*nanH*. In *E. coli* p*nanH* or empty vector (*E. coli* pEmpty) strains were cultured in media supplemented with Neu5Ac, 3'SL, 6'SL, and lactose as sugar sources. The sugar components remaining in the medium after culturing were detected via thin-layer chromatography. (B) Growth curves of *L. salivarius* strain S22 in medium cultured with *E. coli* p*nanH* or *E. coli* pEmpty ($n = 3$). All data represent mean ± SEM. Fig. S4 Growth curves of ETEC 3 strains and *S.* Typhimurium when indicate concentrations of acetate or lactate was added ($n = 3$). All data represent mean ± SEM. Fig. S5. Prediction of sialic acid metabolic enzymes in suckling piglets (SP) and formula-fed piglets (FP). (A) Prediction of sialic acid metabolic enzymes, namely, N-acetylneuraminate lyase (NanA), epimerases (NanE, NanE-II), and kinase (NanK), and (B) the bacterial species (genus level) that possess sialic acid metabolic enzymes in fecal samples from SP and FP, with analysis performed using PICRUSt2 (Table S10). These data correspond to main Fig. 5E. Fig. S6 Composition of microbiota in ex vivo culture of piglet feces. (A) Piglet feces (from suckling piglets) were inoculated at 0.1% (w/v) into a medium supplemented with Neu5Ac, lactose, 3'SL, and 6'SL as carbon sources, and incubated anaerobically for 24 h ($n = 2$). (B) PCoA based on Unweighted and weighted UniFrac distance matrix generated from sequencing 16S rRNA genes form ex vivo culture samples. (C) Shannon index, Observed ASV, and Faith's Phylogenetic Diversity of ex vivo culture samples are plotted. All data represent mean ± SEM. (D) Genus-level bacterial composition of ex vivo culture samples. (E) Concentrations of short-chain fatty acids (SCFAs) in ex vivo culture samples are presented. Fig. S7 Genotyping of *B. thetaiotaomicron* JCM 5827^T *ΔnanH*. (A) Pr_GYS84 was annealed to the 1,128 bp upstream of *nanH*, and Pr_GYS85 was annealed to the 1114 bp downstream of *nanH*. (B) The result of agarose-gel electrophoresis of PCR product using Pr_GYS84 and Pr_GYS85 as the primer. Genomic DNA of WT and *ΔnanH* were used as the templates.

Supplementary Material: Table S1. Analysis of BRAY–CURTIS permanova-pairwise for Fig. 5D. Table S2. Selected Ions for LC/MS analysis in Negative Ion Mode. Table S3. Sequence of oligonucleotide primers used for qPCR. Table S4. Plasmids and Oligonucleotide primers used for generation of *B. thetaiotaomicron ΔnanH* strain. Table S5. Plasmids and oligonucleotide primers used for plasmid construction of *E. coli* NanH-expressing strain. Table S6. Sequence of the codon-optimized sequence of mature *nanH* gene for *E. coli* NanH-expressing strain. Table S7. Composition of mMRS broth. Table S8. The abundance of each EC number in piglets from birth to weaning (Day 0 to Day 56), as predicted by PICRUSt2. Table S9. The estimated gene counts for EC:3.2.1.18 in each ASV of piglets from birth to weaning (Day 0 to Day 56), as predicted by PICRUSt2. Table S10. The abundance of each EC number in of breast-fed piglets (BFP) and formula-fed piglets (FFP), as predicted by PICRUSt2.

Acknowledgements
We thank Kenta Watanabe, Yoshihiko Sugiyama, and Ryo Ishihara for their support with sampling. We are also grateful to Sachiko Murayama, Mihoko Washimi, Lisa Fujisawa, and Kanami Furukawa at the Gifu Prefectural Livestock Research Institute for managing pig fecal samples. We appreciate Luciano Arellano-Arriagada for his helpful suggestions on the manuscript. We acknowledge the National BioResource Project (NIG, Japan) for providing *Escherichia coli* strains JM109 λpir and S17-1 λpir.

Authors' contributions
K.N., F.N., E.Na., Y.S. and H.K. conceptualized the original project; R.H., K.N., F.N., T.Sa., I.F., Y.S., K.O., T.Sh., E.T., S.T., E.Ni. and Y.S. performed the experiments; K.S., T.Sa., R.O., M.F., T.N., G.Y. and Y.S. collected the samples; R.H., K.N., F.N. and T.Sh. analyzed and interpreted the data; R.H., K.N., F.N., I.F., Y.S., K.O., T.Sh., S.S., T.M., T.N., J.V., W.I., E.Na., Y.S. and H.K. wrote, reviewed, and revised the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by the Grant-in-Aid for Scientific Research (B) (23 K27051, K.N.), Challenging Research (Exploratory, 23 K18072, H.K.), Research Activity Start-up (23 K19327, F.N.) from JSPS, Development of Innovative Technology grants (JPJ007097, H.K.) from BRAIN; 2023 research grant from the Japanese Dairy Science Association (K.N.).

Data availability
16S rRNA amplicon sequence data are deposited in the DNA Data Bank of Japan under BioProject No. DRA019686 (https://ddbj.nig.ac.jp/resource/sra-submission/DRA019686) and No. DRA019687 (https://ddbj.nig.ac.jp/resource/sra-submission/DRA019687). The abundance of all Enzyme Commission (EC) numbers in each sample calculated by PICRUSt2 is listed in Table S8-10.

Declarations

Ethics approval and consent to participate
All animal experiments at Miyagi University and Tohoku University were approved by the Animal Care and Management Committee (approval numbers: 2021–17 for Miyagi University and 2021–006, 2021–038-02 for Tohoku University). The animals were raised for meat production following standard husbandry practices at the Gifu Prefectural Livestock Research Institute. All animal sample collection and processing procedures adhered to the regulations of the Biosafety Committee of Tohoku University (approval number: 2016–045-01).

Consent for publication
Not applicable.

Competing interests
Authors T.Sh., E.Na. are employees of Kyowa Hakko Bio Co., Ltd. Authors S.T., E.Ni. are employees of FASMAC Co., Ltd. The remaining authors declare no competing interests.

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Received: 3 January 2025 Accepted: 30 April 2025
Published online: 24 May 2025

References

1. Bartol FF, Wiley AA, Bagnell CA. Epigenetic programming of porcine endometrial function and the lactocrine hypothesis. Reprod Domest Anim. 2008;43(Suppl 2):273–9.
2. Chu DM, Meyer KM, Prince AL, Aagaard KM. Impact of maternal nutrition in pregnancy and lactation on offspring gut microbial composition and function. Gut Microbes. 2016;7:459–70.
3. Keady MM, et al. Ecoevolutionary processes structure milk microbiomes across the mammalian tree of life. Proc Natl Acad Sci U S A. 2023;120: e2218900120.
4. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell. 2014;157:121–41.
5. Bäckhed F, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host Microbe. 2015;17:690–703.
6. Sanidad KZ, Zeng MY. Neonatal gut microbiome and immunity. Curr Opin Microbiol. 2020;56:30.
7. Fujimura KE, et al. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. Nat Med. 2016;22:1187–91.
8. Kim YG, et al. Neonatal acquisition of Clostridia species protects against colonization by bacterial pathogens. Science. 2017;356:315–9.
9. Stewart CJ, et al. Longitudinal development of the gut microbiome and metabolome in preterm neonates with late onset sepsis and healthy controls. Microbiome. 2017;5:75.
10. Peiper AM, et al. Metabolic immaturity and breastmilk bile acid metabolites are central determinants of heightened newborn vulnerability to norovirus diarrhea. Cell Host Microbe. 2024;3:1488-1501.e5.
11. Global Pig Farming Market Report 2024 Edition, Market Size, Share, CAGR, Forecast, Revenue. Available at: https://www.cognitivemarketresearch.com/pig-farming-market-report. Accessed 16 Nov 2024.
12. Saladrigas-García M, et al. An insight into the commercial piglet's microbial gut colonization: from birth towards weaning. Anim Microbiome. 2022;4:68.
13. Beaumont M, et al. The early life microbiota is not a major factor underlying the susceptibility to postweaning diarrhea in piglets. Microbiol Spectr. 2023;11(4):e0069423.
14. Mahmud MR, et al. Impact of intestinal microbiota on growth performance of suckling and weaned piglets. Microbiol Spectr. 2023;11(3):e0374422.
15. Gaukroger CH, et al. Changes in faecal microbiota profiles associated with performance and birthweight of piglets. Front Microbiol. 2020;11:917.
16. Li N, et al. Differences in the gut microbiota establishment and metabolome characteristics between low- and normal-birth-weight piglets during early-life. Front Microbiol. 2018;9:1798.
17. Schokker D, et al. Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets. PLoS ONE. 2014;9: e100040.
18. Frese SA, Parker K, Calvert CC, Mills DA. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome. 2015;3:28.
19. Granger CL, et al. Maternal breastmilk, infant gut microbiome and the impact on preterm infant health. Acta Paediatr. 2021;110:450–7.
20. Moossavi S, et al. Composition and variation of the human milk microbiota are influenced by maternal and early-life factors. Cell Host Microbe. 2019;25:324-335.e4.
21. Urashima T, Saito T, Nakamura T, Messer M. Oligosaccharides of milk and colostrum in non-human mammals. Glycoconj J. 2001;18:357–71.
22. Masterson HK, Urashima T, Owens RA, Hickey RM, Milk oligosaccharides. *Advanced Dairy Chemistry: Volume 3: Lactose, Water, Salts and Minor Constituents* 2022;261–296. https://doi.org/10.1007/978-3-030-92585-7_7.
23. Durham SD, Wei Z, Lemay DG, Lange MC, Barile D. Creation of a milk oligosaccharide database, MilkOligoDB, reveals common structural motifs and extensive diversity across mammals. Sci Rep. 2023;13(1):10345.
24. Bunesova V, Lacroix C, Schwab C. Fucosyllactose and L-fucose utilization of infant Bifidobacterium longum and Bifidobacterium kashiwanohense. BMC Microbiol. 2016;16:1–12.
25. Sakanaka M, et al. Evolutionary adaptation in fucosyllactose uptake systems supports bifidobacteria-infant symbiosis. Sci Adv. 2019;5(8):eaaw7696.
26. Laursen MF, et al. Bifidobacterium species associated with breastfeeding produce aromatic lactic acids in the infant gut. Nat Microbiol. 2021;6:1367–82.
27. Henrick BM, et al. Bifidobacteria-mediated immune system imprinting early in life. Cell. 2021;184:3884-3898.e11.
28. Charbonneau MR, et al. Sialylated milk oligosaccharides promote microbiota-dependent growth in models of infant undernutrition. Cell. 2016;164:859–71.
29. Mudd AT, et al. Porcine milk oligosaccharides and sialic acid concentrations vary throughout lactation. Front Nutr. 2016;3: 213082.
30. Soyyilmaz B, et al. The mean of milk: a review of human milk oligosaccharide concentrations throughout lactation. Nutrients. 2021;13(8):2737.
31. Ren X, et al. Human milk oligosaccharides are associated with lactation stage and lewis phenotype in a Chinese population. Nutrients. 2023;15(6):1408.
32. Vimr ER, Kalivoda KA, Deszo EL, Steenbergen SM. diversity of microbial sialic acid metabolism. Microbiol Mol Biol Rev. 2004;68:132.
33. Hopkins AP, Hawkhead JA, Thomas GH. Transport and catabolism of the sialic acids N-glycolylneuraminic acid and 3-keto-3-deoxy-d-glycero-d-galactonononic acid by Escherichia coli K-12. FEMS Microbiol Lett. 2013;347:14–22.
34. Kleerebezem M, et al. Complete genome sequence of Lactobacillus plantarum WCFS1. Proc Natl Acad Sci. 2003;100:1990–5.
35. Almagro-Moreno S, Boyd EF. Insights into the evolution of sialic acid catabolism among bacteria. BMC Evol Biol. 2009;9:118.
36. Ravcheev DA, Thiele I. Comparative genomic analysis of the human gut microbiome reveals a broad distribution of metabolic pathways for the degradation of host-synthetized mucin glycans and utilization of mucin-derived monosaccharides. Front Genet. 2017;8:111.
37. Marcobal A, et al. Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. Cell Host Microbe. 2011;10:507–14.
38. Ng KM, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature. 2013;502:96.
39. Nishiyama K, et al. Two extracellular sialidases from Bifidobacterium bifidum promote the degradation of sialyl-oligosaccharides and support the growth of Bifidobacterium breve. Anaerobe. 2018;52:22–8.
40. Robinson LS, Lewis WG, Lewis AL. The sialate O-acetylesterase EstA from gut Bacteroidetes species enables sialidase-mediated cross-species foraging of 9-O-acetylated sialoglycans. J Biol Chem. 2017;292:11861–72.
41. Buzun E, et al. A bacterial sialidase mediates early-life colonization by a pioneering gut commensal. Cell Host Microbe. 2024;32:181-190.e9.
42. Kim K, Song M, Liu Y, Ji P. Enterotoxigenic Escherichia coli infection of weaned pigs: Intestinal challenges and nutritional intervention to enhance disease resistance. Front Immunol. 2022;13: 885253.
43. Saha S, Namai F, Nishiyama K, Villena J, Kitazawa H. Role of immunomodulatory probiotics in alleviating bacterial diarrhea in piglets: a systematic review. J Anim Sci Biotechnol. 2024;15(1):112.
44. Fukuda S, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 2011;469(7331):543–7.
45. Huang YL, Chassard C, Hausmann M, Von Itzstein M, Hennet T. Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in mice. Nat Commun. 2015;6:8141.
46. Ding J, et al. Effect of breastmilk microbiota and sialylated oligosaccharides on the colonization of infant gut microbial community and fecal metabolome. Metabolites. 2022;12:1136.
47. Song D, Moon H, Kang B. Porcine epidemic diarrhea: a review of current epidemiology and available vaccines. Clin Exp Vaccine Res. 2015;4:166.
48. Boeckman JX, et al. Effect of chronic and acute enterotoxigenic E. coli challenge on growth performance, intestinal inflammation, microbiome, and metabolome of weaned piglets. Sci Rep. 2022;12(1):5024.
49. Young W, Egert M, Bassett SA, Bibiloni R. Detection of sialic acid-utilising bacteria in a caecal community batch culture using RNA-based stable isotope probing. Nutrients. 2015;7:2109–24.
50. Bell A, Severi E, Owen CD, Latousakis D, Juge N. Biochemical and structural basis of sialic acid utilization by gut microbes. J Biol Chem. 2023;299(3):102989.
51. Klindworth A, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013;41(1):e1.
52. Bolyen E, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37:852–7.
53. Douglas GM, et al. PICRUSt2 for prediction of metagenome functions. Nat Biotechnol. 2020;38:685–8.

54. Ohgi R, et al. In vitro evaluation of the immunomodulatory and wakame assimilation properties of Lactiplantibacillus plantarum strains from swine milk. Front Microbiol. 2024;15:1324999.
55. García-Bayona L, Comstock LE. Streamlined genetic manipulation of diverse bacteroides and parabacteroides isolates from the human gut microbiota. mBio. 2019;10(4):e01762-19.
56. Narita J, et al. Display of active enzymes on the cell surface of Escherichia coli using PgsA anchor protein and their application to bioconversion. Appl Microbiol Biotechnol. 2006;70:564–72.
57. Poo H, et al. Novel high-level constitutive expression system, pHCE vector, for a convenient and cost-effective soluble production of human tumor necrosis factor-α. Biotechnol Lett. 2002;24:1185–9.
58. Tanaka T, Kawabata H, Ogino C, Kondo A. Creation of a cellooligosaccharide-assimilating Escherichia coli strain by displaying active beta-glucosidase on the cell surface via a novel anchor protein. Appl Environ Microbiol. 2011;77:6265–70.
59. Tsujikawa Y, et al. Establishment of porcine fecal-derived ex vivo microbial communities to evaluate the impact of livestock feed on gut microbiome. Biosci Microbiota Food Health. 2024;43:100–9.

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