AUTHOR(S):

KAMBE, Jun; SASAKI, Yu; INOUE, Ryo; TOMONAGA, Shozo; KINJO, Teruo; WATANABE, Gen; JIN, Wanzhu; NAGAOKA, Kentaro

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Analysis of infant microbiota composition and the relationship with breast milk components in the Asian elephant (Elephas maximus) at the zoo

Jun KAMBE[1)#], Yu SASAKI[1)#], Ryo INOUE[2)], Shozo TOMONAGA[3)], Teruo KINJO[4)], Gen WATANABE[1)], Wanzhu JIN[5)] and Kentaro NAGAOKA[1)*]

[1)]Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan
[2)]Laboratory of Animal Science, Department of Applied Biological Sciences, Setsunan University, Osaka 573-0101, Japan
[3)]Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
[4)]Okinawa Zoo Animal Hospital, Okinawa Zoo and Museum, Okinawa 904-0021, Japan
[5)]Institute of Zoology, Chinese Academy of Sciences, 1-5 Beichen West Road, Chaoyang, Beijing, China

ABSTRACT. The prevention of diseases through health control is essential at zoos. Here, we investigated the gut microbiota formation during infancy in an Asian elephant and compared the composition between infant and mother. Besides, we analyzed the components of breast milk and examined the correlation with the infant gut microbiota. Analysis revealed the gut microbiota of the infant contained high amount of *Lactobacillales* and its diversity was relatively low compared to that of the mother. We found several milk components, showed a positive correlation with the change of *Lactobacillales*. The present study revealed the mechanism of gut microbiota formation during infancy in an Asian elephant and provides important insights into the health control of Asian elephants in zoos.

KEY WORDS: blood metabolites, breast milk, *Elephas maximus*, intestinal microbiota

For mammals, symbiotic relationships with bacteria in several organs, such as the skin, reproductive tract, and gastrointestinal tract, are important for their health and survival [16]. Gut microbiota, an ecosystem formed by bacteria living in the gastrointestinal tract, play a critical role in many physiological and immunological processes [7, 9, 15]. Moreover, there is increasing evidence showing that specific aberrations during initial bacterial colonization in infancy can increase susceptibility to several diseases in later life [11, 21, 23]. Breast milk is essential for the optimal colonization and maturation of the infant microbiota; bacteria such as *Bifidobacterium* and *Lactobacillus* are dominant in humans and mice, respectively, and their growth is encouraged by milk compounds such as oligosaccharides and hydrogen peroxide [12, 24, 30]. These lactic acid bacteria show a variety of positive effects on gut microbiota and are widely used as probiotics for increased health benefits [4, 15, 17].

Although we know the importance of gut microbiota and breast milk in human infant health, we have a poor understanding of these relationships in other species. Studies on gut microbiota may be of particular relevance to animals maintained in captive environments, such as zoos, which require preventative health care, as well as veterinary treatment, to combat many kinds of diseases. The importance of a "healthy microbiome" for captive animals is recognized but characterizing the taxonomic and functional attributes of this is in its infancy [1, 2, 22].

The Asian elephant (*Elephas maximus*) is classified as Endangered on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species. In parallel with wild populations, many captive populations are also facing rapid decline and extinction because of current low fertility and high elephant calf mortality rates [29]. It is essential for elephant calves to receive nourishment from their mother's milk for the first 3–6 months, although lactation lasts from 2 to 8 years [14]. The basic components of Asian elephant milk are reported to be 82% moisture, 3.3% protein, 7.7% lipid and 6.7% carbohydrate, and the milk is characterized by having a large amount of glucosamine [25]. The Asian elephant is a hind-gut fermenter that utilizes the developed cecum and colon as a fermenter, but the constitution and formation of the gut microbiota during infancy and its

relationship to milk components are little understood.

In this study, a single elephant calf was born at 9:42 PM on March 4, 2015, and colostrum intake was confirmed next day (March 5; day 1 of lactation). Breast milk was collected from day 1 of lactation and samples of feces from the mother and infant were collected from day 3 of lactation (Fig. 1). During the sampling period, the elephant calf fed only on breast milk, but it was observed several times to eat red soil from its enclosure. Coprophagy was first observed on day 26 of lactation, which is normal behavior in many herbivorous animals. The birth weight of the elephant calf was about 100 kg, and the bodyweight increased by 1 kg or more per day. The body weight on the 31st day of lactation was 163 kg. The contents of the mother's daily feeding are 60 kg of napier grass, 60 kg of reeds, 18 kg of trees, 2–3 kg of hay and supplemented with about 20 g of light calcium carbonate.

To extract the DNA from fecal samples, fecal samples (25 mg) were suspended in 500 μl of Tris-EDTA (TE) buffer (pH 8.0) and centrifuged at 14,000 rpm for 1 min. Fecal pellets were washed three times in TE buffer. Subsequently, the pellets were resuspended in 600 μl of TE buffer containing 300 mg of glass beads (diameter, 0.2 mm) and vortexed vigorously at 5,500 rpm for 20 sec using Micro Smash^{TM}(MS-100; Tomy Digital Biology, Tokyo, Japan). The resultant suspension was incubated with 1.2 μl of 10 mg/ml lysozyme at 37°C for 1 hr. To the mixture, 600 μl of buffer-saturated phenol and 100 μl of 10% sodium dodecyl sulfate (SDS) were added, vortexed at 5,500 rpm for 20 sec using Micro Smash^{TM}, incubated at 70°C for 10 min, centrifuged at 14,000 rpm for 5 min, and the supernatant (600 μl) was collected and subjected to isopropanol precipitation. The precipitated DNA was suspended in 200 μl of TE buffer.

After purification DNA from fecal samples, 16S rRNA metagenome analyses of fecal samples were performed. Briefly, the first PCR targeting the variable regions 3 and 4 (V3-4) of the 16S rRNA gene was performed using the primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) followed by the second PCR for attachment of dual indices. An equal amount of the amplicon was pooled and 10 pM of the library was mixed with phiX control and sequenced using a MiSeq v3 kit (illumina Inc., San Diego, CA, USA) as per the manufacturer's instructions. Processing of sequence data, including the chimera check, operational taxonomic unit (OTU) definition, and taxonomic assignment, was performed using QIIME v1.9, USEARCH v9.2.64, UCHIME v4.2.40, and VSEARCH v2.4.3, respectively. Singletons were dropped in the present study. Taxonomic assignment of the resultant OTU was achieved using the RDP classifier v2.10.2 with the Greengenes database (published in May 2013). The relative abundance of taxa in the samples was analyzed using the MicrobiomeAnalyst with default settings (https://www.microbiomeanalyst.ca). The alpha diversity of the samples, which was measured using Chao1 which is defined as "richness", showed a significant difference between the mother and infant (Fig. 2A). The principal coordinate analysis (PCoA) plot based on the Bray-Curtis index and permutational multivariate analysis of variance (PERMANOVA) test also demonstrated differences in community composition (Fig. 2B). Linear discriminant analysis (LDA) and effect size (LEfSe) analyses identified 15 bacterial taxa at the level of the order with a difference in relative abundance between the mother and infant during lactation (Fig. 2C). *Enterobacteroidales* and *Lactobacillales* were characteristic of the Asian elephant calf microbiota during infancy.

During infancy, the gut microbiota composition of the elephant calf changed. *Enterobacteroidales* and *Lactobacillales* seemed high in the early lactation period; *Closridiales* seemed high in the late lactation period (Fig. 3A). As described above, the abundance of *Enterobacteroidales* and *Lactobacillales* in the mother's gut microbiota was lower than in the infant's, while *Bacteroidales* and *Closridiales* were shown to be constant during lactation (Fig. 3A).

To elucidate change of milk metabolites during lactation period, milk samples were centrifuged at 16,000 rpm at 4°C for 60 min, and the middle layer was collected as skim milk. An untargeted metabolomics analysis was performed using GC-MS, as described previously [27]. In brief, 50 μl of skim milk were mixed with 250 μl of methanol-chloroform-water (2.5:1:1) and 5 μl of 1 mg/ml 2-isopropylmalic acid as an internal standard, and homogenized using a Polytron homogenizer (Micro-tec Co., Ltd., Urayasu, Chiba, Japan). Samples were subsequently mixed in a shaker at 1,200 rpm at 37°C for 30 min and centrifuged at 16,000 × g at 4°C for 5 min. Next, 160 μl of the supernatant was mixed with 200 μl of distilled water and vortexed. This was followed by centrifugation at 16,000 × g at 4°C for 5 min. Afterwards, 250 μl of the supernatant was dried under a vacuum using a centrifugal evaporator (RD-400, Yamato Scientific Co., Ltd., Koto, Tokyo, Japan). Dried samples were pre-treated, derivatized, and analyzed using GC-MS (QP-2010 Ultra; Shimadzu Corp., Kyoto Japan) within 24 hr of derivatization. The Shimadzu Smart Metabolites Database was used to identify metabolites. Peak identification and statistical analysis of the metabolomic analysis was performed

	Day 0 Weight: 100 kg										Day 31 Weight: 163 kg		
Lactation day	1	2	3	4	5	6	7	8	15	22	29	37	38
Milk	○	○	○	○	○	○	○	○	○	○	○	○	
Feces — Mother			○		○	○	○		○	○	○		○
Feces — Infant			○			○	○		○	○	○	○	○

Fig. 1. Breast milk and feces sample collection schedules. "○" represents day of samples collection. Body weight of elephant calf was recorded at day 0 and day 31 of lactation.
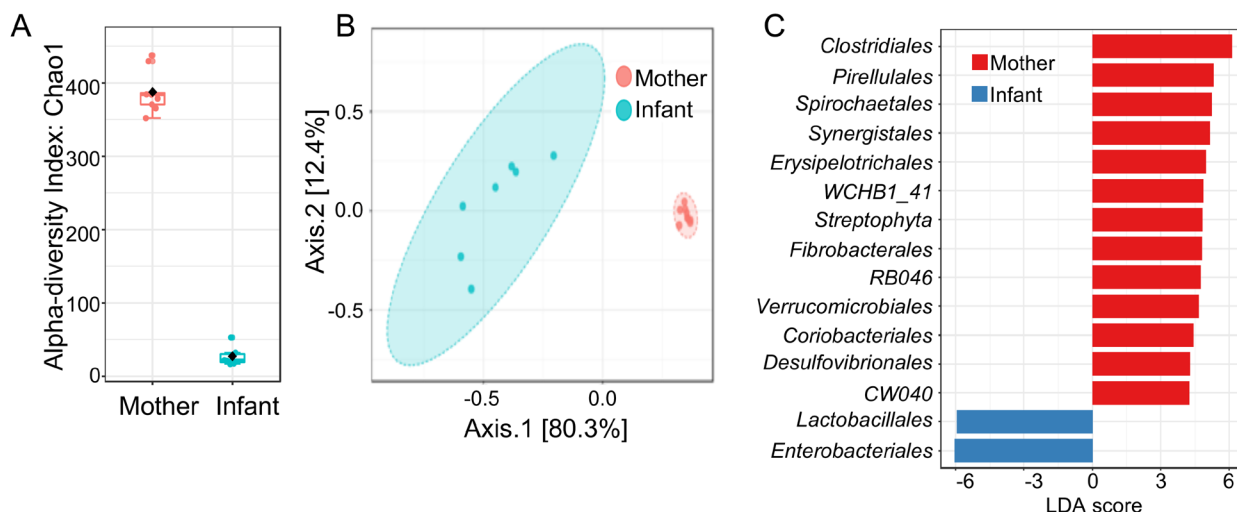
Fig. 2. Gut microbiota composition in mother and infant during lactation. A: Alpha-diversity, measured by Chao1 Index is plotted for the mother (red) and infant (blue) ($P<0.05$). B: Plots of PCoA based on the Bray-Curtis Index of microbial communities in the mother (red) and infant (blue) ($P<0.05$). C: The relative abundance of bacterial taxa in the feces from the mother and infant based on LEfSe comparisons. Red indicates overabundant bacteria in the mother, and blue indicates overabundant bacteria in the infant.
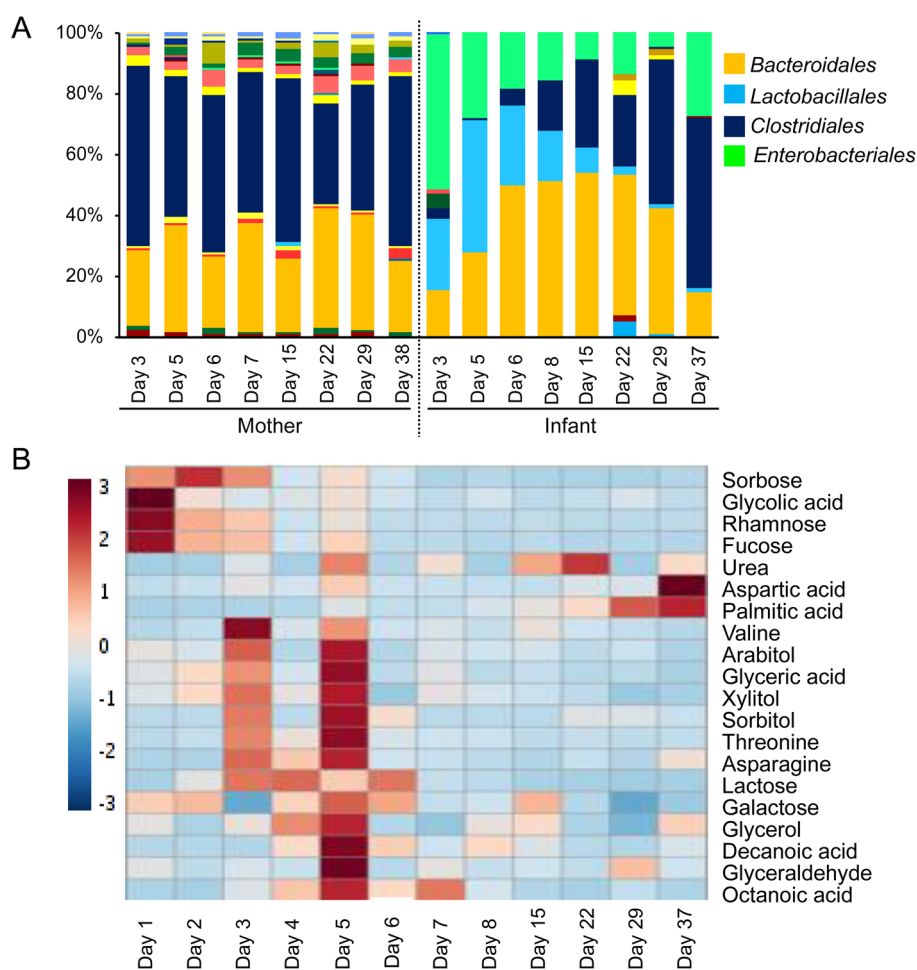

Fig. 3. Change of gut microbiota and milk components during lactation. A: Relative abundance of bacterial order association with the mother (left side) and the infant (right side) during the lactation period (from day 3 to day 38 of lactation). *Bacteroidales* (orange), *Lactobacillales* (light blue), *Clostridiales* (dark blue) and *Enterobacteroidales* (light green) B: Heatmap showing the relative amount of milk components detected by metabolomic analysis during the lactation period. Components with a higher level are displayed in red, while lower levels are displayed in blue.

using MS-DIAL and MetaboAnalyst [26].

An untargeted metabolomic analysis of the breast milk revealed that 20 metabolites were detectable, and their amount changed during lactation (Fig. 3B). To define any relationship between changes in breast milk components and infant gut microbiota composition, we performed a Pearson r correlation test or Spearman rank correlation test. As shown in Table 1, there were positive and negative correlations between milk components and gut microbiota including *Closridiales, Lactobacillales, Enterobacteroidales,* and *Bacteroidales* during infancy. In *Closridiales*, palmitic acid showed a positive correlation and threonine showed the most negative correlation (Fig. 4A). In *Lactobacillales*, lactose showed the most positive correlation (Fig. 4B). In *Enterobacteroidales*, asparagine showed the most positive correlation (Fig. 4C), while in *Bacteroidales*, aspartic acid showed the most negative correlation (Fig. 4D). In addition, we investigated the relationship steroid hormones in breast milk, in particular estradiol-17β, and gut microbiota in the elephant calf. Milk samples (100 μl) were diluted to 400 μl with a phosphate buffer containing 1% BSA, and 2.0 ml of diethyl ether was added to each tube and mixed for 3 min. After mixing, the tubes were immersed in methanol containing dry ice, and the diethyl ether was transferred into glass tubes and evaporated to dryness at 60°C. After cooling, 400 μl of phosphate buffer containing 1% BSA was added into the tube and mixed for 3 min. For the radioimmunoassay (RIA), 100 μl aliquots of the samples were transferred to the assay tubes in triplicate, and milk estradiol-17β concentrations were measured by using a double-antibody RIA system with ^{125}I-labeled radioligands (MP Biomedicals, LLC, Solon, OH, USA). The concentration of estradiol-17β in Asian elephant breast milk showed a positive correlation with the change of *Lactobacillales* during infancy (Fig. 5).

In zoos, preventive health management is very important because providing appropriate treatment to a wide variety of animals is difficult. Probiotics are known to confer health benefits when consumed by human and domestic animals. Nowadays, many studies are seeking effective probiotics for different types of zoo animals, however, this requires definition of the specific composition of gut microbiota in each animal. The gut microbiota is formed during infancy, but the formation mechanism is not well understood in different species. In this study, we investigated the gut microbiota composition of an Asian elephant mother and infant during the lactation period. The infant gut microbiota underwent some changes from early to middle and late lactation. The components of low molecular metabolites and steroid hormones in breast milk showed several correlations with the change in gut bacterial taxa during infancy. Our study is the first to characterize the composition of infant gut microbiota and components of breast milk and to suggest the possibility that breast milk plays an important role in the formation of gut microbiota during infancy in an Asian elephant.

Microbiota analysis revealed that the alpha diversity index Chao1 was lower in the infant than in the mother, which is consistent with previous studies and indicates that gut microbiota richness was low in the infant [10]. In addition, *Enterobacteroidales* and *Lactobacillales* were characteristic of the gut microbiota of the infant Asian elephant. It has been reported that lactic acid bacteria have important functions to stimulate the development of the gut immunity and barriers during infancy. The gut microbiota in infants is dominated by *Bifidobacterium* in humans and *Lactobacillus* in mice, showing a reduced diversity of bacterial species [3, 5, 30]. Recent studies have reported that human milk oligosaccharides (HMOs) are regulators that increase *Bifidobacterium*

Table 1. Correlation between breast milk components and infant gut microbiota

Low molecular metabolites	Bacterial order			
	Clostridiales	*Lactobacillales*	*Enterobacteriales*	*Bacteroidales*
Glycolic acid	ns	0.7949[a]	ns	ns
Urea	ns	ns	ns	ns
Glycerol	ns	ns	ns	ns
Decanoic acid	ns	0.8353[b]	ns	ns
Galactose	ns	ns	ns	ns
Sorbitol	ns	0.8406[b]	ns	ns
Palmitic acid	0.9358[b]	ns	ns	ns
Valine	−0.7619[a]	ns	ns	ns
Glyceraldehyde	ns	ns	ns	ns
Octanoic acid	−0.8095[a]	0.8333[a]	ns	ns
Glyceric acid	−0.8095[a]	0.8095[a]	ns	ns
Threonine	−0.9048[b]	0.8333[a]	0.8095[a]	ns
Aspartic acid	ns	ns	ns	−0.9524[b]
Asparagine	ns	ns	0.9286[b]	−0.7381[a]
Xylitol	ns	ns	ns	ns
Arabitol	ns	ns	ns	ns
Rhamnose	−0.8810[b]	0.9048[b]	ns	ns
Fucose	−0.7857[a]	0.7381[a]	ns	ns
Sorbose	−0.7619[a]	0.8095[a]	0.8571[b]	ns
Lactose	−0.8571[b]	0.9286[b]	ns	ns

R (correlation coefficient) was displayed. ns: not significant, a) *P*<0.05, b) *P*<0.01.
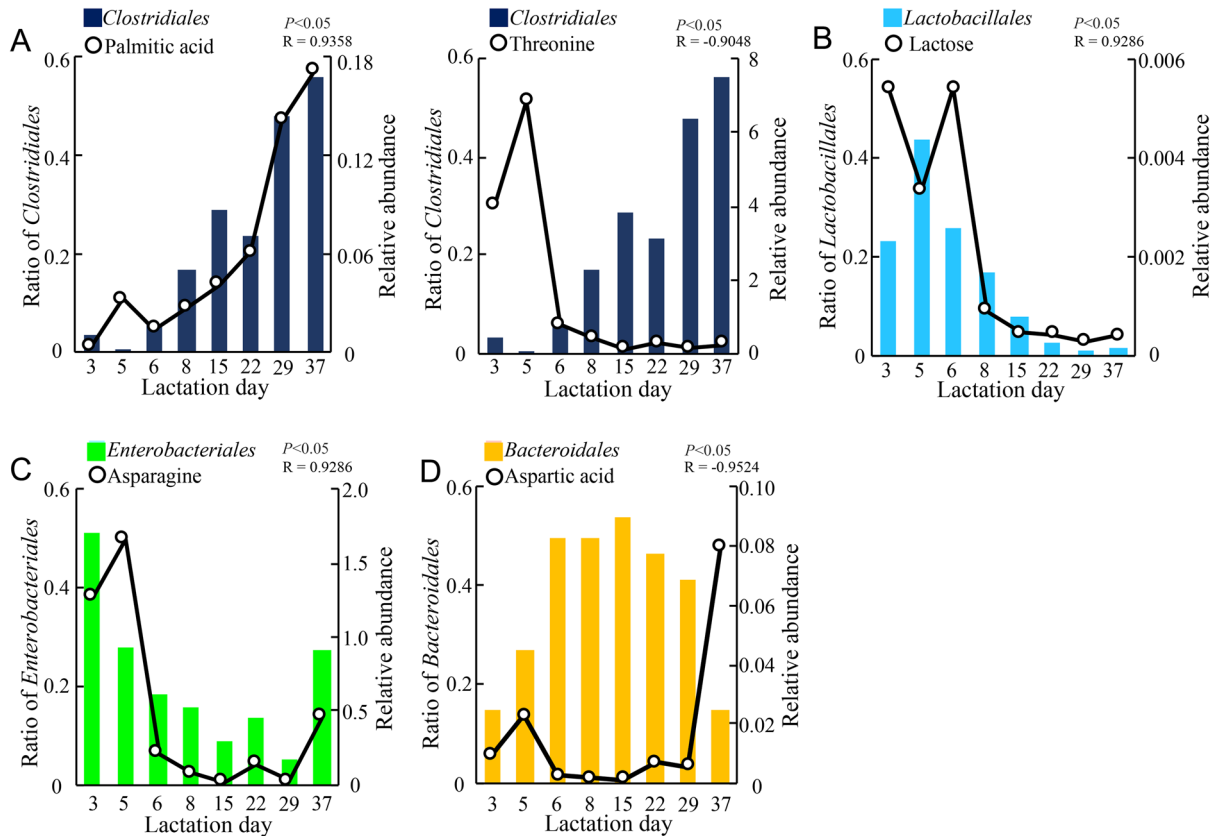
Fig. 4. Relationships between infant microbiota composition and breast milk components. A: Positive correlation between palmitic acid and *Clostridiales* (left histogram), and negative correlation between threonine and *Clostridiales* (right histogram). B: Positive correlation between lactose and *Lactobacillales*. C: Positive correlation between asparagine and *Enterobacteroidales*. D: Negative correlation between aspartic acid and *Bacteroidales*. *Clostridiales* (dark blue), *Lactobacillales* (light blue), *Enterobacteroidales* (light green), and *Bacteroidales* (orange).
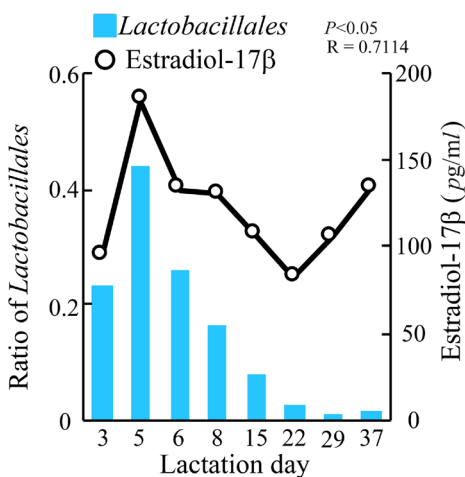

Fig. 5. Relationships between infant microbiota composition and breast milk hormones. Positive correlation between estradiol-17β and *Lactobacillales*. R (correlation coefficient) is 0.7114 (*P*<0.05).

in infant gut microbiota and that hydrogen peroxide is involved in the increase of the *Lactobacillus* population in mouse infant gut microbiota by metabolizing amino acids in milk [12, 18, 24]. These observations suggest that breast milk should contain several factors that regulate *Lactobacillales* composition during infancy.

Here, we identified that milk components in the Asian elephant were positively correlated with the change of *Lactobacillales* during the lactation period, including lactose, rare sugars, fatty acids and threonine. It is well known that mammalian milk is rich in lactose which is used as the primary energy source for *Lactobacillales* growth. This indicates that the high correlation between lactose levels in milk and *Lactobacillales* composition in the infant gut microbiota is convincing. It is also reported that rhamnose, one of the rare sugars, is a constituent of the cell wall of *Lactobacillales* and threonine is a metabolic substrate for *Lactobacillales* [6, 28]. Interestingly, we found that estradiol-17β also showed a positive correlation with *Lactobacillales*. Estradiol-17β, as a lipophilic substance, easily passes through the blood–milk barrier and its presence in milk is in direct correlation with its levels in blood [20]. The good or bad effect of milk estradiol-17β on infant health in humans is as yet undecided, but our data indicate that it plays a role in advancing *Lactobacillales* growth during the formation of infant gut microbiota in Asian elephants.

In humans and mice, the gut microbiota of infants is less diverse than that of adult individuals and tends to lack *Clostridiales*, the dominant taxa found in the adult [8, 19]. Likewise, in Asian elephant, the *Clostridiales*

population was low at the early lactation stage and increased to the same level as adults during the late lactation period. Moreover, a positive correlation was observed between milk palmitic acid and the changes in *Clostridiales* composition. Palmitic acid is a saturated fatty acid that accounts for 20% to 25% of the fatty acids in breast milk and is considered to be the primary energy source for infants [13]. In the Asian elephant, milk palmitic acid may function to increase bacterial diversity and develop the gut microbiota during the late lactation period. There were also correlations between Asparagine and *Enterobacteroidales* or between aspartic acid and *Bacteroidales*, but the amino acid metabolic pathway was complicated, and the details remain unclear.

In conclusion, it was possible to see changes over time in the gut microbiota and breast milk components of the Asian elephant. The gut microbiota of the mother elephant was relatively stable, but large fluctuations were observed in the infant. More importantly, in the Asian elephant, *Lactobacillales* but not *Bifidobacteriales* is the dominant lactic acid bacteria, which is important information when we choose commercial probiotic products for supporting the health care of Asian elephants in zoos. Several correlations were found between breast milk components and infant gut microbiota, however, further studies are required to understand the full mechanism of the gut microbiota formation during infancy. Furthermore, in order to characterize each bacterium from elephant gut microbiota and use it as a probiotic, it is necessary to clarify the lower taxa classification and separate bacterium *in vitro*.

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