Original Article

Profiles of commensal and opportunistic bacteria in human milk from healthy donors in Taiwan

Po-Wen Chen [a,*], *Yi-Ling Lin* [b], *Mao-Sheng Huang* [c]

[a] *Department of Nursing, St. Mary's Junior College of Medicine, Nursing and Management, Taiwan*
[b] *Department of Obstetrics Central, St. Mary's Hospital Luodong, Taiwan*
[c] *Department of Medicine Laboratory, St. Mary's Hospital Luodong, Taiwan*

ABSTRACT

Recent studies indicate that milk from healthy mothers may harbor potential probiotics. Nonetheless, the distribution of bacterial profiles in human milk samples in Taiwan is not fully understood. Therefore, with the aim to address this question, in this study, milk samples were collected from 33 healthy mothers (D1 to D33) visiting our hospital during a 6-month period. The milk microbiota was analyzed by a molecular approach (Illumina MiSeq sequencing). The results indicate that the milk samples have a unique profile and patterns of bacterial abundance levels. Moreover, in colostrum and transitional-milk samples, we detected 154 and 127 bacterial species, respectively, and these sets shared 42.6% of the bacterial species. The most common bacterial species among all milk samples were *Staphylococcus epidermidis*, *Streptococcus lactarius*, and *Staphylococcus hominis*, suggesting that the skin contamination route plays an important role in the composition of the milk microbiota. Nevertheless, four *Lactobacillus* species, *Lactobacillus helveticus*, *Lactobacillus iners*, *Lactobacillus zeae,* and *Lactobacillus gasseri*, were present in only 7 samples (21% prevalence), and bifidobacterial species were quite rare taxa among the present samples. The *Staphylococcus aureus* was detected in a total of 15 samples (45% prevalence), suggesting that this species may be commonly present in milk samples. In conclusion, each milk sample revealed a unique profile and patterns of bacterial abundance levels, and our data do not support the idea that lactobacilli and bifidobacteria are common and abundant in modern milk samples. Because none of the donors of the milk samples showed mastitis or any discomfort during the sampling process or at follow-up inspection, the microbiota of these milk samples is not likely to negatively affect its host. This study provides new information on the proportions of commensal bacteria in human milk in Taiwan.

* *Corresponding author*. No. 100, Lane 265, Sec 2, Sanxing Rd., Sanxing Township, Yilan County 26644, Taiwan. Fax: +886 3 9897235.
E-mail address: pwchen@smc.edu.tw (P.-W. Chen).

1. Introduction

Recent studies showed that human milk contains commensal or probiotic bacteria [1–3], and several additional reports have suggested that human milk is a direct source of commensal or probiotic bacteria found in the infant gut [4–7]. Commensal bacteria in human milk may have a positive effect on the health of breast-fed babies by shaping their gut microbiotas [8]. Thus, research on the milk microbiota and the factors that can shape it is important.

Several studies have identified diverse bacteria in human milk, and their presence and abundance in human milk seem to vary among individuals [9,10]. In addition, the mode of delivery, lactation time, gestational age, and maternal health, weight, exposure to chemotherapy, diet, and geographical location have all been found to play roles in bacterial profiles of human milk [1,4,11,12]. Nonetheless, individual factors that shape the milk microbiota are still unclear because several contradictory findings have been reported [4]. For instance, most studies have indicated that human milk may be a source of probiotic bacteria for the infant because lactobacilli and bifidobacteria are common, albeit less abundant (2%–3% relative abundance), constituents of the milk microbiota [1,9,13,14]. In contrast, a complete absence of lactobacilli or bifidobacteria in human milk has been reported [15,16]. Previously, we investigated the bacterial profiles of human milk samples collected in Taiwan via a culture-based approach. Of 19 milk samples, only one was found to be colonized with *Lactobacillus gasseri*, and little *Bifidobacterium* species were not isolated from any of the samples [17]. Moreover, a recent study assessed the bacterial composition of milk from mothers in Taiwan and China, and they reported the predominant bacterial family to be *Lactobacillaceae* (at 6.2% relative abundance), but *Bifidobacterium* seems not to be a predominant genus in human milk samples [18].

Some reports have provided contradictory data on the abundance of lactobacilli and bifidobacteria in human milk samples, and composition of the microbiota in human milk in Taiwan is not fully understood. In the aforementioned study on the milk microbiota in Taiwan [18], 31 milk samples from seven cites have been obtained, and only several milk samples were collected per city. Moreover, these milk samples had been collected during quite a long sampling period (from donors 0.5–2.7 months after a delivery). As described above, many factors can shape the milk microbiota. Therefore, in the current study, using a molecular approach, we investigated the bacterial patterns in human milk samples harvested from donors mostly within 12 days after delivery. This study clarified the proportions of lactobacilli and bifidobacteria in human milk collected locally. The potential roles of milk-isolated lactobacilli are discussed.

2. Materials and methods

2.1. Collection of milk samples

Ethical approval for this study and all experimental protocols was provided by the Institutional Review Board (IRB) of Saint Mary's Hospital, Lundong (IRB104011). All the methods were carried out in accordance with relevant guidelines and regulations of the IRB. Briefly, milk samples were donated from January to June 2016 by mothers visiting Saint Mary's Hospital, which is located in eastern Taiwan (Yilan County). As shown in Table 1, 33 healthy Taiwanese mothers (age range 17–43 years; samples D1 to D33) without mastitis or any infectious diseases were randomly recruited to donate milk samples. Written informed consent was obtained from all the participants. Thirty participants provided their milk samples within 12 days after delivery, and 3 participants provided their milk samples between 120 and 320 days after delivery (Table 1).

Milk samples were collected following the protocol used in a previous report [10], with several modifications. Briefly, milk samples were collected into sterile tubes by manual expression using sterile gloves after nipples and areolas were cleaned with a swab soaked in sterile water or saline; the first 1–2 mL of milk was discarded to avoid contamination from the environment. Then, 5–15 mL of milk was collected and was immediately frozen and stored at −20 °C until DNA was extracted for the microbial diversity analysis. The collected milk samples were also categorized according to the length of time postpartum, including colostrum (within 5 days postpartum; D1 to D20), transitional milk (between 6 and 15 days

Table 1 – Information of milk sample and donor.		
Sample (donor)	Age	Sampling Day[a]
D1	31	3
D2	43	5
D3	28	5
D4	33	5
D5	28	3
D6	26	5
D7	34	2
D8	34	5
D9	24	5
D10	23	5
D11	33	5
D12	19	5
D13	17	2
D14	32	4
D15	32	3
D16	26	3
D17	32	3
D18	32	5
D19	22	5
D20	22	5
D21	26	7
D22	25	7
D23	30	7
D24	22	7
D25	28	7
D26	35	7
D27	25	12
D28	33	6
D29	35	6
D30	33	12
D31	28	120
D32	35	120
D33	31	320

[a] Days after delivery.

postpartum, n = 10), and mature milk (more than 15 days postpartum; n = 3) in accordance with a previously published definition [16].

2.2. DNA isolation and microbial diversity analysis

DNA was directly extracted from milk samples as previously described using the QIAsymphony® Virus/Bacteria Mini Kit [4,19,20], but with some modifications. First, the 1 mL milk sample was centrifuged at 5000 × g for 30 min, and the supernatant was discarded. The harvested bacterial pellet was resuspended in 300 μL of an enzyme solution (20 mg/mL lysozyme or 200 μg/mL lysostaphin in a buffer consisting of 20 mM Tris-HCl pH 8.0, 2 mM EDTA, and 1.2% Triton X-100) at 37 °C for at least 30 min. Then, the bacterial DNA was extracted according to manufacturer's instructions. Finally, the obtained DNA was subjected to microbial diversity analysis as described below.

As for the microbiota analysis, an initial 30-cycle PCR was carried out using AccuPrime Hifi Polymerase (Invitrogen, Carlsbad, CA, USA). The cycling conditions were as follows: initial denaturation at 94 °C for 2 min; followed by 30 cycles at 94 °C for 20 s, 56 °C for 30 s, 68 °C for 60 s; and final storage at 4 °C. Amplicons of the variable regions V3 to V4 of the 16S rRNA gene were generated with primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGG-TATCTAATCC-3′) (Illumina, Carlsbad, CA, USA). Libraries were purified using AMPure XP beads (LABPLAN; Naas, Ireland) according to the Illumina 16S sequencing library protocol, and the libraries were tested for purity and quantity on a Nanodrop 1000 spectrophotometer. The barcoded amplicon libraries were combined in equal concentrations into a single pool according to their Nanodrop quantification. The size was assessed with an Agilent DNA 1000 Kit (Agilent Technologies Ireland Ltd., Dublin, Ireland) on an Agilent 2100 Bioanalyser (Agilent Technologies Ireland Ltd., Dublin, Ireland). Pooled amplicon libraries were sequenced using the MiSeq system, producing 2 × 300 bp paired-end reads. For the microbial diversity analysis, the steps to obtain final reads were performed according to the manufacturer's protocol. The Illumina-generated FASTQ files (.fastq) and quality files were acquired as raw and mapped sequence data. Briefly, data were analyzed in the latest version of bioinformatics software packages Mothur v.1.33.3 and QIIME v1.80 for the 16S rDNA analysis (e.g., selection of operational taxonomic units [OTUs] and taxonomic assignment) by means of the Greengenes 16S rRNA database (gg_13_8). Pairwise distances between aligned DNA sequences for all effective reads were calculated with a cutoff of 0.03, then clustered into OTUs by the average neighbor algorithm with a hard cutoff of 0.03, and, finally, the OTUs were classified by taxonomic assignment.

2.3. Statistical analysis

To compare the bacterial profiles between milk samples from different lactation periods, Venn diagrams (Venny 2.1) and principal coordinate analysis (PCoA) plots were generated by previously reported methods [4,16]. Venn diagrams show mathematical or logical sets as circles or closed curves with an enclosed area, with common elements of the sets represented by the areas of overlap between the circles. PCoA is a distance-based multivariate analysis that combines location and dispersion effects [21]. In this analysis, not only the pattern of bacteria but also the abundance of bacterial genera in each milk sample were all estimated and counted. Briefly, when two samples were found to have similar patterns of bacterial genera, they were placed in the same location in multidimensional space.

2.4. Results and discussion bacterial genus composition of human milk

Fig. 1 shows the profiles of bacterial genera in the milk samples from healthy mothers in Taiwan. To identify and compare the major bacterial populations in each milk sample, bacterial taxa with relative abundance less than 1% in individual samples were categorized into the "others" group as suggested recently [16]. Here, profiles of the milk microbiota in each sample revealed a unique profile and patterns of bacterial abundance. Briefly, each milk sample had been colonized with one to three common or abundant bacterial genera, and the top five most abundant bacterial genera were *Staphylococcus*, *Streptococcus*, *Enhydrobacter*, *Enterococcus*, and *Rothia*. Among these most prevalent (i.e., common) bacterial genera, *Staphylococcus* and *Streptococcus* are the two most prevalent and abundant genera among our milk samples. On the other hand, as partly mentioned above, in each sample, we detected some minor bacterial genera (less than 1% abundance in each sample) that were categorized into the "other" group, and overall, bacterial genera of this group in individual samples ranged in abundance from 1.1% to 13.3%, and the median abundance for this group was approximately 4.9%. Therefore, each milk sample contained both major (relatively abundant) and minor groups of commensal bacteria, and this finding further supports the notion that each milk sample can have a unique and quite complex pattern of bacterial genera.

As for the prevalence of potential probiotic bacteria in milk samples, small counts of genera *Lactobacillus* (0.6% relative abundance) and *Bifidobacterium* (<0.01%) were found in these milk samples. Because these minor bacterial genera were categorized into the "other" group in each sample as described above, to understand the distribution of these minor lactobacilli among milk samples, the detected *Lactobacilli* are further summarized and discussed below (Fig. 4 and Table 2).

One report indicates that milk samples form three lactation periods (from the same milk donors) manifest different bacterial patterns and diversity but no statistically significant differences have been recognized [16]. Thus, we also compared the bacterial genera among lactation periods. Because we obtained only three mature milk samples here, only colostrum and transitional milk samples were subjected to this analysis. As presented in Fig. 2, the two milk types showed similar proportions and distributions of *Staphylococcus*, *Streptococcus*, and *Rothia*. Moreover, colostrum contained much more *Enhydrobacter* (10% relative abundance) than transitional milk did (1%). Conversely, transitional milk contained ~8% *Enterococcus*, and colostrum contained ~0.5%. Therefore, colostrum and transitional milk seem to show different tendencies on specific bacterial genera. At present, the factors that could contribute to the different bacterial
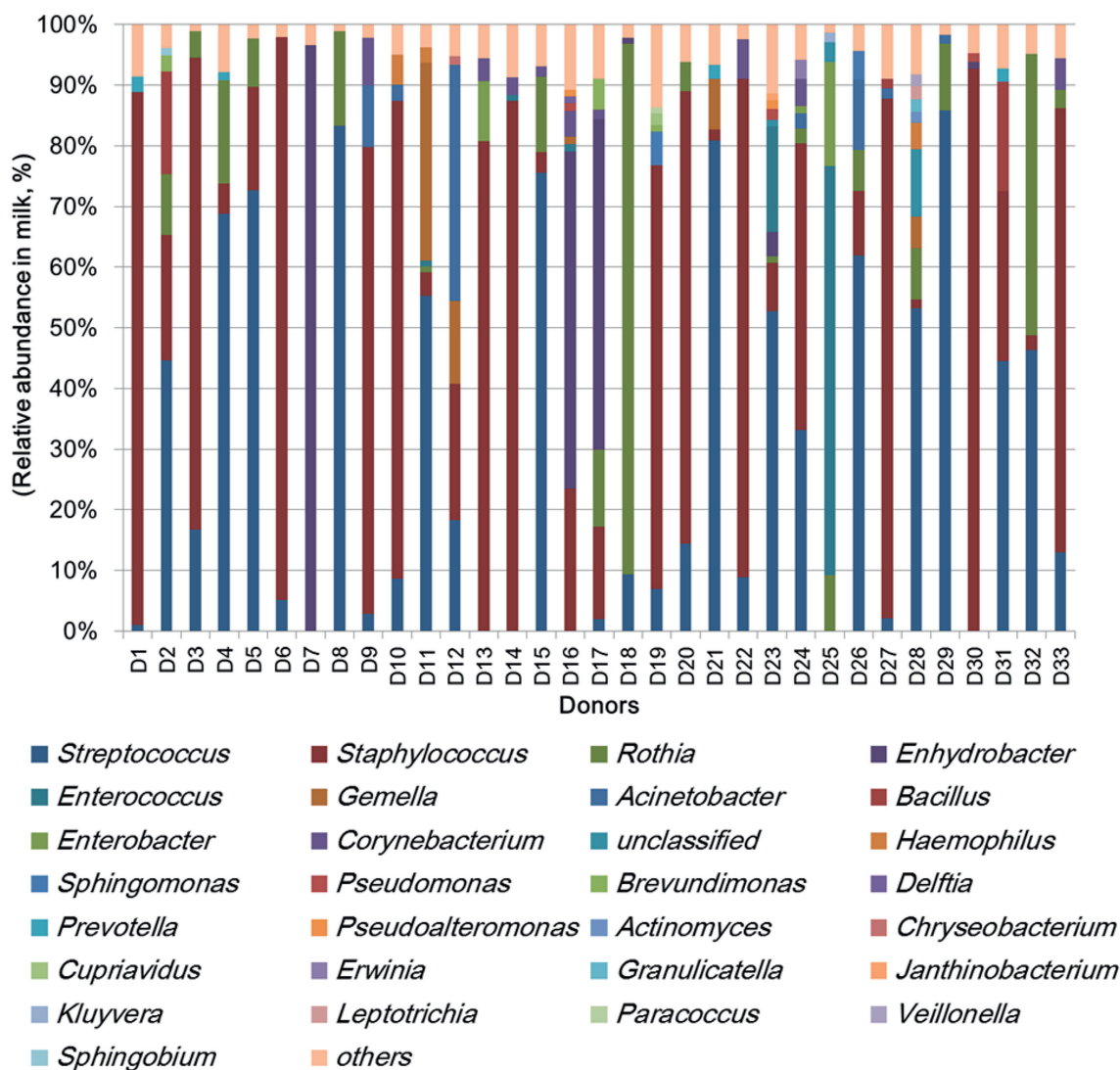
Fig. 1 — Bacterial genus composition in human milk. The bars show the proportion of each bacterial genus detected by 16S rRNA sequencing of milk samples from healthy donors (n = 33). Genera that represented less than 1% of all bacterial genera were grouped into an "others" category.

profiles between the two milk types are still unknown, and this topic needs to be investigated further.

2.5.   PCoA of bacterial genera

The above findings indicate that colostrum and transitional milk may show common and also different tendencies in bacterial taxa. Next, we analyzed the milk microbiotas by PCoA. As illustrated in Fig. 3A, most milk samples were distributed without clustering in the multidimensional space of this analysis except for nine colostrum samples. These nine colostrum samples, namely D1, D3, D6, D9, D10, D13, D14, D19, and D20, clustered together (Fig. 3B), implying that these samples shared similar profiles of bacterial genera. Consistent with the above PCoA findings, the nine colostrum samples had similar bacterial profiles, as shown in Fig. 1. Moreover, C7 (D7) was an outlier in relation to the other colostrum samples, and this sample indeed revealed a unique bacterial profile in

comparison to the other milk samples because this sample was dominated by *Enhydrobacter* (>90% relative abundance; Fig. 1).

As for transitional and mature milk samples, almost all these samples were scattered in the multidimensional space without clusters, indicating that these samples had substantially distinct profiles of bacterial genera (Fig. 3A). Some researchers limit the definition of colostrum to the period until 2—3 days postpartum and confine transitional milk to the period 3—4 to 10—14 days postpartum. Thus, among the above-mentioned nine clustered colostrum samples, only two milk samples (D1 and D13) were harvested within 3 days after delivery (Table 1). As a result, the contrasting bacterial profiles between colostrum and transitional milk here may not be associated with the nutrient-rich fluid in colostrum milk [22]. Notably, given the uneven sampling, such as 20 colostrum samples and 10 transitional-milk samples, and only one sample per subject, accurate evaluation of temporal
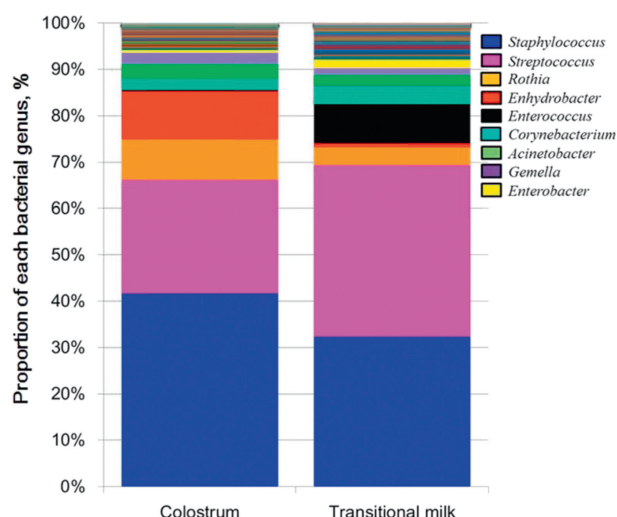
Fig. 2 — Proportions of each bacterial genus in colostrum (n = 20) and transitional milk (n = 10). The relative abundance of all bacterial genera were determined using 16S rRNA gene amplicon sequencing. The proportion of each bacterial genus is indicated in a specific color. Only the nine most common bacterial genera are shown.

postpartum effects is unlikely. Nevertheless, our results from PCoA also suggest that most milk samples have a unique bacterial profile and abundance levels.

2.6. Bacterial-species composition of human milk

The profiles of bacterial genera in milk samples were evaluated above. Next, we compared the bacterial species detected in the present milk samples, as shown in Fig. 4. In agreement with the above findings, which showed that bifidobacterial genera (<0.01%) are quite rare in the present milk samples, bifidobacterial species, namely *B. adolescentis*, *B. longum*, and *B. pseudolongum*, were detected in several milk samples here, but with counts ranging between 1 and 36. Collectively, these data indicate that bifidobacterial species are quite rare in these milk samples. As for the distribution of lactobacilli in milk samples, *L. gasseri* was found to be abundant in samples D27 (1.5%) and D31 (17.7%). Moreover, several other *Lactobacillus* species were detected in some samples, but they constituted less than 1% of the bacterial community in these milk samples. Because most of the above-mentioned minor bacterial taxa cannot be evaluated in Fig. 4 (less than 1% relative abundance), the prevalence of these minor *Lactobacillus* species in milk samples is further summarized and compared in Table 2. Here, in contrast with the higher abundance of *L. gasseri* in samples D27 (1.5%) and D31 (17.7%), this species was also detected in low abundance in D21 (0.1%), D1 (0.1%), D33 (0.6%), and D19 (0.1%). Moreover, *Lactobacillus helveticus* was detected in D23 (0.5%); *Lactobacillus iners* was detected in D23 (0.2%); and *Lactobacillus zeae* was present in D1 (0.1%). To sum up, bifidobacterial species are quite rare among our milk samples, and *Lactobacilli* species are present in 7 samples amounting to ~21% prevalence; and *L. gasseri* is highly prevalent in our milk samples (18% prevalence rate).

Various reports have suggested that lactobacilli and bifidobacteria are both commonly present but less abundant bacterial taxa in human milk, accounting for approximately 2–3% of the bacterial cells in human milk [1,9,13,14]. In contrast to those reports, we found very little lactobacilli in most of the samples and rarely detected bifidobacteria, and they accounted for less than 1% of the bacterial cells in most milk samples in the present study. Nonetheless, we believe that these minor lactobacilli in milk may still play important roles in the human intestine, in that the capacity for colonization, adherence, or survival of potential probiotics in the gastrointestinal tract is quite important, and a small inoculum may have a great impact as explained elsewhere [23–25]. Besides, human milk has been reported to display prebiotic or bifidogenic activities, i.e., can promote the growth of probiotics. For instance, it has been documented that oligosaccharides in human milk strongly promote a bifidobacteria-dominant microflora in infants [26]. Moreover, the prebiotic effects of human milk are attributed to a complex of interacting substances within milk, including the low concentration of proteins and phosphorus, lactose, lactoferrin, nucleotides, and oligosaccharides [26]. In addition, a number of reports indicate that the major classes of bacterial metabolites, e.g., short-chain fatty acids, not only have diverse effects on host health but also exert prebiotic and bifidogenic actions [27–29]. Collectively, the above information suggests that the small amounts of milk-derived bifidobacteria or lactobacilli could be later boosted by human milk or short-chain fatty acids in the infant intestine. Nevertheless, whether this low abundance of lactobacilli and bifidobacteria is a universal phenomenon in human milk in Taiwan should be investigated further, including monitoring studies. Partially, socioeconomic, cultural, genetic, dietary, and chemotherapeutic factors may contribute to differences in the prevalence of lactobacilli and bifidobacteria in human milk among studies [1,30].

As partly mentioned in the Introduction section, a recent study evaluated the milk microbiota in milk samples collected in Taiwan and China [18]. Notably, almost all of their samples were mature-milk samples, in that their milk was collected from mothers between 0.1 and 21.7 months after delivery (n = 133). In that study, ~31 samples were harvested from 7 cities in Taiwan (several samples per city), and the sampling time ranged from 0.5 to 21.7 months after a delivery. Although the wide range of sampling time and the small numbers of samples per city may not reflect the true distribution of the milk microbiota, a total of four *Lactobacillus* species, such as *Lactobacillus paracasei*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, and *Lactobacillus vaginalis* and five members of bifidobacteria, *B. adolescentis*, *B. dentium*, *B. longum*, *B. longum subsp. infantis*, and *B. stercoris*, were detected. Of note, high prevalence of *L. paracasei* (prevalence: 78.2%, relative abundance: 3.2%) were observed among those human milk samples. Moreover, *B. longum* (prevalence: 62.4%, abundance: 0.3%) was found to be the predominant bifidobacterial species. This recent study indicates that *Lactobacillaceae* (6.2%) is one of the 17 most predominant bacterial families in human milk but bifidobacteria seem not to be abundant in milk samples [18]. Collectively, partially in line with our findings, their data suggest that the high prevalence of lactobacilli may be expected in milk
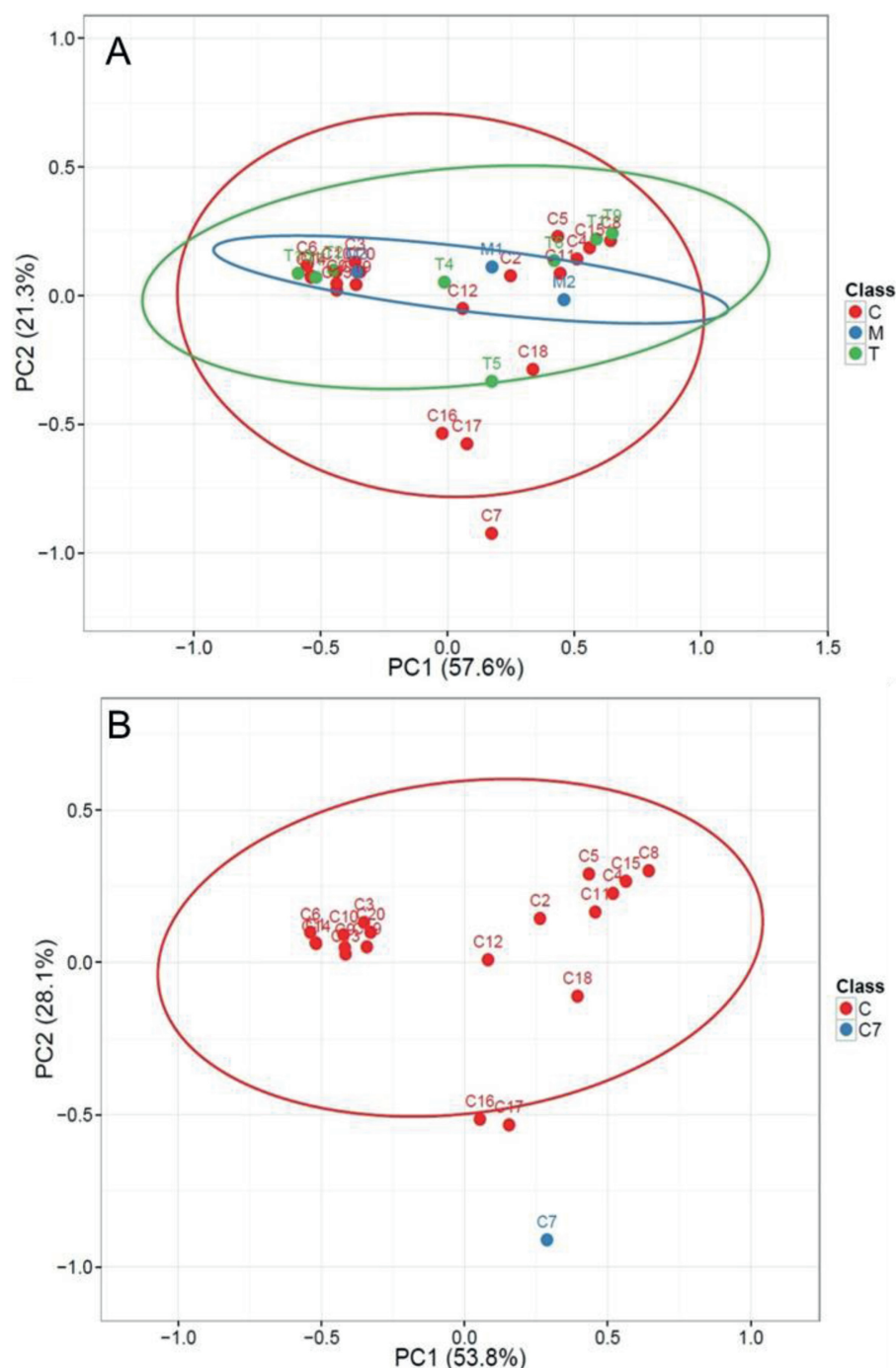
Fig. 3 — Principal coordinate analysis based on the bacterial genera identified in milk samples. Percentages shown along the axes represent the proportions of dissimilarity. Each circle represents the 16S rRNA gene sequences from a sample. Different colors and shapes represent the three lactational stages. C: Colostrum (C1 to C20); T: transitional (T1 to T10); M: mature milk (M1 to M3).

samples from Taiwan but bifidobacteria may not be abundant in human milk in Taiwan or China. On the other hand, in that study, mature milk samples from mothers who had undergone Caesarian section showed significantly higher abundance of *Lactobacillus* ($P < 0.05$) and a larger number of unique unclassified OTUs ($P < 0.001$) in comparison with mothers who had a vaginal delivery (vaginal group) [18]. In the present study, 13 milk samples were associated with a vaginal delivery, and the other samples with Caesarian section (n = 20). The prevalence rates of lactobacilli in vaginal and Caesarian section groups are ~30% (4/13) and 15% (3/20), respectively. Thus, in contrast to the previous report, our study suggests that donors who have had a vaginal delivery may have higher abundance of lactobacilli in milk, as revealed mostly by colostrum and transitional milk samples here. Nonetheless, these findings need to be tested with a large sample size.
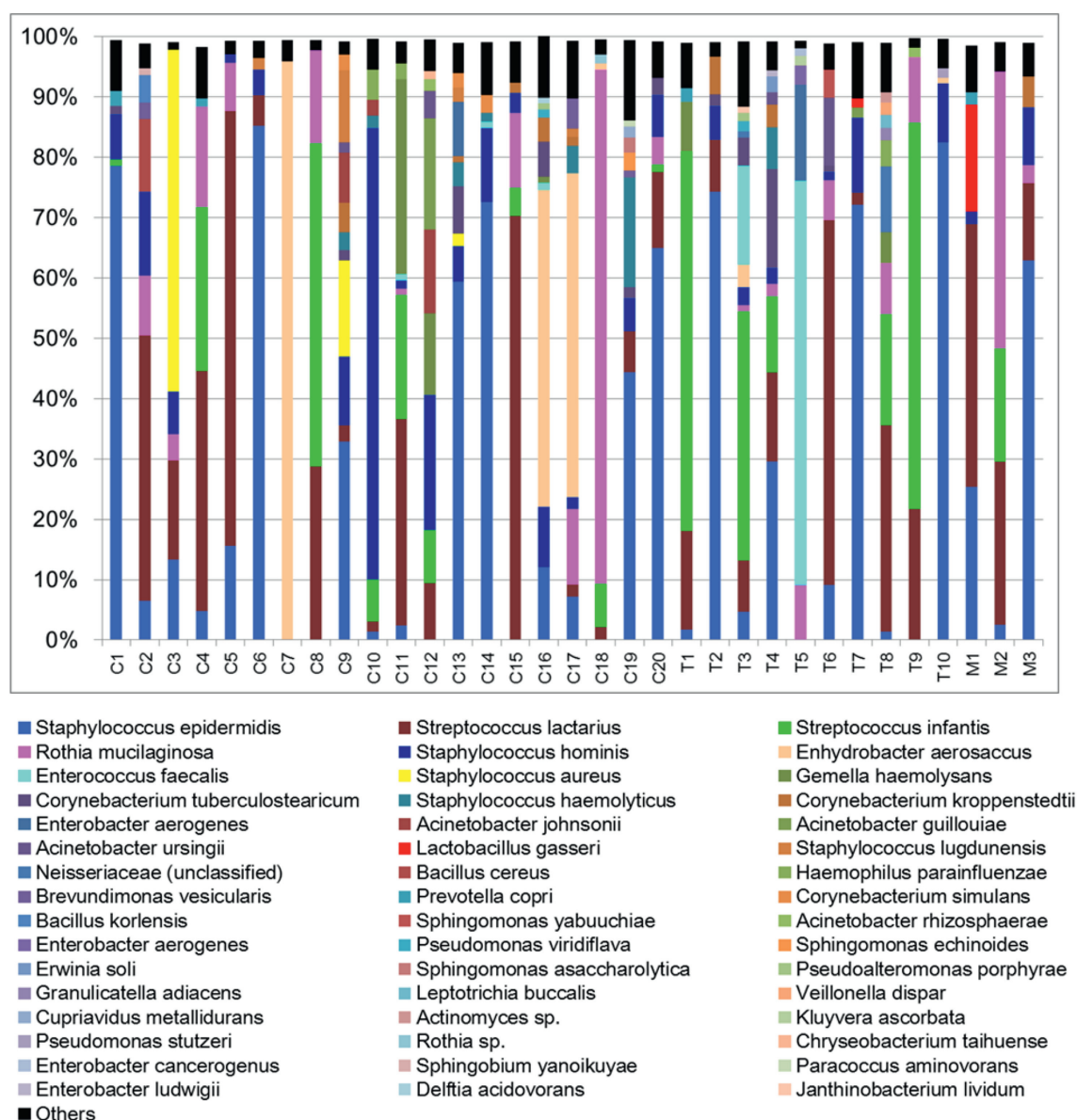
Fig. 4 – Bacterial species composition in human milk. The bars show the proportions of each bacterial species detected by 16S rRNA sequencing of milk samples from healthy donors (n = 33). Species that represented less than 1% of all bacterial species were grouped in an "others" category.

As for the other commensal bacterial species in milk samples (Fig. 4), the most abundant and widely distributed bacterial species were *Staphylococcus* (*Sta.*) *epidermidis* (79% prevalence), *Streptococcus* (*Str.*) *lactarius* (79% prevalence), *Sta. hominis* (73% prevalence), *Rothia mucilaginosa* (55% prevalence), and *Str. infantis* (45% prevalence). Moreover, most milk samples were dominated by one to 10 common and major bacterial species (Fig. 4). In agreement with the PCoA results (Fig. 3), samples from donors D1, D3, D6, D9, D10, D13, D14, D19, and D20 (corresponding to colostrum samples C1, C3, C6, C9, C10, C13, C14, C19, and C20) indeed manifested much more similar bacterial profiles than did the other milk samples. On the other hand, as shown in Fig. 4, all the samples contained ~1.3%–13.3% (median: 5.10%) of "other" or minor bacterial species, implying that each milk sample had been colonized with minor (low abundance) and major (high abundance)

bacterial taxa. Collectively, these data all suggest that each human milk sample has a rather unique bacterial-species pattern.

As described above, the most common and abundant bacterial species in our milk samples are *Sta. epidermidis*, *Str. lactarius*, and *Sta. hominis*. Notably, these bacteria often colonize human and animal skin and are known to be commensal and harmless bacteria. Thus, it appears that most of the bacteria in human milk samples here were skin bacteria, leading to a concern about whether it is possible to collect human milk aseptically. On the other hand, we took several precautions to avoid contamination from the environment, as described in the Materials and Methods section. In fact, various skin- or human-body–related bacteria are often isolated from (or detected in) human milk samples [3], and it is difficult to rule out the possibility that all the bacteria detected

Table 2 — Distribution of *Lactobacillus* and *Staphylococcus aureus* species in milk samples.

Lactobacillus species	Sample (relative abundance in each sample, %)
Lactobacillus helveticus	D23 (0.5%)
Lactobacillus iners	D23 (0.2%)
Lactobacillus zeae	D1 (0.1%)
Lactobacillus gasseri	D21 (0.1%), D1 (0.1%), D31 (17.7%), D27 (1.5%), D33 (0.6%), D19 (0.1%)
Staphylococcus aureus	D3 (56.6%), D9 (15.9%), D13 (2%) D31 (0.4%) D1 (0.3%), D19 (0.3%), D2 (0.2%), D4 (0.2%) D7 (0.1%), D10 (0.1%), D20 (0.1%) D21 (0.2%), D22 (0.1%), D24 (0.1%), D26 (0.1%)

Fig. 5 — **Common and different bacterial genera and species between two milk types. Venn diagrams showing the number of bacterial genera (upper) or species (bottom) shared between or unique to two milk types, excluding bacterial genera or species present at an abundance of <1%.**

actually colonize the milk because of this possibility of environmental contamination or contamination during sampling. Nevertheless, these bacteria all can be ingested by breast-fed infants.

Moreover, *Enhydrobacter aerosaccus* was found to be highly abundant in three milk samples, namely D7 (95.9%), D16 (52.5%), and D17 (53.7%), and was also detected in seven other samples, but at much lower abundance levels (Fig. 4). This species is one of the most prevalent bacteria in the oral microbiota during deciduous dentition in children [31]. Therefore, our findings imply that *E. aerosaccus* may have originated in the oral cavity of infants and then invaded breast tissues during breast feeding. To the best of our knowledge, this is the first study to detect high prevalence and even the dominance of *Enhydrobacter* species in human milk samples. Notably, none of the donors of the above-mentioned three milk samples showed mastitis or any discomfort during the sampling process or at the follow-up inspection, suggesting that *E. aerosaccus* in these milk samples may not negatively affect its host. Therefore, it will be interesting to dissect the roles or mechanisms of action of this species when it is the dominant bacterial genus in specific milk samples in our next study.

As indicated above, most of the collected milk samples here had been colonized with various major and minor bacterial taxa. In other reports, several mechanisms have been proposed for the establishment of this human milk microbiota. For instance, one study has described a convincing mechanism that involves contamination routes, with bacteria in milk originating from the mother's skin or the infant's oral cavity [1]. In support of this notion, the most dominant bacteria in milk samples in the present study are bacteria that commonly colonize the human body, oral cavity, and skin.

2.7. Common and different bacterial taxa in different milk types

The above data revealed that colostrum and transitional-milk samples can be colonized with different and common bacterial taxa (Fig. 2). Next, by Venn analysis, we determined the whole sets of bacterial taxa shared between (and unique to)
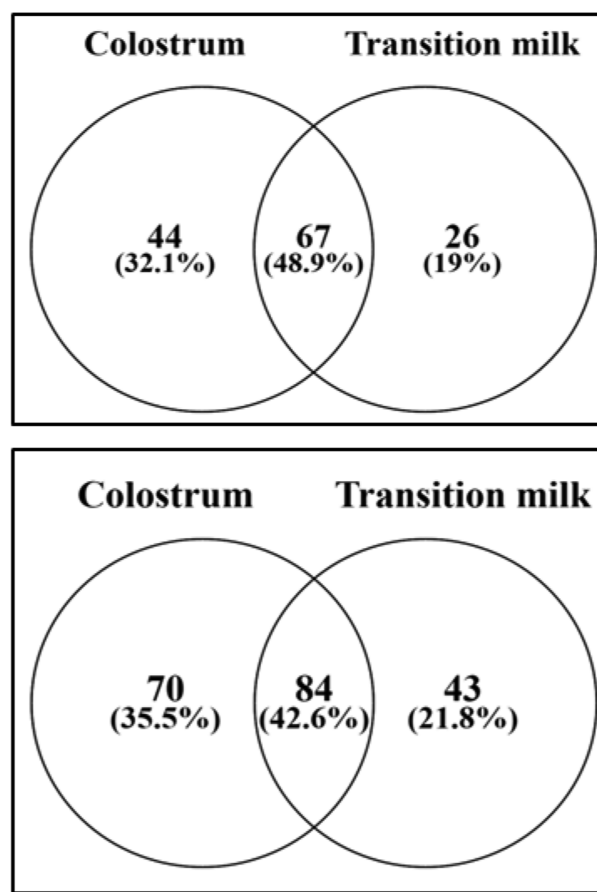
colostrum and transitional-milk samples (Fig. 5). All the detected bacterial genera and species (both major and minor taxa) were subjected to this analysis. As shown in Fig. 5, the colostrum and transitional milk samples shared 48.9% of bacterial genera, and in these milk types, we detected a total of 111 and 93 abundant bacterial genera, respectively. Furthermore, the colostrum and transitional milk samples shared 42.6% of bacterial species, and in these milk types, we detected a total of 154 and 127 bacterial species, respectively. Notably, it should be mentioned that the MiSeq approach is known to not be sensitive enough to detect all bacteria to the species level. Thus, the 154 and 127 bacterial species above are only some of the bacteria that were identified to the species level. Nevertheless, these data also reveal that there are common as well as unique bacterial genera and species between the two milk types.

2.8. Roles and prevalence of opportunistic pathogens in milk

A recent study shows that *Sta. aureus* can be a causative agent of mastitis or subclinical mastitis, but this bacterial species

may be commonly found in human milk as well [1]. As in the other reports, this bacterial species was frequently found in the present milk samples. For reference, this species was detected in 15 milk samples (45% prevalence). By contrast, this taxon was only a minor bacterial species in 12 milk samples, with relative abundance of less than 1%, and was categorized into the "other" group in most of individual samples (Fig. 4). Because the distribution of Sta. aureus in milk samples cannot be evaluated in Fig. 4, the distributions (abundance levels) of this species in milk samples are summarized in Table 2. As shown in the table, Sta. aureus was abundant in three milk samples, namely D3 (56.6%), D9 (15.9%), and D13 (2%), but the abundance of this species was quite low in the other 12 samples: below 0.4%. Taken together, the data obtained in the present study suggest that Sta. aureus can be a common (i.e., prevalent) but minor bacterial species in human milk samples. Notably, although Sta. aureus was quite abundant in two milk samples here (D3 and D9), these bacterial isolates did not seem to negatively affect its host. In support of this finding, none of our milk donors (including D3 and D9) showed clinical mastitis or any discomfort during the sampling process or at follow-up inspection. These findings imply that the Sta. aureus detected here represents a common but nonpathogenic strain(s) in the milk samples under study. On the other hand, partially supporting the idea that Sta .aureus may be nonpathogenic in most milk samples, another report revealed that other commensal bacteria in milk may help to constrain the growth of Sta. aureus [2]. Indeed, in the present study, in addition to abundant Sta. aureus, the D3 sample contained four more abundant bacterial species, namely Sta. epidermidis, Str. lactarius, R. mucilaginosa, and Sta. hominis; similarly, D9 also contained the other highly abundant commensal bacteria (Fig. 4).

Several criteria have been adopted for the use of donated breast milk or for milk sharing [32]. For instance, when the donor milk has a total bacterial count in the range of 10^3 to 10^5 colony-forming units (cfu) per milliliter, it is used only if the prevalent microorganisms are common commensal bacteria such as Sta. epidermidis, Viridans streptococci, and diphtheroids. Moreover, the donor milk is not used if the total bacterial count is greater than 10^3 cfu/mL and includes Sta. aureus, any gram-negative rod species (like Pseudomonas sp.), β-hemolytic streptococci, or Sta. faecalis [32,33]. Therefore, according to the aforementioned criteria, regarding feeding of preterm infants or milk sharing, the high prevalence of Sta. aureus in the present milk samples is a cause for concern.

In conclusion, this study characterized the microbiota in milk samples collected from healthy mothers in Taiwan. In contrast to other reports, lactobacilli and bifidobacteria were not found to be common or abundant commensal bacteria in our milk samples. In addition, Sta. aureus was found to be quite common and prevalent in the milk from our healthy donors. Nevertheless, none of the donors of the milk samples showed mastitis or any discomfort during the sampling process or at follow-up inspection. These results suggest that the microbiota in these milk samples may not negatively affect its host. This study provides new information on the abundance of commensal bacteria in human milk in Taiwan.

Conflicts of interest

No conflict of interest exists.

Acknowledgements

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