HPV infection and bacterial microbiota in breast milk and infant oral mucosa

Heidi Tuominen [1]*, Samuli Rautava[2], Maria Carmen Collado[3], Stina Syrjänen[1,4], Jaana Rautava[1,4]

1 Department of Oral Pathology and Oral Radiology, Institute of Dentistry, Faculty of Medicine, University of Turku, Turku, Finland, 2 Department of Paediatrics, University of Turku & Turku University Hospital, Turku, Finland, 3 Department of Biotechnology, Institute of Agrochemistry and Food Science, Spanish National Research Council (IATA-CSIC), Valencia, Spain, 4 Department of Pathology, Turku University Hospital, Turku, Finland

* heemtu@utu.fi

Abstract

Objective

We investigated the association between bacterial microbiota in breast milk and the infant mouth. The influence of human papilloma virus (HPV) infection on infant oral microbiota was also assessed.

Material and methods

Altogether 35 breast milk and 35 infant oral samples with known HPV status were selected from the Finnish Family HPV Study cohort. In total, there were 31 mother-infant pairs. The microbiota composition was characterized by 16S rRNA gene sequencing (V3-V4 region).

Results

HPV DNA was present in 8.6% (3/35) of the breast milk and 40% (14/35) of the infant oral samples. Eight shared genera between breast milk and infant oral were found; these included *Streptococcus*, *Staphylococcus*, *Unclassified Gemellaceae*, *Rothia*, *Veillonella*, *Haemophilus*, *Propionibacterium* and *Corynebacterium*. HPV status was not associated with either microbiota richness or diversity in the infant mouth. However, the infant oral microbiota clustered in different groups according to HPV status. We detected higher abundance of *Veillonella dispar* (p = 0.048) at species level in HPV negative infant oral samples. We did not detect differences in the breast milk microbiota composition related to HPV infection due to only three HPV positive milk samples.

Conclusions

HPV infection is associated with distinct oral bacterial microbiota composition in infants. The direction of causality underlying the phenomenon remains unclear.

Introduction

The role of the bacterial microbiota in human health and disease has attracted considerable research interest during the last two decades. Much less is known about the interaction between viruses and the indigenous bacterial microbiota. Human papillomavirus (HPV) is a carcinogenic virus which has been causally associated with carcinomas in both the anogenital [1] and the head and neck region[2]. In order to understand oral HPV-related carcinogenesis, there is a need to understand the natural history of HPV infection.

Breast milk is colonized with bacteria. Despite high intra- and interindividual variability, each mother harbors a unique and personalized microbial profile in her breast milk. The most predominant bacteria in human milk include *Streptococcaceae* and *Staphylococcaceae* groups followed by *Proteobacteria*, *Pseudomonadaceae*, *Bifidobacteriaceae*[3–7]. The presence of HPV has been found in breast milk in 2.5–28.8% of the samples[8–11]. However, Mammas[8] et al. (2011) were not able to detect any high-risk HPV types (HR-HPV, including HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) in breast milk samples whereas Sarkola[9] et al. (2008) and Yoshida[10] et al. (2011) both found HPV16 in 4.0% and 2.5%, of the studied milk samples, respectively. Interestingly, the presence of HPV DNA in mother's milk is not a risk factor for oral HPV infection in the infant[8,10,11].

HPV has been reported to be detectable in 3.2–21% of infant oral mucosa samples[12–16]. Specific cell-mediated immunity against HPV16 has also been detected in sexually naïve infants [17]. Vertical transmission of HPV from the mother has been reported to occur prenatally via the placenta and during vaginal delivery[15,18,19]. HPV infection has also been detected in conjunctival, pharyngeal and genital sites of infants[16]. The reported maternal risk factors for vertical HPV transmission include persistent cervical HR-HPV infection, infection with multiple HPV genotypes and HPV positive placenta as well as hand warts, young maternal age at the onset of sexual activity and oral contraceptive use[12,14,15,20,21]. In addition, cord blood positive for HPV increases the risk of HPV infection in the infant[21]. In contrast, the mode of delivery has not been a predictor for vertical transmission in most studies [13]. In addition to acquiring HPV from the mother, HPV infections can also be transmitted horizontally since we have previously shown that persistent infant oral HPV infections are associated with oral carriage of HR-HPV in both the mother and the father[14,20].

The infant oral microbiota is delicate and primarily influenced by the mothers' or care givers' own microbiota composition particularly in breast milk, the mammary areola and in the oral cavity[22]. The development of the infant oral cavity microbiota begins directly after delivery. The first colonizers, studied from 10 minutes to 53 hours after birth, include *Staphylococcus epidermidis*, followed by *Staphylococcus aureus*, *Streptococcus mitis* and *Streptococcus oralis*[23,24]. During the first year of life the levels of anaerobic bacteria increase steadily: in two-month-old infants, the most abundant anaerobic bacteria are *Veillonella* spp. and early colonizers also include *Prevotella melaninogenica*. *Prevotella intermedia* is a late colonizer and already at one year of age the primary anaerobic species has been reported to be *Fusobacterium nucleatum*[25].

We have previously shown that the presence of HPV infection has an effect on microbiota composition in the placenta, cervix and in the oral cavity in women[26]. In this study, we extended the research to mother's breast milk and their infant's oral microbiota. Our aim was to investigate the similarities in the overall microbiota profile between the breast milk and the infant mouth and the association of HPV infection with these two microbiota profiles.

Methods

The subjects and samples of the present study are a sub-set from the Finnish Family HPV Study collected during the years 1998–2001[14,20]. The original study was designed to evaluate

the interactions of HPV infection within families. Altogether 329 women were enrolled to this study in their final trimester, together with their male spouses (n = 131) and offspring to come (n = 313) with a 6-year follow-up period. Written informed consent was obtained from all participants and the study design was found acceptable by the Ethics Committee of the Intermunicipal Hospital District of Southwest Finland (#3/1998, #2/2006, 45/180/2010).

We selected a total of 40 infant oral and 39 breast milk samples from the Finnish Family HPV study from our previously published study population described in detail by Tuominen et al. (2018)[26]. Because some of the originally selected samples had low DNA quality for further analyses, we were forced to exclude 5 infant oral and 4 breast milk samples from microbiota analyses. Therefore, the final study population consisted of 35 infant oral and 35 breast milk samples. Because not all of the excluded samples were from the same mother-infant pairs, we ended up with 31 paired samples. The samples were collected at two different time points due to family-related reasons. Thirteen infant oral samples were collected at birth and 22 samples at the age of two months. Altogether, 31 breast milk samples were obtained at birth and four of them two months postpartum.

HPV detection and genotype

HPV DNA was extracted by the high-salt method as described in Miller[27] et al. (1988). HPV genotyping was performed by Multimetrix (Progen Biotechnik GmbH, Heidelberg, Germany) as described in Koskimaa[28] et al.(2012) and Louvanto[11] et al. (2017). The HPV genotypes of all of the samples has been analyzed and reported earlier from infant oral[28] and from breast milk[11].

16S bacterial gene sequencing and analysis

The sample analyzing have previously been described in detail[26]. Briefly, the V3-V4 region of the 16S rDNA gene was amplified following Illumina protocols using Nextera XT Index Kit (Illumina, San Diego, CA, USA). Five infant oral samples and four breast milk samples were excluded from the analyses due to the poor quality of the DNA. Thus, the final cohort comprised of 35 breast milk and 35 infant's oral samples. The libraries were sequenced using a 2x300pb paired-end run (MiSeq Reagent kit v3) on a MiSeq-Illumina platform (Lifesequencing sequencing service, Valencia, Spain). The PCR amplification and library controls were sequenced along as controls to exclude possible contaminations.

After primer removal, sequences with <300 nucleotides read length were trimmed and quality filtered. Chimeric sequences were filtered using UCHIME program version 4.2. An open reference OTU picking method using 99% identity to the Greengenes 13_8 database was performed using QIIME pipeline (version 1.9.0)[29]. Singletons and OTUs with a relative frequency below 0.01 were removed. Sequences, that could not be classified to domain level, or were classified as *Cyanobacteria*, *Chloroplasts* and *Rhizobiales* were removed.

Alpha diversity indices (Chao1 index: richness and Shannon index: diversity) and beta diversity using UNIFRAC (phylogenetic) and Bray Curtis distance (non-phylogenetic) were obtained. The Analysis of similarities (ANOSIM) test was used to test for statistically significant differences between the groups. Calypso software version 8.24 (http://cgenome.net/calypso/) was used with total sum normalization (TSS) for the statistical analysis. Linear discriminant analysis effect size (LEfSe) was used to detect unique biomarkers (linear discriminant analysis (LDA) score>3.0) in relative abundance of bacterial taxonomy. Inter and intraindividual variability was identified by pair-level analyses (paired t-test). We considered p-values ≤0.05 as statistically significant.

Results

HPV status of the samples

The HPV status of the complete Finnish family HPV study samples has been previously reported by Koskimaa[28] et al. (2012) and Louvanto[11] et al. (2017). In the present sub cohort of 31 mother-infant pairs, HPV DNA was detected in 8.6% of the mother's breast milk and 40% of the infant oral samples. The genotypic distribution of all of these samples is presented in Table 1. The study subjects included one set of twins. Infection with multiple HPV types was found in one infant oral sample and in none of the milk samples. Table 2 depicts the HPV status according to the sex of the infant, delivery mode and duration of gestation within the mother-infant pairs.

Bacterial microbiota composition in breast milk and the infant oral cavity

The most abundant phyla in breast milk and infant oral cavity were *Firmicutes* (79.7% vs 82.6%, respectively), *Proteobacteria* (14.1% vs 4.5%, p = 0.0082), *Actinobacteria* (5.2% vs 6.4%) and *Bacteroides* (0.5% vs 6.0%, p = 0.009) (Fig 1A). The predominant microbial families belonged to *Streptococcaceae* (50.7% vs 59.4%), *Gemellaceae* (15.3% vs 4.2%, p = 0.0012), *Staphylococcaeae* (11% vs 3.9%, p = 0.0078) (Fig 1B). A core of eight genera was observed between breastmilk and infant oral cavity as shown by Venn diagram (Fig 1C). This core microbiota was composed of *Streptococcus*, *Staphylococcus*, *Unclassified Gemellaceae*, *Rothia*, *Veillonella*, *Haemophilus*, *Propionibacterium* and *Corynebacterium*.

The effect of age on infant oral microbiota

Infant age had a substantial impact on infant oral microbiota composition. Two distinct clusters in relation to age are seen when samples obtained at birth (n = 13) are compared to those collected two months (n = 22) after birth (Fig 2A). Furthermore, higher richness (p = 0.026, Chao 1 index, Fig 2B) was observed in the infant oral samples collected at two months of age when compared to infant oral samples taken at birth. No difference in diversity was detected (p = 0.18, Shannon index, Fig 2C). With LEfSe (linear discriminant analysis effect size) test, higher abundance of *Streptococcus*, *Veillonella* and *Rothia* (LDA score>4.0, p<0.05) was found in the two-month-old infant oral samples, whereas *Lactobacillus*, *Bacteroides* and *Megasphaera* (LDA score>4.0, p<0.05) were higher in the infant oral samples collected at birth (Fig 2D).

Table 1. HPV genotype distribution among samples.

HPV genotype/s	Infant oral (one set of twins) n = 14/35 (40%)	Mother's breast milk n = 3/35 (8,6%)
HPV 16	6/35 (17%)	1/35 = 3%
HPV 6	1/35 (3%)	2/35 = 6%
HPV 18	2/35 (6%)	
HPV 66	2/35 (6%)	
HPV 70	1/35 (3%)	
HPV 58	1/35 (3%)*	
multiple types (HPV 33 and 59)	1/35 (3%)	

*mother had HPV 6 in her breast milk, other breast milk HPV positive mothers had HPV negative infants

Table 2. HPV status of the samples.

ID	Infant sex	Delivery mode (VD vaginal delivery, CS caesarean section)	Gestation weeks	Infant oral (HPV type)	Timepoint when infant oral samples were collected	Mother's milk (HPV type)	Timepoint when breast milk samples were collected
1	female	VD	42.0	(-)	at 2 months		at birth
2	female	VD	41.0	33,59	at birth		at birth
3	female	CS	35.4	(-)	at birth		at birth
4	female	VD	40.1		at 2 months		at birth
5	male	VD	37.6		at 2 months		at 2 months
6	female	VD	40.1		at 2 months		at birth
7	male	VD	42.0		at 2 months		at birth
8	female	VD	39.4		at 2 months		at birth
9	female	VD	38.3		at 2 months	(-)	at birth
10*	male	VD	38.0		at 2 months		at birth
11*	male	VD	38.0	18	at 2 months		at birth
12	male	CS	38.5		at 2 months		at birth
13	male	CS	42.2		at 2 months		at birth
14	female	VD	41.5	18	at 2 months		at birth
15	male	VD	41.0		at 2 months		at birth
16	male	VD	39.0		at birth		at birth
17	female	CS	40.0	16	at birth		at birth
18	male	CS	42.1		at 2 months		at birth
19	female	CS	42.4	6	at 2 months		at birth
20	female	CS	41.1		at 2 months		at birth
21	female	VD	39.6		at 2 months		at birth
22	male	CS	38.4	66	at birth	(-)	at birth
23	female	VD	39.0	16,33(-)	at birth		at birth
24	male	VD	42.0		at birth	16	at birth
25	female	VD	40.4	16	at birth		at birth
26	female	CS	39.0		at 2 months		at birth
27	male	VD	40.4	16	at birth		at birth
28	female	VD	41.0	70	at 2 months		at birth
29	female	CS	39.1		at 2 months		at birth
30	female	VD	41.6	16	at 2 months		at birth
31	female	CS	40.6		at 2 months		at birth
32	male	VD	38.3	66	at birth		at 2 months
33	female	VD	40.6		at 2 months		at 2 months
34	female	VD	40.0	58	at birth	6	at birth
35	female	CS	39.0		at birth	6	at birth
36	female	CS	41.4		at birth	(-)	at birth
37	male	VD	39.6	6(-)	at birth		at birth
38	male	CS	35.3	16	at birth	(-)	at birth
39	male	VD	36.6	6(-)	at birth		at birth
40	male	CS	42.0	16	at birth		at 2 months

*twins

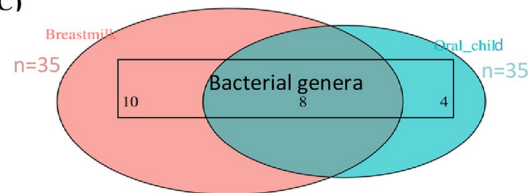(-) samples not included in the analyses because of the poor DNA quality

A)
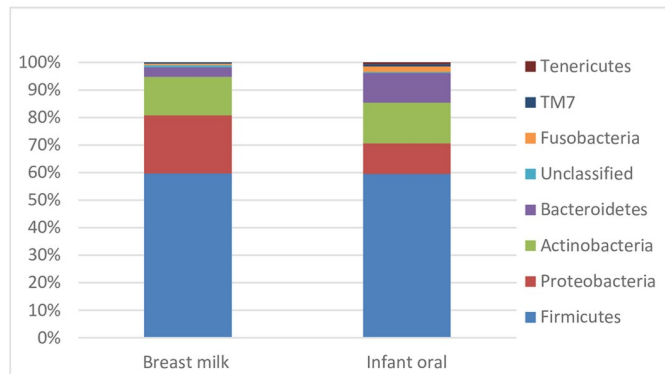
B)
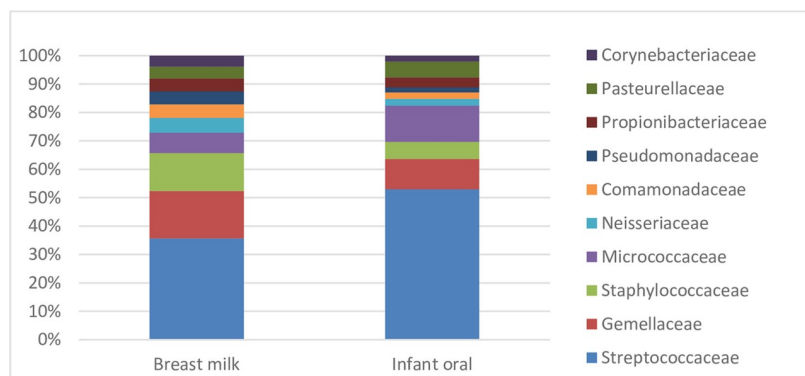
C)

| Taxa	type ▾	details
Veillonella	core	Breastmilk&Oral_child
Unclassified.Gemellaceae	core	Breastmilk&Oral_child
Streptococcus	core	Breastmilk&Oral_child
Staphylococcus	core	Breastmilk&Oral_child
Rothia	core	Breastmilk&Oral_child
Propionibacterium	core	Breastmilk&Oral_child
Haemophilus	core	Breastmilk&Oral_child
Corynebacterium	core	Breastmilk&Oral_child
Unclassified.Weeksellaceae	unique	Oral_child
Unclassified.Planococcaceae	unique	Breastmilk
Unclassified.Comamonadaceae	unique	Breastmilk
Unclassified	unique	Breastmilk
Sphingomonas	unique	Breastmilk
Pseudomonas	unique	Breastmilk
Prevotella	unique	Oral_child
Neisseria	unique	Breastmilk
Lysinibacillus	unique	Breastmilk
Lactobacillus	unique	Breastmilk
Delftia	unique	Breastmilk
Burkholderia	unique	Oral_child
Agrobacterium	unique	Breastmilk
Actinomyces	unique	Oral_child

Fig 1. Bacterial microbiota composition in breast milk and the infant oral cavity. The relative abundance of bacteria are presented on the phylum (Fig 1A) and family levels (Fig 1B) for both groups. A Venn diagram with the list of shared and unique bacterial genera both in breast milk and infant oral samples (Fig 1C).

At phylum level, the levels of *Micrococcaceae* (p = 0.000051) and *Streptococcaceae* (p = 0.0051) were higher in samples taken two months after birth when compared to elevated level of *Tissierellaceae* (p = 0.033) in samples taken at birth. Furthermore, at species level *Rothia mucilaginosa* (p = 0.000043), *Unclassified Streptococcus* (p = 0.0051), *Unclassified Weeksellaceae* (p = 0.0095), *Unclassified Haemophilus* (p = 0.032) and *Veillonella dispar* (p = 0.034), *Unclassified Anaerococcus* (p = 0.030) and *Unclassified Finegoldia* (p = 0.052) levels were all higher at the age of two months compared to the samples taken at birth.
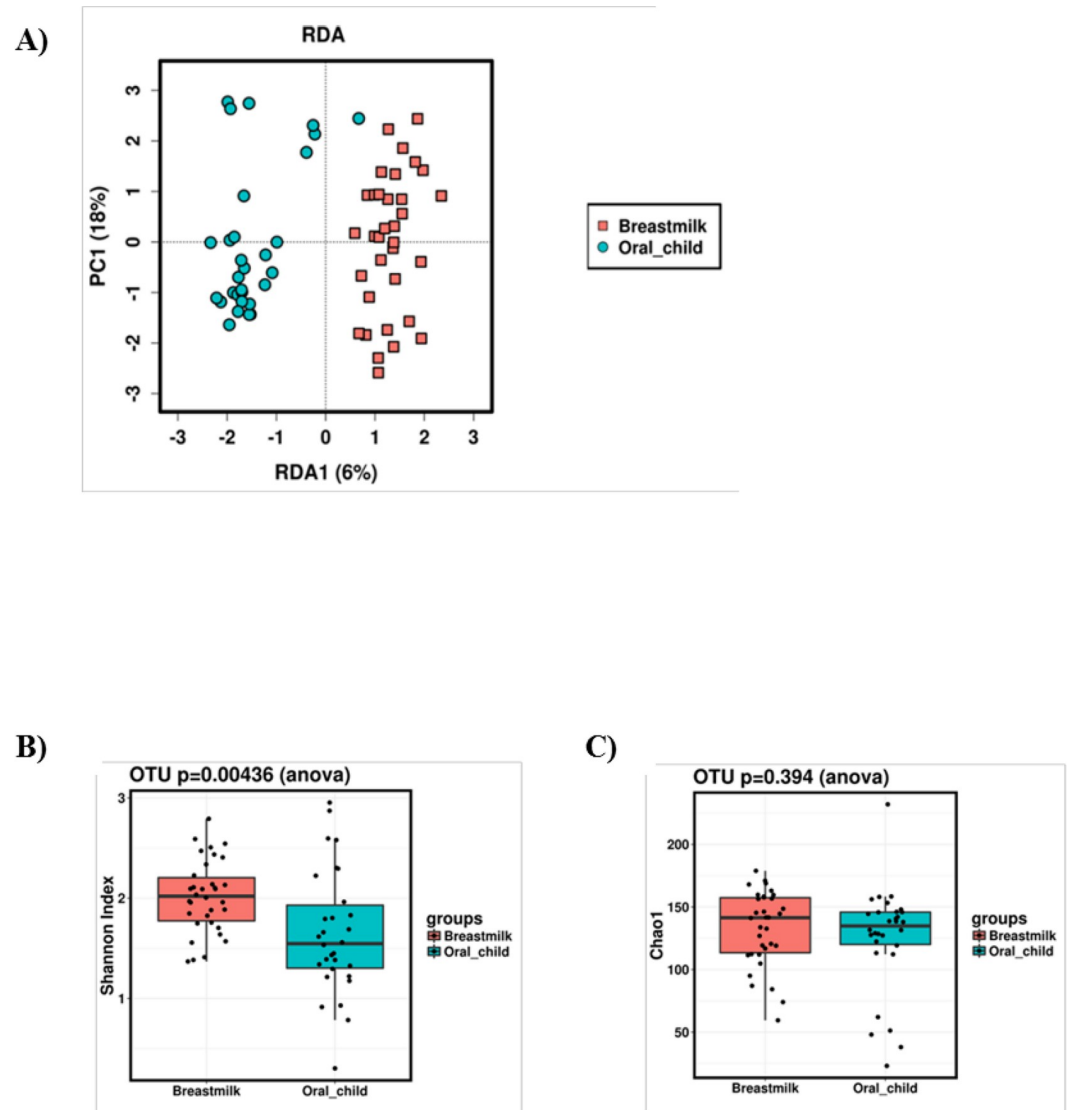
Differences and similarities between breast milk and infant oral microbiota

Significant differences in the microbiota profile were found between breast milk and infant oral cavity using multivariate Redundant Discriminant Analysis (RDA) (p = 0.0001, Fig 3A). The comparisons were performed by clustering all mothers' breast milk microbiome data together and compared it to the clustered infant oral microbiota data. Furthermore, breast milk showed higher microbial diversity as compared to the infant oral microbiota (p = 0.0043, Shannon index, Fig 3B) but no differences in bacterial richness (p = 0.394, Chao1 index, Fig 3C) were detected.
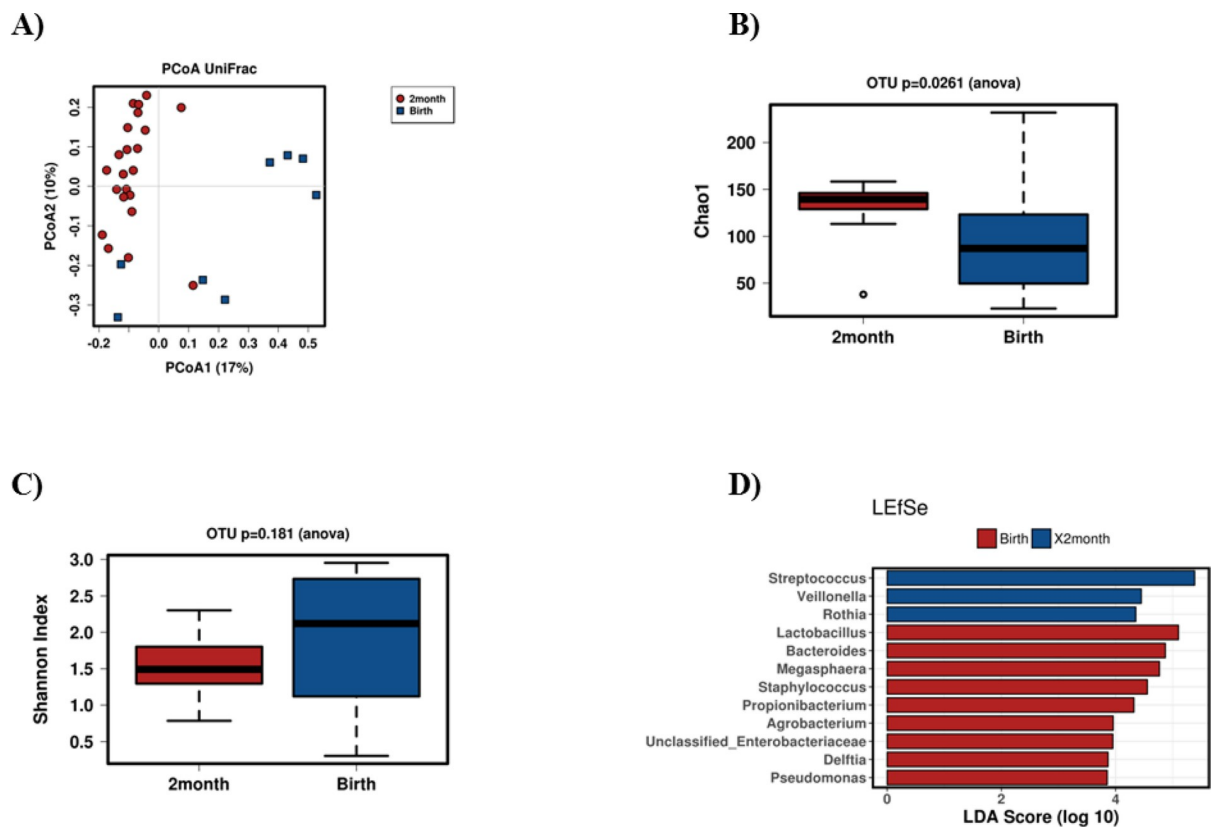
Fig 2. The effect of age on infant oral microbiota. PCoA chart depicting two different clusters in relation to the time (at birth = blue, at two months of age = red, Fig 2A). Higher richness (p = 0.026, Chao 1 index; Fig 2B) was observed in infant oral samples collected at 2 months of age (red) versus samples collected at birth (blue) but no difference in diversity were evident (Shannon index; Fig 2C). LEfSe test showing different abundances between the two different time groups (blue = two months, red = birth, Fig 2D).

Fig 3. Differences and similarities between breast milk and infant oral microbiota. Multivariate redundancy discriminant analysis (RDA) showing differences between breast milk (light red) and infant oral cavity (light blue) microbiota profiles (Fig 3A). Higher diversity (p = 0.0044, Shannon index, Fig 3B) was detected in breast milk (light red) as compared to infant oral (light blue) samples, but no differences in richness were observed (Chao 1 index, Fig 3C).

No statistical differences were observed in breast milk or infant oral microbiota composition according to the mode of delivery either with PCoA or with RDA (p = 0.570 and p = 0.456, respectively). When analyzing the microbiota data of the 31 mother- infant pairs we were not able to identify pairwise similarities or to connect mother to her infant by microbiota profile.

Breast milk microbiota and HPV
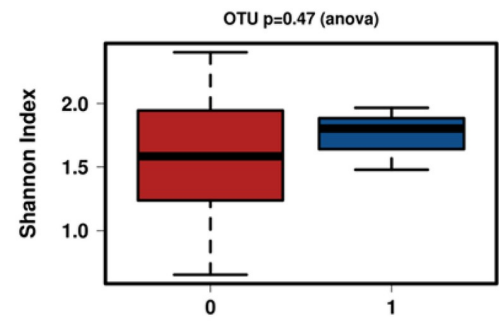
There were only three HPV positive breast milk samples. No statistically significant differences in species richness (Fig 4A) or diversity (Fig 4B) were detected between the HPV positive and HPV negative breast milk samples, nor did we observe any statistical differences in microbial compositions at phylum or at family levels in the breast milk samples (Fig 4C and 4D).
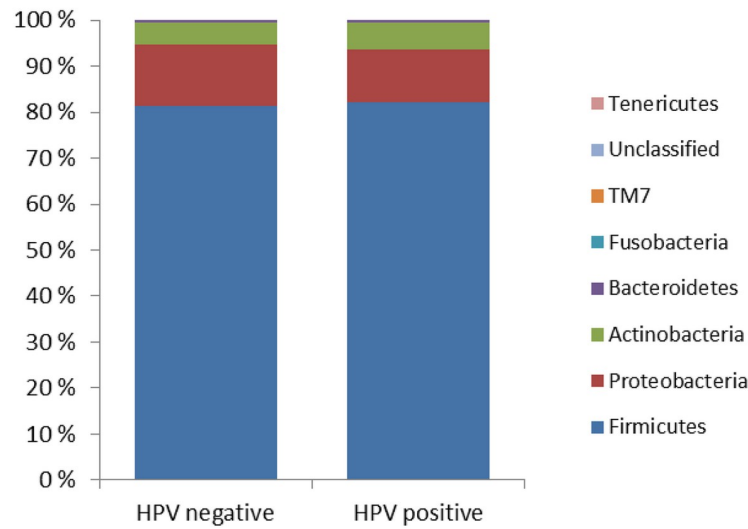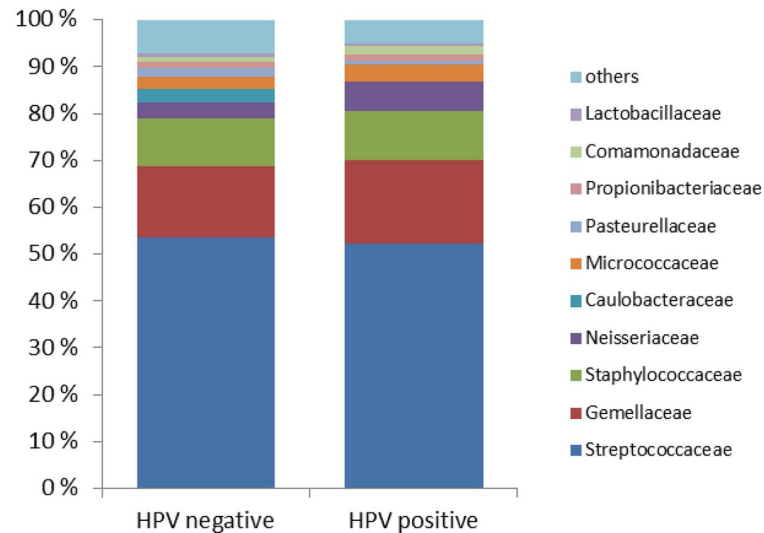
A)
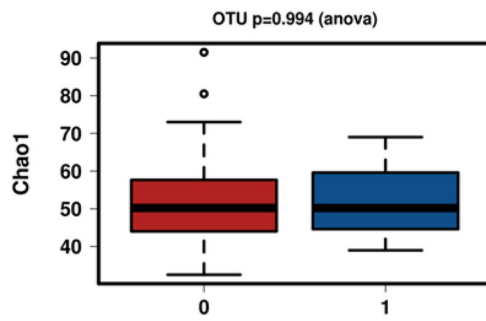
B)

C)

D)

Fig 4. Breast milk microbiota and HPV. No differerence in richness (Chao 1 index, Fig 4A) or diversity (Shannon index, Fig 4B) were detected between HPV postitive (blue) and HPV negative (red) breast milk samples. The relative abundances of bacteria are presented on the phylum (Fig 4C) and family levels (Fig 4D).

Infant oral HPV infection and bacterial microbiota

There were no differences between richness (Fig 5A) or diversity (Fig 5B) in infant oral samples grouped by the HPV status of the samples. Nonetheless, statistically significant clustering of the oral microbiota from HPV positive and negative infants was detected by RDA (p = 0.036) (Fig 5C). No statistically significant differences in microbial composition at phylum level were detected between HPV positive and HPV negative infant oral samples (Fig 5D). However, *Proprionibacteriaceae* (p = 0.095) was increased at family level, *Propionibacterium* (p = 0.095) at genus level and *Propionibacterium acnes* (p = 0.093) at species level in HPV positive infant oral samples. In the HPV negative infant oral samples, higher abundance of *Veillonella* (p = 0.025) at genus level and *Veillonella dispar* (p = 0.048) at species level was detected. (Fig 5E).

With LefSE tests, *Lysinibacillus* and *Ureaplasma* (LDA scores>4.0, p<0.05) were significantly increased in the HPV positive group compared to the HPV negative infant oral sample group, whereas *Veillonella* (LDA score>4.0, p<0.05) was higher in HPV negative infant oral samples (Fig 5F).

The impact of HPV infection on infant oral microbiota was investigated at two different time points albeit the sample size was relatively small. No differences in bacterial richness, diversity or composition were detected when comparing the infant oral HPV negative (n = 4) and the infant oral HPV positive (n = 8) samples collected at birth. However, we observed higher richness in the infant oral HPV negative samples collected two months after birth (n = 17) when comparing to the HPV positive infant oral samples at two months of age (n = 5). No differences in diversity were seen. *Veillonellaceae* (p = 0.025) and *Veillonella dispar* (p = 0.052) levels were higher in the HPV negative infant oral samples collected two months after birth when compared to the HPV positive two months old infant's oral samples.

Discussion

Our results suggest for the first time that HPV infection is associated with alterations in the microbiota in the infant oral cavity. The changes are more pronounced at genus and family levels, but they do not influence species richness or diversity as a whole. Nonetheless, two distinct infant oral microbiota clusters dependent on HPV status were observed with RDA analysis. In contrast, we were not able to detect differences in breast milk microbiota composition associated with HPV infection, possibly because the total number of HPV positive breast milk samples was insufficient. Nevertheless, we still performed microbiota analyses, because the number of HPV positive milk samples (8.6%) were in line with previous studies[8–10].

The infant oral microbiota observed in this study corresponds to previous studies, which indicate that the most abundant taxa in the infant oral microbiota belong to *Streptococcus*, *Veillonella*, *Neisseria* and *Firmicutes*[5,22]. The early infant oral microbiota differs from the oral microbiota at later stages of childhood, which may in part be attributable to the absence of hard dental tissues in early infancy. Our data demonstrated that the oral microbial community evolves during the first two months of extra uterine life. Previously, the changes in microbial communities have been reported to stabilize within the first few months of life[30].

While factors including prematurity, maternal intrapartum antibiotics, delivery mode and formula feeding all affect infant oral microbiota composition[30–32], the microbes from the maternal mouth as well as the mammary areola and breast milk are the primary sources of
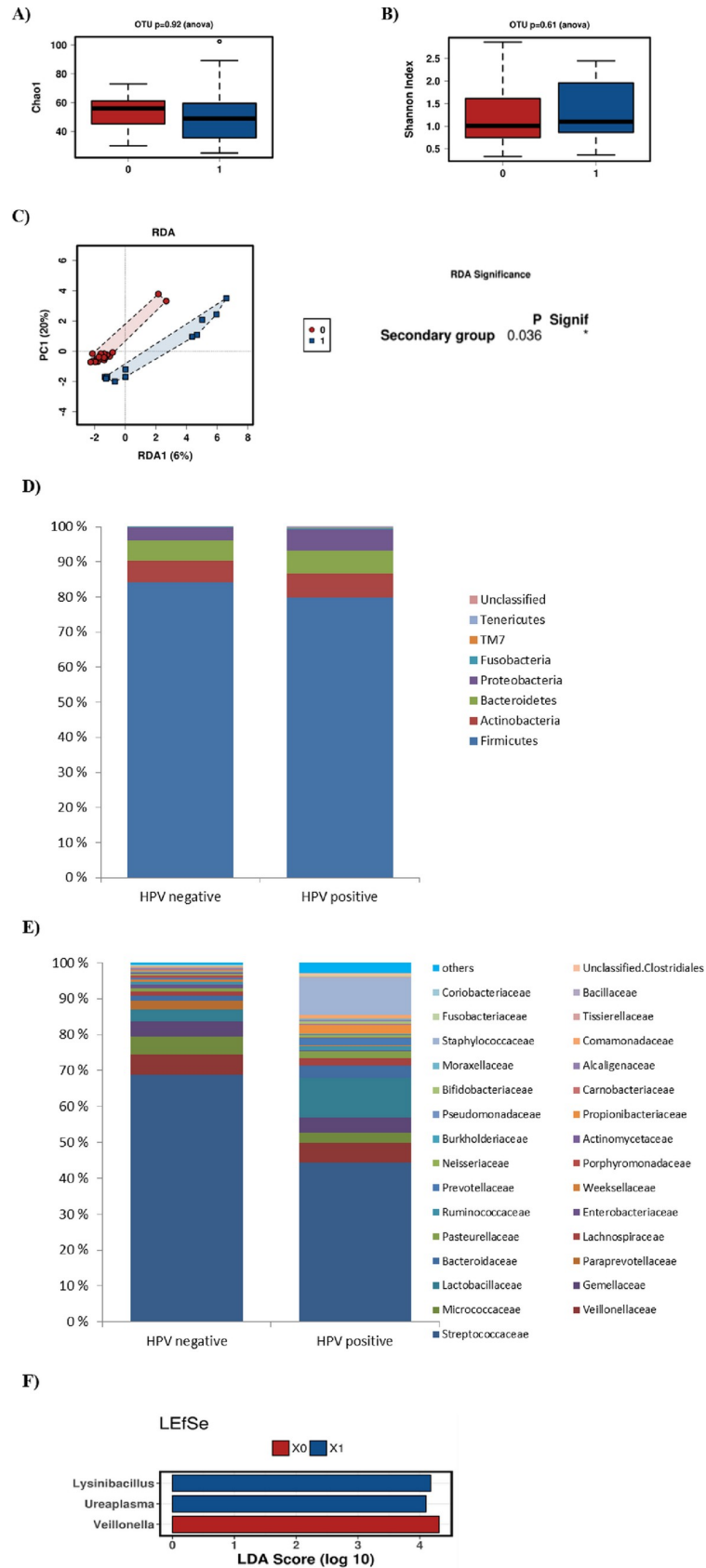
Fig 5. Infant oral HPV infection and bacterial microbiota. No differences in bacterial richness (Chao 1 index, Fig 5A) or diversity (Shannon index, Fig 5B) between HPV positive (blue) and HPV negative (red) infant oral samples were detected. RDA analysis (Fig 5C) shows significant differences between HPV positive (blue) and HPV negative infant oral samples (red). The relative abundances of bacteria are presented on the phylum (Fig 5D) and family levels (Fig 5E). By LEfSe analysis (Fig 5F), *Lysinibacillus* and *Ureaplasma* were significantly enriched in HPV positive (blue) infant oral samples (LDA score >4.17 and 4.10 respectively, p<0.05), while *Veillonella* (LDA score>4.31, p<0.05) were enriched in HPV negative (red) compared to HPV positive infant oral samples.

bacteria for the infant mouth[5,22]. In our study population, we detected a shared core of eight bacterial genera between breast milk and infant oral mucosa microbiota. We did not observe any correlation between infant oral microbiota and the mode of birth in oral mucosa samples obtained at birth or at two months of age despite a previous report indicating that delivery affects the infants oral microbial composition directly after birth[33].

The breast milk microbiota is predominantly composed of bacteria which are thought to contribute to the infant's newly developing gut microbiota and favorable microbiota prevents pathogens from colonizing the infant gut[3,5,34]. The bacterial composition of breast milk and the infant oral microbiota seem to have a connection, which is quite easy to understand since breast milk is often the first food source for the infant. This was also one of the main findings in our study; we were able to recognize a core of eight bacteria which were shared between mother's breast milk and the infant oral cavity. This core microbiota was composed of *Veillonella*, Unclassified *Gemellaceae*, *Streptococcus*, *Staphylococcus*, *Rothia*, *Haemophilus* and *Cornyebacterium*. These data are consistent with previous study by Biagi[5] et al. who concluded that passage of breast milk through infant's mouth has an effect on the oral microbiota composition as they also found *Staphylococcus*, *Streptococcus* and unclassified *Gemellaceae* shared between infant oral mucosa and breast milk microbiota. In our study, all of the infants received breast milk, though we are not aware, if there have been any additional food supply alongside breast feeding during the time when samples were collected. Because the main food supply has been from mother's milk, we are confident that the results present an actual interaction between infant oral and breast milk microbiota.

The genera *Propionibacterium*, *Lysinibacillus* and *Ureaplasma* were enriched in HPV positive infant oral mucosal samples when compared to HPV negative samples. *Propionibacterium* has been found in the gut microbiota of preterm infants[35]. Oral *Ureaplasma* has previously been linked to HPV positivity in sexually active men[36] and *Ureaplasma* found in the cervix and placenta is connected to preterm birth without the presence of HPV[37]. Recently, *Ureaplasma* has also been discovered in infant oral cavity collected 15 minutes after vaginal birth[38]. Despite the fact that *Ureaplasma* and *Propionibacterium* have previously been connected to preterm birth, our study had only total of three infants born slightly preterm and all of them were born after 35 weeks of gestation. Still it seems that HPV infection affects oral microbiota composition by favoring the growth of pathogens, which may have clinical significance later in life.

An increase in the level of oral *Veillonella* spp. was observed in HPV negative infant oral samples as compared to positive samples at two months of age. This finding is line with previous studies indicating that *Veillonella* is one of the most abundant bacteria in infant oral cavity [25]. Later in life, oral *Veillonella* has been associated with higher caries activity[39] and poor oral hygiene[40].

Breastfeeding confers several significant health benefits to the infant. In addition, breast milk serves as one of the most important sources of microbes for the infant[5,34,41]. Healthy breast milk microbiota is mainly composed of *Proteobacteria*, *Streptococcaceae*, *Pseudomonadaceae*, *Bifidobacteriaceae* and *Staphylococcaceae*[5–7], but the composition seems to be very

variable with geographical differences between individuals[3,4,6]. Even delivery mode has been reported to have effect on the microbial composition of breast milk. In particular, levels of *Lactobacillus* have been reported to be higher in milk samples from mothers who delivered by caesarean section compared to vaginal delivery[6]. In our breast milk samples, however, we did not observe differences in neither of the groups regarding the microbial composition according to the birth mode, possibly because of the low number of samples studied. Even though we did take the birth mode into account and selected samples according to it, the overall numbers of vaginal delivery and caesarean section probably remained too few to reach to statistical significance between the two groups.

The Finnish family HPV study population and samples are exceptional. The study has been designed to evaluate HPV interactions within families, and consequently all of the samples have been collected within the same family. Nonetheless, our study has limitations which include the fact that only three breast milk samples were HPV DNA positive. The observed prevalence of 8.6% HPV positivity in breast milk is in line with previous studies[8–10]. Still, possibly because of the low number of HPV positive breast milk samples we failed to reach statistical significance between microbial compositions in HPV positive and HPV negative breast milk samples. Furthermore, we were forced to exclude number of infant oral and breast milk samples from further analysis, because of the poor quality of DNA or the low number of sequences (n = 5, n = 4, respectively). Only samples that reached 1000 reads were included in the analyses. This study concentrated on investigating only selected HPV genotypes (low-risk HPV including HPV6, 11, 42, 43, 44 and 70 and high-risk HPV types HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) because these mucosal HPV genotypes are the most relevant to the pathogenesis of human disease according to present knowledge[42].

We have taken into consideration possible contamination and sequenced negative controls from PCR amplification and libraries. Furthermore, we aimed to verify our results by excluding singletons and OTUs with a relative frequency below 0.01 when analyzing of the data. In addition to that, sequences that were classified as *Cyanobacteria*, *Chloroplasts* and *Rhizobiales* or sequences that could not be classified to domain level were removed from the dataset.

In conclusion, present HPV infection in infant oral cavity is connected to detectable changes in microbial composition and it also seems to favor pathogens. Still, we do not know whether present HPV infection induces these changes in microbiota composition or whether the altered microbiota colonization predisposes to HPV infection. We were not able to find alterations in breast milk samples when taken into account present HPV infection. Finally, we conclude that infant oral and breast milk share similar features, as we found a core of eight shared bacteria.

Acknowledgments

Author Contributions

Conceptualization: Samuli Rautava, Stina Syrjänen, Jaana Rautava.

Data curation: Stina Syrjänen.

Formal analysis: Heidi Tuominen.

Funding acquisition: Heidi Tuominen, Stina Syrjänen.

Investigation: Heidi Tuominen, Samuli Rautava, Maria Carmen Collado, Jaana Rautava.

Methodology: Heidi Tuominen, Maria Carmen Collado.

Project administration: Samuli Rautava, Maria Carmen Collado, Jaana Rautava.

Resources: Heidi Tuominen, Stina Syrjänen.

Software: Maria Carmen Collado.

Supervision: Samuli Rautava, Maria Carmen Collado, Jaana Rautava.

Writing – original draft: Heidi Tuominen.

Writing – review & editing: Samuli Rautava, Maria Carmen Collado, Stina Syrjänen, Jaana Rautava.

References

1. Giuliano AR, Nyitray AG, Kreimer AR, Campbell CMP, Goodman MT, Sudenga SL, et al. EUROGIN 2014 Roadmap: Differences in HPV infection natural history, transmission, and HPV-related cancer incidence by gender and anatomic site of infection HHS Public Access. Int J Cancer Int J Cancer June. 2015;

2. Betiol J, Villa LL, Sichero L. Impact of HPV infection on the development of head and neck cancer. Brazilian J Med Biol Res = Rev Bras Pesqui medicas e Biol [Internet]. 2013 Mar [cited 2018 Jan 17]; 46 (3):217–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23532264

3. Aakko J, Kumar H, Rautava S, Wise A, Autran C, Bode L, et al. Human milk oligosaccharide categories define the microbiota composition in human colostrum. Benef Microbes [Internet]. 2017 Aug 24 [cited 2018 Jan 17]; 8(4):563–7. Available from: http://www.wageningenacademic.com/doi/10.3920/BM2016.0185 PMID: 28726512

4. Cacho NT, Harrison NA, Parker LA, Padgett KA, Lemas DJ, Marcial GE, et al. Personalization of the Microbiota of Donor Human Milk with Mother's Own Milk. Front Microbiol [Internet]. 2017 Aug 3 [cited 2018 Jan 17]; 8:1470. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01470/full PMID: 28824595

5. Biagi E, Quercia S, Aceti A, Beghetti I, Rampelli S, Turroni S, et al. The Bacterial Ecosystem of Mother's Milk and Infant's Mouth and Gut. Front Microbiol [Internet]. 2017 Jun 30 [cited 2018 Jan 17]; 8:1214. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01214/full PMID: 28713343

6. Li S-W, Watanabe K, Hsu C-C, Chao S-H, Yang Z-H, Lin Y-J, et al. Bacterial Composition and Diversity in Breast Milk Samples from Mothers Living in Taiwan and Mainland China. Front Microbiol [Internet]. 2017 May 30 [cited 2018 Jan 17]; 8:965. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00965/full PMID: 28611760

7. Urbaniak C, Cummins J, Brackstone M, Macklaim JM, Gloor GB, Baban CK, et al. Microbiota of Human Breast Tissue. Macfarlane GT, editor. Appl Environ Microbiol [Internet]. 2014 May 15 [cited 2018 Jan 17]; 80(10):3007–14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24610844 https://doi.org/10.1128/AEM.00242-14 PMID: 24610844

8. Mammas IN, Zaravinos A, Sourvinos G, Myriokefalitakis N, Theodoridou M, Spandidos DA. Can 'high-risk' human papillomaviruses (HPVs) be detected in human breast milk? Acta Paediatr [Internet]. 2011 May [cited 2018 Jan 17]; 100(5):705–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21214884 https://doi.org/10.1111/j.1651-2227.2010.02129.x PMID: 21214884

9. Sarkola M, Rintala M, Grénman S, Syrjänen S. Human papillomavirus DNA detected in breast milk. Pediatr Infect Dis J [Internet]. 2008 Jun [cited 2017 Jan 2]; 27(6):557–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18449059 https://doi.org/10.1097/INF.0b013e318169ef47 PMID: 18449059

10. Yoshida K, Furumoto H, Abe A, Kato T, Nishimura M, Kuwahara A, et al. The possibility of vertical transmission of human papillomavirus through maternal milk. J Obstet Gynaecol (Lahore) [Internet]. 2011 Aug 8 [cited 2018 Jan 17]; 31(6):503–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21823849

11. Louvanto K, Sarkola M, Rintala M, Syrjänen K, Grenman S, Syrjänen S. Breast Milk Is a Potential Vehicle for Human Papillomavirus Transmission to Oral Mucosa of the Spouse. Pediatr Infect Dis J [Internet]. 2017 Jul [cited 2018 Jan 17]; 36(7):627–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28604561 https://doi.org/10.1097/INF.0000000000001546 PMID: 28604561

12. Hahn HS, Kee MK, Kim HJ, Kim MY, Kang YS, Park JS, et al. Distribution of maternal and infant human papillomavirus: risk factors associated with vertical transmission. Eur J Obstet Gynecol Reprod Biol

[Internet]. 2013 Jul [cited 2017 Jan 2]; 169(2):202–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23578811 https://doi.org/10.1016/j.ejogrb.2013.02.024 PMID: 23578811

13. Hong Y, Li S-Q, Hu Y-L, Wang Z-Q. Survey of human papillomavirus types and their vertical transmission in pregnant women. BMC Infect Dis [Internet]. 2013 Feb 27 [cited 2018 Jan 17]; 13(1):109. Available from: http://bmcinfectdis.biomedcentral.com/articles/10.1186/1471-2334-13-109

14. Rintala MAM, Grénman SE, Järvenkylä ME, Syrjänen KJ, Syrjänen SM. High-risk types of human papillomavirus (HPV) DNA in oral and genital mucosa of infants during their first 3 years of life: experience from the Finnish HPV Family Study. Clin Infect Dis [Internet]. 2005 Dec 15 [cited 2018 Jan 17]; 41 (12):1728–33. Available from: https://academic.oup.com/cid/article-lookup/doi/10.1086/498114 PMID: 16288396

15. Park H, Lee SW, Lee IH, Ryu HM, Cho AR, Kang YS, et al. Rate of vertical transmission of human papillomavirus from mothers to infants: relationship between infection rate and mode of delivery. Virol J [Internet]. 2012 [cited 2016 Aug 19]; 9:80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22497663 https://doi.org/10.1186/1743-422X-9-80 PMID: 22497663

16. Trottier H, Mayrand M-H, Coutlée F, Monnier P, Laporte L, Niyibizi J, et al. Human papillomavirus (HPV) perinatal transmission and risk of HPV persistence among children: Design, methods and preliminary results of the HERITAGE study. Papillomavirus Res [Internet]. 2016 Dec [cited 2017 Mar 15]; 2:145–52. Available from: http://linkinghub.elsevier.com/retrieve/pii/S240585211630026X https://doi.org/10.1016/j.pvr.2016.07.001 PMID: 29074173

17. Koskimaa H-M, Paaso A, Welters MJP, Grénman S, Syrjänen K, van der Burg SH, et al. Human papillomavirus 16-specific cell-mediated immunity in children born to mothers with incident cervical intraepithelial neoplasia (CIN) and to those constantly HPV negative. J Transl Med [Internet]. 2015 Nov 25 [cited 2018 Jan 17]; 13(1):370. Available from: http://www.translational-medicine.com/content/13/1/370

18. Chatzistamatiou K, Sotiriadis A, Agorastos T. Effect of mode of delivery on vertical human papillomavirus transmission—A meta-analysis. J Obstet Gynaecol [Internet]. 2016 Jan 2 [cited 2017 Nov 22]; 36 (1):10–4. Available from: http://www.tandfonline.com/doi/full/10.3109/01443615.2015.1030606 PMID: 26367314

19. Lee SM, Park JSJW, Norwitz ER, Koo JN, Oh IH, Park JSJW, et al. Risk of vertical transmission of human papillomavirus throughout pregnancy: a prospective study. PLoS One [Internet]. 2013 Jan [cited 2016 Aug 19]; 8(6):e66368. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23785495 https://doi.org/10.1371/journal.pone.0066368 PMID: 23785495

20. Rintala MAM, Grénman SE, Puranen MH, Isolauri E, Ekblad U, Kero PO, et al. Transmission of high-risk human papillomavirus (HPV) between parents and infant: A prospective study of HPV in families in Finland. J Clin Microbiol. 2005;

21. Sarkola ME, Grénman SE, Rintala MAM, Syrjänen KJ, Syrjänen SM. Human papillomavirus in the placenta and umbilical cord blood. Acta Obstet Gynecol Scand [Internet]. 2008 Jan [cited 2016 Aug 19]; 87 (11):1181–8. Available from: http://doi.wiley.com/10.1080/00016340802468308 PMID: 18972230

22. Drell T, Štšepetova J, Simm J, Rull K, Aleksejeva A, Antson A, et al. The Influence of Different Maternal Microbial Communities on the Development of Infant Gut and Oral Microbiota. Sci Rep [Internet]. 2017 Aug 30 [cited 2018 Jan 17]; 7(1):9940. Available from: http://www.nature.com/articles/s41598-017-09278-y https://doi.org/10.1038/s41598-017-09278-y PMID: 28855595

23. Nelson-Filho P, Borba IG, Mesquita KSF de, Silva RAB, Queiroz AM de, Silva LAB. Dynamics of microbial colonization of the oral cavity in newborns. Braz Dent J [Internet]. 2013 Jul [cited 2018 Jan 17]; 24 (4):415–9. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0103-64402013000400415&lng=en&tlng=en https://doi.org/10.1590/0103-6440201302266 PMID: 24173267

24. Pearce C, Bowden GH, Evans M, Fitzsimmons SP, Johnson J, Sheridan MJ, et al. Identification of pioneer viridans streptococci in the oral cavity of human neonates. J Med Microbiol [Internet]. 1995 Jan 1 [cited 2018 Jan 17]; 42(1):67–72. Available from: http://jmm.microbiologyresearch.org/content/journal/jmm/10.1099/00222615-42-1-67 PMID: 7739028

25. Könönen E, Kanervo A, Takala A, Asikainen S, Jousimies-Somer H. Establishment of oral anaerobes during the first year of life. J Dent Res [Internet]. 1999 Oct 18 [cited 2018 Jan 17]; 78(10):1634–9. Available from: http://journals.sagepub.com/doi/10.1177/00220345990780100801 PMID: 10520968

26. Tuominen H, Rautava S, Syrjänen S, Collado MC, Rautava J. HPV infection and bacterial microbiota in the placenta, uterine cervix and oral mucosa. Sci Rep [Internet]. 2018 Dec 28 [cited 2018 Jul 5]; 8 (1):9787. Available from: http://www.nature.com/articles/s41598-018-27980-3 PMID: 29955075

27. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988;

28. Koskimaa H-M, Waterboer T, Pawlita M, Grénman S, Syrjänen K, Syrjänen S. Human papillomavirus genotypes present in the oral mucosa of newborns and their concordance with maternal cervical human papillomavirus genotypes. J Pediatr [Internet]. 2012 May [cited 2017 Jan 2]; 160(5):837–43. Available

from: http://www.ncbi.nlm.nih.gov/pubmed/22137368 https://doi.org/10.1016/j.jpeds.2011.10.027 PMID: 22137368

29. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;

30. Chu DM, Ma J, Prince AL, Antony KM, Seferovic MD, Aagaard KM. Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. Nat Med [Internet]. 2017 Mar 23 [cited 2018 Jan 17]; 23(3):314–26. Available from: http://www.nature.com/doifinder/10.1038/nm.4272 PMID: 28112736

31. Gomez-Arango LF, Barrett HL, McIntyre HD, Callaway LK, Morrison M, Dekker Nitert M. Antibiotic treatment at delivery shapes the initial oral microbiome in neonates. Sci Rep [Internet]. 2017 Feb 27 [cited 2017 Jul 28]; 7:43481. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28240736 https://doi.org/10.1038/srep43481 PMID: 28240736

32. Timby N, Domellöf M, Holgerson PL, West CE, Lönnerdal B, Hernell O, et al. Oral Microbiota in Infants Fed a Formula Supplemented with Bovine Milk Fat Globule Membranes—A Randomized Controlled Trial. Topcuoglu N, editor. PLoS One [Internet]. 2017 Jan 18 [cited 2018 Jan 17]; 12(1):e0169831. Available from: http://dx.plos.org/10.1371/journal.pone.0169831 PMID: 28099499

33. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A [Internet]. 2010 Jun 29 [cited 2018 Jan 17]; 107(26):11971–5. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1002601107 PMID: 20566857

34. Xu W, Judge M, Maas K, Hussain N, McGrath JM, Henderson WA, et al. Systematic Review of the Effect of Enteral Feeding on Gut Microbiota in Preterm Infants. J Obstet Gynecol neonatal Nurs JOGNN [Internet]. 2017 Oct 14 [cited 2018 Jan 17]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/29040820

35. Aujoulat F, Roudière L, Picaud J-C, Jacquot A, Filleron A, Neveu D, et al. Temporal dynamics of the very premature infant gut dominant microbiota. BMC Microbiol [Internet]. 2014 Dec 31 [cited 2018 Jan 28]; 14(1):325. Available from: http://bmcmicrobiol.biomedcentral.com/articles/10.1186/s12866-014-0325-0

36. Nakashima K, Shigehara K, Kawaguchi S, Wakatsuki A, Kobori Y, Nakashima K, et al. Prevalence of human papillomavirus infection in the oropharynx and urine among sexually active men: a comparative study of infection by papillomavirus and other organisms, including Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma spp., and Ureaplasma spp. BMC Infect Dis [Internet]. 2014 Jan 27 [cited 2018 Jan 19]; 14(1):43. Available from: http://bmcinfectdis.biomedcentral.com/articles/10.1186/1471-2334-14-43

37. Doyle RM, Harris K, Kamiza S, Harjunmaa U, Ashorn U, Nkhoma M, et al. Bacterial communities found in placental tissues are associated with severe chorioamnionitis and adverse birth outcomes. PLoS One. 2017;

38. Otgonjargala B, Becker K, Batbaatar G, Tsogtsaikhan S, Enkhtsetseg J, Enkhjargal A, et al. Effect of Mycoplasma hominis and cytomegalovirus infection on pregnancy outcome: A prospective study of 200 Mongolian women and their newborns. Balish MF, editor. PLoS One [Internet]. 2017 Mar 3 [cited 2018 Jan 19]; 12(3):e0173283. Available from: http://dx.plos.org/10.1371/journal.pone.0173283 PMID: 28257513

39. Jiang S, Gao X, Jin L, Lo E. Salivary Microbiome Diversity in Caries-Free and Caries-Affected Children. Int J Mol Sci [Internet]. 2016 Nov 25 [cited 2018 Feb 21]; 17(12):1978. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27898021

40. Mashima I, Theodorea CF, Thaweboon B, Thaweboon S, Scannapieco FA, Nakazawa F. Exploring the salivary microbiome of children stratified by the oral hygiene index. Alekseyenko A V., editor. PLoS One [Internet]. 2017 Sep 21 [cited 2018 Feb 21]; 12(9):e0185274. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28934367 PMID: 28934367

41. Mosca F, Giannì ML. Human milk: composition and health benefits. La Pediatr Medica e Chir [Internet]. 2017 Jun 28 [cited 2018 Jan 17]; 39(2):155. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28673076

42. Trottier H, Burchell AN. Epidemiology of mucosal human papillomavirus infection and associated diseases. Public Health Genomics. 2009; 12(5–6):291–307. https://doi.org/10.1159/000214920 PMID: 19684442