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The infant pharyngeal microbiomes: origin, impact and manipulation

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The infant pharyngeal microbiomes: origin, impact and manipulation

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Abstract

Purpose of review: There has been an exponential increase in research into infant microbiome evolution, and it appears that pharyngeal microbiota are associated with clinical phenotypes (e.g. infection and asthma). While broad consensus views are emerging, significant challenges and uncertainties remain.

Recent findings: Infant pharyngeal microbiome research is limited by low biomass, high temporal diversity, and lack of agreed standards for sampling, DNA sequencing, and taxonomic reporting. Analysis of amplicon sequence variants and improved cost and availability of whole genome sequencing are promising options for improving taxonomic resolution of such studies. Infant respiratory microbiomes arise, at least in part, from maternal flora (e.g. the respiratory tract and breastmilk), and are associated with environmental and clinical factors (e.g. mode of feeding and delivery, siblings, daycare attendance, birth season, and antibiotic usage). Interventional research to modify the infant pharyngeal microbiota has recently been reported, using dietary supplements.

Summary: Further work is needed to improve characterisation of the infant pharyngeal microbiomes, including routes of bacterial acquisition, role of environmental factors, and associations with disease phenotypes. Methodological standards are desirable to facilitate more reproducible, comparable research. Improved understanding may enable manipulation of infant pharyngeal microbiota to improve clinical outcomes.

Keywords: microbiota, infant, pharynx, respiratory

Introduction

Over the past decade, improvements in the efficiency of and access to genomics-based technologies have seen an explosion in the field of microbiome research. To date, the majority of human microbiome studies focus on adults rather than infants, and on the gastrointestinal tract rather than the upper respiratory tract. However, there is growing interest in the complex interplay between maternal and infant microbiota, and emerging evidence suggests that evolution of the nasopharyngeal, oropharyngeal and hypopharyngeal microbiomes in the first year of life is associated with clinical outcomes in childhood, including respiratory tract infections (RTI) and asthma[1]. Further, modifiable variables such as mode of delivery and feeding appear to associate with infant microbiome evolution[2]. Such findings raise the exciting question of whether infant pharyngeal microbiomes could be manipulated to prevent colonisation and infection by pathobionts (potentially pathogenic commensal flora), and whether doing so could foster more favourable clinical outcomes in childhood[3].

This narrative review explores the evidence to date on the acquisition, clinical and environmental associations, and manipulation of the nasopharyngeal, oropharyngeal and hypopharyngeal microbiomes in the first year of life, and summarises the ongoing challenges in studying these microbial niches. Recent advances are highlighted, including the only published interventional study reporting on manipulation of infant pharyngeal microbiota[3]; and scope for amplicon sequence variant analysis and whole genome sequencing to improve taxonomic resolution of microbiome studies[4].

Methods

Given the broad remit of this review, we focus on the pharyngeal bacteriome (rather than virome or fungome), and only refer to studies of adjacent sites (e.g. the oral and nasal cavities) where infant pharyngeal data are lacking. We searched the PubMed database (from inception to August 2020) using the MESH-based search: "(microbiota) AND (infant OR newborn OR pregnancy OR maternal-fetal Relations) AND (respiratory system OR pharynx OR nasopharynx OR oropharynx OR hypopharynx OR mouth OR nasal cavity)". Relevant articles were used to identify further papers for review, using published references and the PubMed Similar Articles and Cited By tools. Unless otherwise specified, the studies cited refer to results of 16s ribosomal RNA (rRNA) sequencing, and bacterial nomenclature quoted refers to operational taxonomic units (OTUs).

Studying the infant pharyngeal microbiomes: approaches, challenges and limitations

Unlike traditional culture-based techniques, and even targeted molecular technologies, next generation sequencing allows taxonomic characterisation of entire microbial communities[4]. The majority of pharyngeal microbiome research has employed amplicon sequencing; i.e. sequencing highly conserved genetic loci possessing predictable variability between genera (and even species). The most widely used of these are the hypervariable regions of the pan-prokaryotic 16s rRNA genes, initially employed in the ground-breaking work of Woese et al. in the 1980s[5].

Although powerful, this approach has several limitations. Rather than using traditional binomial classification, 16s rRNA sequencing typically reports OTUs, by clustering sequences within a pre-defined similarity threshold (conventionally 97%)[6]. OTUs usually

offer genus- rather than species- or strain-level resolution, such that a single OTU can include both harmless commensals and pathogens, and identical OTUs in mother-infant pairs may not represent bacterial transmission[7]. Choice of hypervariable region may improve resolution; for example, V4 sequencing alone fails to resolve *Enterobacteriaceae* to even genus-level, while this is overcome by sequencing both V3 and V4 regions[4]. More recently, 16s rRNA microbiome studies have reported amplicon sequence variants (ASVs), which are sequences of true biological origin identified after algorithmic removal of presumed errors[8]. Unlike OTUs, ASV analysis can offer species-level resolution and comparison between unrelated datasets, and has recently been used in microbiome studies of the neonatal mouth[9] and hypopharynx[4].

Non-amplicon based whole genome shotgun sequencing (WGSS) offers reliable strain-level resolution, although it remains more financially and computationally resource-intensive. In WGSS, all genetic material in a sample is non-selectively sequenced, and contiguous overlapping reads are aligned, yielding a comprehensive metagenomic library. As the pharynx is a low-biomass site (compared with e.g. the gastrointestinal tract), this approach is prone to contamination bias[10], and has been largely avoided in the study of the pharyngeal microbiomes, although it may have a role in diagnostics (e.g. undifferentiated fever[11]). Inclusion of negative controls, analysis of mock communities, and in silico denoising and decontamination all attempt to reduce contamination bias in both amplicon-based and WGSS pharyngeal microbiome studies[6,12].

As with all molecular technologies, microbiomic profiles do not indicate organism viability (unlike traditional culture), so may include dead microbial debris. Parallel culture of microbiome samples[4] and transcriptomic analysis (e.g. characterising gene

expression associated with pathogen-specific host immune responses[13]), attempt to overcome such limitations.

These issues are compounded when comparing the pharyngeal microbiomes in infants and their mothers (or primary care-givers), as upper respiratory microbial density is much lower and diversity higher in infants than adults. Thus, applying the same sequencing method and depth to samples from infants and adults may lead to over- and underestimation of OTUs, respectively. Pharyngeal anatomy varies with age, and infants display less marked microbiome differences between adjacent niches than adults[14]. Further, the nasopharyngeal, oropharyngeal, nasal and oral niches differ in epithelial and environmental interfaces, and it remains unclear to what extent results from one niche can be extrapolated to an adjacent niche. Indeed, the oral and nasopharyngeal microbiomes overlap significantly at birth, becoming distinct by one week, and continuing to diverge over three months[15]. Modest but significant correlation is noted between the nasopharyngeal and nasal microbiomes, although evidence is limited – Luna et al.[16] report only on infants hospitalised for bronchiolitis; Wang et al.[17] include only 21 infants.

Defining the infant pharyngeal microbiomes is further hampered by significant methodological variability between studies, including sampling site and technique, choice of 16s rRNA gene hypervariable region (V1-V9), DNA extraction and sequencing method, use of controls, and bioinformatic analysis strategy[18]. While the growing body of infant respiratory microbiome research is valuable and informative, these limitations must be borne in mind, and unfounded inference of causality or extrapolation between datasets and adjacent niches must be avoided. There have been calls for the introduction of gold standards in microbiome research[19,20], although none have yet been agreed.

Microbiome evolution and stability

Despite such limitations, significant progress has been made in recent years towards defining the acquisition and evolution of the infant microbiome, although the majority of such research has been into gastrointestinal microbiota. Broadly speaking, the consensus view is that neonates become rapidly colonised at birth with a highly diverse pioneer microbiome, of presumed maternal and environmental origin, with very little site-specific differentiation[7]. Within the first few days of life, and certainly by one week, differences appear between discrete anatomical niches (e.g. skin, gastrointestinal tract, mouth), with reduced species diversity and increased microbial density at each niche[14]. Indeed, WGSS confirms that samples taken from different 6 week old infants at the same site are more homologous than samples from the same infant at different sites, supporting the principle that anatomical niche is a key microbiome determinant[21]. Upper respiratory niche differentiation continues over at least the first year of life, with increasing biomass[12] and alpha-diversity (indicating species richness and evenness[9,22,23]) at each site. The rate of change slows progressively over the first year[2,24], although it remains unclear at what point the infant microbiomes approximate their stable adult-like counterparts, especially for the under-researched pharyngeal microbiomes.

A small number of longitudinal birth cohort studies characterise pharyngeal microbiome development, with a greater number reporting instead on the oral and nasal cavities. The Microbiome Utrecth Infant Study cohort[15] of 112 infants reports that the nasopharyngeal microbiome is dominated by *Streptococcus* on day one, with *Staphylococcus* overtaking and dominating from 1 to 4 weeks. Beyond the first month, these Firmicutes are progressively replaced by Actinobacteria (*Corynebacterium*) and Proteobacteria (*Moraxella* and *Haemophilus*). Certain 'keystone' genera, such as *Corynebacterium* and

Dolosigranulum are consistently associated with a more stable nasopharyngeal microbiome, while *Streptococcus* (particularly *S. pneumoniae)* and *Haemophilus* are associated with a more dynamic profile[14].

In contrast with the nasopharynx, the developing oropharyngeal microbiome displays lower within-sample alpha-diversity and between-sample beta-diversity, with a *Streptococcus*-dominated profile throughout the first year[25]. Lower-abundance colonisers like *Staphylococcus* appear in early life, and are replaced with *Neisseria* and *Veillonella* in older infants. The Danish COPSAC-2010 cohort of 700 infants[22] characterises the hypopharynx, reporting early *Staphylococcus* dominance (49% relative abundance in 1 week old infants), and gradual replacement by *Streptococcus* and *Moraxella* (29% and 23% by 3 months, respectively, compared with only 10% for Staphylococcus). OTUs making up nearly two thirds of hypopharyngeal biomass at 3 months are already detectable at one week, suggesting early establishment of persistent resident flora.

Thus, while microbiome studies differ in reported OTU richness and relative abundance, there is broad consensus of evolution from diverse pioneer microbiomes to discrete niche-differentiated communities, and of key genera across time and space.

<u>Routes of microbiome acquisition</u>

Despite these longitudinal characterisations of infant flora, it remains unclear where and how infant microbial strains are acquired. To assess maternal contribution, a number of studies compare microbiome samples from mother-infant pairs (Figure 1), although to our knowledge, there are no published reports comparing matched mother-infant oropharyngeal or hypopharyngeal samples. We identified only one study comparing

infant nasopharyngeal samples with maternal samples: Dominguez-Bello et al.[26] report no significant overlap between infant nasopharyngeal pioneer microbiota and maternal oral flora at birth, although this study lacks longitudinal results from later niche-differentiated infant microbiomes.

While pharyngeal data are lacking, longitudinal cohort studies comparing oral flora in mother-infant pairs suggest potential mother-to-infant microbial transfer[27]. Using WGSS, Ferretti et al.[7] demonstrate greater overlap between the neonatal oral cavity and that of its own mother than unrelated mothers. By 3 days old, over 95% of neonatal tongue species are also present on their own mother's tongue, although these account for less than 7% of maternal oral flora. Strains present in both mother and infant are more long-lived in the infant mouth (70.5% detected at more than one time point) than infant strains not found in the mother (only 27%). Taken together, these results suggest that a subset of maternal oral bacteria are particularly well-suited to colonising the infant mouth. Interestingly, Drell et al.[28] report that, while infant and maternal oral microbiomes overlap significantly from birth to 6 months, an infant's oral microbiome is no more similar to its own mother's than to unrelated mothers (although it is unclear whether the study is powered to detect such differences, with a sample of only 7 mother-infant pairs).

In addition to maternal oral samples, microbiome studies have also compared maternal milk, skin, gastrointestinal and vaginal flora with their infants' oral and nasal microbiota. Saliva samples from 84 infants over the first year of life clustered distinctly from their mothers' peripartum vaginal, skin and saliva samples[29], while Drell et al.[28] report minimal overlap between infant oral flora and maternal gut or vaginal flora.

Turning to breastmilk, the source of this microbiome remains inconclusive, although an enteromammary pathway has been suggested, whereby viable maternal gastrointestinal bacteria translocate haematogenously to the breast[30]. Due to very low biomass and contamination from breast pumps and skin, the core breastmilk microbiome is less well-characterised than that of the infant pharynx, mouth, or nose, with conflicting results reported in recent studies[31]. Nonetheless, there is some evidence that maternal milk microbiota contribute to the evolving infant oral microbiome. Ruiz et al.[30] compare pre-colostrum from 17 pregnant women with oral samples from their one-week old infants, reporting 19-65% shared OTUs. Using targeted culture and WGSS of suspected shared bacteria, they demonstrate strain-sharing (>99.9% homology) in 8 out of 10 paired isolates. Further, Williams et al.[32] note that *Streptococcus*, *Staphylococcus*, *Gemella*, *Rothia* and *Veillonella* were the most abundant genera in both breastmilk and infant oral samples from 21 mother-infant pairs sampled longitudinally over the first six months of life.

More controversial still is the suggestion that some infant respiratory flora may be acquired prenatally from the placenta[33]. From the late 1880s, traditional culture-based research concludes that the womb is free of microorganisms[34]. Although more recent microbiomic studies suggest in-utero colonisation (with maternal gut flora as the suspected source), there are significant issues with studying this very low biomass site, including contamination bias from placental passage through the birth canal and from molecular reagents, and no relevant studies to date have included adequate negative controls.

Finally, there is some evidence that the infant's oral microbiota overlap with its own gut flora, although such evidence is currently lacking for the pharyngeal niches. Indeed, Ferretti et al.[7] report more significant homology between infant tongue and stool samples

over the first four months of life than between paired maternal tongue and stool samples. Further, strain-sharing (>99.5% homology) is reported for a third of *Bifidobacterium* isolates identified in paired oral and faecal samples from 15 one-month old infants[35]. It is unclear whether the gut seeds the infant mouth through microaspiration, or whether oral microbiota seed the gut through swallowing, although the less acidic infant gut may be more amenable to seeding from oral flora than the adult gut[7]. Conversely, Drell et al.[28] note very little similarity between the infant oral and gut microbiomes, although this finding is limited by a small sample of only 9 babies.

Although some studies report significant differences between maternal and infant flora, this does not necessarily disprove horizontal transfer of microbiota. Indeed, maternally-derived bacteria may influence infant flora by complex niche-differentiation rather than simple migration, with a subset of maternal flora exerting a disproportionately large effect on evolving infant microbiomes.

Environmental and clinical associations

As discussed, the greatest contributor to microbial evolution is anatomical niche. Using multivariate analysis to account for the observed differences between samples taken from the same niche from different infants, the greatest contributors are the individual (R^2 18.2% for the nasopharynx) and age (10.4%)[2]. Antecedent environmental and clinical factors (Figure 1) appear to contribute far less, including co-habiting siblings (1.6%), day care attendance (0.9%), birth season (0.7%), breastfeeding (0.5%), birth mode (0.4%), and recent infant antibiotic usage (0.3%).

Comparing by mode of delivery, the pioneer nasopharyngeal microbiome of a vaginally-delivered neonate overlaps significantly more with its own mother's rather than unrelated mothers' vaginal flora, while the same does not hold true for Caesarean-delivered neonates and maternal skin flora[26] (suggesting that the predominant source of pioneer microbiota in Caesarean-delivered neonates is not maternal skin). Beyond the pioneer microbiome, however, Caesarean-delivered infants demonstrate a higher abundance and more long-lived presence of nasopharyngeal *Staphylococcus* than vaginally-delivered infants, with lower abundance and delayed appearance of genera associated with microbiome stability (e.g. *Corynebacterium*, *Dolosigranulum* and *Moraxella*)[36]. Similarly, breastfed infants demonstrate greater nasopharyngeal abundance of *Corynebacterium* (relative effect size 1.98) and *Dolosigranulum* (2.61) but reduced *Staphylococcus* (0.48), *Prevotella* (0.25) and *Veillonella* (0.33) than formula-fed infants[3]. Interestingly, Teo et al.[38] report no significant effect of feeding or delivery modality on the nasopharyngeal microbiome at 2 months, although over half of their samples were taken during episodes of RTI. Other studies also identify significant differences associated with feeding or delivery modality for infant oral flora[9,29], as well as a reduction in infant oral *Lactobacillus* and increased abundance of the phyla Bacteroidetes and Proteobacteria associated with intrapartum vaginal disinfection[39].

Co-habiting siblings and daycare attendance are both associated with reduced abundance of infant nasopharyngeal *Staphylococcus* and *Corynebacterium* but increased *Haemophilus* and *Moraxella*[38], although these variables are not significantly associated with oral microbiome profiles[15]. Further, nasopharyngeal microbiota vary with season, with greater bacterial richness and greater abundance of Proteobacteria and gram-positive Bacilli in summer-born children[40]. Recent infant antibiotic use correlates with depleted *Corynebacterium* and *Dolosigranulum* in the nasopharynx[2], while administration of the

ten-valent pneumococcal vaccine does not appear to significantly impact the nasopharyngeal microbiome[41]. Finally, distinct oropharyngeal microbiomes are demonstrated for premature versus term infants[25], and for infants of gestational diabetics versus euglycaemic mothers[17].

The conclusions of such association studies are limited by potential confounding variables. For example, Caesarean section is associated with increased use of intrapartum antibiotics, reduced breastfeeding, higher rates of maternal obesity, younger gestational age at delivery, and less exposure to the hormones, cytokines and contractions of labour, each of which may contribute to the microbial profiles observed[42].

The infant pharyngeal microbiomes and respiratory disease

In addition to these environmental and clinical associations, the infant pharyngeal microbiomes appear to correlate with respiratory disease phenotypes. During acute RTI, including bronchiolitis requiring hospitalisation, the nasopharyngeal microbiome is dominated by either *Moraxella*, *Streptococcus*, or *Haemophilus*, and is less likely to be enriched for *Corynebacterium*, *Staphylococcus*, or *Dolosigranulum* (also reported as *Alloiococcus*)[38,43]. Nasopharyngeal microbiome profiles correlate with RTI severity, with a *Haemophilus*-dominant microbiome predicting intensive care admission (odds ratio [OR] 6.43) and longer length of hospitalisation (OR 4.31)[16]. Presence of RSV in severe bronchiolitis is also associated with reduced alpha-diversity and greater perturbation from baseline microbiota[44].

RTI-associated microbiota has been shown to precede the development of RTI[38], as has loss of topography (i.e. blurring of the distinction between nasopharyngeal and adjacent

oral microbiota)[15], suggesting that microbial changes may have a causal role in developing infection. Such pathobionts are associated with respiratory inflammation[45], which may increase vulnerability to infection. However, up to 93% of infants are colonised with at least one of these pathobionts at any time, even in the absence of infection[46], pointing away from a strictly causal role in RTI. Rather, the presence of pathobionts may indicate a state of dysbiosis, in which disease-causing bacteria and/or viruses may cause infection in a previously resilient ecological niche.

As well as microbial changes preceding and during RTI episodes, recurrent RTIs are associated with long-term microbial and phenotypic changes. Recurrent RTIs appear to accelerate microbiome development, approximating that seen in older infants[2] Further, the development of childhood chronic wheeze is more common if preceded by a pathobiont-rich respiratory microbiome in infancy (such as early nasopharyngeal *Streptococcus*, oropharyngeal *Haemophilus* and *Neisseria*, and hypopharyngeal *Veillonella*, *Prevotella* and *Gemella*), as well as febrile lower RTIs and early allergic sensitisation[38,43,45,47].

Inter-individual variation makes it difficult to define a 'normal' microbiome, and therefore to define dysbiosis by comparison, and seemingly inconsistent results suggest that our understanding remains incomplete. For example, nasopharyngeal *Staphylococcus* enrichment is negatively associated with intercurrent RTI, although early nasopharyngeal *Staphylococcus* dominance is a strong predictor of earlier first RTI[38]. Further, *Moraxella*-enrichment has been associated with less stable RTI-prone microbiomes[38,43,48], but also with more stable RTI-resilient microbiomes[24] and less severe RTIs than *Haemophilus*-dominated profiles[16]. It is unclear why this is, although methodological differences are noted, including sampling (site and timing) and RTI definition (parent- versus clinician-

reported). Although many of the studies described reported large sample sizes, prospective longitudinal design, and attempts to control for confounding environmental variables, it is nonetheless difficult to interpret their significance in the absence of interventional studies.

Manipulating the infant pharyngeal microbiome

In light of these association studies, it is worth considering whether the developing infant microbiome could be manipulated to encourage favourable commensalisation, limit pathobiont colonisation, and even reduce disease. We could identify only one interventional study reporting on the infant pharyngeal microbiome: a nested factorial double-blind placebo-controlled randomised trial involving hypopharyngeal sampling (from 1 week to 3 months of life) from 695 infants following maternal administration of high-dose vitamin D3 (2400 IU per day), n-3 long chain fatty acids (LCFA), both or placebo (from 24 weeks gestation until 1 week postpartum)[3]. One month old infants whose mothers received LCFA demonstrate reduced hypopharyngeal *Gemella* and *Veillonella* abundance, and reduced putative *S. pneumoniae* for vitamin D3; these microbiota are reported to correlate with increased risk of later asthma development, and the authors speculate that dietary supplements could be used to manipulate early airway colonisation and even modify asthma risk.

Past studies employing conventional microbiological techniques have demonstrated reduction in infant pathobiont carriage following intranasal inoculation with alpha-haemolytic streptococci (strain 215)[49] or low-pathogenicity *Staphylococcus aureus* (strain 502A)[50]. Further, a handful of randomised double-blind placebo-controlled trials have reported on the use of oral probiotics in infants to prevent RTI[51], although none have

included pharyngeal microbiome sampling. We identified only three interventional respiratory microbiome studies in adults, including using a nasal probiotic spray containing *Streptococcus salivarius* 24SMBc *S. oralis* 89a[52], and controlled intranasal inoculation with *S. pneumoniae*[53]. There have been more numerous attempts to capture changes in infant gastrointestinal microbiota following probiotic administration (e.g. bifidobacteria and/or lactobacilli) to neonates and/or their mothers[54]. Finally, Dominguez-Bello et al.[55] reported on anal, oral, and skin microbiota in Caesarean-born neonates following interventional exposure to maternal vaginal fluids. However, as none of above studies involve manipulation of the infant pharyngeal microbiome, further details are beyond the scope of this review.

Conclusions

Amidst a boom in microbiome research, broad consensus has emerged regarding evolution from pioneer to niche-differentiated communities, including identification of key genera across time and anatomical space. However, inconsistencies remain, as well as challenges inherent to studying low biomass, highly dynamic microbial niches. Attempts to manipulate the infant pharyngeal microbiome, by controlled inoculation or probiotic administration to infants and/or their mothers, require large sample sizes and randomisation to control for potential confounders (e.g. mode of delivery and feeding, intrapartum antibiotics, and inter-current RTI). Looking ahead, future work must be informed by the pitfalls discussed in this review, and methodological gold standards are desirable to facilitate reproducible, comparable research.

Key points

- Studies comparing infant and maternal pharyngeal microbiota are lacking, although sampling from mother-infant pairs suggest infant oral microbiota overlap with maternal oral and breastmilk flora (including at strain-level).
- Anatomical niche and age are the most significant contributors to microbial evolution, although differences in pharyngeal microbiota are also associated with environmental and clinical factors (e.g. mode of feeding and delivery, older siblings and daycare attendance, and antibiotic usage).
- Infant pharyngeal microbial changes precede respiratory disease phenotypes (e.g. infection and asthma), and predict disease severity, suggesting a possible causal role in modifying susceptibility to disease.
- A recent study has reported on the use of dietary supplements to modify the infant pharyngeal microbiome. Probiotics and controlled bacterial inoculation may offer the possibility of microbial manipulation to improve health outcomes.
- There are ongoing challenges in the study of infant pharyngeal microbiomes, including low biomass, temporal variability, and lack of gold standards for sampling, DNA sequencing, and taxonomic reporting.

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Conflicts of interest

None

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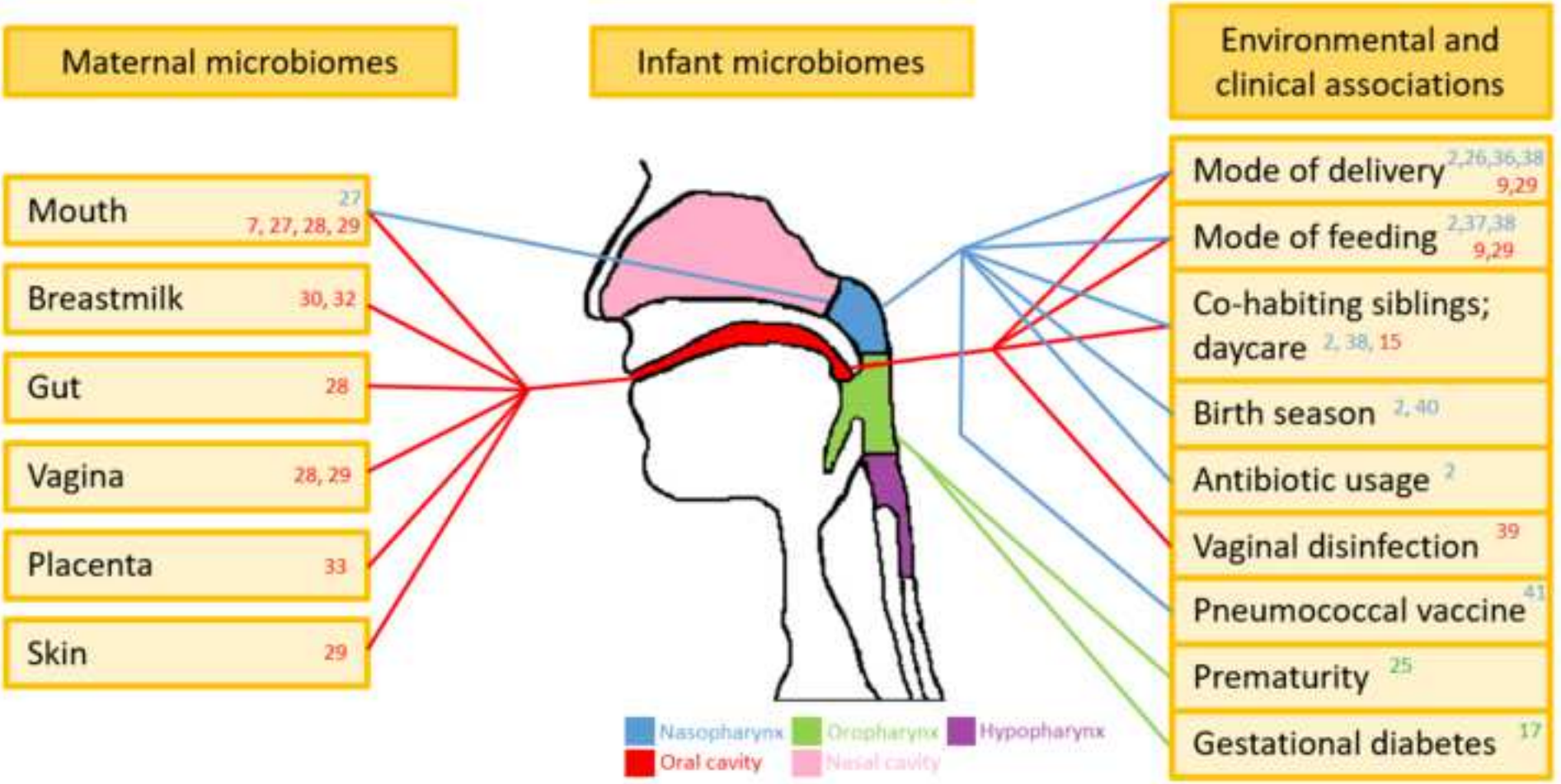
Figure 1. Maternal, environmental and clinical factors associated with infant pharyngeal microbiome profiles. Arrows indicate reported associations (rather than directional / causal relationships). Colours used for arrows and numerical references correspond to relevant infant anatomical niche.

Figure


Maternal microbiomes
Infant microbiomes
Environmental and clinical associations
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Gut 28
Vagina 28, 29
Placenta 33
Skin 29
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Mode of feeding 2,37,38 9,29
Co-habiting siblings; daycare 2, 38, 15
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Antibiotic usage 2
Vaginal disinfection 39
Pneumococcal vaccine 41
Prematurity 25
Gestational diabetes 17
Nasopharynx
Oropharynx
Hypopharynx
Oral cavity
Nasal cavity