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A reconceptualized framework for human microbiome transmission in early life

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Human development and physiology are fundamentally linked with the microbiome. This is particularly true during early life, a critical period for microbiome assembly and its impact on the host. Understanding microbial acquisition in early life is thus central to both our basic understanding of the human microbiome and strategies for disease prevention and treatment. Here, we review the historical approaches to categorize microbial transmission originating from the fields of infectious disease epidemiology and evolutionary biology and discuss how this lexicon has influenced our approach to studying the early-life microbiome, often leading to confusion and misinterpretation. We then present a conceptual framework to capture the multifaceted nature of human microbiome acquisition based on four key components: what, where, who, and when. We present ways these parameters may be assigned, with a particular focus on the 'transmitted strain' through metagenomics to capture these elements. We end with a discussion of approaches for implementing this framework toward defining each component of microbiome acquisition.

The human microbiome, the assembly of microorganisms living in and on the human body, and the genes and products of these microbes, has emerged as a pivotal determinant of host physiology and disease, influencing multiple tissues and organ systems. Early life, defined here as spanning from pregnancy to infancy, is a critical period of host-microbe interactions. The interactions range from the impact of maternally derived microbial metabolites on fetal development *in utero* to serving as a blueprint of current and future health[1]. Disturbances to the source, order of arrival, and succession of microbes during early life have been linked to infections and different physiological disorders, including cancer[2].

The terminologies describing microbiome acquisition, mainly that of vertical and horizontal transmission, have roots in infectious disease epidemiology and evolutionary biology. The origin of using *"transmission"* to describe the transfer of microorganisms from one host to another originates from Koch's works[3,4] and studies of

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invertebrates causing "*vector-born*" infectious diseases of animals and plants[5–9]. Applied to mother-to-child transmission, the term "*vertical*" can be traced to the early 1950s and the transmission of microbial pathogens[10–15]. In the coming decades, microbial transmission between parent and offspring remained in the realm of infectious diseases, being referred to as "*inheritance of infection*" or "*hereditary transmission*" (Box 1). Although the used definitions consider single infectious agents and may give information on cross-generation microbial transfer, the microbiome field has adapted the terminology for mother-to-child microbial transmission without major modifications.

Compared to the infectious disease lens, in which the term vertical transmission focuses on generational inheritance (namely from a parent), we see an added focus from the fields of ecology and evolutionary biology. In addition to generational inheritance, these scientific fields have emphasized the mechanism and, relatedly, timing of transmission. Here, the added distinction between vertical and horizontal transmission is based on *when* and, by extension, *where* transmission occurs (Box 1). A prime example of vertical transmission is the transovarial transmission of the intracellular bacteria *Wolbachia* in the fruit fly *Drosophila*[16]. In comparison, horizontal transmission of microbes, in essence, is everything else, with a focus on routes such as sexual, vector-borne, and attendant-borne transmission[17]. Well-studied examples focus on environmental sources of microbes in open ecosystems, such as the colonization of the light organ by the marine free-living symbiont *Vibrio fischeri* by the Hawaiian bobtail squid *Euprymna scolopes*, and the acquisition of the rhizosphere microbiome from soil.

Yet, within studies of the microbiome acquisition along animal and plant life cycles, there are gray areas between vertical and horizontal transmission. For example, during trophallaxis in termites, offspring may acquire the maternal fecal microbiome after hatching. In stinkbugs of the family *Plataspidae*, endocellular *γ-Proteobacteria* are transmitted to the offspring via symbiont capsules that females produce upon oviposition[18]. Such scenarios have invoked the terms "*social transmission, pseudo-vertical transmission, external maternal transmission, and postnatal vertical transmission*" to describe such microbial acquisition routes[17].

In this Perspective, we discuss how the above-described historical lexicon is often ambiguous when used in the context of human microbiome acquisition and limited in capturing the multidimensional features of microbial transmission. Given the imprecision and limitations of this status quo lexicon, we propose a conceptual framework termed 4 W to describe microbiome transmission in early life, centered on assigning four critical features: what, where, who, and when (Box 1). We then follow with a discussion of how we can capture these features in a human microbiome cohort design.

Central to an accurate description of early-life microbiome acquisition is the ability to define from '*who*', '*where*', and '*when*' transmission occurs by methods enabling tracking of the '*what*'. We define the "*transmitted microbial strain*" based on metagenomic resolution as currently the most precise unit to determine the transmission of microbes over space and time, and discuss how it can be used to assign the parameters of microbiome acquisition. We then introduce a fifth question, '*why*', discussing how a 4 W framework can address both mechanisms of early-life acquisition, and while not the focus of this Perspective, aspects of microbial assembly, such as succession and colonization. We end by providing recommendations for the design of studies aiming to capture the 4Ws of microbiome acquisition. The proposed framework will empower an expanded understanding of the transmission and factors shaping the human microbiome and the mechanisms governing the impact of the early-life microbiome on health and disease.

Ambiguity of existing terms for human microbiome acquisition

The term "vertical" has been widely used for human microbiome acquisition. In similar contexts, "vertical transmission" is broadly and ambiguously defined as 1) transmission from the mother or both parents, 2) from and to different body sites (in contact or not with the open environment), and 3) transmission during or also after birth. Although a commonly used term originating from infectious disease epidemiology, the description of "vertical transmission" in scientific reports often lacks important information, including simultaneous reporting of timing, source of transmission, and the microbial commodity being transmitted.

With a few exceptions, the term "*horizontal transmission*" is rarely used in the early-life microbiome field. Instead, other terms such as "*microbial taxa dispersal from different sources*" and "*horizontal dispersal of microbes*" have been applied. The few studies that used the term "*horizontal transmission*" generally indicated that it is not mother-to-infant microbial transmission; however, it is unclear whether the transmission of the microbiome is from other family members, the community, or the environment. That this term is not commonly used in the context of early-life microbiome acquisition and the absence of its definition shows that transmissions of microbes from the environment and others than the mother remain understudied.

BOX 1

Early-life microbial transmission: Historical definitions and the 4 W framework

Infectious disease epidemiology

"*Vertical transmission*" - The direct transfer of infection from a parent organism to progeny.

"*Horizontal transmission*" - Any transfer of infection between host individuals, except that which occurs directly from parent to progeny.

Ecology and evolutionary biology[70]

"*Vertical transmission*" - Inheritance of the symbiont from the parental generation. The emphasis has been on transmission through the female germ line, without contact with the external environment.

"*Horizontal transmission*" - From an environmental, often "free-living" symbiont source, anew by each host generation.

Proposed 4 W framework for microbial transmission in early life

"*What*" - Microbes with replicative potential, microbial structural elements, and products, such as metabolites.

"*Where*" - Both the host location/site of the origin of the microbe (*e.g.*, maternal skin), the site of infant colonization (*e.g.*, the infant gut), and the route (*e.g.*, ingestion via the alimentary tract).

"*Who*" - Who did the microbe come from? Parents, household members, pets/other animals, and the abiotic environment.

"*When*" - The timing of the event of transmission, such as during pregnancy, at birth, or at what age.

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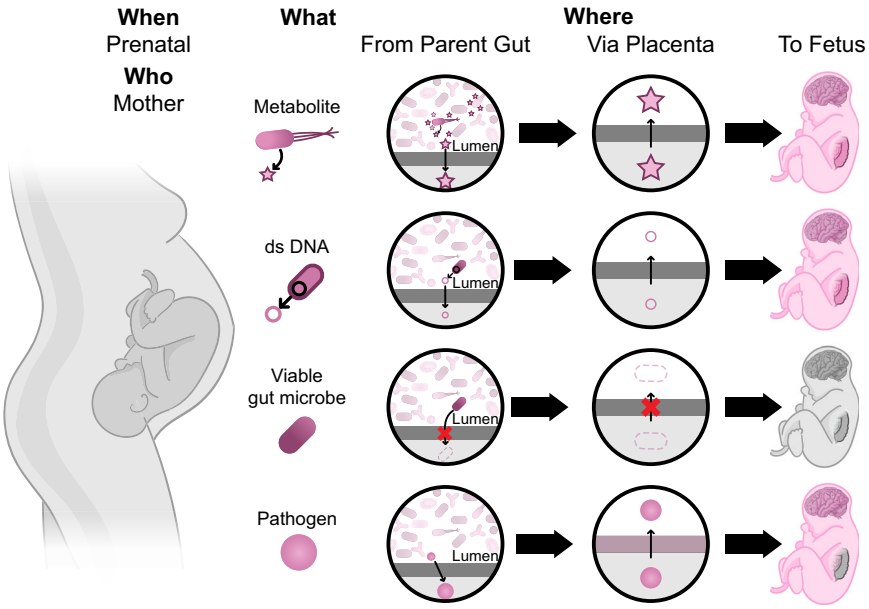

Fig. 1 | Prenatal transmission of microbes, microbial DNA and metabolites, highlighting "*what*" can be transferred and the other parameters of the 4 W framework. Different microbes colonize the mother at multiple body sites, although the focus is on gut bacteria here. Microbially derived metabolites (*what*) can translocate from the mother gut (*from who*) during pregnancy (*when*) into the intestinal lamina propria and blood circulatory system. Metabolites can also cross via the placenta and affect the developing brain of the fetus (*where to*). Microbially derived DNA from the parent gut can translocate into circulation, cross via the placenta, and may enter different sites in the fetus, including the gut. Sequencing-based techniques may detect the presence of microbial DNA, even when live microbes are not occurring in the system. Live organisms are not expected to translocate outside the gut or cross the placental barrier into the fetus. Pathogenic microorganisms like *L. monocytogenes* can translocate from the gut and infect the placenta and fetal brain tissue.

What terms, if any, should we use to describe early-life microbiome acquisition? If based on an evolutionary biology definition focused on the timing of transmission, the use of "*vertical*" and "*horizontal*" transmission in reference to early-life microbiome transmission may similarly be imprecise and potentially inappropriate. If defined as transmission before birth, the acquisition of microbes with replicative potential is limited to pathogens in an uncomplicated pregnancy, therefore excluding "*vertical transmission*" from an evolutionary framework. Similarly, as we will describe below, when considering microbial transmission from a broader perspective, including microbe-derived factors such as metabolites across the placenta, indeed, "*vertical transmission*" from an evolutionary definition may apply, yet this situation is seldom considered in the common use of the term. For vaginal delivery, do we consider this vertical, horizontal, or rather use quasi-terms as in the evolutionary literature? As such, one of the fundamental features distinguishing vertical and horizontal transmission is whether transmission occurs in a closed (transplacental/transovarial) or open ecosystem (consider whale vaginal birth) and thus varies in ecological competition and fidelity of transmission[19]. We are faced with a common problem in lexicology: the misuse of commonly accepted terms or their acceptance, explicit statements of definition, and even redefinition of terms.

A conceptual framework of microbial acquisition and transmission

As reviewed above, precise terminology for different mechanisms of transmission is lacking, and the existing terms referring to the transmission of microbial strains from mother to infant fail to capture the multifaceted nature of microbial acquisition. Ultimately, what do we want to know about early-life microbiome acquisition?

Here, we provide a conceptual and operational framework of early-life microbial transmission structured around four central components (4 W): what, where, who, and when. Characterizing transmission events according to each of these components is critical to our understanding of the assembly of the human microbiome and the mechanisms by which the microbiome impacts human physiology and disease. This precise characterization should inform study design, methodology, and results interpretation when studying the early-life microbiome. Here, we lay out this conceptual framework and provide examples of how these parameters may characterize new and unanticipated transmission events.

What. When considering transmission of the microbiome, we must consider "*what*" is the transmitted commodity. These might include cells (or, in the case of viruses, virions, viroids or even viroid-like) that have replicative potential (inclusive of microbes in dormant phases such as spores), microbially derived components, such as different structural elements of the cells (proteins/peptides, nucleic acids, mobile genetic elements, lipids and sugars), and their metabolites (Fig. 1). Classically, when thinking about the assembly of the infant microbiome, the operative "*what*" are microbial cells that can "*seed*" and subsequently replicate for "*colonization*". Importantly, the workhorse of identifying the microbial "*what*" of the microbiome is next-generation sequencing (NGS), such as amplicon (*i.e.*, bacterial 16S rRNA gene or fungal internal transcribed spacer region) and shotgun metagenomic sequencing, which is based on DNA present in the sample and thus not indicative of "*colonization*" per se. Advances in single-cell microbial genomics hold the potential for bridging this discrepancy. At present, metagenome-defined strains are the units most accessible to infer transmission and thus assess microbial acquisition in early life (Box 2; Fig. 2).

A weak signal of the nucleic acid translocation might be detected by NGS and considered as a fetal microbiome. Relatedly, with the exception of colonization of the placenta and fetus with pathogens (such as Group B streptococci; GBS, see "*when*" below[20]), contamination with microbial DNA during post-fetal sampling has also been misinterpreted as evidence of placental or fetal colonization[21]. Because of the latter challenges, one must control for contamination, which is omnipresent in microbial studies and can happen at any stage of

BOX 2

The 'transmitted strain' as a tool to define microbial acquisition

The 4 W framework provides a conceptual approach to deconstruct microbial transmission, whether in early life or throughout the lifespan. Operationally, defining the 4 W parameters of transmission requires both a toolkit and methods to identify specific microorganisms at the strain level, the lineages they share, the source, and the timing of transmission. In essence, this can be achieved in two ways. First, using observational design, we can identify whether the '*same*' strain is present in samples across the 4Ws (i.e., present in maternal vagina, absent in maternal stool, and detected at specific timepoints after birth in infant stool). A second method of microbial tracking is interventional. A defined strain is introduced as a '*pre-source*' to populate a defined index case '*source*' for both '*who*' and '*where*'. The strain can be traced over time in the infant samples by metagenomic sequencing, genome tag-based identification, or whole genome sequencing upon isolation. Central to this is our ability to identify a '*strain*' using metagenomics readouts and computational approaches. In the following, we discuss the conceptual basis (referring the reader to other papers outlining computational methods)[71,72] of defining the "*transmitted strain*" as a tool to capture the early-life microbial transmission (Fig. 2).

Strain-level resolution metagenomics

Thanks to developments in metagenomics, researchers now have the throughput and ability to accurately profile hundreds of the microbial inhabitants of the human body, together with the genomic resolution to infer transmission between individuals. The human microbiome is person-specific, and multiple tools are now available for profiling genomic variants of microorganisms with strain-level resolution[73,74]. When applying these tools to infer microbial transmission, common questions remain: "*How similar should such variants be to consider that a variant in an infant is the same as that in the mother/other individual and thus presumably of their origin*?". An overly stringent threshold means missing the detection of some of such events, while one that is too lenient causes false positives, i.e., assuming transmission when that is not the case.

Transmitted strain - key unit to determine microbial transmission

When considering microbial transmission among individuals, the term "*strain*" can be used to describe genomic variants of microorganisms that are, in principle, unique to individuals, so that when sharing of a strain between individuals is detected, one can infer a transmission event[36]. This use of "*strain*" differs between fields, for example, in classic microbiology, "*strain*" represents "*a set of genetically similar descendants of a single colony or cell*", while in phylogenetics, the term is sometimes used for leaf nodes[71]. In metagenomics, metagenome-assembled genomes (MAGs) are also sometimes called "*strains*"[4,72,75]. Similarly, to the earlier discussed terms for microbial transmission, the above definitions of "*strain*" are not suitable for describing transmission in the context of the human microbiome. Therefore, we propose an operational definition of a "*transmitted strain*" as a "*person-specific genetic cluster within a species that allows transmission among individuals to be inferred*" (Fig. 2).

Beyond arbitrary: Refining microbial strain definitions for transmission

While we acknowledge that different methods can give different results (e.g., profiling marker genes or comparing assembled genomes, building phylogenetic trees, or analyzing a multiple sequence alignment), the suggested operational definition of transmitted strain serves the purpose of inferring microbial transmission. The definition of "*strain*" has often been arbitrary, consisting of a given percentile of a distribution of (phylo-)genetic distances and not based on the identification of person-specific genomic variants. Still, the field is moving forward to identify thresholds for genomic similarity that allow for the inference of transmission more accurately[36]. In addition, as the multiple species in the microbiome evolve at different rates, the thresholds for strain identity are best defined on a species-by-species basis[36]. For non-bacterial members of the microbiome, other specific thresholds are being defined[65]. For ecology and evolution studies of the early-life microbiome, the same definition of "*strain*" holds; however, more detailed genomic knowledge is necessary than for transmission studies[76].

Experimental methods for microbial source tracking

In addition to advancements in bioinformatic approaches for microbial source tracking, definitive approaches to define sources of microbial transmission have been applied, such as controlled transmission experiments and strain-tagging. When ethical and feasible to conduct, these studies provide a distinct advantage of minimizing ambiguity in inferring sources of transmission.

Controlled transmission experiments. Administration of specific strains (*e.g.*, probiotic) to focal people (such as mothers) and tracking in offspring, for example, by strain-specific PCR or genome sequencing of cultured isolates. Examples include evaluating the transmission of *Bifidobacterium* probiotic candidates (Probio-M8) ingested by lactating mothers, after which detection was assayed in maternal fecal and breast milk samples and feces of infants[31].

Strain-tagging. Genetic tagging of bacteria, such as integrated random barcodes [sequence tag-based analysis of microbial population dynamics (STAMP) and similar approaches[77]] and wild-type isogenic standardized hybrid (WISH) tags, has been used to label pathogens and trace infections in animal models. Such an approach, combined with the controlled introduction of tagged strains, can be used to define both the sources of transmission and additionally determine the population bottlenecks during early-life transmission.

sample collection and processing, with well-to-well contamination being a major culprit in microbiome studies. Stringent negative and positive controls need to be included, especially in the case of low microbial biomass samples (e.g., human milk, skin). Several approaches are now available to prevent and detect contamination, including sterile sampling and spike-in quantitative approaches for low biomass communities[20,22]. Similarly, a variety of open-source tools exists, from lists of common contaminants and guidelines on how to reduce well-to-well effects to reports on technical biases in general.

In addition to using NGS approaches for defining the "*what*" of transmission, advances in analytical techniques such as metabolomics and metaproteomics have enabled the characterization of other essential units of the microbiome (e.g., proteins and metabolites) that may be transmitted in early life. Metaproteomics seeks to comprehensively define the proteins in a given sample. Thus, proteins or shorter peptides detected in infant samples and assigned as of microbial origin may serve both as evidence of microbiome transmission and the potential for function. At the same time, the use of metaproteomics for the study of microbial transmission in early life has been limited to a few pioneering studies so far. For example, bacterial peptides have been detected in the amniotic fluid derived from uncomplicated pregnancies, as well as within extracellular vesicles isolated from amniotic fluid[23] and human milk[24]. The metabolome, the collection of small molecules within a given sample, can also be

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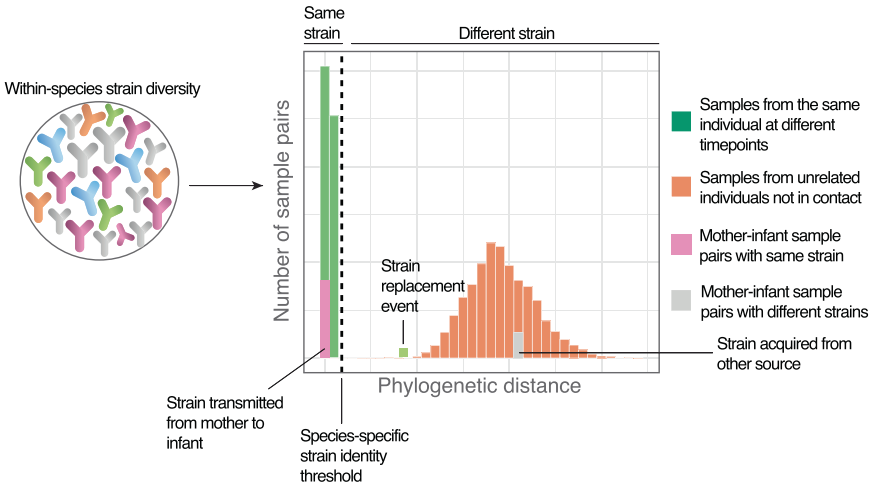

Fig. 2 | Species-specific operational definition of a transmitted strain. Strain boundaries should be identified on a species-by-species basis and based on a comparison of (phylo-) genetic distance distributions of strains detected in longitudinal samples from the same individual (same strain; green distribution) to those between unrelated individuals who have never been in contact (different strain; orange distribution). While some strain replacement events might occur within an individual's microbiome even without any intervention (e.g., antibiotic treatment, diet changes), these are a limited minority in samples taken less than six months apart[36]. Once such thresholds are established, the origin of a strain in the infant can be inferred (maternal: pink distribution; from an unknown source: gray distribution). Sampling of more environments, individuals, and body sites thus adds to the "*from where/who*" and "*to where/who*" dimensions, while the collection of samples from multiple time points allows to establish "*when*" the transmission event took place.

used to define microbiome transmission. Microbial-derived metabolites, such as short-chain fatty acids or 4-ethylphenylsulfate produced by bacteria in the maternal gut during gestation, may enter circulation and cross the placenta into fetal circulation[25]. Microbially-derived metabolites have been reported so far in human amniotic fluid[26], fetal intestine[27], and breast milk[28]. In addition, free nucleic acids derived from maternal microbes (most notably pathogens) may interact with the placenta, acting as agonizts of innate immunity. Effects of such microbial products can be entry into fetal blood/tissues, and subsequent activation of inflammatory programs impacting the fetus or stimulation of fetal intestinal lymphoid system (Fig. 1).

Critically, while some proteins and metabolites can be clearly defined as of microbial origin [such as distinct primary (e.g., acetate) or secondary metabolites (e.g., lantibiotics)], other proteins or metabolites can be synthesized by both host and microbes (e.g., acetate or secondary amino acid metabolites such as serotonin and polyamines). Thus, ascribing such proteins or metabolites as of microbial origin and transmitted by the microbiome requires specialized approaches to trace the source of such molecules. Two approaches, limited to animal studies, have been used to date. This first has been using 13 C or 15 N labeled substrates, such as dietary fibers or proteins, respectively, in mice with (conventionally reared) or without (germ-free) a resident microbiome. This strategy is followed by sampling of mouse tissues and detection of labeled proteins and/or metabolites, whereby comparison of labels between conventional and germ-free mice defines microbial origin. To date, such an approach has not been used to define early-life transmission. Alternatively, bacteria can be labeled isotopically in vitro, before introduction to mice, followed by sampling of mouse tissues, in essence a 'pulse-chase' experiment. Such an approach using auxotrophic bacteria to eliminate colonization of labeled bacteria at different timepoints has been used to define 'when' (gestational, postnatal) and '*what*' (amino acid metabolite, nucleic acid, protein) of early-life transmission of microbial products in mouse models.

Where. The "*where*" of microbial transmission can be thought of as the route of transmission of the "*what*", i.e., from where/to where, often occurring "via" conduit(s). During gestation, pathogens, classically such as *Listeria monocytogenes*, which originate "*from*" the maternal gut, translocate via the epithelial barrier to the maternal blood and the placenta, eventually disseminating "*to*" fetal systemic and neurovascular circulation[29,30]. Another example is the intracellular protozoan *Toxoplasma gondii*, a parasite of cats with a range of intermediate hosts (e.g., rodents) that transmits from mother to fetus at the placenta, resulting in congenital infection. In vaginal delivery, the birth canal is the main route of microbial transmission, with microbes "*from*" the maternal vaginal and fecal microbiomes, skin, and nearby environment, being transmitted "*to*" the newborn's skin, oral cavity, and gut, the latter "via" oral ingestion (Fig. 3A). During delivery by Cesarean section, skin microbes from the mother are transferred to the infant, in addition to microbes transferred from the medical staff or operating room (Fig. 3B). Breast milk and transmission of breast milk microbiota to the infant during lactation (Fig. 4A) is another example of "*where*", such as maternal bacteria transmission via the oral-entero-mammary route[31,32].

Who. Related to where is the "*who*". Here, the "*to who*" is defined as the biological offspring. The "*from who*" can vary from both human (mother, father, parent, siblings, health-care providers, etc.) and non-human (pets) and sources from the abiotic environment (food, air, built environment, such as a newborn nursery). "*Who*" can also involve secondary actors or routes, for example, microbes transmitted from the environment via a secondary carrier. As a hypothetical example, imagine a family dog that carries goat stool microbes on its mouth and transfers them by licking an infant's mouth or skin (Fig. 4B).

When. Finally, the time of acquisition is captured by the "*when*". This can be broken down into discrete, operational periods, such as at which gestational week, at which stage of delivery (e.g., premature rupture of membranes), and post-delivery, and including important transitions in diet (suckling and weaning). This final parameter defining early-life microbial transmission emphasizes that the specific timing of when microbes and their products are transmitted is critical to ecological succession, microbial competition, and immune tolerance windows[33]. An example where the timing of microbial transmission/colonization has been shown to be important is microbiome disturbance by antibiotics in infancy, which is linked to an increased disease risk later in life[34]. Since exposures to antibiotics might have a different effect if occurring exclusively during pregnancy or after

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delivery, this points to distinct time windows by which microbiome transmission impacts infant health.

The importance of microbial transmission timing can also be derived from infectious diseases, with examples such as congenital, perinatal, and postnatal acquisition of pathogens, such as cytomegalovirus, human immunodeficiency virus, and herpes simplex[35]. There, the timing of pathogenic microbe transmission affects the disease risk, with multiple sources having an additive effect on the rate of transmission. The "*when*" parameter of microbial acquisition also illustrates the multidimensional nature of the early-life microbiome since determining only "*when*" without any information on the microbial sources ("*where*" and "*who*") is bound to give incomplete information. For example, GBS, which may cause severe neonatal infections such as sepsis and meningitis, is considered of maternal origin (acquired during birth) if it causes early onset infection (< 72 h after delivery). Yet for late-onset sepsis, GBS could be acquired from the mother via various routes, including breast milk or other sources. Hence, to identify the GBS source ("*what*") with high certainty, samples from the infant, mother, and their environment ("*where*" and "*who*") would have to be collected during pregnancy and the first month of life ("*when*").

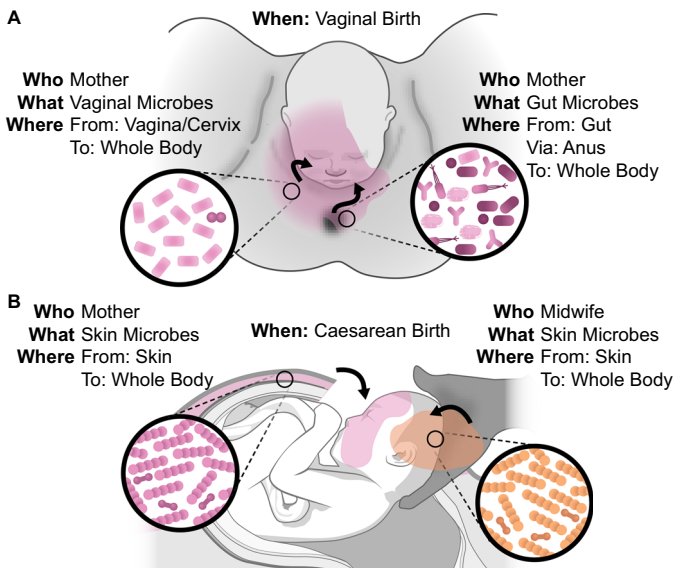

Fig. 3 | Microbial acquisition during birth, highlighting *"from where"* microbes can be transferred as well as other parameters of the 4 W framework. A During vaginal birth (*when*), microbes (*what*) are transferred from the mother's (*who*) vaginal and fecal communities (*where from*) to the child's oral, gastrointestinal, and skin communities (*to where*). **B** During Cesarean section, skin microbes are transferred from the skin of the mother and health personnel to the infant.

Everything, everywhere, from everyone, all the time

Unlike the movie, it is not feasible nor are agencies likely to fund a prospective study encompassing the multiverse of samples and methods of analysis, powered to capture the 4Ws in the scope and depth required to answer the who (from and to), what, where, and when of each microbe and microbial product of early-life transmission. Ultimately, the design of a prospective study of microbiome acquisition (although not limited to early life period) will depend on a balance of the constraints of a study (e.g., budget, sample collection infrastructure, storage, and technical/analytic capacity), the primary and secondary aims of the study, and which and how many of the 4Ws should be captured to answer specific questions. In Table 1, we highlight a selection of representative studies of early-life microbiome acquisition, presenting these studies through the lens of the 4 W framework, defining which aspects of 4 W were captured, focused on, and how these allowed for the definition of early-life microbial transmission. In Box 3, we provide case studies in which specific questions can be answered and prioritized (and 'future-proofed' for the potential to address additional and forthcoming questions and aspects of transmission) through weighing sample collection and analysis for specific aspects of the 4Ws. Below, we discuss theoretical approaches to capture the 4Ws in a prospective study of early-life microbial transmission. Although we focus our discussion of the 4 W framework on microbial acquisition at early life, the lens of 4 W can be applied for microbial transmission, colonization, and succession throughout a person's lifetime[36], such as defining the 4Ws of microbiome ecology during travel, hospital admissions, and microbiome repopulation through and after antibiotic use.

For the "*what*" component, the status quo, allowing definition and tracking of the 'transmitted strain', is shotgun metagenomics. As most (but not all and not all the time[22]) human microbiomes are dominated by bacteria, metagenomics will bias to a bacterial *'what'*. However, it is crucial to understand that microbial transmission is not restricted to

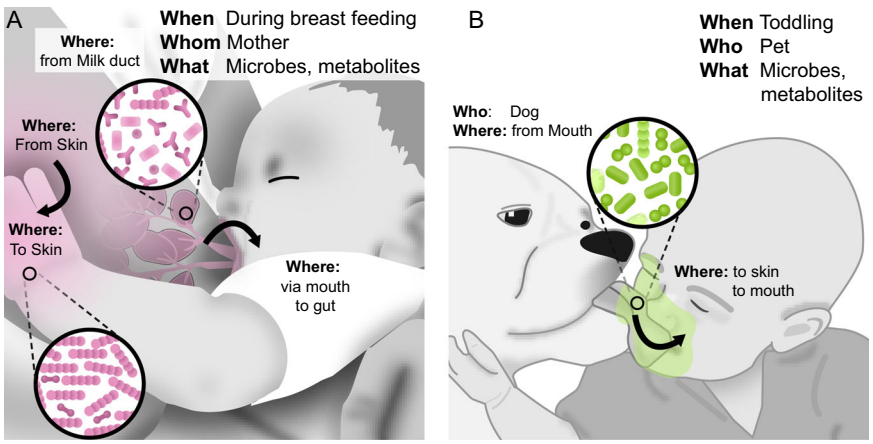

Fig. 4 | Microbial acquisition after birth, highlighting the parameters of the 4 W framework. A The mother (*who*) transfers microbes and metabolites (*what*) to the child via breastfeeding (*when*) from breast milk (*from where*) to the mouth, gut, or skin of the infant (*to where*). **B** Transfer of microbes from the dog's skin and mouth to the baby's skin and mouth.

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Table. 1 | Presentation of 12 representative studies investigating microbiome transmission and acquisition through the lens of the 4 W framework

Study	What	Who	Where	When	4 W focus and Main findings
Ferretti et al., 2018[62]	Shotgun metagenomic sequencing **Analysis focus:** Bacterial microbiome	25 mother-infant pairs (Italy)	Maternal samples from stool, vagina, skin, and tongue. Infant samples from stool and tongue.	Sampling at delivery for mothers, and at days 1, 3, 7, and months 1 and 4 for infants.	**4 W focus: Who** Among the first studies providing evidence of bacterial strains transmission from mother to infant, focusing on the "*who*". **Maternally- transmitted strains were more likely to persist in the infant gut than non-maternally acquired strains**. The study identified a high inter-subject variability of strains in the general population and suggested transmission only for cases in which a mother and her infant have a strain similarity substantially higher than that found between unrelated subjects.
Feehily et al., 2023[51]	Shotgun metagenomic sequencing and cultivation + whole genome sequencing (WGS) of focal taxa **Analysis focus:** Bacterial microbiome at the population and single-isolate level	135 mother-infant pairs (Ireland)	Maternal samples from stool, vagina, breast milk, and oral rinse. Infant samples from stool.	Sampling at 16 and 34 weeks of pregnancy, and at 1- and 3-months post-partum for mothers. Breast milk samples collected at 1 month. Infant stool samples collected at delivery, within 1 week, 1 month, and at 3 months.	**4 W focus: What and Where** The study combined metagenomic sequencing with isolation and sequencing of bifidobacteria from mother-infant pairs, highlighting the method effects in the analysis of "*what*". The authors showed that several bacterial strains are commonly transferred, with a focus on the *Bifidobacterium* genus. **Strain transfer was found in about 50% of dyads, with some transfer events uniquely detected either by cultivation or metagenomic sequencing**. The authors used phylogenetic analysis rather than strict nucleotide identity thresholds alone to identify mother and infant strains that belonged to the same lineage.
Manara et al. 2023[63]	Shotgun metagenomic sequencing **Analysis focus:** Bacterial and protozoal microbiome	Mother-infant cohorts from Ethiopia Locally produced fermented food.	Fecal samples from infants and mothers.	Different longitudinal study designs.	**4 W focus: Where and When** The study investigated **variations in mother-infant strain sharing in westernized and non-westernized communities**, with the latter having reduced microbiome sharing. Further, **locally produced fermented food provided an important microbial source for the infants**. The study also explored the potential transmission of the eukaryotic fraction of the infant microbiome ("*what*"). The study highlighted the need to analyze transmission in non-Westernized countries due to the high level of uncharacterized bacteria.
Mitchell et al. 2020[64]	Shotgun metagenomic sequencing and 16S rRNA sequencing **Analysis focus:** Bacterial microbiome	75 mother-infant pairs (USA)	Maternal rectal and vaginal samples. Neonatal stool samples.	Maternal rectal and vaginal samples were collected 24 h before delivery. Newborns' stool samples were collected daily at days 0-4 and at 2 weeks of life	**4 W focus: Where and When** This study investigated two maternal sources (vagina or rectum) and found evidence for mother-to-child transmission of rectal rather than vaginal strains, *i.e.*, from "*where*". Further, investigations of the timing of microbial signatures ("*when*") associated with delivery mode suggested that **differences in colonization stability of transmitted strains are an important factor shaping infant gut microbiome composition**.
Zhog et al. 2022[31]	Shotgun metagenomic sequencing and cultivation + whole-genome sequencing of probiotic strain **Analysis focus:** Bacterial microbiome at the population and single-isolate level	11 mother-infant pairs (China)	Fecal (infants and mothers) and breast milk (mothers) samples.	Breast milk and feces of mothers and infants were collected continuously once or twice a week for 8–15 weeks after starting the Probio-M8 intervention.	**4 W focus: What and Where** A biomarker strain, *Bifidobacterium animalis* subsp. *lactis* Probio-M8 was given to lactating mothers to evaluate the bacteria transmission by integrated culture-dependent and independent methods. **First study to provide strain-level evidence of mother-to-infant bacterial transmission** via **the oral-entero-mammary route**. A strong focus on the "*where*" and "*when*" by controlling the "*what*".
Sprockett et al. 2020[58]	16S rRNA sequencing **Analysis focus:** Bacterial microbiome	47 mother–child dyads from Tsimane people, Bolivian Amazon	Stool samples and tongue swabs were collected from infants and adults.	Samples collected between September 2012 and March 2013 using a mixed longitudinal design.	**4 W focus: Who and Where** The authors assessed how transmission dynamics affect community assembly in a unique population of an indigenous forager-horticulturalist population ("*who*"). **First study to highlight the importance of neutral forces during microbiota assembly**, whose patterns were consistent with infant care-associated behaviors.

Table 1 (continued) | Presentation of 12 representative studies investigating microbiome transmission and acquisition through the lens of the 4 W framework

Study	What	Who	Where	When	4 W focus and Main findings
Garmaeva et al. 2024[65]	Virus-like particles extraction + Shotgun metagenomic sequencing of stool **Analysis focus:** Virome and bacterial microbiome	30 women and their 32 infants (Netherlands)	Maternal and infant stool samples.	Maternal samples collected during pregnancy (weeks 12, 28, and close to birth) and the first 3 months. Infant samples collected at 1, 2, 3, 6, 9, and 12 months of age.	**4 W focus: What and When** First study to show that the composition of maternal gut virome remains stable during late pregnancy and after birth. On the other hand, **infant gut virome contains a higher abundance of active temperate phages, which decreases over the first year of life. The study also provides evidence of co-transmission of viral and bacterial strains from mothers to infants**. The focus is on "*what*", but this study also includes a strong "*when*" with longitudinal sampling of both mother and infant.
Dubois et al. 2024[66]	Shotgun metagenomic sequencing **Analysis focus:** Bacterial microbiome	81 families, including 81 babies, 80 mothers, and 70 fathers (Finland)	Stool samples from infants, mothers, and fathers.	Infant samples collected at 3 weeks, and 3, 6, and 12 months. Parent samples mostly collected before birth (89%,127/144) with a median of 8 days before birth.	**4 W focus: Who** The study for the first time documented **stable bacterial transmission from fathers to infants ("*who*")**. The father complemented microbiome assembly independently of mode of delivery, and **fecal microbiota transplantation successfully restored maternal seeding in Cesarean births**.
Selma-Royo et al. 2024[67]	Shotgun metagenomic sequencing **Analysis focus:** Bacterial microbiome	34 mother-infant pairs from the MAMI cohort (Spain). Two birth environments: hospital and home.	Maternal and infant stool samples. Breast milk.	Infant samples collected at 7 days and 1, 6, and 12 months. One faecal and one milk sample collected 1-month postpartum from mothers.	**4 W focus: When** **First study to describe how place of birth impacts mother-to-infant microbiota transmission timing** ("*when*"). Strain-level analysis of *B. longum* highlighted subspecies replacement patterns mainly explained by breastfeeding practices (focus on particular "*what*" and "*where*").
Shei et al. 2019[68]	18S/ITS rRNA sequencing **Analysis focus:** Fungal microbiome	298 mother-offspring pairs (Norway)	Maternal and infant stool samples	Maternal samples collected at 35–38 weeks of gestation and 3 months postpartum. Infant samples obtained at 10 days, 3 months, 1 year, and 2 years.	**4 W focus: What** **One of the first studies to describe the gut fungal microbiota establishment on a relatively high number of mother-infant pairs**, with respect to the quantity, diversity, and association with the maternal gut mycobiome ("*what*"). The diversity and composition of gut mycobiome showed a succession towards the maternal mycobiota as the child aged ("*when*").
Kaisanlahti et al. 2023[23]	Extracellular vesicles (EVs) analyses by transmission electron microscopy, nanoparticle tracking, proteomics, 16S rRNA amplicon **Analysis focus:** Bacterial microbiome	28 pregnant women undergoing elective C-section delivery (Finland)	Maternal amniotic fluid and stool samples.	Amniotic fluid samples collected under sterile conditions during the Caesarean section. Fecal samples obtained from the mothers before the Caesarean section.	**4 W focus: What** **First study to describe the presence of bacterial EVs in the fetal environment during healthy pregnancies ("*what*")**. The EVs detected in amniotic fluid exhibited similarities to EVs found in the maternal gut microbiota.
Vatanen et al. 2022[69]	Shotgun metagenomic sequencing, LC-MS **Analysis focus:** Bacterial microbiome, fecal metabolome	70 mother-infant dyads (Finland)	Infant and maternal stool samples. Breast milk.	Infant samples collected at 2 weeks and monthly between 1 and 12 months. Breast milk collected between days 13–29 after delivery. Maternal stool collected in late pregnancy.	**4 W focus: Where and What** **First study to show that the maternal microbiome also shapes the infant gut microbiome through horizontal gene transfer** events by describing the discovery of hundreds of mother-to-infant gene transmission events in the absence of maternal carrier strains in the infant gut. Further, the study included the analysis of metabolites as a "*what*". Infant gut metabolomes were less diverse than maternal but featured hundreds of unique metabolites and microbe-metabolite associations not detected in mothers.

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BOX 3

A theoretical and logistical study design using the 4 W framework

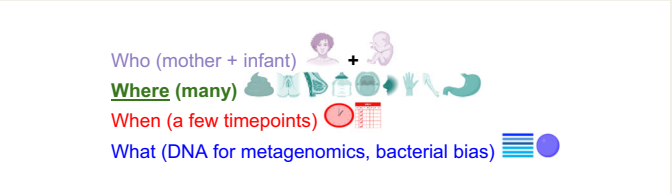
In theory, a prospective study of early-life transmission would collect (as we joke) everything (‘*what*’) from everyone and every place (‘*who*’), everywhere (‘*where*’, duh), all the time (‘*when*’). In theory, unless you know from whom, to where, and when transmission occurs, then anything is possible, and the sample collection and analysis strategy needs to be nearly open-ended. But in practice, (1) not all possibilities are likely (yes, the Yoga instructor or kitchen sink could be a source to the baby), (2) not all data is relevant, and (3) most importantly, there are limitations. These range from budget and sample volume to various logistical, technical, and computational capacity constraints. Ultimately, one must ask what aspect(s) of early-life acquisition one wants to know. From there, based on balancing the limitations outlined above and with a certain degree of future proofing, *i.e.*, sample collection to address alternative hypotheses/ additional questions, one can design generalist (capture all 4Ws broadly) or specialist (capture one or two 4Ws with breadth and depth) studies. Below, we describe four study designs emphasizing distinct questions and focusing on the capture of distinct 4 W.

‘Who’ are the sources of the early-life microbiome? Data suggests that a majority of the strains of the gut bacterial microbiome of infants come from origins other than the mother. What are the sources of these microbes? This study will focus on the parameter space of ‘*who*’, sampling potential sources of microbial transmission to the infant: the birthing parent, non-birthing parent, individuals present during labor and delivery, home environment (including pets), built environments like hospitals, and dietary sources. From there, one would decide the restricted parameters of the other 4Ws to address from where the infant gut microbes derive: ‘*where*’ (feces, vaginal, skin, or mouth) and which microbial commodity (‘*what*’). Or one can flip this to address which oral sources across ‘*who’s*’ are the source for the infant gut microbiome. In the restricted parameter space of ‘*when*’ would be a decision of the time period and frequency of sampling the defined ‘*what*’. ‘*Who*’ focused studies will benefit by defining a contact index of the time, distance, and nature of ‘*who*’ - infant interactions (touch, kiss, hold) to associate the strength of such indices with acquisition.

Example sampling design:

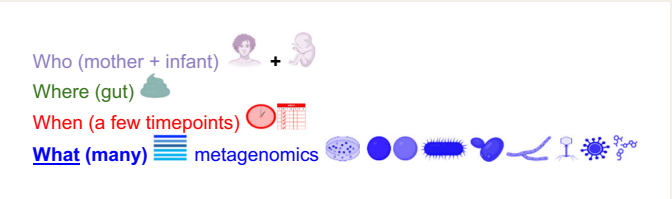
From ‘where’ on the mother is the infant gut microbiome acquired? This study would focus on a restricted ‘*who*’ (mother and infant), ‘*when*’ (cross-sectional or minimal time series from birth), ‘*what*’ (microbial focus of choice), while emphasizing multiple ‘*where’s*’ (oral, multiple skin sites, mother’s milk, gut, vagina, from one or both the mother and the infant). If one finds strain-matching from mother’s milk or breast skin to the infant gut, one can infer transmission from these origins. Couple this by expanding the ‘*when*’ component, one can find strains that first are detected in the infant (presumably from a non-maternal source) and then in the mother, for reverse acquisition. A quantitative ‘*what*’ (absolute abundance of such a strain) can weigh the probability of transmission of one possible source from the other (*i.e.*, higher abundance of the same strain in the maternal gut versus vagina). Sampling of intermediate route ‘*where’s*’ such as detection of strains in an oral and/or gastric samples (i.e., pre-term infants in intensive care units) can define routes of transmission based on absence or presence at each site, such as direct maternal to infant skin without oral-gut seeding versus a route from maternal skin to infant mouth and subsequently the gut via the stomach.

Example sampling design:



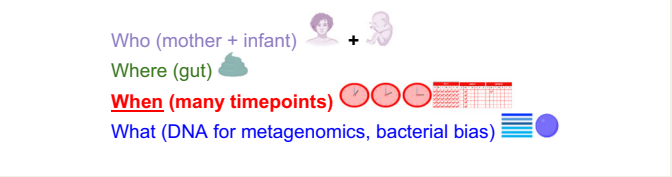
What is transmitted at birth? Here, the emphasis is placed on defining as many what’s simultaneously as possible, critical to defining ecological mechanisms (‘*why*’) of early-life transmission, across a restricted space of ‘*who*’, *where*’, and ‘*when*’. For example, a study could seek to define as many ‘*what’s*’ as feasible for a given mass/ volume of a sample. How many ‘*what’s*’ can one ascertain from the same sample will depend on microbial load (bacteria vs. fungi) and detection limits of different methods (extracted DNA amount, cultivation). Key is the choice of the ‘*where*’ (feces vs skin swab) and the technical capacities such as the ability to perform semi-quantitative measurements across the ‘*what’s*’ (cross-kingdom) and capabilities to build a metagenomic library from low amounts of DNA.

Example sampling design:



When does transmission occur in early life? The dominant parameter is ‘*when*’ optimizing dense and/or extended periods and focusing on a restricted (or relaxed, such as to capture more ‘*who’s*’ at focal time points) sampling space for the other 4Ws. A dense time series of a maternal and infant dyad (‘*who*’) of a relaxed ‘*where*’ (either oral, vaginal, or fecal), with focal ‘*what*’ (metagenomics on strain-level) can define when acquisition events from distinct sites may occur. Similarly, coupled with a relaxed ‘*who*’ parameter, with a dominant yet focused ‘*when*’ parameter space, such as by collecting samples at birth and over time from multiple ‘*who’s*’ can enable determination of specific windows.

Example sampling design:



bacteria, which have been the most studied to date. Viruses, fungi, and protozoa, as well as different microbial metabolites and structural components, play significant roles in early life development and deserve sufficient attention from the research community. Current and future studies need to expand the description of "*what*" in terms of whether targeting microorganisms or metabolites, including the resolution level of the identified organism or metabolite. This will require improved sequencing techniques, such as the use of long-read sequencing to provide better strain-specific identification, tools like Hi-C cross-linkage to map mobile elements and viruses to their host organisms, deep sequencing to allow full coverage of rare community members, and targeted enrichment of hard-to-capture organisms and clades[37].

Related to the '*what*' is '*how much of what*'. Both NGS workflows and omics-based measurements (such as metabolomics and proteomics to a certain extent) are semi-quantitative, providing the relative abundance of the '*what*'. Such readouts limit our understanding of critical aspects of microbial transmission. First, our ability to determine microbial growth and colonization versus passing through without replication is limited when we measure the relative abundance of DNA through NGS. Second, measurement of one domain of life (as above), let alone the relative abundance of this domain, curtails our ability to determine the role of interkingdom interactions in early-life transmission. Relatedly, and third, approaches have been proposed to utilize absolute quantification from meta-omics data[38], such as metabolomics and proteomics, that can aid in deciphering the mechanisms of microbe-microbe and microbe-host interactions. Future studies focusing on technological and analytical quantitation of the '*how much*' of the microbiome[22,39–41] during early life will allow us to turn the '*what*' of microbial transmission into the '*how*'.

The role of the "*where*" factor has largely been recognized as a critical influence on early microbiome development. Babies delivered vaginally vs Cesarean delivery show pronounced differences in their microbiome compositions during the first year of life[42]. Similarly, the effect of breastfeeding on the gut microbiome is substantial[43]. To address the "*where*" aspect comprehensively, it is essential to collect multiple potential sources such as feces, vaginal fluid, skin, saliva, and breast milk and detailed information on delivery and feeding, including specifics such as the time the amniotic sac was broken, the use of a vacuum during delivery, the use of a breast pump, intermittent formula feeding, and finally, multiple samples from the environment.

For the "*who*" aspect, sampling should not be restricted to babies and mothers. Samples from family members and other proximate individuals, including pets, can also carry valuable information. Specific questions around "*who*" could also be addressed through study designs encompassing the diversity of familial relationships: studies of microbial acquisition in children born by surrogacy or children breastfed by parents who did not give birth to them may provide insight into differences in microbial source and transmission timing. "*Who*" should also include health care providers, especially in the newborn nursery and neonatal intensive care units, in such critical early life periods.

Lastly, addressing the "*when*" aspect necessitates the longitudinal collection of samples of both the infant and other individuals; however, the frequency of sampling will depend on the life stage. Compared to a rather stable microbiome of a healthy adult, the infant microbiome experiences dynamic changes during its establishment. In contrast, the mother's microbiome gradually changes during pregnancy and returns to a pre-pregnancy state after delivery[44,45]. Therefore, although challenging, samples should be collected at least once from the mother before and after birth, from all individuals involved in birth, and more frequently from the infant in the first weeks of life. Such a sampling strategy will offer the most detailed insights into the establishment of the infant microbial ecosystem. After this initial stage, monthly sampling from the infant, family members, and environment would be ideal for capturing the different sources contributing to the microbiome development. The frequency of sampling should also increase during life events that alter the microbiome composition, such as the introduction of new food and weaning in general, vaccination, or medical treatment. Finally, while some reports suggest that the infant gut microbiome starts to resemble an adult one after the age of two to three years, others show that a child's microbiome does not reach an adult-like state until later[46]. These discrepancies underline the need for more frequent and longer follow-ups on the child's microbiome development.

Practical aspects of 4 W study design

In practical terms, conducting large cohorts with extensive metadata collection, comprehensively sampling infants and their household members, plus the environment in a longitudinal design, and finally, sample characterization via multi-omics, is currently unrealistic. This issue is challenging for cohort studies in general, but is pronounced for early-life microbiome studies due to the dynamic nature of microbial communities, which are continuously adapting to the fast-developing human physiology and display a large inter-individual variability. In addition, the specifics of the country where the research takes place will have a major influence on the study design, as one must account for differences in ethical norms, jurisdiction, and geographical proximity/ remoteness that affect logistics. Because of the economic, logistic, and other practical challenges, here we present examples of how researchers have been employing strategies to describe, at least partly, the 4 W parameters (Table 1) and a theoretical approach to study design along a 4 W framework (Box 3).

Firstly, one can design nested zoom-in studies within cohorts that have a large number of participants and employ extensive longitudinal sampling[47]. Depending on the research question, one may apply a cross-sectional, nested case-control, or matched cohort design within a larger population study, using samples from selected timepoints (defining "*when*"), sources ("*who*" and "*where*"), and focusing on particular "*what*" (e.g., type of microorganisms or their products). From an epidemiological perspective, there will be a difference if the research aim is to study how early life microbial acquisition and transmission are affected by a rare exposure (e.g., formula contamination) or a rare outcome (e.g., necrotizing enterocolitis in premature infants). In the first case, large cohorts are necessary, yet one can opt to characterize only those with the exposure and several controls (matched cohort design). In the case of a rare outcome, one can choose to select and characterize all samples of those with the outcome, matched to one or more controls without the outcome (nested case-control design). The advantages of both approaches compared to characterizing all collected samples include lower cost and comparator groups that are more equal in size, facilitating more statistically robust bioinformatics analyses, as some analytic measures do not perform well if group sizes are unbalanced. Finally, clinical intervention studies, ideally designed as randomized control trials (RCT), can confirm associations between microbial transmission and selected early life factors, as shown in a study using maternal fecal microbiota transplantation to restore the intestinal microbiota of Cesarean section-born infants[48]. However, RCTs performed in the infant population require extensive ethical approvals compared to adults, which significantly extends the project planning period. Current examples of large population studies that have investigated specific questions related to early life microbial transmission include the Canadian CHILD, Swedish SweMaMi, Danish COPSAC, Dutch Lifelines-NEXT, and Finnish FinnBrain and HELMi studies. Many of these studies are still ongoing and including more 4 W aspects into their designs would help obtain novel insight into early-life microbial transmission and assembly. For empirical evidence, see Table 1 with several case studies that illustrate how the 4 W framework can guide research on human microbiome acquisition and development, when used to

identify which of the components of microbial transmission have been closely investigated and which remain largely uncharacterized.

Secondly, another study design is to focus only on some of the 4 W parameters, but study them in greater depth. For instance, the BabyBiome project from Oslo sampled 12 infants daily throughout the first year of life, providing high resolution into the dynamics of gut bacterial community over time and describing a strong temporal structure and specific developmental stages of the community maturation[49]. More of such studies with densely sampled mother-infant pairs would give insight into the "*when*" of microbial transfer and the effect of the transferred microbes on the colonization dynamics of the infant microbiome. However, this is not feasible for large cohorts. Examples of other focused cohorts include the Diabimmune and DIPP studies[50], designed to pinpoint specific microbial profiles in individuals predisposed to immune diseases, and the MicrobeMom study, which studies the transmission of specific bacteria from mother to infant[51].

Lastly, robust findings on early life microbiome can and will be derived from joint analysis of results from various studies with differing designs[52–54]. This is indeed a good example of the way forward, which lies in collaboration, allowing researchers to connect resources (e.g., economic, know-how, personnel) and combined datasets to tackle research questions. To this end, ensuring the transparent sharing of metagenomic data and metadata is crucial[55].

"*Why*" is this microbiome different from all other microbiomes

Defining the 4Ws of microbiome transmission is crucial to understanding microbiome acquisition in early life. Still, defining '*what*', '*when*', from '*who*' and '*where*' microbes are transmitted does not allow for full comprehension of the acquisition process and aspects of microbial ecology outside of early-life acquisition, such as primary (assembly) and secondary (after perturbation) succession and colonization over time. Indeed, a fifth question on '*why*' is fundamental to understanding which members of the microbiome are acquired and successfully colonize specific habitats in the infant (and beyond). Ultimately, our understanding of '*why*' requires ecological and evolutionary frameworks, and for which we can turn to the four tenets of community ecology and assembly: stochastic processes (dispersal, drift, diversification) and selection (*i.e.*, the niche[56,57]). To date, most studies have focused on the latter, defining the roles of selection on microbial acquisition and colonization in early life. Many of these selective factors are intimately related to the 4Ws. For example, dietary factors such as milk-derived oligosaccharides, which may selectively feed transmitted microbes in the gut, or the developmental expression of host secreted innate (antimicrobial peptides) and adaptive (antibodies in milk or produced in the gut over time) factors specific for distinct microbes, are examples whereby '*when*' combine with '*why*' to determine transmission. Microbe-microbe interactions, spanning negative (e.g., antibiotics) and positive (e.g., cross-feeding), provide examples where two distinct '*whats*' (i.e., microbial strains) act as selective factors for (co-) acquisition.

While selective (or deterministic) pressures define the microbial niche and potential for success of acquisition, stochastic (or probabilistic) processes, i.e., neutral processes, are likely to play instrumental roles in early-life microbiome transmission and assembly. At present, however, as the default or null hypothesis of transmission, dispersal (random movement of microbes across space), drift (random changes in fitness of microbial populations), and diversification (genomic changes), all defined as '*random*', are difficult to assign a weight in early-life microbial transmission.

How can the 4 W framework offer new insights into defining stochastic versus selective aspects of microbial ecology, assembly, and succession? Indeed, combining the power of population genetic approaches with microbial source tracking will allow us to test the null hypothesis and the roles of sources and sinks by modeling neutral processes shaping the microbiota acquisition[58]. Operationally, a broad sampling and quantification across the domains of life of the '*who*' and '*where*' will allow definition of the '*sources*' and '*sink*'. Here also lies a research potential in the use and integration of strain-tagging and controlled transmission experiments[31,59].

For example, source tracking has been used to identify and categorize transmission events[60]. The algorithms assume a somewhat unidirectional transmission, where the infant community is the '*sink*' and it acquires microbiomes for a set of '*source*' reference communities. The algorithm can then either predict similarity to a source state or predict the portion of the microbial community contributed by each source[61]. Modeling of neutral processes during microbiota assembly can reveal the contribution of dispersal and demographic stochasticity, which has been found to explain the prevalence of a majority of infant-colonizing microbes[58]. With comprehensive sampling across the 4 W and population genetic modeling approaches, we can define if the transmission of a microbe obeys the features of a stochastic process. When this null hypothesis is not met, we can begin to define the contribution of selection (and then what features of microbes are selected) in the assembly and definition of the microbial niche of transmission and successful colonization. For example, by quantifying absolute abundances of a specific microbe ('*what*') in a source (e.g., vagina at delivery) and in infant feces ('*who*' and '*where*') at birth and over time ('*when*'), we could determine if such transmission was related to the microbial abundance in that particular source (stochastic) versus the microbial properties, which facilitate successful acquisition and colonization (selection).

Such a 4 W framework can be readily applied to aspects of microbial acquisition outside of the early-life period, such as repopulation of the gut microbiome after antibiotic perturbation. By capturing the 4 W of potential '*who's*' (the person taking the antibiotics and their contacts), '*where's*' (the sources of the microbes to repopulate the gut, *i.e.*, their own oral microbiome or the gut microbiome of their household partner), '*when*' (the timing and relation to the abundance and composition of the focal person's gut microbiome before, during and after use of antibiotics), and '*what*' (strain-level tracking via metagenomics or whole-genome sequencing of cultivated isolates), we can use the 4 W framework to capture the parameters needed to test the null hypothesis and by extension define role of the ecological niche in transmission. Ultimately, a combination of both deterministic and probabilistic modeling will allow a quantitative and predictive understanding of the '*why*' of early-life microbiome transmission.

Conclusions

The current lexicon of early-life microbiome acquisition originating from the fields of infectious disease epidemiology and evolutionary biology, and namely vertical and horizontal transmission, often shows limited resolution. To achieve a deep ecological understanding of the early life microbial dynamics, we propose that efforts should be centered on deciphering the what-where-who-when aspects of microbial acquisition. By transitioning from the vertical/ horizontal transmission language toward using precise terminology of the 4 W framework, researchers can systematically examine different components of microbiome acquisition. Currently, metagenomics is the workhorse for describing microbial transmission. But also wider adoption of other methods for tracking microbial cells and their components, such as single cell-based microbial genomics, metabolomics, metaproteomics, and high-throughput culturomics, has the potential to significantly contribute to these goals, when affordable and accessible to non-specialists.

Importantly, the interpretation of transmission events carries a large degree of uncertainty and necessitates considering alternative microbial sources. A broader implementation of computational approaches, which can resolve microbial patterns and minimize degrees of freedom in interpreting transmission events, is thus a prerequisite for the study of human microbiome acquisition. Defining which aspects of the 4Ws and how we can feasibly capture these in

studies of the early-life human microbiome, and human microbiome at-large, will be instrumental to future-proofing and comprehensively understanding the dynamics and significance of early-life microbial colonization and when, where, and how we should and can intervene for human health.

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This perspective was conceived during a research sabbatical, with contributions from S.R.N., J.D., M.V.C., H.T.N., M.E.T., A.Z., N.B., and V.K.P. All of these authors contributed to the writing, editing, and critical review of the manuscript. MVC created Fig. 2, while JD designed Figs. 1, 3, and 4. J.D. finalized all figures for publication. S.R.N. used his institution's professional BioRender account (Boston Children's Hospital) to illustrate the 4 W study designs in Box 3. The outlines for the pregnant person and fetus used in Figs. 1, 3b were obtained from BioArt, an NIH-funded public repository accessible to J.D. The bacteria's shapes and colors were created by J.D. V.K.P. secured the funding from the Center for Advanced Study (CAS).

Competing interests

The authors declare no competing interests.

Additional information

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