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Breastfeeding as a regulating factor of the development of the intestinal microbiome in the early stages of life

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Abstract

Since the first bacterial inhabitants of the human gastrointestinal tract were identified a lot of research into the study of the human microbiome and its effects on health has been conducted. Currently, it is accepted that humans have a symbiotic relationship with the gut microbiome, though the specifics of this relationship are not well understood. The microbiome of neonates constantly changes and appears to influence many facets of the infant's health and predisposition later in life. This review aims to show how the microbiome develops over time. We discuss it is composition, origins and stages of development of microbiota, the possible health benefits of a proper neonatal microbiome, and the dangers associated with dysbiosis. We emphasize the shielding, modulating, and stimulating effects breast milk has on the infant microbiota. The methods commonly used for the study of microbiota are also discussed.

Keywords: microbiota, neonatal gut, breast milk, dysbiosis, enterotypes, probiotics

Statements and Declarations

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The importance of the gut microbiome

The human microbiota has been investigated thoroughly and its effects on human health are firmly established even though the precise relationship is yet to be understood [1,2]. The gut microbiome is seen as an integral part of the human body and contributes to metabolic functions, protects against pathogens, and educates the immune system [3]. It is often seen as an extension of the human genetic pool, with the gut microbiome encoding over 3 million genes, which eclipses the 23 thousand genes present in the human genome [1]. The microbiome is flexible and can be affected by dietary ingredients and the resulting changes can affect the health of the host. Transplantation of a microbiome from healthy individuals to sick patients can effectively treat *Clostridium difficile* infections [4] and other applications for this procedure are emerging [5]. Research on mice has linked the composition of the microbiome to obesity [6].

Microbes present in the gut, metabolize substrates present in consumed food creating nutrients that are usable for the host while also producing bioactive compounds that modulate the immune system, physiology, and gene expression of host cells [7]. Humans only produce a few hydrolases capable of hydrolyzing starches and rely on the enzymes produces by the microbiome to gain energy from complex carbohydrates[8]. Short-chain fatty acids (SCFAs) produced by bacterial metabolism of carbohydrates contributes to approximately 10% of the caloric requirement of humans. Additionally, these fatty acids provide anti-inflammatory effects. Butyrate, a SCFA, improves the integrity of the host's intestinal epithelial cells [9].

The gut also hosts microorganisms capable of utilizing the gaseous byproducts of fermentation such as carbon dioxide and hydrogen, and through the removal of these waste products, helps drive metabolism forward [10,11]. The fermentation of amino acids by these bacteria provides additional SCFAs, that can be used as fuel.[12] However, the metabolism of aromatic, sulphur-containing , and basic amino acids produces pro-inflammatory, cytotoxic, and neuroactive compounds [7]. Only a small portion of dietary fat reaches the colon[13] and the relationship between microbial lipid metabolism and the host's health is unknown. However, it is known that free lipids have antimicrobial properties [14]. Saturated fatty acids promote inflammation [15], which might be one reason for the chronic inflammation present in obesity [7], while omega-3 unsaturated fatty acids are anti-inflamatory [16]. .

Interactions with various antigens play an important role in immune system maturation. It is suggested that exposure to certain microorganisms early in life is a factor in preventing the development of allergies and aids in regulating immune system activity. The gut has the greatest concentration of microorganisms that humans have contact with in their lives. Therefore, it is natural to assume that the gut microbiome plays an important role in immune system regulation [17].

The microbiome is known for modulating the secretion of antibodies and interleukins and the functions of other immune cells [18]. As suggested by recent studies, the early establishment of symbiosis between the immune system and the gut microbiome has a large influence on the susceptibility or the resistance to diseases later in life [19]. During the weaning period, the immune system of infants undergoes rapid development. It has been shown that the microbiome takes an important part in the development of isolated lymphoid follicles and the regulation of intraepithelial lymphocytes, macrophages, and invariant killer T cells [18].

Changes within the microbiome during pregnancy

Although the adult microbiome differs between persons, it is fairly stable during life and research has revealed some generalities. The most common phyla present in healthy individuals are Firmicutes (22.2 +/- 18.66%) and Bacteroidetes (73.13 +/- 22.16%) followed by Proteobacteria (2.15 +/- 10.39%) and Actinobacteria, which is mostly represented by the *Bifidobacterium* genus (1.82+/- 3%). A vast majority of Bacteroidetes are members of the *Bacteroides* genus, with *Bacteroides dorei* being the most dominant (17.44 +/- 8.74%), while *Bacteroides fragilis* is the most widespread species. The abundance of *Bifidobacteria* varies between 0.004% and 12.21%. In regards to Firmicutes*,* the genus *Clostridium* appears to be the most common [2].

During pregnancy, the mother's vaginal, oral, and gut microbiota undergo significant changes, the origin of which is unknown. Changes in hormonal regulation, immunity, energy homeostasis, and fat storage likely have a role in influencing the microbiome [20]. The changes in the microbiome happen gradually during pregnancy. An increase in the abundance of Proteobacteria and Actinobacteria is seen at the cost of *Faecalibacterium* and other SCFA producers [21].

During the third trimester, mothers showed a lower diversity within a single sample, while having the largest diversity between different mothers. This suggests that pregnancy causes the depletion of microbial diversity, however, it increases the diversity between individuals. The increased diversity between mothers lasted for up to one month postpartum [21]. When transferred to germ-free mice, third-term microbiota caused more weight gain, insulin resistance, and inflammatory responses than first-term microbiota. This shows that the microbiota contributes to the changes occurring during pregnancy [21]. There is also evidence suggesting that an alternation in maternal microbiota during pregnancy such as during exposure to antibiotics, influences the neonate's immunity and health [22]. Changes to the vaginal microbiota, such as the presence of certain fungi like *Candida albicans* [23], a lower *Lactobacillus* abundance, and an increased *Gardnerella* and *Ureaplasma* abundance[24] are associated with preterm birth .

The “in utero” origin of the microbiome

The exact source of the early microbiome is unknown. The proposed sources of early life gut bacteria are the mother’s vagina during birth, breast milk, and the mother’s gut microflora, however, the mechanism of such transfer is unknown. Recent studies have proposed the idea of in utero colonization [25].

The placenta and the amniotic fluid have always been considered sterile, however recent studies have raised doubts about this assumption. Bacteria have been isolated from the placenta and studies have shown the presence of the microorganisms in amniotic fluid [26, 27]. However, the detected biomass remains low suggesting the detected microbiota are contaminates rather than native inhabitants [28]. It has also been suggested that polymerase chain reaction (PCR) based detection might identify DNA of dead bacteria instead of living ones [29].

Bacteria have also been found in the umbilical cord suggesting the transfer of microbiota between mother and fetus [30]. However, the mechanism for such a transfer is not understood. One theory is that the bacteria are transferred from the mother’s intestine. An experiment in mice showed *Enterococcus faecium* strains fed to the mother orally were later detected in the amniotic fluid supporting this claim [31].

It has also been shown that microbial exposure of the mother during pregnancy might have a significant impact in preventing allergies [32]. Children, whose mothers were exposed to farm animals during pregnancy are less likely to develop allergies, as well as an exposure to other allergens reduced the symptoms of asthma, hay fever, and eczema in the children [22]. However, the evidence supporting the existence of a placental microbiome is still controversial.

The changes in the microbiome associated with type of delivery

One of the first big shifts in the microbial composition of the infant’s gut happens during birth, and the birth mode seems to be a major factor influencing the early microbiome. Children born from cesarean section have lower *Bifidobacteria* abundance and the colonization by *Bifidobacteria* is delayed. This delay is not affected by the form of feeding. They also have an abundance of potentially harmful *Klebsiella* and *Enterococcus.* This increase in *Klebsiella* and *Enterococcus* is also independent of antibiotic exposure, hospitalization time, and feeding.

There is evidence suggesting that children delivered vaginally are seeded by the mother’s fecal microbiota. Furthermore, these children have a more stable early microbiota than children born by cesarean section, who are inhabited by more strains associated with respiratory tract infections during the first year of life. This suggests that the passage through the vaginal canal has an important role in the early colonization of the infant’s gut [33].

Vaginal seeding is a procedure in which a gauze swab is used to transfer vaginal fluid, and the microorganisms within it, onto an infant born via cesarean section. In theory, this should alter the infant's microbiota towards a more "natural" composition. However, the evidence regarding the health benefits of this procedure is limited and harbors the possibility of transferring pathogenic microorganisms. Due to the absence of evidence of the benefits and potential risks, performing this procedure is currently not recommended [34].

Breast milk composition and bioactive components

Breast milk is the most optimal source of nutrients for newborns, but the evidence for its role in preventing health problems and disease in early childhood is prevalent. Some suggest that the benefits might also apply later in life, though this is inconclusive [3]. Although the artificial formula has improved since it was first introduced, it is still unable to provide the same health benefits as natural human breast milk. Breastfed children have lower risks of respiratory tract infections, neonatal necrotizing enterocolitis (NEC), and gastrointestinal illnesses [35]. As such, breastfeeding remains the recommended feeding method of newborns, however, in certain cases, such as babies with lactose intolerance or mothers who cannot breastfeed due to health reasons, it is not possible and must be replaced or supplemented by artificial formula.

The composition of breast milk changes over time and is considered fully mature 4 to 6 weeks after birth. The colostrum, which is produced in low quantities during the first few days following birth is rich in IgA, lactoferrin, leukocytes, developmental factors, sodium, magnesium, and chloride[36]. However, it contains relatively lower concentrations of lactose, calcium, and potassium. This suggests that the main function of colostrum is immunogenic rather than nutritional [37,38,39]. The composition of macronutrients in breast milk varies between mothers, however, remains similar across populations despite differences in maternal nutrition [40]. In preterm mothers, breast milk contains higher concentrations of secretory IgA, likely to compensate for the underdeveloped neonatal immune system [41].

The protein content of breast milk is estimated to be around 0.9 to 1.2 g/dL [36] and can be grouped into 3 major classes based on where they can be found: caseins (α-casein, β-casein, and κ-casein), whey (α-lactalbumin, lactoferrin, lysozyme, and secretory IgA), and mucins. Caseins are aggregated in micelles while whey proteins are present in solution and mucins are incorporated into the milk fat globule membrane (MFGM) [42]. In addition to proteins, breast milk contains free amino acids with higher concentrations of glutamic acid and glutamine, thought to have an appetite-regulating effect [43].

Lipids represent 44% of the total energy provided by human milk, being the major contributor. The most common fatty acids in breast milk are palmitic acid and oleic acid. Palmitic acid is mostly concentrated in the 2nd position of triglycerides, which allows for increased absorption and decreased calcium malabsorption [42]. The fat in human breast milk is concentrated in globules surrounded by a MFGM, which contains a high amount of bioactive compounds that play a role in neurocognitive development and immune function [44]. The content of long-chain polyunsaturated fatty acids (LCPUFA) is largely affected by the mother's diet, and is negatively affected by the high omega-6/omega-3 ratio present in western diets. [45,46]A higher ratio of omega-6/omega-3 is positively associated with higher body fat percentages between 2 weeks and 4 months of age and may contribute to adiposity [47].

The main carbohydrate in human breast milk is lactose [36]. It appears at a concentration of 6.7g/100 ml exceeding the concentration of other species [48]. The concentration of lactose increases in mothers with a higher volume of milk production [49]. The micronutrient composition of breast milk varies by maternal diet and body stores. Breast milk contains vitamins A, B1, B2, B6, B12, and D along with iodine and other micronutrients.[50,51] Regardless of diet, vitamin K is low in human breast milk and should be supplemented [23d]. The effects of the micronutrients in human breast milk on infant growth are not well known [43].

In addition to macro- and micronutrients, breast milk contains numerous bioactive components including hormones, growth factors, cytokines, and immune cells. The growth factors present in milk stimulates the development of the intestines, growth and maturation of neurons, repair of tissues, and protection against damage from hypoxia and ischemia . T cells, stem cells, lymphocytes, and macrophages are all present in breast milk along with non-cellular immune components such as immunoglobulins and cytokines. Additionally, it contains compounds such as lactoferrin, lactadherin, bile salt-stimulating lipase, and mucins which serve a role in protecting the infant against bacteria and viruses [36].

The infant microbiome, health risks and benefits associated with microorganisms found in the neonatal gut

The composition of the neonatal microbiome has substantially more plasticity than adults. It changes rapidly with ageing and depends on various factors such that it is significantly different between formula-fed and breastfed babies [1,9,14]. Table 1 contains a comparison of the neonatal gut and breast milk microbiota.

During the first week of life, the microbiome is dominated by facultative anaerobes, such as those from the Proteobacteria family. These bacteria consume oxygen and shape the intestinal environment to be more habitable for obligatory anaerobes which appear later [54].

Not only does breast milk contain factors that shield the underdeveloped immune system of newborns but it also appears to promote the growth of certain microbes, such as *Bifidobacterium* species, due to their ability to metabolize human milk oligosaccharides (HMOs), and *Lactobacilli*. *Enterococci* are also more prevalent in breastfed infants, while formula-fed children show an increase in the presence of *Clostridium*, *Escherichia*, and *Bacteroides* [1]. The microbiome has a big effect on infant development. Studies show that mice grown in germ-free environments have poor growth, decreased weight, intestinal problems, and altered neurodevelopment [55].

Overall, neonates are characterized by lower bacterial diversity than adults with breast-fed infants having less diversity in their gut than formula-fed infants [19]. It also appears that children on a mixed diet have the bacterial diversity profile of formula-fed infants. The microbiome shifts quite dramatically when solid foods are introduced to the infants' diet, with a shift in dominance towards fiber-fermenting *Bacteroides* and *Firmicutes* and moving towards a composition similar to that of adults [19]**.**

The most studied members of the gut microbiome are the model organism *Escherichia coli* along with the genera *Lactobacillus*, *Bifidobacterium,* and potential pathogens such as *Clostridium*. *Escherichia coli* is a microorganism commonly found in the lower intestine of mammals. Although most strains are harmless and even aid in the health of the host by producing exogenous vitamins such as K vitamins [56]. Unfortunately, there exist *E.coli* strains that can cause diarrhoea, respiratory tract infections, pneumonia, and urinary tract infections 57]. The pathogenicity of *E.coli* is dependent on several virulence factors such as fimbriae, adhesions, toxins, and other elements which can directly interact with epithelial cells of the intestinal, respiratory, and urinary tract [58]. It has been shown that *E.coli* strains in breast-fed infants have fewer virulence factors such as the K-capsule and have increased type 1 fimbriae expression. The IgA contained in the mothers' milk can bind to the same type of fimbriae [59]. It appears that *E.coli* isolated from breast-fed infants show higher adherence to epithelial cells of the colon compared to those in formula-fed children [60]. Type 1 fimbriae expression has been shown to enhance the virulence of *E.coli* in the urinary tract [61]. However, breast-fed infants have shown a lower risk of urinary tract infection [59].

Bacteria from the *Lactobacillus* genus belong to a broad group called the lactic acid bacteria, defined by their ability to produce lactic acid as the sole or main byproduct of carbohydrate metabolism. They are known to colonize oral cavities, gastrointestinal tracts, and vaginas of humans and animals. The presence of *Lactobacilli* in the gut is commonly regarded as beneficial to the host and are frequently used as probiotics. However, there is little evidence supporting any major role this genus might have on the human gastrointestinal tract. On the contrary, evidence suggests only a small number of *Lactobacilli* are true residents of the mammalian gastrointestinal tract, and that most are instead allochthonous and derived from food or the oral cavity. Recent research, based on the amplification of 16S rRNA genes, shows that *Lactobacilli* make up only a small fraction of the total microbiota [62]. Attempts to treat infant colic with *Lactobacilli* supplementation have shown no benefit [63]. However, it has been shown that supplementation with *Lactobacillus* *rhamnosus* reduces the duration of diarrhea [64]. Studies in animals have shown that treatment with *Lactobacillus* can improve enteriditis recovery [65] and inhibit the colonization of the pathogenic *E.coli* K1 strain [66].

Clostridium is a genus of Gram-positive, anaerobic, and spore-forming bacilli. *C. difficile* is a major cause of diarrhoea and potentially lethal nosocomial infections, especially in the elderly [67]. However, its pathogenicity in infants is still debated [68]. Up to70% of healthy newborns can be colonized by *C. difficile* during the first months of life and most lack any symptoms of infection even when large numbers of toxin-producing bacteria are present. The underdeveloped intestinal mucosa may lack *C. difficile* toxin receptors or other factors such as the immaturity of the immune system might also play a role, although the true reason is unknown [68]. It is important to note that *C. difficile* infections still occur especially in infants with hematological malignancies, inflammatory bowel disease, and cystic fibrosis following lung transplantation [67]. Colonization by *C. difficile* is more common among formula-fed infants than among breastfed ones [67] due to the lack of IgA in the formula [69]. The presence of *C. difficile* decreases with ageing and reaches the prevalence levels similar to adults by 3 years of age [70].

Bifidobacterium is a genus of Gram-positive, anaerobic bacteria that commonly inhabit the gastrointestinal tract, vagina, and oral cavities of mammals, including humans. Their presence in the gastrointestinal tract is deemed beneficial, thus they are commonly added to probiotics and functional foods . *Bifidobacteria* rapidly colonize the infant gut during the first weeks after birth. *Bifidobacteria* have been associated with protection from carcinogens, reduction in inflammation, and regulation of gut function. They are more prevalent in babies born vaginally suggesting they are acquired from the vaginal tract of the mother. Furthermore, breastfeeding supports the growth of this genus due to its ability to digest human breast milk oligosaccharides. As a result, *Bifidobacteria* are a major part of the newborn microbiome. However, their presence decreases rapidly with ageing and remains low but stable during adulthood [71].

Enterotypes in infants and stages of gut microflora development

In recent years, metagenomic studies have suggested that the intestinal microbiome of each human belongs to one of three types based on the dominating microorganism. These genera are *Bacteroides* (Enterotype 1), *Prevotella* (Enterotype 2), and *Ruminococcus* (Enterotype 3). These enterotypes do not differ in functional abundance and do not correlate with any factors relating to the host. However, the prevalence of certain genera indicates the use of different routes to generate energy from fermentation [72]. Although the possible benefits of using the enterotype model are high, there are certain points of contest when it comes to the theory. The enterotypes are not sharply delineated [72], and apparent clusters may arise from certain methods of data processing even when they are not factual [73]. Additionally, by focusing on the enterotype model it is possible to miss smaller changes and individual differences in the microbiota. The long-term stability of a human's enterotype also comes into question [73]. Some research suggests that there are only two enterotypes, the *Prevotella* and *Bacteroides* genera [74].

Certain studies seeking to evaluate the presence and importance of enterotypes in infants have been performed. This research has identified four distinct enterotypes with the dominant microorganisms being either the Firmicutes phylum, *Bifidobacterium*, *Bacteroides*, or *Prevotella* [75]. Unlike adults, the differences in enterotypes seem to be dependent on the stage of gut development and can transition from a less mature into a more mature one. In particular, the strains associated with Firmicutes and *Bifidobacterium* were correlated with the early developmental stages of the gut microbiota, while *Bacteroides* and *Prevotella* were correlated with later stages [75]. While the enterotypes did not seem correlated with antepartum or postpartum factors, certain clinical factors seemed to influence them to an extent. Type Firmicutes were more common in infants delivered by C-section and in infants with lower gestational age, although these factors often appear together. The duration of breastfeeding was also a factor with Firmicutes being more common in infants breastfed for shorter durations while breastfeeding longer seemed to promote *Bifidobacterium* [75]. A different study, using two enterotype models failed to detect a negative correlation between *Prevotella* and *Bacteroides* in infants 9 to 18 months of age. However, such a correlation appeared at 36 months suggesting stable enterotypes develop between 18 and 36 months of age [76].

The dangers of microbial dysbiosis and factors contributing to its occurrence

Multiple factors affect the composition of the infant microbiome, including but not limited to the mother's diet, feeding type, and medication [55]. Dysbiosis is a term used to describe a breakdown in the balance between "protective" and "harmful" intestinal bacteria [77]. Dysbiosis is associated with multiple diseases, such as obesity, type 2 diabetes, hypertension, NEC, and inflammatory bowel disease, autoimmune diseases [18], asthma, food allergies, autism, and opportunistic infections [19].

One of the most common causes of dysbiosis is antibiotic treatment. Antibiotics are the most common medication prescribed for children. Studies have shown that the use of antibiotics in early life is associated with obesity and the occurrence of diseases later in life. Antibiotic treatment has a long-term effect on the microbial composition and diversity in the gut. Antibiotic treatment in early life has been associated with allergies, atopic diseases, autoimmune diseases, and infections such as NEC [77]. Acid blockers are also associated with dysbiosis and NEC [55]. Children of obese mothers have a different bacterial colonization profile than those born to nonobese mothers. These differences are maintained during the first few years of life. However, the development of obesity may begin in utero due to the obesogenic and inflammatory maternal environment [78].

Gestational diabetes mellitus (GDM) is associated with changes to the microflora of both mother and child. Samples taken from GDM positive subjects exhibited less diversity than those from GDM negative patients. In addition, the meconium of GDM positive mothers exhibits a higher abundance and prevalence of eukaryotic viruses possibly exposing the child to a greater number of viruses [79].

Preterm infants are especially susceptible to dysbiosis due to their underdeveloped intestines. The immaturity of the gastrointestinal tract and immune system coupled with altered gut microbiota can have severe health consequences. Moreover, pre-terms require hospital treatment which further disturbs the microbiome and exposes the infant to the influences of the hospital's environmental microbiome [41].

Antibiotics are routinely prescribed for preterm children to prevent infections. Although this treatment decreases mortality it also alters the microbiota causing reduced bacterial diversity[80], delaying *Bifidobacteria* colonization [81] and increased presence of multi-drug resistant strains [80] . Furthermore, the time required for the recovery from such disruptions is positively correlated with the length of antibiotic treatment [80,81]. Additionally, artificial respiration shifts the microbiome towards aerobic and facultative anaerobic bacteria due to the introduction of oxygen to an otherwise anoxic gastrointestinal tract [82]. This can result in the weakening of the mucosal barrier [83] and reduced production of energy, nutrients, and bioactive components [84].

Modulation of the gut microbiota by probiotics and breast milk

Probiotics are live microorganisms promoted as having health benefits when taken as food supplements, while prebiotics are compounds that promote the growth or activity of beneficial microorganisms. There have been several studies investigating the benefits of pre- and probiotic supplementation for infants.

Studies on animal models show that *Bifidobacteria* supplementation might counteract the effect of carcinogens, help reduce diarrhea caused by viral infections or antibiotic treatment, and prevent constipation [71]. There is also evidence that supplementation with *Bifidobacteria* reduces the occurrence and severity of NEC in low birth or preterm infants [85]. It also has the potential to reduce the spread of gastroenteritis and diarrhea in infants in residential care units [86].

Attempts to treat infant colic with *Lactobacilli* supplementation has shown no benefit [63]. However, supplementation with *Lactobacillus rhamnosus* reduces the duration of diarrhea [64]. *Lactobacillus* GG has been shown to prevent and reduce the duration of diarrhea causes by rotavirus infections in animals [87, 88]. Animal studies have shown that treatment with *Lactobacillus* can improve enteritidis recovery [65] and inhibit the colonization of the pathogenic *E.coli* K1 strain [66]. *Lactobacilli* have also been shown to modulate Th1/Th2 cytokine balance [89,90] which might help in the prevention of atopic disease and supplementing breastfeeding mothers or infants has been shown to reduce the incidence of atopic dermatitis (eczema) [91].

In the absence of a mother's breast milk, donor human milk (DHM) appears to be the best substitute for helping the development of preterm babies. The microbiota of children fed DHM is similar to breastfed infants, although it shows a decrease in Bifidobacteriaceae and an increase in Staphylococcaceae, Clostridiaceae, and Pasteurellaceae. The pasteurization of donated breast milk and the different composition of preterm milk and donated milk might contribute to this effect [92].

HMOs are a type of carbohydrate present in breast milk and although they don't have any nutritional value, they serve as a prebiotic stimulating the growth of proper microbiota and modulating several infant mucosal and systemic immune functions [36]. These oligosaccharides differ between mothers, but this does not cause any incompatibility issues [93, 94, 95]. However, it has been shown that one type of HMO, specifically disialyllacto-N-tetraose (DSLNT) is protective against the risk of NEC in rats, which point to the conclusion that the protective effects of these compunds are dependant on specific HMO structured [96]. A study in piglets has also shown that HMOs can reduce the symptoms of rotavirus infections [97].

Research on the benefits of probiotics in infants has been promising and they appear to be safe. However, the studies have used different strains and administration strategies thus more studies are needed to identify the ideal combination. As of today, feeding breast milk from either the mother or that has been donated appears to be the best method of stimulating a beneficial microbial composition**.**

Methods for studying the microbiome

Historically the study of human and animal microbiota has been based around traditional non-molecular methods involving the isolation of microbes, microscopic observation, and growing them in culture. Although these methods have been incredibly useful in the early study of the microbiome, they have several limitations. Traditional cultures tend to underestimate the true variety of microorganisms present as a large number of bacteria cannot be cultivated using currently known methods or require artificially created environmental conditions for that organism to grow [98,99]. Temperature, pH, oxygen, and nutrient levels [98] and cultivation time [99] need to be tuned towards the studied microorganism. Furthermore, the existence of mutual relationships between different bacteria further complicates the issue. In particular, the creation of a biofilm, which is composed of many different microorganisms, is difficult to replicate in a lab. This limits the variety of microorganisms that can be studied using traditional methods, which provides a biased view of the microbiome composition with an overrepresentation of aerobic organisms [100].

Even with these limitations, culture methods have the unique advantage of allowing living microorganisms to be studied in regards to antibiotic response and susceptibility, antigens, microorganism relationships, biofilm formation, and the creation of experimental models [99]. New culture methods are still being developed to allow for the growing of microorganisms previously considered uncultivable. Examples of such methods for the cultivation of hard-to-culture microorganisms are the use of gnotobiotic animals [101] or the creation of artificial environments simulating the intestinal environment, such as the SHIME system [102]. These methods come with the additional benefit of being able to study gut microbe-host and microbe-microbe relationships [103, 104].

In response to the limitations of traditional methods, molecular methods for studying microorganisms were developed. These methods involve the study of a microorganism’s molecular components such as DNA, RNA, proteins, and metabolites. These methods are culture-independent, meaning that the studied microorganisms do not need to be isolated and cultivated in a medium. Rather, they allow for the *in vitro* study of microorganisms considered impossible to be grown. The basis for most molecular methods is a variant of the DNA PCR [98]. By using PCR, the amount of DNA in a sample can be increased exponentially allowing for further analysis with techniques such as Southern blotting [105]. With modifications of the PCR method by using different starters, conditions, or pre-preparation techniques on the samples it is possible to turn it into a diagnostic method itself. For example, ligation-mediated PCR techniques utilize the selective amplification of DNA fragments generated by enzymatic restrictions creating a genetic fingerprint for a sample [106]. Other methods can also provide certain insights, for instance, terminal restriction fragment length polymorphism (T-RFLP) has suggested that *Clostridium* plays an important role in the pathogenesis of NEC [107]. While variants of gradient gel electrophoresis have revealed the disruption of the human microbiome by antibiotic administration and identified a correlation between *Sphingomonas* and NEC in human children [108,109]

With the rise of DNA sequencing technology, the ability to study complex microbial communities has increased. Although its use was initially limited due to costs, improvements in the technology have allowed for cheaper, faster, and more sensitive identification technologies. The increased availability of bioinformatic tools has allowed for the creation of modern new generation sequencing (NGS) technology and allowed for the development of metagenomics, which is the study of the total genetic material within an environmental sample. Metagenomics can be used to study microbial diversity and dysbiosis of the intestine, identify new genes and microbial pathways and identify relationships between the microbiome and the host’s health [110]. Metagenomics aims to catalog all the genes from a microbial community by random sequencing of all DNA present in a sample [110]. The gene most commonly used for sequencing is the 16S rRNA gene as it is present in various microorganisms while having highly variable regions allowing for its differentiation between species [111]. For fungi the 18S rRNA gene or the Internal Transcribed Spacer sequence is used [112].

Another method of sequencing is whole-genome shotgun sequencing which allows for the identification of viruses [58g,58h] whose genetic data is missed by 16S sequencing as they lack such sequences [98]. This method can also provide information regarding gene content and metabolic pathways [113]. However, a major disadvantage of this technique is that the DNA from the host is also amplified and can often overwhelm the bacterial DNA. Additionally, analysis of the acquired data is complex and requires a lot of computational power [114]. The sequences obtained by either method can be analyzed with the assistance of bioinformatic tools and methods, such as databases stemming from data acquired by the Human microbiome project or the MetaHIT project. This enables a broader understanding of the structure and function of microbial communities. Metagenomic methods have revealed the relative stability of a healthy individual's microbiome and identified multiple factors that affect its composition [110].

Although molecular methods are incredibly useful in the study of microorganisms they do have limitations. DNA sequencing provides information on the presence of genes but doesn't give any insight into gene expression [98,99]. Additionally, some DNA sequences may amplify more efficiently under given conditions introducing bias to the results. Furthermore, the PCR reaction does not discriminate between living and dead bacteria or their fragments [114]. The DNA samples used for metagenomic analysis must be of high quality and in sufficient quantity. However, microbiome samples are almost universally contaminated by human DNA. Additionally, not all identified sequences can be matched due to the lack of reference and determining function on sequence homology introduces ambiguity to the results [110, 115].

A supplementary method to DNA sequencing is RNA sequencing, which allows researchers to look directly at the transcriptome of microorganisms and gain insight into gene expression [116]. RNA sequencing can be used to study the effects of environmental perturbations and factors on the function of the gut microbiome and identify a functional change before a composition change occurs. This could allow one to preemptively detect the signs of dysbiosis [117, 118]. Metatranscriptomics can be used to determine the activity of genes in a defined environment, such as the human gut. However, this method requires high-quality RNA samples, which are difficult to obtain and often difficult to separate mRNA from other types of RNA. Additionally, mRNA is unstable and the reference databases are still insufficient [110].

Methods for studying protein (metaproteomics) or metabolite (metabolomics) profiles are also being developed and can supplement metagenomic analysis. Metaproteomics has greatly benefited from improved methods of protein separation, high throughput mass spectrometry, increased computing power, and the growth of metagenomic databases. However, such methods are in their infancy and their development is difficult due to the high complexity of human samples and difficulties in analyzing the data [114]. Meanwhile, metabolomic profiles of the human gut microbiota combined with other methods can be used to predict the appearance of dysbiosis [119]. Methods for studying the microbiome are presented in Fig 1.

Conclusion

The human intestinal microbiome is an incredibly complex subject to study. Not only is it one of the richest microbial ecosystems found on earth but the relationships between the host, the microbiome, and one's health are often not straightforward, with each influencing the other. Furthermore, the microbiome of babies displays significant plasticity and is influenced by multiple factors such as mode of birth, type of feeding, medical conditions and treatments, and is shaped by the development of the infant's gut (Fig 2). However, research has identified several health effects associated with the microbiome and found ways to influence the developing microbiome, with some of these methods being put into practice. Although several issues remain unclear.

The origins and roles of pre- and postpartum factors on the development of an infant's microbiome are still inconclusive. The specific roles certain classes of microorganisms assume in the gut and the importance of their metabolic products have yet to be discovered. New methods for studying microorganisms have been crucial in enhancing our current knowledge base and in conjunction with traditional methods have provided further insight into the ecosystem of the human gut. With such knowledge, new ways of treating illnesses and improving an infant's health may appear.

Author contributions

Bartosz Ostrowski: Conceptualization, Collected date, Writing - Original Draft

Beata Krawczyk: Conceptualization, Visualization, Supervision, Writing – Review & Editing

All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Table 1. Comparison of bacteria commonly found in breast milk [52] and the infant's intestine [53].

Phyllum	Genera	Breast milk	Neonatal intestine
Firmicutes	*Staphylococcus*	+	+
	Streptococcus	+	+
	Veillonella	+	+
	Enterococcus	+	+
	Gemella	+	-
	Clostridium	+	+
	Lactobacillus	+	+
	Eubacterium	-	+
	Ruminococcus	-	+
	Peptostreptococcus	-	+
Actinobacteria	*Propionibacterium*	+	+
	Actinomyces	+	-
	Corynebacterium	+	+
	Bifdobacterium	+	+
	Streptomyces	-	+
Proteobacteria	*Pseudomonas*	+	-
	Sphingomonas	+	-
	Serratia	+	-
	Escherichia	+	+
	Enterobacter	+	+
	Ralstonia	+	-
	Bradyrhizobium	+	-
	Klebsiella	-	+
	Acinetobacter	-	+
	Desulfovibrio	-	+
Bacteroidetes	*Prevotella*	+	+
	Bacteroides	-	+

Fig 1. Methods used for the study of microbiome. Legend: T-RFLP - Terminal restriction fragment length polymorphism; WGS - Whole Genome Sequencing

Fig 2. Source of the infant microbiome. The figure shows the influence of the mother's microbiota and the environmental microbiota on the bacterial colonization of newborns and infants.

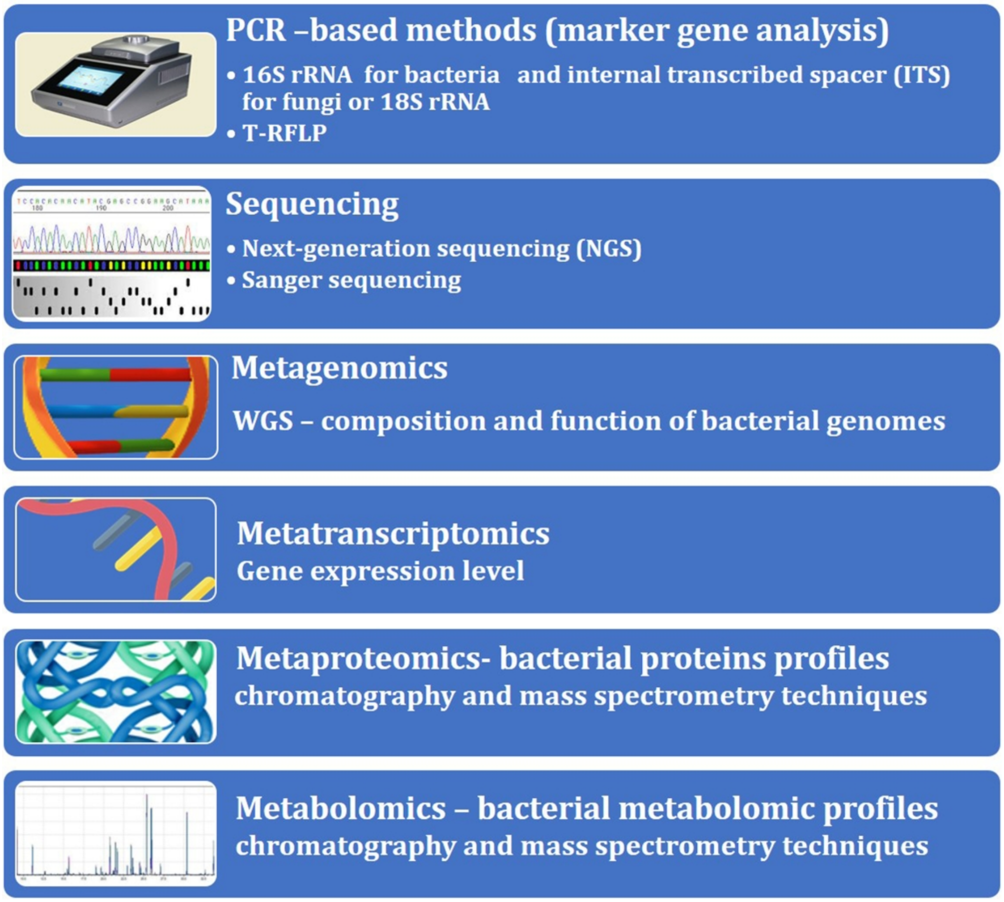

Fig.1 Methods used for the study of microbiome. Legend: T-RFLP - Terminal restriction fragment length polymorphism; WGS - Whole Genome Sequencing

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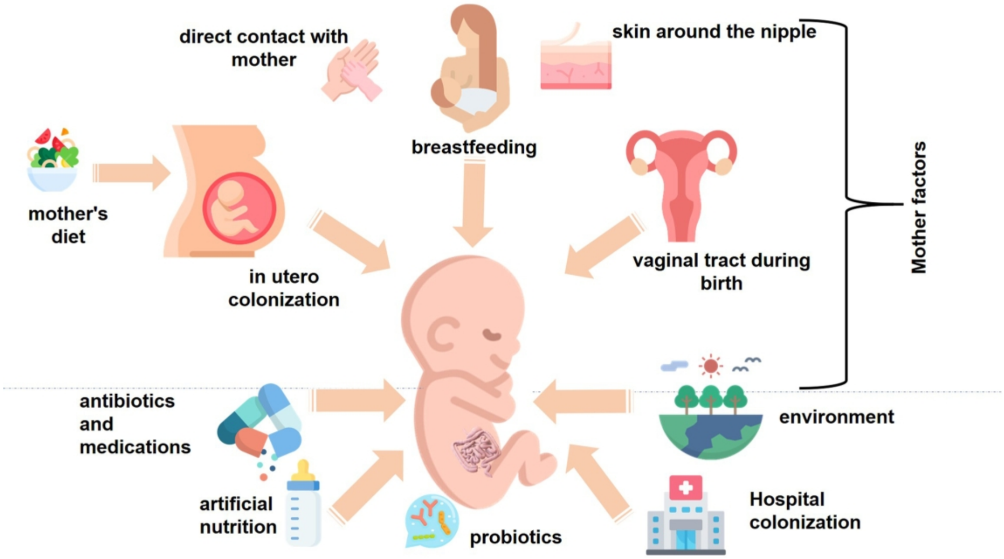

Fig 2. Source of the infant microbiome. The figure shows the influence of the mother's microbiota and the environmental microbiota on the bacterial colonization of newborns and infants.

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