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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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# 1 **Breastfeeding as a regulating factor of the development of the intestinal**

## 2 **microbiome in the early stages of life**

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16 9

### 17 **Abstract**

18 10  
19  
20 11 Since the first bacterial inhabitants of the human gastrointestinal tract were identified a lot of research into the study of the  
21  
22 12 human microbiome and its effects on health has been conducted. Currently, it is accepted that humans have a symbiotic  
23  
24 13 relationship with the gut microbiome, though the specifics of this relationship are not well understood. The microbiome of  
25  
26 14 neonates constantly changes and appears to influence many facets of the infant's health and predisposition later in life. This  
27  
28 15 review aims to show how the microbiome develops over time. We discuss its composition, origins and stages of  
29  
30 16 development of microbiota, the possible health benefits of a proper neonatal microbiome, and the dangers associated with  
31  
32 17 dysbiosis. We emphasize the shielding, modulating, and stimulating effects breast milk has on the infant microbiota. The  
33  
34 18 methods commonly used for the study of microbiota are also discussed.  
35  
36 19

37 20 **Keywords:** microbiota, neonatal gut, breast milk, dysbiosis, enterotypes, probiotics  
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## 30 **The importance of the gut microbiome**

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

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

46 The gut also hosts microorganisms capable of utilizing the gaseous byproducts of fermentation such as carbon  
47 dioxide and hydrogen, and through the removal of these waste products, helps drive metabolism forward [10,11]. The  
48 fermentation of amino acids by these bacteria provides additional SCFAs, that can be used as fuel.[12] However, the  
49 metabolism of aromatic, sulphur-containing, and basic amino acids produces pro-inflammatory, cytotoxic, and neuroactive  
50 compounds [7]. Only a small portion of dietary fat reaches the colon[13] and the relationship between microbial lipid  
51 metabolism and the host's health is unknown. However, it is known that free lipids have antimicrobial properties [14].  
52 Saturated fatty acids promote inflammation [15], which might be one reason for the chronic inflammation present in obesity  
53 [7], while omega-3 unsaturated fatty acids are anti-inflammatory [16]. .

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

1  
2 58 The microbiome is known for modulating the secretion of antibodies and interleukins and the functions of other  
3  
4 59 immune cells [18]. As suggested by recent studies, the early establishment of symbiosis between the immune system and  
5  
6 60 the gut microbiome has a large influence on the susceptibility or the resistance to diseases later in life [19]. During the  
7  
8 61 weaning period, the immune system of infants undergoes rapid development. It has been shown that the microbiome takes  
9  
10 62 an important part in the development of isolated lymphoid follicles and the regulation of intraepithelial lymphocytes,  
11  
12 63 macrophages, and invariant killer T cells [18].  
13  
14 64

### 15 65 **Changes within the microbiome during pregnancy**

16  
17 66 Although the adult microbiome differs between persons, it is fairly stable during life and research has revealed  
18  
19 67 some generalities. The most common phyla present in healthy individuals are Firmicutes (22.2 +/- 18.66% ) and  
20  
21 68 Bacteroidetes (73.13 +/- 22.16% ) followed by Proteobacteria (2.15 +/- 10.39%) and Actinobacteria, which is mostly  
22  
23 69 represented by the *Bifidobacterium* genus (1.82 +/- 3%). A vast majority of Bacteroidetes are members of the *Bacteroides*  
24  
25 70 genus, with *Bacteroides dorei* being the most dominant (17.44 +/- 8.74%), while *Bacteroides fragilis* is the most  
26  
27 71 widespread species. The abundance of *Bifidobacteria* varies between 0.004% and 12.21%. In regards to Firmicutes, the  
28  
29 72 genus *Clostridium* appears to be the most common [2].  
30

31 73 During pregnancy, the mother's vaginal, oral, and gut microbiota undergo significant changes, the origin of which  
32  
33 74 is unknown. Changes in hormonal regulation, immunity, energy homeostasis, and fat storage likely have a role in  
34  
35 75 influencing the microbiome [20]. The changes in the microbiome happen gradually during pregnancy. An increase in the  
36  
37 76 abundance of Proteobacteria and Actinobacteria is seen at the cost of *Faecalibacterium* and other SCFA producers [21].  
38

39 77 During the third trimester, mothers showed a lower diversity within a single sample, while having the largest  
40  
41 78 diversity between different mothers. This suggests that pregnancy causes the depletion of microbial diversity, however, it  
42  
43 79 increases the diversity between individuals. The increased diversity between mothers lasted for up to one month postpartum  
44  
45 80 [21]. When transferred to germ-free mice, third-term microbiota caused more weight gain, insulin resistance, and  
46  
47 81 inflammatory responses than first-term microbiota. This shows that the microbiota contributes to the changes occurring  
48  
49 82 during pregnancy [21]. There is also evidence suggesting that an alternation in maternal microbiota during pregnancy such  
50  
51 83 as during exposure to antibiotics, influences the neonate's immunity and health [22]. Changes to the vaginal microbiota,  
52  
53 84 such as the presence of certain fungi like *Candida albicans* [23], a lower *Lactobacillus* abundance, and an increased  
54  
55 85 *Gardnerella* and *Ureaplasma* abundance[24] are associated with preterm birth .  
56  
57 86

## 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 contaminants 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 ( $\alpha$ -casein,  $\beta$ -casein, and  $\kappa$ -casein), whey ( $\alpha$ -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

1  
2 144 triglycerides, which allows for increased absorption and decreased calcium malabsorption [42]. The fat in human breast  
3  
4 145 milk is concentrated in globules surrounded by a MFGM, which contains a high amount of bioactive compounds that play a  
5  
6 146 role in neurocognitive development and immune function [44]. The content of long-chain polyunsaturated fatty acids  
7  
8 147 (LCPUFA) is largely affected by the mother's diet, and is negatively affected by the high omega-6/omega-3 ratio present in  
9  
10 148 western diets. [45,46] A higher ratio of omega-6/omega-3 is positively associated with higher body fat percentages between  
11  
12 149 2 weeks and 4 months of age and may contribute to adiposity [47].

13 150 The main carbohydrate in human breast milk is lactose [36]. It appears at a concentration of 6.7g/100 ml exceeding  
14  
15 151 the concentration of other species [48]. The concentration of lactose increases in mothers with a higher volume of milk  
16  
17 152 production [49]. The micronutrient composition of breast milk varies by maternal diet and body stores. Breast milk contains  
18  
19 153 vitamins A, B1, B2, B6, B12, and D along with iodine and other micronutrients.[50,51] Regardless of diet, vitamin K is low  
20  
21 154 in human breast milk and should be supplemented [23d]. The effects of the micronutrients in human breast milk on infant  
22  
23 155 growth are not well known [43].

24  
25 156 In addition to macro- and micronutrients, breast milk contains numerous bioactive components including  
26  
27 157 hormones, growth factors, cytokines, and immune cells. The growth factors present in milk stimulates the development of  
28  
29 158 the intestines, growth and maturation of neurons, repair of tissues, and protection against damage from hypoxia and  
30  
31 159 ischemia . T cells, stem cells, lymphocytes, and macrophages are all present in breast milk along with non-cellular immune  
32  
33 160 components such as immunoglobulins and cytokines. Additionally, it contains compounds such as lactoferrin, lactadherin,  
34  
35 161 bile salt-stimulating lipase, and mucins which serve a role in protecting the infant against bacteria and viruses [36].

## 36 162 37 38 163 **The infant microbiome, health risks and benefits associated with microorganisms found in the** 39 40 41 164 **neonatal gut**

42  
43 165 The composition of the neonatal microbiome has substantially more plasticity than adults. It changes rapidly with  
44  
45 166 ageing and depends on various factors such that it is significantly different between formula-fed and breastfed babies  
46  
47 167 [1,9,14]. Table 1 contains a comparison of the neonatal gut and breast milk microbiota.

48  
49 168 During the first week of life, the microbiome is dominated by facultative anaerobes, such as those from the Proteobacteria  
50  
51 169 family. These bacteria consume oxygen and shape the intestinal environment to be more habitable for obligatory anaerobes  
52  
53 170 which appear later [54].

54 171 Not only does breast milk contain factors that shield the underdeveloped immune system of newborns but it also appears to  
55  
56 172 promote the growth of certain microbes, such as *Bifidobacterium* species, due to their ability to metabolize human milk  
57  
58  
59  
60

1  
2 173 oligosaccharides (HMOs), and *Lactobacilli*. *Enterococci* are also more prevalent in breastfed infants, while formula-fed  
3  
4 174 children show an increase in the presence of *Clostridium*, *Escherichia*, and *Bacteroides* [1]. The microbiome has a big effect  
5  
6 175 on infant development. Studies show that mice grown in germ-free environments have poor growth, decreased weight,  
7  
8 176 intestinal problems, and altered neurodevelopment [55].

9  
10 177 Overall, neonates are characterized by lower bacterial diversity than adults with breast-fed infants having less  
11  
12 178 diversity in their gut than formula-fed infants [19]. It also appears that children on a mixed diet have the bacterial diversity  
13  
14 179 profile of formula-fed infants. The microbiome shifts quite dramatically when solid foods are introduced to the infants' diet,  
15  
16 180 with a shift in dominance towards fiber-fermenting *Bacteroides* and *Firmicutes* and moving towards a composition similar  
17  
18 181 to that of adults [19].

19 182 The most studied members of the gut microbiome are the model organism *Escherichia coli* along with the genera  
20  
21 183 *Lactobacillus*, *Bifidobacterium*, and potential pathogens such as *Clostridium*. *Escherichia coli* is a microorganism  
22  
23 184 commonly found in the lower intestine of mammals. Although most strains are harmless and even aid in the health of the  
24  
25 185 host by producing exogenous vitamins such as K vitamins [56]. Unfortunately, there exist *E.coli* strains that can cause  
26  
27 186 diarrhoea, respiratory tract infections, pneumonia, and urinary tract infections [57]. The pathogenicity of *E.coli* is dependent  
28  
29 187 on several virulence factors such as fimbriae, adhesions, toxins, and other elements which can directly interact with  
30  
31 188 epithelial cells of the intestinal, respiratory, and urinary tract [58]. It has been shown that *E.coli* strains in breast-fed infants  
32  
33 189 have fewer virulence factors such as the K-capsule and have increased type 1 fimbriae expression. The IgA contained in the  
34  
35 190 mothers' milk can bind to the same type of fimbriae [59]. It appears that *E.coli* isolated from breast-fed infants show higher  
36  
37 191 adherence to epithelial cells of the colon compared to those in formula-fed children [60]. Type 1 fimbriae expression has  
38  
39 192 been shown to enhance the virulence of *E.coli* in the urinary tract [61]. However, breast-fed infants have shown a lower risk  
40  
41 193 of urinary tract infection [59].

42 194 Bacteria from the *Lactobacillus* genus belong to a broad group called the lactic acid bacteria, defined by their  
43  
44 195 ability to produce lactic acid as the sole or main byproduct of carbohydrate metabolism. They are known to colonize oral  
45  
46 196 cavities, gastrointestinal tracts, and vaginas of humans and animals. The presence of *Lactobacilli* in the gut is commonly  
47  
48 197 regarded as beneficial to the host and are frequently used as probiotics. However, there is little evidence supporting any  
49  
50 198 major role this genus might have on the human gastrointestinal tract. On the contrary, evidence suggests only a small  
51  
52 199 number of *Lactobacilli* are true residents of the mammalian gastrointestinal tract, and that most are instead allochthonous  
53  
54 200 and derived from food or the oral cavity. Recent research, based on the amplification of 16S rRNA genes, shows that  
55  
56 201 *Lactobacilli* make up only a small fraction of the total microbiota [62]. Attempts to treat infant colic with *Lactobacilli*  
57  
58 202 supplementation have shown no benefit [63]. However, it has been shown that supplementation with *Lactobacillus*



1  
2 203 *rhamnosus* reduces the duration of diarrhea [64]. Studies in animals have shown that treatment with *Lactobacillus* can  
3  
4 204 improve enteritis recovery [65] and inhibit the colonization of the pathogenic *E.coli* K1 strain [66].

5  
6 205 *Clostridium* is a genus of Gram-positive, anaerobic, and spore-forming bacilli. *C. difficile* is a major cause of  
7  
8 206 diarrhoea and potentially lethal nosocomial infections, especially in the elderly [67]. However, its pathogenicity in infants is  
9  
10 207 still debated [68]. Up to 70% of healthy newborns can be colonized by *C. difficile* during the first months of life and most  
11  
12 208 lack any symptoms of infection even when large numbers of toxin-producing bacteria are present. The underdeveloped  
13  
14 209 intestinal mucosa may lack *C. difficile* toxin receptors or other factors such as the immaturity of the immune system might  
15  
16 210 also play a role, although the true reason is unknown [68]. It is important to note that *C. difficile* infections still occur  
17  
18 211 especially in infants with hematological malignancies, inflammatory bowel disease, and cystic fibrosis following lung  
19  
20 212 transplantation [67]. Colonization by *C. difficile* is more common among formula-fed infants than among breastfed ones  
21  
22 213 [67] due to the lack of IgA in the formula [69]. The presence of *C. difficile* decreases with ageing and reaches the  
23  
24 214 prevalence levels similar to adults by 3 years of age [70].

25 215 *Bifidobacterium* is a genus of Gram-positive, anaerobic bacteria that commonly inhabit the gastrointestinal tract,  
26  
27 216 vagina, and oral cavities of mammals, including humans. Their presence in the gastrointestinal tract is deemed beneficial,  
28  
29 217 thus they are commonly added to probiotics and functional foods. *Bifidobacteria* rapidly colonize the infant gut during the  
30  
31 218 first weeks after birth. *Bifidobacteria* have been associated with protection from carcinogens, reduction in inflammation,  
32  
33 219 and regulation of gut function. They are more prevalent in babies born vaginally suggesting they are acquired from the  
34  
35 220 vaginal tract of the mother. Furthermore, breastfeeding supports the growth of this genus due to its ability to digest human  
36  
37 221 breast milk oligosaccharides. As a result, *Bifidobacteria* are a major part of the newborn microbiome. However, their  
38  
39 222 presence decreases rapidly with ageing and remains low but stable during adulthood [71].

## 40 223 41 42 224 **Enterotypes in infants and stages of gut microflora development**

43  
44  
45 225 In recent years, metagenomic studies have suggested that the intestinal microbiome of each human belongs to one  
46  
47 226 of three types based on the dominating microorganism. These genera are *Bacteroides* (Enterotype 1), *Prevotella* (Enterotype  
48  
49 227 2), and *Ruminococcus* (Enterotype 3). These enterotypes do not differ in functional abundance and do not correlate with any  
50  
51 228 factors relating to the host. However, the prevalence of certain genera indicates the use of different routes to generate energy  
52  
53 229 from fermentation [72]. Although the possible benefits of using the enterotype model are high, there are certain points of  
54  
55 230 contest when it comes to the theory. The enterotypes are not sharply delineated [72], and apparent clusters may arise from  
56  
57 231 certain methods of data processing even when they are not factual [73]. Additionally, by focusing on the enterotype model it

1  
2 232 is possible to miss smaller changes and individual differences in the microbiota. The long-term stability of a human's  
3  
4 233 enterotype also comes into question [73]. Some research suggests that there are only two enterotypes, the *Prevotella* and  
5  
6 234 *Bacteroides* genera [74].

7  
8 235 Certain studies seeking to evaluate the presence and importance of enterotypes in infants have been performed.  
9  
10 236 This research has identified four distinct enterotypes with the dominant microorganisms being either the Firmicutes phylum,  
11  
12 237 *Bifidobacterium*, *Bacteroides*, or *Prevotella* [75]. Unlike adults, the differences in enterotypes seem to be dependent on the  
13  
14 238 stage of gut development and can transition from a less mature into a more mature one. In particular, the strains associated  
15  
16 239 with Firmicutes and *Bifidobacterium* were correlated with the early developmental stages of the gut microbiota, while  
17  
18 240 *Bacteroides* and *Prevotella* were correlated with later stages [75]. While the enterotypes did not seem correlated with  
19  
20 241 antepartum or postpartum factors, certain clinical factors seemed to influence them to an extent. Type Firmicutes were more  
21  
22 242 common in infants delivered by C-section and in infants with lower gestational age, although these factors often appear  
23  
24 243 together. The duration of breastfeeding was also a factor with Firmicutes being more common in infants breastfed for  
25  
26 244 shorter durations while breastfeeding longer seemed to promote *Bifidobacterium* [75]. A different study, using two  
27  
28 245 enterotype models failed to detect a negative correlation between *Prevotella* and *Bacteroides* in infants 9 to 18 months of  
29  
30 246 age. However, such a correlation appeared at 36 months suggesting stable enterotypes develop between 18 and 36 months  
31  
32 247 of age [76].  
33

### 34 249 **The dangers of microbial dysbiosis and factors contributing to its occurrence**

35  
36 250 Multiple factors affect the composition of the infant microbiome, including but not limited to the mother's diet,  
37  
38 251 feeding type, and medication [55]. Dysbiosis is a term used to describe a breakdown in the balance between "protective"  
39  
40 252 and "harmful" intestinal bacteria [77]. Dysbiosis is associated with multiple diseases, such as obesity, type 2 diabetes,  
41  
42 253 hypertension, NEC, and inflammatory bowel disease, autoimmune diseases [18], asthma, food allergies, autism, and  
43  
44 254 opportunistic infections [19].  
45

46 255 One of the most common causes of dysbiosis is antibiotic treatment. Antibiotics are the most common medication  
47  
48 256 prescribed for children. Studies have shown that the use of antibiotics in early life is associated with obesity and the  
49  
50 257 occurrence of diseases later in life. Antibiotic treatment has a long-term effect on the microbial composition and diversity in  
51  
52 258 the gut. Antibiotic treatment in early life has been associated with allergies, atopic diseases, autoimmune diseases, and  
53  
54 259 infections such as NEC [77]. Acid blockers are also associated with dysbiosis and NEC [55]. Children of obese mothers  
55  
56 260 have a different bacterial colonization profile than those born to nonobese mothers. These differences are maintained during  
57  
58  
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1  
2 261 the first few years of life. However, the development of obesity may begin in utero due to the obesogenic and inflammatory  
3  
4 262 maternal environment [78].

5  
6 263 Gestational diabetes mellitus (GDM) is associated with changes to the microflora of both mother and child.  
7  
8 264 Samples taken from GDM positive subjects exhibited less diversity than those from GDM negative patients. In addition, the  
9  
10 265 meconium of GDM positive mothers exhibits a higher abundance and prevalence of eukaryotic viruses possibly exposing  
11  
12 266 the child to a greater number of viruses [79].

13 267 Preterm infants are especially susceptible to dysbiosis due to their underdeveloped intestines. The immaturity of  
14  
15 268 the gastrointestinal tract and immune system coupled with altered gut microbiota can have severe health consequences.  
16  
17 269 Moreover, pre-terms require hospital treatment which further disturbs the microbiome and exposes the infant to the  
18  
19 270 influences of the hospital's environmental microbiome [41].  
20

21 271 Antibiotics are routinely prescribed for preterm children to prevent infections. Although this treatment decreases  
22  
23 272 mortality it also alters the microbiota causing reduced bacterial diversity[80], delaying *Bifidobacteria* colonization [81] and  
24  
25 273 increased presence of multi-drug resistant strains [80]. Furthermore, the time required for the recovery from such  
26  
27 274 disruptions is positively correlated with the length of antibiotic treatment [80,81]. Additionally, artificial respiration shifts  
28  
29 275 the microbiome towards aerobic and facultative anaerobic bacteria due to the introduction of oxygen to an otherwise anoxic  
30  
31 276 gastrointestinal tract [82]. This can result in the weakening of the mucosal barrier [83] and reduced production of energy,  
32  
33 277 nutrients, and bioactive components [84].  
34  
35 278

### 36 279 **Modulation of the gut microbiota by probiotics and breast milk**

37  
38  
39 280 Probiotics are live microorganisms promoted as having health benefits when taken as food supplements, while  
40  
41 281 prebiotics are compounds that promote the growth or activity of beneficial microorganisms. There have been several studies  
42  
43 282 investigating the benefits of pre- and probiotic supplementation for infants.

44 283 Studies on animal models show that *Bifidobacteria* supplementation might counteract the effect of carcinogens,  
45  
46 284 help reduce diarrhea caused by viral infections or antibiotic treatment, and prevent constipation [71]. There is also evidence  
47  
48 285 that supplementation with *Bifidobacteria* reduces the occurrence and severity of NEC in low birth or preterm infants [85]. It  
49  
50 286 also has the potential to reduce the spread of gastroenteritis and diarrhea in infants in residential care units [86].  
51

52 287 Attempts to treat infant colic with *Lactobacilli* supplementation has shown no benefit [63]. However,  
53  
54 288 supplementation with *Lactobacillus rhamnosus* reduces the duration of diarrhea [64]. *Lactobacillus* GG has been shown to  
55  
56 289 prevent and reduce the duration of diarrhea caused by rotavirus infections in animals [87, 88]. Animal studies have shown  
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1  
2 290 that treatment with *Lactobacillus* can improve enteritidis recovery [65] and inhibit the colonization of the pathogenic *E.coli*  
3  
4 291 K1 strain [66]. *Lactobacilli* have also been shown to modulate Th1/Th2 cytokine balance [89,90] which might help in the  
5  
6 292 prevention of atopic disease and supplementing breastfeeding mothers or infants has been shown to reduce the incidence of  
7  
8 293 atopic dermatitis (eczema) [91].

9  
10 294 In the absence of a mother's breast milk, donor human milk (DHM) appears to be the best substitute for helping the  
11  
12 295 development of preterm babies. The microbiota of children fed DHM is similar to breastfed infants, although it shows a  
13  
14 296 decrease in Bifidobacteriaceae and an increase in Staphylococcaceae, Clostridiaceae, and Pasteurellaceae. The  
15  
16 297 pasteurization of donated breast milk and the different composition of preterm milk and donated milk might contribute to  
17  
18 298 this effect [92].

19 299 HMOs are a type of carbohydrate present in breast milk and although they don't have any nutritional value, they  
20  
21 300 serve as a prebiotic stimulating the growth of proper microbiota and modulating several infant mucosal and systemic  
22  
23 301 immune functions [36]. These oligosaccharides differ between mothers, but this does not cause any incompatibility issues  
24  
25 302 [93, 94, 95]. However, it has been shown that one type of HMO, specifically disialyllacto-N-tetraose (DSLNT) is protective  
26  
27 303 against the risk of NEC in rats, which point to the conclusion that the protective effects of these compounds are dependant on  
28  
29 304 specific HMO structured [96]. A study in piglets has also shown that HMOs can reduce the symptoms of rotavirus  
30  
31 305 infections [97].

32  
33 306 Research on the benefits of probiotics in infants has been promising and they appear to be safe. However, the  
34  
35 307 studies have used different strains and administration strategies thus more studies are needed to identify the ideal  
36  
37 308 combination. As of today, feeding breast milk from either the mother or that has been donated appears to be the best method  
38  
39 309 of stimulating a beneficial microbial composition.

## 40 310 41 42 311 **Methods for studying the microbiome**

43  
44  
45 312 Historically the study of human and animal microbiota has been based around traditional non-molecular methods  
46  
47 313 involving the isolation of microbes, microscopic observation, and growing them in culture. Although these methods have  
48  
49 314 been incredibly useful in the early study of the microbiome, they have several limitations. Traditional cultures tend to  
50  
51 315 underestimate the true variety of microorganisms present as a large number of bacteria cannot be cultivated using currently  
52  
53 316 known methods or require artificially created environmental conditions for that organism to grow [98,99]. Temperature, pH,  
54  
55 317 oxygen, and nutrient levels [98] and cultivation time [99] need to be tuned towards the studied microorganism. Furthermore,  
56  
57 318 the existence of mutual relationships between different bacteria further complicates the issue. In particular, the creation of a

1  
2 319 biofilm, which is composed of many different microorganisms, is difficult to replicate in a lab. This limits the variety of  
3  
4 320 microorganisms that can be studied using traditional methods, which provides a biased view of the microbiome composition  
5  
6 321 with an overrepresentation of aerobic organisms [100].

7  
8 322 Even with these limitations, culture methods have the unique advantage of allowing living microorganisms to be  
9  
10 323 studied in regards to antibiotic response and susceptibility, antigens, microorganism relationships, biofilm formation, and  
11  
12 324 the creation of experimental models [99]. New culture methods are still being developed to allow for the growing of  
13  
14 325 microorganisms previously considered uncultivable. Examples of such methods for the cultivation of hard-to-culture  
15  
16 326 microorganisms are the use of gnotobiotic animals [101] or the creation of artificial environments simulating the intestinal  
17  
18 327 environment, such as the SHIME system [102]. These methods come with the additional benefit of being able to study gut  
19  
20 328 microbe-host and microbe-microbe relationships [103, 104].

21 329 In response to the limitations of traditional methods, molecular methods for studying microorganisms were  
22  
23 330 developed. These methods involve the study of a microorganism's molecular components such as DNA, RNA, proteins, and  
24  
25 331 metabolites. These methods are culture-independent, meaning that the studied microorganisms do not need to be isolated  
26  
27 332 and cultivated in a medium. Rather, they allow for the *in vitro* study of microorganisms considered impossible to be grown.  
28  
29 333 The basis for most molecular methods is a variant of the DNA PCR [98]. By using PCR, the amount of DNA in a sample  
30  
31 334 can be increased exponentially allowing for further analysis with techniques such as Southern blotting [105]. With  
32  
33 335 modifications of the PCR method by using different starters, conditions, or pre-preparation techniques on the samples it is  
34  
35 336 possible to turn it into a diagnostic method itself. For example, ligation-mediated PCR techniques utilize the selective  
36  
37 337 amplification of DNA fragments generated by enzymatic restrictions creating a genetic fingerprint for a sample [106]. Other  
38  
39 338 methods can also provide certain insights, for instance, terminal restriction fragment length polymorphism (T-RFLP) has  
40  
41 339 suggested that *Clostridium* plays an important role in the pathogenesis of NEC [107]. While variants of gradient gel  
42  
43 340 electrophoresis have revealed the disruption of the human microbiome by antibiotic administration and identified a  
44  
45 341 correlation between *Sphingomonas* and NEC in human children [108,109]

46 342 With the rise of DNA sequencing technology, the ability to study complex microbial communities has increased.  
47  
48 343 Although its use was initially limited due to costs, improvements in the technology have allowed for cheaper, faster, and  
49  
50 344 more sensitive identification technologies. The increased availability of bioinformatic tools has allowed for the creation of  
51  
52 345 modern new generation sequencing (NGS) technology and allowed for the development of metagenomics, which is the  
53  
54 346 study of the total genetic material within an environmental sample. Metagenomics can be used to study microbial diversity  
55  
56 347 and dysbiosis of the intestine, identify new genes and microbial pathways and identify relationships between the  
57  
58 348 microbiome and the host's health [110]. Metagenomics aims to catalog all the genes from a microbial community by  
59  
60

1  
2 349 random sequencing of all DNA present in a sample [110]. The gene most commonly used for sequencing is the 16S rRNA  
3  
4 350 gene as it is present in various microorganisms while having highly variable regions allowing for its differentiation between  
5  
6 351 species [111]. For fungi the 18S rRNA gene or the Internal Transcribed Spacer sequence is used [112].

7  
8 352 Another method of sequencing is whole-genome shotgun sequencing which allows for the identification of viruses  
9  
10 353 [58g,58h] whose genetic data is missed by 16S sequencing as they lack such sequences [98]. This method can also provide  
11  
12 354 information regarding gene content and metabolic pathways [113]. However, a major disadvantage of this technique is that  
13  
14 355 the DNA from the host is also amplified and can often overwhelm the bacterial DNA. Additionally, analysis of the acquired  
15  
16 356 data is complex and requires a lot of computational power [114]. The sequences obtained by either method can be analyzed  
17  
18 357 with the assistance of bioinformatic tools and methods, such as databases stemming from data acquired by the Human  
19  
20 358 microbiome project or the MetaHIT project. This enables a broader understanding of the structure and function of microbial  
21  
22 359 communities. Metagenomic methods have revealed the relative stability of a healthy individual's microbiome and identified  
23  
24 360 multiple factors that affect its composition [110].

25 361 Although molecular methods are incredibly useful in the study of microorganisms they do have limitations. DNA  
26  
27 362 sequencing provides information on the presence of genes but doesn't give any insight into gene expression [98,99].  
28  
29 363 Additionally, some DNA sequences may amplify more efficiently under given conditions introducing bias to the results.  
30  
31 364 Furthermore, the PCR reaction does not discriminate between living and dead bacteria or their fragments [114]. The DNA  
32  
33 365 samples used for metagenomic analysis must be of high quality and in sufficient quantity. However, microbiome samples  
34  
35 366 are almost universally contaminated by human DNA. Additionally, not all identified sequences can be matched due to the  
36  
37 367 lack of reference and determining function on sequence homology introduces ambiguity to the results [110, 115].

38 368 A supplementary method to DNA sequencing is RNA sequencing, which allows researchers to look directly at the  
39  
40 369 transcriptome of microorganisms and gain insight into gene expression [116]. RNA sequencing can be used to study the  
41  
42 370 effects of environmental perturbations and factors on the function of the gut microbiome and identify a functional change  
43  
44 371 before a composition change occurs. This could allow one to preemptively detect the signs of dysbiosis [117, 118].  
45  
46 372 Metatranscriptomics can be used to determine the activity of genes in a defined environment, such as the human gut.  
47  
48 373 However, this method requires high-quality RNA samples, which are difficult to obtain and often difficult to separate  
49  
50 374 mRNA from other types of RNA. Additionally, mRNA is unstable and the reference databases are still insufficient [110].

51  
52 375 Methods for studying protein (metaproteomics) or metabolite (metabolomics) profiles are also being developed and  
53  
54 376 can supplement metagenomic analysis. Metaproteomics has greatly benefited from improved methods of protein separation,  
55  
56 377 high throughput mass spectrometry, increased computing power, and the growth of metagenomic databases. However, such  
57  
58 378 methods are in their infancy and their development is difficult due to the high complexity of human samples and difficulties

1  
2 379 in analyzing the data [114]. Meanwhile, metabolomic profiles of the human gut microbiota combined with other methods  
3  
4 380 can be used to predict the appearance of dysbiosis [119]. Methods for studying the microbiome are presented in Fig 1.

## 5 6 381 7 382 **Conclusion**

8  
9 383 The human intestinal microbiome is an incredibly complex subject to study. Not only is it one of the richest  
10  
11 384 microbial ecosystems found on earth but the relationships between the host, the microbiome, and one's health are often not  
12  
13 385 straightforward, with each influencing the other. Furthermore, the microbiome of babies displays significant plasticity and is  
14  
15 386 influenced by multiple factors such as mode of birth, type of feeding, medical conditions and treatments, and is shaped by  
16  
17 387 the development of the infant's gut (Fig 2). However, research has identified several health effects associated with the  
18  
19 388 microbiome and found ways to influence the developing microbiome, with some of these methods being put into practice.  
20  
21 389 Although several issues remain unclear.

22 390 The origins and roles of pre- and postpartum factors on the development of an infant's microbiome are still  
23  
24 391 inconclusive. The specific roles certain classes of microorganisms assume in the gut and the importance of their metabolic  
25  
26 392 products have yet to be discovered. New methods for studying microorganisms have been crucial in enhancing our current  
27  
28 393 knowledge base and in conjunction with traditional methods have provided further insight into the ecosystem of the human  
29  
30 394 gut. With such knowledge, new ways of treating illnesses and improving an infant's health may appear.

## 31 32 395 33 34 396 **Author contributions**

35  
36 397 Bartosz Ostrowski: Conceptualization, Collected data, Writing - Original Draft

37  
38 398 Beata Krawczyk: Conceptualization, Visualization, Supervision, Writing – Review & Editing

39  
40 399 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].

Phylum	Genera	Breast milk	Neonatal intestine
<b>Firmicutes</b>	<i>Staphylococcus</i>	+	+
	<i>Streptococcus</i>	+	+
	<i>Veillonella</i>	+	+
	<i>Enterococcus</i>	+	+
	<i>Gemella</i>	+	-
	<i>Clostridium</i>	+	+
	<i>Lactobacillus</i>	+	+
	<i>Eubacterium</i>	-	+
	<i>Ruminococcus</i>	-	+
	<i>Peptostreptococcus</i>	-	+
<b>Actinobacteria</b>	<i>Propionibacterium</i>	+	+
	<i>Actinomyces</i>	+	-
	<i>Corynebacterium</i>	+	+
	<i>Bifidobacterium</i>	+	+
	<i>Streptomyces</i>	-	+
<b>Proteobacteria</b>	<i>Pseudomonas</i>	+	-
	<i>Sphingomonas</i>	+	-
	<i>Serratia</i>	+	-
	<i>Escherichia</i>	+	+
	<i>Enterobacter</i>	+	+
	<i>Ralstonia</i>	+	-
	<i>Bradyrhizobium</i>	+	-
	<i>Klebsiella</i>	-	+
	<i>Acinetobacter</i>	-	+
	<i>Desulfovibrio</i>	-	+
<b>Bacteroidetes</b>	<i>Prevotella</i>	+	+
	<i>Bacteroides</i>	-	+

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2 706 **Fig 1. Methods used for the study of microbiome.** Legend: T-RFLP - Terminal restriction fragment length  
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4 707 polymorphism; WGS - Whole Genome Sequencing

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6 708 **Fig 2. Source of the infant microbiome.** The figure shows the influence of the mother's microbiota and the environmental  
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8 709 microbiota on the bacterial colonization of newborns and infants.  
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For Peer Review

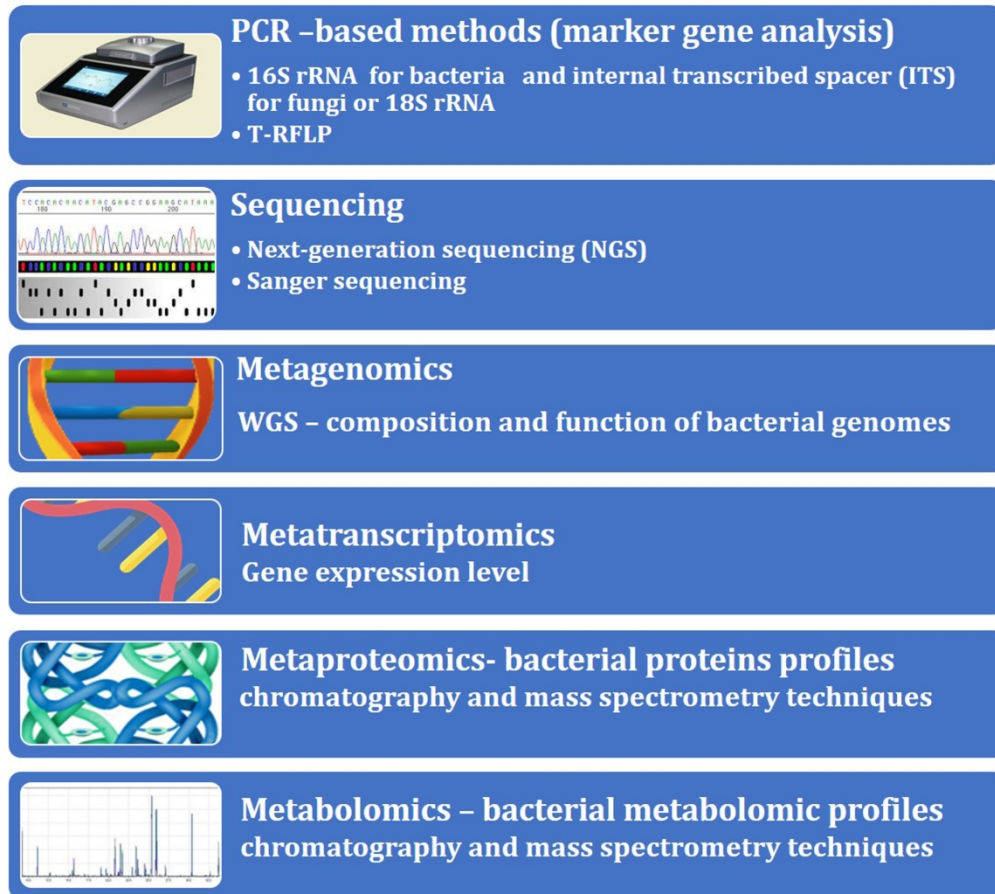


Fig.1 Methods used for the study of microbiome. Legend: T-RFLP - Terminal restriction fragment length polymorphism; WGS - Whole Genome Sequencing

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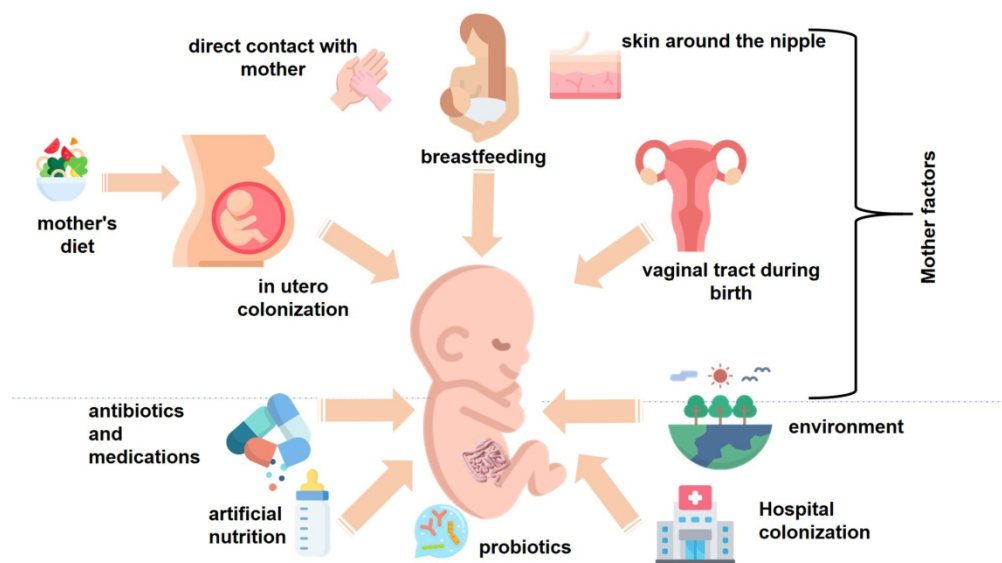


Fig 2. Source of the infant microbiome. The figure shows the influence of the mother's microbiota and the environmental microbiota on the bacterial colonization of newborns and infants.

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