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Review

Gut perturbation and probiotics in neonatology

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Abstract

Recent studies suggest that foetal colonisation begins prior to birth. There are other major determinants for neonatal gut colonisation other than that of a possible prenatal transfer of maternal bacteria to the foetus, including the delivery and feeding mode, as well as perinatal antibiotic exposure. Generally, vaginally born infants are first colonised by bacteria from the maternal vagina, whereas the gut microbiota of infants born by caesarean section (CS) more often resembles that of maternal skin and oral microbiota. Indeed, CS delivered babies seem to have a higher incidence of obesity, type 1 diabetes and asthma. The mode of feeding also plays an important role in influencing early intestinal microbiota. A more eubiotic microbiota composition is conferred to breastfed infants than to their formula-fed counterparts. Nowadays, we have evidence of antibiotic induced intestinal dysbiosis, which is, in turn, associated to an increased risk of developing overweight/obesity, as well as asthma, wheezing and/or inflammatory bowel disease, later in life. Overall, the early gut dysbiosis may have long-term negative effects on an infant's healthy immunological, hormonal and metabolic development. There has been extensive evaluation of how probiotic supplementation early in life may re-establish gut eubiosis and reduce the negative long-term effects of early dysbiosis. The most commonly used and studied probiotic strains and species include *Lactobacilli*, *Bifidobacteria* and *S. boulardii*. Accumulated evidence in neonatology suggests that some probiotic strains may be effective in preventing antibiotic associated diarrhea, necrotizing enterocolitis in premature infants and/or eczema. *L. reuteri* may also be effective in treating infantile colic.

Keywords

Eubiosis, dysbiosis, neonatal microbiota, probiotics, infants.

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Introduction

Publications on gut microbiota and microbiome were few and far between before the year 2000. From then on, as various authors elucidated the potential causal impact of microbiota on some human diseases, interest in this field has increased significantly. This is reflected by an extraordinary surge in the number of publications on microbiota over the last 5 years, with more than 4,000 publications to date [1].

As humans, our microbial load exceeds the total number of our own cells by about 10 fold [2]. However, recent estimates suggest that the ratio bacterial/human cells is closer to 1:1 [3]. The largest collection of microbes resides in the intestine, encompassing around 100,000 billion microbes of more than 1,200 different species. Their genome includes 3 million genes, which is a figure 100 fold higher than that of the human genome.

The advent of culture-independent approaches, such as gene sequencing of the bacterial 16S ribosomal RNA (rRNA) gene, has facilitated a more precise definition of the various gut microbial species. Since this gene is present in all bacteria and contains nine variable regions, it has become a widespread technique, permitting an easy distinction of the various species [4]. As the sequencing of the entire 16S rRNA gene has a low sensitivity, the approach has more recently shifted to a more in depth analysis of shorter sub-regions of this gene, even if this technique is not infallible [5]. The current high resolution techniques, like whole-genome shotgun metagenomics, which identified 2,172 species isolated from humans, classified into 12 different phyla, has led to a complete identification of microbiota composition [6, 7].

The most common bacteria (93.5% of all species identified) found in the neonatal intestine belong to four different phyla: *Actinobacteria*, *Prtoeobacteria*, *Firmicutes* and *Bacteroidetes*. Within the phylum itself, the microbes are further classified on the basis of a given subclass, genus, species and strain. However, microbial colonisation does not have an equal distribution along the intestinal tract, with the proximal and distal colon being the two most densely populated segments [8].

Factors affecting early gut microbiota

Numerous changes in microbial composition occur early on, during pregnancy. One such example of these modifications is the fact that there is a highly viable microbial count in the oral cavity, with a predominance of *P. gingivalis*, *A. actinomycetemcomitans* and *Candida spp.* [9]. Oral microbiota is of utmost importance as a strong phylum-level similarity has been observed between the placenta and tongue, tonsils, saliva and sublingual plaque taxonomic profiles. A unique placental microbiota, mainly made up of non-pathogenic commensal aerobic and anaerobic bacteria from the *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes* and *Fusobacteria* phyla, has been characterized [10]. Other major changes take place in vaginal microbiota composition during pregnancy e.g. in the gut and vagina, which show an increased *Actinobacteria*, *Proteobacteria* and *Lactobacilli* count, respectively. Therefore, the neonatal gut composition is influenced during pregnancy by a haematogenous spread and ascending bacteria colonisation, as well as by maternal genetics and/or other epigenetic factors, such as lifestyle, diet, hygiene and pharmaceutical intake [2, 11].

At birth, the delivery mode is a major discriminant affecting the newborn's gut microbiota. Indeed, the neonatal colonisation in a vaginal delivery (VD) is mainly influenced by the mother's vaginal and intestinal microflora, whereas the mother's skin microflora mostly modulates the microbiota of babies born by caesarean section (CS). This difference is reflected by both a lower abundance and diversity of *Actinobacteria* and *Bacteroidetes* phyla and a higher abundance and diversity of *Firmicutes* in CS delivered babies.

At a genus level, *Bifidobacteria* and *Bacteroides* seem to be significantly more frequent in VD infants than in CS infants who tend to harbor *Clostridia*, but lack *Bifidobacteria*. However, the delivery mode seems to have less effect on the colonisation and diversity of *Bifidobacteria*, *Lactobacilli*, *Bacteroides* and *Clostridia* from the age of 6 to 12 months of life [12-14]. Indeed, the microbial diversity increases during the first year of life and the microbiota composition converges towards a distinct adult-like microbial profile by around 2.5 years of age [2].

The type of feeding also plays a key role in the development of an infant's gut microbiota. Indeed, breastfeeding is undoubtedly the best way to promote the healthy development of a human offspring, modulating the infant's early gut colonization, both by human milk microbioma and other unique nutritional components contained in human milk, e.g. oligosaccharides and lactoferrin, also known as prebiotic or bifidogenic factors. Consequently, breastfed infants are more colonised by *Bifidobacteria* species than their formula-fed counterparts, who have a higher microbial diversity, including *Clostridium*, *Bacteroides*, *Prevotella*, *Lactobacillus* and *E. coli* species [8]. The human milk microbiota composition may, in turn, be influenced by the bacterial entero-mammary pathway, as well as by the infant coming into contact with the mother's breast skin [15].

In summary, host genetics, maternal positive epigenetic factors, VD and breastfeeding work in concert to shape the neonatal gut microbiota homeostasis, also known as "eubiosis".

The role of gut eubiosis

The last few years have witnessed a rise in the interest the role early gut eubiosis plays in health. Indeed, gut microbiota can be considered an organ with immuno-metabolic functions. It also has several immunological effects, i.e. it balances Th-1, Th-2, Th-17 and T-regulatory (T-reg) cell responses, giving rise to a reduced predisposition towards both atopic and auto-immune diseases, prevents pathogens binding to cellular receptors, releases bacteriocins inhibiting pathogen development, produces short-chain fatty acids (SCFA) with trophic and anti-inflammatory properties, stimulates the tight junction proteins and modulates mucosal cells growth [16]. The bacterial species mainly responsible for these functions are segmented filamentous bacteria, *Bifidobacteria*, *Lactobacilli* and *Bacteroides* [17].

Anti-inflammatory properties play a pivotal role in the prevention of endocrine and metabolic diseases, such as type 2 diabetes and obesity, which have been shown to be associated with changes in the gut microbiota leading to increased mucus degradation, gut permeability and metabolic endotoxaemia. Consequently, inflammation and macrophage infiltration of the adipose tissue are triggered predisposing to insulin resistance. The glucose homeostasis regulation and insulin sensitivity is also regulated by the gut microbiota. It modulates the intestinal content of the endocannabinoid system as well as the mRNA expression of cannabinoid type 1 specific receptors, which, when activated, induce gut permeability [18].

More recently, there is emerging evidence as to a bidirectional route that communicates with the brain, the so-called brain-gut microbiota (BGM) axis [1]. Indeed, the production of neuro-endocrine-immune mediators in the intestine may affect the brain by modulating the psychological, behavioural and cognitive functions. Through the production of tryptophan, a key neurotransmitter of the BGM axis and other yet only partially understood mechanisms, the gut microbiota exerts control over the hypothalamic-pituitary-adrenal axis, which, in turn, regulates psychological stress responses in the host organism [19].

Factors perturbating gut microbiota homeostasis in the newborn

As the transfer of bacteria from the mother to the foetus and the newborn contributes to the development of the neonatal gut eubiosis, every factor that interferes with this physiological process may be responsible for the gut microbiota perturbation. The loss of native host beneficial specific micro-organisms leads to a disruption in neonatal gut microbiota defined as "dysbiosis" which, in turn, has been associated with detrimental effects on health, either in early or later life [20, 21].

As aforementioned, CS delivery may play a role in early dysbiosis. Indeed, meta-analyses of cohort and case-control studies observed a positive association between CS delivery and the development of type 1 diabetes, asthma and/or obesity, with an increased risk ratio (RR) of about 20%. Noteworthy is the fact that all these meta-analyses reported the association of CS delivery with these outcomes [22]. Moreover, CS delivered infants have a higher risk of developing diseases associated with the immune function, mainly those involving the mucosal immune system [23]. CS delivery also seems to upregulate the immune response to food allergens, thus predisposing infants delivered by CS to the development of food allergy, but not atopic dermatitis, in early childhood [24]. Moreover, a systematic review and meta-analysis recently reported an association between CS delivery and childhood obesity, with a RR of 1.29 (CI: 1.16-1.44) after adjustment

for maternal pre-pregnancy weight [25]. These data have been confirmed by other cohort studies reported in literature [26].

According to literature, the early colonisation with *Clostridia spp.*, namely *C. difficile*, the lower microbial diversity and the reduced count of *Bifidobacteria* seem to be the microbiota perturbations most commonly associated with the development of immuno-metabolic diseases [18, 27].

Perinatal antibiotic exposure is another relevant "dysbiotic" factor [20, 28], particularly in preterm infants, as they are already prone to dysbiosis. This predisposition is a consequence of numerous factors, including: higher rates of CS deliveries, maternal infections and the use of anti H-2 drugs; the infant's intestinal immaturity and altered motility; lower rates of breastfeeding. The fact that prematurity affects the microbiota is reflected by a reduced percentage of the *Bacteroides* family during the first months of life and a higher initial percentage of *Lactobacilli* in preterm infants, than in full term infants [8]. It has been shown that perinatal antibiotics, including intrapartum antimicrobial prophylaxis, affect the gut microbiota by increasing *Enterobacteria* and *Clostridia* organisms in infants, reducing *Bifidobacteria* and *Lactobacilli* and lowering bacterial diversity [29]. Preterm infants administered early empiric antibiotics are at higher risk of necrotizing enterocolitis (NEC), sepsis and death, than are those not exposed to antibiotics [30]. Of interest is also how infants' fecal microbiota composition may differ at 30 days of life, depending on maternal and/or infant antibiotic exposure. A recent functional inference study [31] shows an absolute predominance of *Proteobacteria* for an isolated maternal antibiotic exposure; along with a predominance of *Proteobacteria*, *Firmicutes* and *Actinobacteria*, which are also detectable when there is an isolated infant antibiotic exposure. Should both mother and infant be exposed to antibiotics, then the infant's gut is predominantly colonised by *Proteobacteria* and, to a lesser extent, *Firmicutes*. This microbial composition is particularly relevant as several studies have reported that *Proteobacteria* and/or *Firmicutes* predominance may be associated with early- or late-onset of NEC [32-34]. Furthermore, literature reports evidence that gut microbiota plays a role in the predisposition to colic. Indeed, colicky infants' gut microbiota is characterized by a predominance of specific *Proteobacteria*, i.e. *Escherichia spp.*, *Klebsiella spp.*, *Serratia spp.*, *Vibrio spp.*, *Yersinia spp.* and *Pseudomonas spp.* and a reduced abundance of *Bifidobacteria*, *Lactobacilli* and *Bacteroides* species [35, 36].

In summary, perinatal antibiotic exposure is one of the major determinants of neonatal gut dysbiosis and is, in turn, related to a higher risk of the development of a dysregulation of the immune response and related diseases, e.g. metabolic syndrome, asthma, atopic and auto-immune diseases and brain disorders.

The role of probiotics

As early gut dysbiosis seems to be related to a higher risk of developing various chronic diseases, the possible role and effect of early probiotic supplementation to restore microbiota homeostasis has been extensively investigated [37]. Probiotics are defined as "live non-pathogenic microorganisms (bacteria or yeasts) that, when administered in adequate amounts, may replicate and colonise in sufficient numbers in the gastrointestinal tract to confer a health benefit on the host" [38]. These microrganisms must be of human origin and be able to resist gastric acid pH and colonize the gastrointestinal tract in a sufficient cell concentration, i.e. at least 105-1,010/g. If they are to be considered probiotics, they must also be able to produce SCFA, antimicrobial substances and vitamins and to have immunomodulating and immunostimulating properties [39]. Although they mostly colonise the large intestine by adhering to the colon mucosa, there is no scientific evidence for probiotic replication or colonisation in the small intestine. Should there be any microbiota perturbation, probiotics may limit the magnitude and/or duration of the resulting dysbiosis [40].

Probiotics exert beneficial intestinal effects in the gut through microbiological, epithelial and immunological actions. The mechanisms involved include: microbiota composition modulation, prevention of pathogen invasion and growth by competitive adhesion to the cell receptors and bacteriocin production; cell barrier modulation and growth by expression of the tight junction proteins and SCFA production, respectively; innate immunity and Th-1/Th-2 ratio modulation and an increase in the number and activity of T-reg cells [41, 42].

The most commonly used and studied of all the several hundreds of different strains and species of probiotics on the market to date are: *Lactobacilli*, *Bifidobacteria* and *S. boulardii* [43].

The indications for the use of probiotics may be limited to the prevention of antibiotic-associated and nosocomial diarrhea, allergy prevention and NEC, during the neonatal period, [44]. A specific *Lactobacillus* species, i.e. *reuteri*, has also been evaluated in the management of infantile colic.

A 2015 *Cochrane* review reported that there is a moderate "quality of evidence" (QE) supporting the fact that probiotics have a protective effect against antibiotic-associated diarrhea (AAD), with a pooled data RR of 0.46 (95% CI: 0.35-0.61) with a number needed to treat (NNT) of 10. It was reported that, amongst the various probiotics evaluated, *L. rhamnosus GG* (*LGG*) or *S. boulardii* at 5 to 40 billion CFU/day may be appropriate, given the low NNT and the very rare occurrence of adverse events. This review also stated that it was premature to draw any conclusions as to the efficacy and/or safety of other probiotic strains [45]. In 2016, the ESPGHAN Working Group for Probiotics/Prebiotics also recommended the use of either *LGG* or *S. boulardii* in AAD prevention, both supported by a moderate QE, but a strong "strength of recommendation" (SR). *S. boulardii* was also indicated as being able to prevent *C. difficile*-associated diarrhea, based on low QE and conditional SR [46]. Moreover, a 2016 meta-analysis of 15 placebo-control trials concluded with the same recommendations i.e. the use of either *LGG* or *S. boulardii* was associated to lower AAD rates, without an increase in adverse events (moderate QE) [47]. A recent *Cochrane* systematic review and meta-analysis of 31 randomized controlled trials (RCTs), on a total of 8,672 patients, concluded that there is moderate certainty evidence to suggest that probiotics are effective in the prevention of *C. difficile*-associated diarrhea (NNT: 42 patients; 95% CI: 32-58). Their short-term use appears to be safe and effective when administered in combination with antibiotics, as long as the patients are not immunocompromised or severely debilitated [48].

Lastly, there is current evidence (moderate QE; strong SR) that *LGG* has a protective effect against nosocomial diarrhea, as recently assessed by two different systematic reviews [42, 49]. When considering the question of allergy, a recent systematic review of randomized trials assessed the effect/s of any probiotic administered to pregnant women, breastfeeding mothers or infants and demonstrated that although probiotics were able to reduce the risk of developing eczema, they had no effect on the risk of asthma [41].

The role of probiotics in the prevention of NEC in premature infants has also been extensively investigated. A 2014 *Cochrane* systematic review including 24 trials demonstrated a significant reduction in stage II NEC events (RR 0.43) in 20 studies with an NNT of 30, in mortality rate (RR 0.65) in 17 studies, and in time to full enteral feeding and duration of hospitalization. Conversely, no significant effect on the occurrence of sepsis in general or weight gain was demonstrated. That is to say, statistically significant preventive effects have been proven both for specific *Lactobacilli* strains alone and for *Lactobacilli* and *Bifidobacteria*/*Saccharomycetes* mixtures, but not for *Bifidobacteria* or *Saccharomycetes* alone [50]. A 2015 systematic review and meta-analysis, carried out by an expert panel of the Italian Society of Neonatology, also supported the beneficial role probiotics play in the prevention of NEC in premature infants (RR 0.47; 95% CI: 0.36-0.60; $p < 0.00001$). However, this preventive effect was evident only in very low birth weight (VLBW) infants, but not in extremely low birth weight infants and was mainly related to the use of *Bifidobacteria* or probiotic mixtures [51]. Multiple-strain probiotic mixtures, including *Lactobacilli*, *Bifidobacteria* and *Saccharomycetes*, have also been proven to be the most effective in reducing NEC incidence and mortality (pooled odds ratio: 0.36; 95% CI: 0.24-0.56; $p < 0.00001$), as was reported in a 2017 updated meta-analysis of early probiotic supplementation in premature and/or VLBW infants. Conversely, no beneficial effects were observed in trials using single strains of *Bifidobacteria* and/or *S. boulardii* alone [52]. A very interesting observational study [53] investigated the effect of routine probiotic (*L. acidophilus* and *B. bifidum*) supplementation on the occurrence of NEC and/or death. Preterm infants (birth weight < 1,500 g), enrolled before and after the introduction of the use of early probiotic administration, were compared. The preventive effect of probiotics was evident only in exclusively breastfed infants (OR 0.43; 95% CI: 0.21-0.93; $p = 0.03$). This may be explained by the symbiotic effect of the administered probiotics and the prebiotic oligosaccharides in breast milk. However, this study does have major limitations due to its observational design and the relatively short treatment duration, as well as dosage and composition of the probiotic mixture. Moreover, it seems that, to date, there is no convincing evidence that probiotic supplementation is

efficacious in the prevention of late onset sepsis (LOS). Indeed, numerous studies have reported conflicting data, which may, however, be due to the heterogeneity of the trials, in as much as they used different probiotic strains, doses and duration of administration [54]. Nevertheless, results from a recent systematic review and meta-analysis of 25 trials seem to confirm that probiotic mixtures reduce LOS incidence in exclusively breastfed VLBW preterm infants (RR 0.75; 95% CI: 0.65-0.86; $p < 0.0001$) only [55]. Moreover, the role probiotics play in the treatment of infantile colic has recently been assessed in a systematic review [56]; data from five RCTs showed that breastfed infants receiving *L. reuteri* had a significant decrease in crying/fussing time compared to controls ($p < 0.01$). These data have also been confirmed in a more recent meta-analysis of double-blind trials that compared *L. reuteri DSM17938* and placebo in a population of 345 colicky infants. After 21 days, the probiotic-supplemented group had a 25 min average reduction in crying and/or fussing time compared to the placebo group. Intervention effects were consistent and significant in breastfed infants, but not in formula-fed colicky infants [57]. According to a 2014 Italian RCT, *L. reuteri DSM17938* is also significantly effective ($p < 0.01$) in the prevention of infantile colic and other functional gastrointestinal disorders [58].

Conclusions

The neonatal early gut microbial colonisation seems to be a crucial step, that takes place at a critical age, for the modulation of an infant's healthy immunological, hormonal and metabolic development. Therefore, according to the "perinatal programming hypothesis" [59, 60], its perturbation might well be a negative epigenetic factor which could lead to long-term negative health effects. In the light of this hypothesis, gut immaturity, maternal infections, perinatal antibiotic exposure, CS deliveries, as well as formula-feeding, may expose infants to a higher risk of being overweight and/or obese, developing asthma, type 1 diabetes, NEC and/or having infantile colic. Overall, a reduced colonization with *Bacteroides* and *Lactobacilli* and a predominance of *Enterobacteriaceae* may characterize the so-called dysbiotic microbiota related to the negative health effects reported in literature to date. This has led to extensive investigation into the feasibility that early probiotic supplementation plays a pivotal role in the modulation and reduction of gut perturbations and dysbiosis. Some probiotics, i.e. those of the *Lactobacilli*, *Bifidobacteria* and *Saccharomycetes* genera, have been reported to be of utmost relevance in restoring early gut eubiosis. However, it must be stressed that the effects of a given probiotic strain in a given population cannot automatically be generalized to other strains or different populations and that the beneficial effects reported in the vast majority of clinical trials refer to a very limited number of strains. In conclusion, although accumulated evidence suggests that probiotics do not significantly impact the faecal microbiota of healthy subjects, they may well be useful in re-establishing gut microbiota homeostasis after a dysbiotic stress.

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Declaration of interest

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