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Crying in infants

On the possible role of intestinal microbiota in the development of colic

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Up to around a quarter of all infants cry excessively and unsoothably during their first months of life. This phenomenon has been termed "infant colic." In most cases, physicians are unable to determine the cause of the colicky behavior. In a recent study, and by means of comprehensive and deep analyses of more than 1,000 intestinal phylotypes, we found that infants with colic showed lower microbiota diversity and stability than control infants in the first weeks of life. Colic-control differences in the abundance of certain bacteria were also found at 2 weeks. These microbial signatures possibly explain the colic phenotype. In this addendum we discuss other recent publications on the subject and present previously unpublished analyses of our own. We address possible mechanisms behind the links between microbiota and crying, and present future directions that could further help elucidate the hypothesized relations between intestinal microbiota and infant colic.

Keywords: colic, intestinal microbiota, infants, excessive crying, development

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Introduction

Up to around a quarter of all infants spend a great part of their first months of life crying excessively and not responding to parental soothing attempts. This phenomenon has been termed "infant colic" and is often a source of great worry for parents, even though it mostly resolves by 3 or 4 mo of age. Many parents seek professional help for their colicky infant, and the situation produces exhaustion and considerable strain, frequently preventing parents from functioning optimally both at home and at the workplace. In 5–10% of the infants with colic, an underlying cause of the crying can be found (e.g., cow milk allergy), but in most cases, physicians are unable to determine the cause of the colicky behavior. There is accumulating evidence that the intestinal microbiota in infants with colic differs from that of healthy controls. In studies that were mostly based on traditional culturing approaches, the stools of colicky infants were found to display reduced diversity in microbiota, lower counts of lactobacilli and higher numbers of gram-negative bacteria. However, these reports described differences in infants already diagnosed with colic and usually of over 6 weeks of age. In a recent study, and by means of comprehensive and deep analyses of more than 1,000 intestinal phylotypes in over 200 samples, we found that infants with colic showed lower microbiota diversity and stability than control infants in the first weeks of life. Already as early as at 2 weeks after birth, specific differences in the abundance of certain bacteria were found when fecal samples of colic and control babies were compared. Although the study was aimed to unravel associations, it is tempting to assume that the observed microbial signatures may be, at least partially, causative of many colicky infants' excessive crying. In this addendum we further discuss our results in the light of other recent publications on the subject and present previously unpublished analyses of our own. Additionally, we address the possible mechanisms for the observed specific microbial signatures and infant crying, providing support for a theoretical causal

model. Finally, we discuss future directions that could further help elucidate the hypothesized relations between intestinal microbiota and infant colic.

Microbial Signatures of Infants with Colic

At birth, the intestines of the infant are virtually sterile. Within minutes, the colonization by bacteria begins. The bacteria originate mainly from the mother and the environment, and the colonization is influenced by factors such as prematurity, mode of delivery, sanitary conditions of hospital and home environments, feeding type, antibiotic use, and presence of siblings or pets.[1-4] A well-balanced colonization of the neonatal intestine is important for the development of the immune system, such that a late acquisition of intestinal bacteria or a reduced complexity of the microbiota, may delay its maturation.[1,5] In addition to affecting the development of the immune system, delays and abnormalities in the colonization process may have other, immediate effects in the young infant. In our study, we compared nine fecal samples of the first 100 d of life from infants that at 6 weeks of age were determined to have colic with those of a control group of infants with low levels of crying behavior.[6] DNA was isolated from all samples and analyzed for their global bacterial composition by using the Human Intestinal Tract Chip (HITChip), a phylogenetic microarray platform that allows a comprehensive and deep analysis of over 1,000 known intestinal bacteria and has been used to analyze over 5,000 intestinal samples (Salojarvi et al., unpublished observations).[7] The main results of our study relating to colic babies are summarized in **Figure 1**. We observed that fecal microbiota diversity gradually increased after birth in the control group but not in the colic group. In the first postnatal weeks, the diversity of the colic group microbiota was found to be significantly lower than that of the control group. Additionally, the stability of the microbiota of successive samples was significantly lower in the colic infants for the first weeks.

Significantly distinct microbial signatures were found in the colic group at 2 or 4 weeks of age, while early indications for the observed aberrations were already detectable in the first week of life. Proteobacterial DNA was increased more than 2-fold in colic as compared with control infants, especially specific groups of bacteria related to *Escherichia, Klebsiella, Serratia, Vibrio, Yersinia* and *Pseudomonas*. Notably, *Escherichia* and *Klebsiella* spp are known for their gas-producing properties as well as the potential production of inflammatory lipopolysaccharides (LPS). Contrarily, the DNA levels of bacteria belonging to the Bacteroidetes and Firmicutes were reduced, the latter including some canonical groups known to produce butyrate. We have evidence that the latter group of strict anaerobes is present in early life and includes bacteria notably related to *Eubacterium halli* (unpublished observations). These members of the *Clostridium* cluster XIVa group are capable of producing butyrate from acetate and lactate.[8] While butyrate is known for its anti-inflammatory, enterocyte-fuelling and pain-reducing effects in the adult intestine,[9] lactate and acetate do not have this positive connotation although these acids are produced in ample amounts by the abundant bifidobacteria. The decreased DNA levels of *Bacteroides* in colic babies are of specific interest, as recently these were found to be reduced as well in infants suffering from atopic eczema.[10,11] Finally, bifidobacteria and lactobacilli DNA was reduced in infants with colic. Detailed analysis of our data indicated that the reduced level of lactobacilli notably related to relatives of *L. plantarum* that also includes *L. rhamnosus*.[7] In line with this, we observed relatively high levels of *L. rhamnosus* in healthy babies of the studied cohort based on deep metagenomic analysis (De Been et al., unpublished observations). In conclusion, our present results confirm and extend those of earlier studies in older infants, which mostly employed traditional culturing methods and focused on specific bacteria.[12-16]

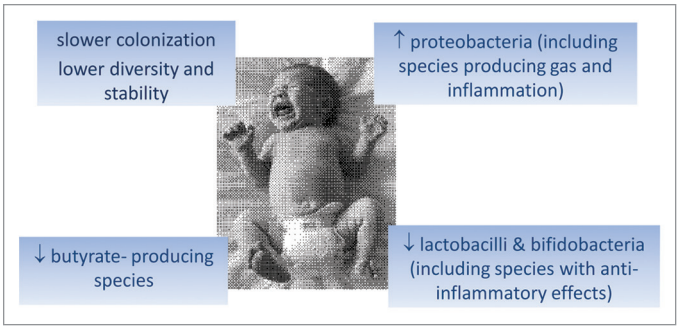

Figure 1. Microbial signatures of infants with colic at 7 or 14 postnatal days.

The microbial differences between the colic and control groups described above were only explained by crying but not by other factors, such as infant sex, mode and place of delivery, breast-feeding, birth weight and attendance of center-based childcare. Finally, the differences between the colic and control groups disappeared by 3–4 mo of age, indicating that the colic microbial signatures may be only temporary and not indicative of a permanently altered intestinal microbiota. This contrasts with the studies on atopic eczema where differences persist for over a year of age.[10,11]

Since the publication of our colic study, two papers have appeared describing the results of randomized double-blind placebo-controlled probiotic trials in colicky infants in two European countries, Italy and Czech Republic.[17,18] Both studies used designs with *Lactobacillus reuteri* as a probiotic, administering the same dose (10^8 colony-forming units) for the same 21-d period. The mean ages of the infants upon entering the study were 30 d (Italy study, n = 29) and 35 d (Czech Republic study, n = 80). Importantly, in both studies the crying decreased by 2-fold or more, significantly more often in the group of infants receiving probiotics than in those receiving placebo. The study in the Czech Republic reported these significant differences in crying behavior already 7 d

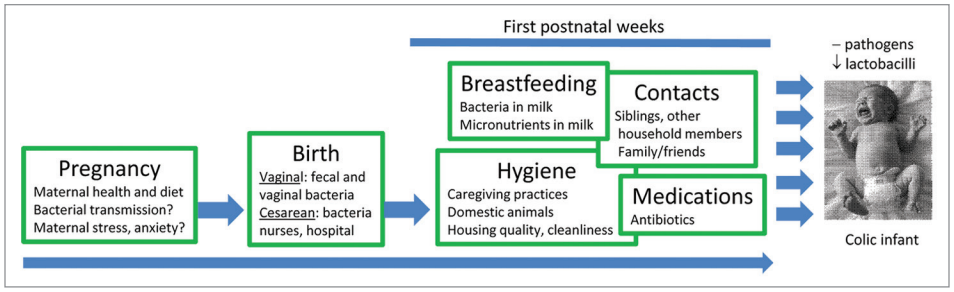

Figure 2. Major factors affecting a full-term infant's microbial colonization of the gut in the first weeks of postnatal life.

after starting the treatment.[18] The study by Roos et al.[17] reanalyzed an earlier *L. reuteri* trial by addressing the intestinal microbiota of the colicky infants by medium depth pyrosequencing. No effect of the probiotic on the global composition of the intestinal microbiota was found, but when the responders to the probiotic intervention were analyzed they had a higher level of *Bacteroides* spp. abundance than the non-responders. The results from these independent but comparable studies point in the direction of a causal relation between intestinal microbiota and excessive crying. Modification of the infant's microbiota by means of an oral probiotic treatment apparently directly affects the crying behavioral phenotype.

Another recent study on the links between microbiota and colic reported a highly increased odds ratio for the presence of *Helicobacter pylori* in feces of colicky infants as compared with controls [odds ratio, 15.3 (95% CI, 17.9–29.8)].[19] The infants were between 2 weeks and 4 mo of age, and of the 55 infants with colic, 45 (81.8%) tested positive for *H. pylori*, while of the 30 healthy controls, 7 (23.3%) tested positive for *H. pylori*. When revisiting this species in our earlier study, we found on average a 2-fold increased level of *Helicobacter*-related bacteria in colic babies as compared with the healthy control babies at 2 weeks (p = 0.01).

Finally, a recently published study by Gelfand et al.[20] reports that 2-mo-old infants of mothers with a history of migraine are 2.6 times as likely to have colic than infants whose mother do not suffer from migraine. The authors propose that colic may be an early life precursor of migraine, as migraine has a strong genetic underpinning. However, the fact that the excessive crying that characterizes colic disappears at 3–4 mo of age, places questions about the crying representing an early life manifestation of migraine. An alternative, and perhaps even provocative, explanation may lie in a possible relation between maternal migraine and intestinal microbiota. Both explanations may be related, perhaps in an indirect way, such as through maternal diet. To our knowledge, there are no studies relating adult migraine to intestinal microbiota. However, a report addressing 12-y-old children suffering from migraine by endoscopic techniques found evidence of inflammatory lesion in 29 out of 31 children.[21] The authors speculate that recurrent abdominal pain may be implicated in the etiology of migraine. In this line, it is possible to hypothesize that abnormalities in the microbiota of the maternal gastrointestinal tract may underlie both the maternal migraine as well as the infant colicky phenotype.

Possible Mechanisms Linking Intestinal Microbiota and Infant Crying

Remarkably, the differences between colic and control microbiota that we found in our study were all in the first month of life, before the colic peak of 6 weeks.[6] Based on these results, we therefore propose that early increased levels of pathogenic bacteria and reductions of lactobacilli, bifidobacteria or butyrate-producing bacteria produce intestinal pain and inflammation in the infant, and that this in turn causes excessive crying. However, it is not possible to determine causality in our study, and we cannot discard the possibility that other, unknown factors are behind both the microbial signatures and the crying. In any case, we can consider the observed patterns (**Fig. 1**) as early warning signals. Remarkably, these patterns share some features with those recently observed in adults with type 2 diabetes.[22]

It is appealing to take a closer look at the process of early colonization, as this process in many cases may hold the answer over the origin of the colic and possibly of other phenotypes. As stated before, infants are born with virtually sterile intestines,[23] and in our work we observed a low bacterial load in the meconium samples that were analyzed.[6] The subsequent colonization by microbiota commences almost immediately and proceeds at a rapid pace, with the infant intestinal tract becoming the habitat of dozens of bacterial species after only a few days. **Figure 2** presents a model of the factors affecting an infant's microbial colonization of the intestinal tract in the first weeks of postnatal life.

Abnormalities in any of these factors, together with fortuitous encounters with pathogens may result in the colic phenotype. It is yet unknown whether bacteria may reach the fetal intestine during pregnancy, although there are speculations on early colonization processes taking place through regular fetal swallowing of amniotic fluid.[24] However, there are other indications that maternal state during pregnancy may influence the neonate's intestinal colonization. Bailey et al.[25] found that prenatal stress reduced the number of bifidobacteria and lactobacilli in offspring of prenatally stressed rhesus monkeys (especially those stressed in late pregnancy). These authors also found a trend for stressed offspring to have more *Shigella flexneri* than non-stressed offspring. Whether human infants' intestinal colonization is similarly influenced by maternal (psychological) stress and anxiety during pregnancy remains to be determined, and we are currently exploring this

area by investigating the effects of late pregnancy maternal stress on the infant's colonization process.

During delivery, infants encounter the first major source of microbes: the maternal fecal and vaginal communities. The quality of the maternal vaginal and intestinal microbiota will therefore play a major role in the initial phase of colonization and perhaps even have long-term consequences for infant development and health.[26] In healthy pregnant women as compared with non-pregnant control women, the diversity and richness of the vaginal microbiome are reduced, with dominance of *Lactobacillus* species.[27] However, women suffering from unbalances or (subclinical) infections in their vaginal or intestinal microbiota will most probably transmit these to their infants, constituting a non-optimal basis for the further development of the intestinal microbiota. In infants delivered by cesarean section, the natural colonization process is disrupted, and these neonates will acquire their intestinal microbiota from the environment (e.g., ward staff, other infants and children, and family and friends) and from the maternal skin.[28] Studies show that these infants are characterized by low bacterial richness and diversity, lower bifidobacteria, lower numbers of *Bacteroides fragilis* and higher numbers of *Clostridium difficile*.[29,30] According to a recent review, the delivery mode has an important role in the numbers and diversity of both lactobacilli and bifidobacteria, and these effects may persist for some time, affecting the infant's subsequent health and development.[31]

Feeding has a crucial impact on the infant's intestinal microbiota, as the composition of the early diet will guide the colonization process.[31,32] Bifidobacteria are more dominant, and often more diverse, in the intestine of breastfed infants as compared with formula fed infants. These differences may be due to the unique composition of maternal milk, but also to the direct presence of bacteria in breast milk. In addition to containing appropriate nutrients for the growing infant, maternal milk is a source of oligosaccharides that selectively promote the growth of *Bifidobacterium* in the infant intestine,[33] and of lactoferrin (antimicrobial agent), and lysozyme (enzyme capable of digesting bacterial cell walls).[31] Human milk energy content may depend on the sex of the infant: a recent study found that mothers of male infants produced milk with a 25% greater energy content than mothers of female infants.[34] These sex-biased differences in quality of milk may in turn affect bacterial colonization of the intestines. With respect to bacteria, human breast milk contains over 360 prokaryotic genera,[35] and may be an important direct source of lactobacilli and bifidobacteria,[36] as well as of other bacteria for the infant.[37-39] These findings are relatively new, and many questions about the role of maternal milk remain. For example, is the colonization process influenced by the frequency and duration of each breastfeeding episode? Cultures around the world vary greatly in their frequency of feeding, from at least twice an hour (e.g., Kung San)[40] to Western patterns of once every 3 or 4 h. Whether and how these differences affect an infant's colonization process is as yet unknown. Also, how does the maternal peripartum psychological state affect the (bacterial) composition of her breast milk? There is a high prevalence of perinatal mental disorders, most commonly depression and anxiety, with rates of 10–13% in high-income countries and of 16–20% in low- and lower-middle-income countries.[41] These mental disorders are closely related to the physiological stress system and to diet. Again, how these states are related to breast milk composition is a matter of further research.

Hygienic properties of the early environment—such as housing characteristics—and cleanliness will most probably play a role in the colonization process. The presence of companion animals may influence hygienic conditions of the house, while at the same time playing an indirect role in transmission of bacteria. Dog-owning adults namely share more skin-like microbiota with their own dogs than with other dogs, and having a dog also increases the shared skin microbiota in cohabiting adults.[4] Parental hygienic and caregiving practices will constitute a direct link between the environment and the bacteria reaching their infant. A recent example of this parental influence comes from a Swedish study on pacifier cleaning practices.[42] The researchers studied two methods for cleaning a pacifier during an infant's first 6 mo of life: boiling vs. parental sucking of the pacifier and found that the latter was related to a reduced risk for allergy development and an altered oral microbiota in the child. The authors speculate that microbes transferred to the infant via the parent's saliva may stimulate the infant's immune system by influencing the development of the oral/pharyngeal microbiota, but also by being swallowed and influencing the development of the intestinal microbiota. However, the oral microbiota was only characterized by profiling and hence one may also envisage that the sucking of the pacifier by the parents is associated with other hygienic conditions leading to altered microbiota in oral and possibly intestinal microbiota.

The structure of the family an infant is born into is likely to influence the colonization of the intestinal tract. Family members share more of their skin, oral and intestinal microbiota than individuals from different households, with stronger effects of cohabitation on skin microbiota.[4] Also, 1-mo-old infants without siblings tend to have lower counts of bifidobacteria in the intestinal tract than infants with older siblings.[30] First-born infants are also more rapidly colonized by *Clostridium* species and by Enterobacteriaceae other than *E. coli*.[43] Fortuitous contacts of the infant with further family and friends could influence the establishment of the intestinal microbiota, but to date little is known about these environmental influences on the colonization process.

In the first postnatal weeks, infectious illnesses together with the use of antibiotics can have major effects on the development of the infant's intestinal microbiota, producing large shifts in taxonomic groups and altering overall diversity.[32] For example, oral antibiotics taken in the first month of life result in a decreased count of fecal bifidobacteria and *B. fragilis*-group species and increased *Enterococcus*.[30,44]

Finally, and for simplicity reasons not included in **Figure 2**, a first factor that may naturally play a role in the microbial colonization is the infant's genetic and epigenetic make-up that may affect the physical and physiological state of the intestinal

tract, in turn making it more or less attractive for different bacterial species.

In summary, many factors affecting the early colonization of the infant intestinal tract have been identified. As such these factors may have a smaller or larger influence in the origins of unbalances in microbiota and hence be implicated in the development of the colic phenotype. However, several gaps in the knowledge about the colonization process remain. Specifically information about the factors that play a determining role in the development of the colic signatures is lacking. Further prospective research carrying out intensive sampling designs, interventions and other cause–effect studies should help shed light on the mechanisms that underlie colic and promote therapies that can prevent this disruptive condition.

Challenges and Future Directions

Although the development of early fecal tests to detect microbial signatures predictive of future colic would seem an attractive advance in preventive medicine, it does not appear to be a feasible endeavor for the near future. The laboratory analyses used to detect the characteristic microbial signatures of infants with colic are presently too complex, time consuming and expensive for application as a diagnostic screening instrument. A more reasonable and pragmatic approach appears to lie in the realm of maternal and infant ingestion of pre- and pro-biotics to reinforce the chances of the infant developing a diverse and normal intestinal microbiota. Modulating maternal intestinal microbiota during pregnancy and lactation could directly diminish the infant's chances of developing colic. Moreover, given the importance of the intestinal microbiota for the maturation and development of the intestinal tract, the immune system and perhaps even the brain, these effects may extend far beyond those of the first couple of months, having a direct effect on the infant's future health. For example, recent studies indicate how interactions between the early intestinal microbiome and the environment may affect metabolic programming already from infancy, leading in some cases to childhood obesity.[45]

With respect to infant colic, the two studies described above,[17,18] together with an earlier study[46] present promising results as they indicate that administration of *L. reuteri* to colicky infants reduced their crying. A next step is to investigate whether the excessive crying can be prevented by giving these or other lactobacilli to the infant from birth (i.e., **before** the colic is apparent) and even to the mother toward the end of the pregnancy and during the breastfeeding period. Such treatments might in many cases help prevent the excessive crying altogether, at the same time giving the infant a boost of beneficial bacteria for the intestinal colonization. Problems that remain to be solved, however, are that the "ideal" composition of the infant intestinal microbiota is yet to be determined, as well as the identity of the core microbes that will lead to the most favorable health outcomes.[31] In the same line, researchers still face the challenge of determining the best (preventive) pre- and probiotics. A recent study by Aloisio et al.[47] investigated the features of 46 strains of *Bifidobacterium* and identified four promising strains for functioning as probiotics for the treatment of infant colic. The authors plan to test the selected strains in a validation clinical trial. Endeavors of this type are necessary to further identify the best strains for probiotic treatments. Similarly, the optimal dose, frequency and timing of administration for the infants and pregnant women are some aspects that need to be evaluated.[48] For example, there are indications that administration of a single dose of probiotics to low birth weight infants may have long-term effects on the microbiota,[49] raising the question about the necessity for a pattern of daily administration.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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