The Microbiological Risk

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Abstract

Microbiological risk in the first part of life is endowed with peculiar features when compared to the same risk in adulthood. The purpose of this review is to highlight these age-related traits. While pathogens harmful for neonates and infants have been reviewed, less attention has been paid to the role played by the infant gut as battle field between pathogens and protecting bacteria or between pathogens and the immune system. Immediately after birth a race for colonizing the gut begins; the main tool for neonates to select good bacteria is represented by mother’s milk. Quite surprisingly, this milk carries potentially harmful bacteria, but antibodies, oligosaccharides and the whole breast milk composition provide a powerful selective tool. Nevertheless this selective action is deeply influenced by the type and/or time (i.e. premature) of delivery or in premature subjects; recent data also show that breast milk could have a different potential in selecting bacterial species. The hygienic conditions of parents and, more generally, of the surrounding environment play a role in the selection of the intestinal biota of infants. It is then possible to group neonates according to the composition of the microbiota. Results of ecological studies suggest that neonates with a different microbiota could have a different microbiological risk.

Introduction

Once the germ theory of disease was accepted, microbes were considered to be pathogens if they met the stipulations of Koch’s postulate [1]. However, it rapidly became apparent that bacteria that are harmless to adults can be harmful in different hosts, such as infants; virulence, despite being a microbial characteristic, is differentially expressed in a susceptible host [2]. Thus, the question, ‘What is a pathogen?’ begs the question, ‘What is the outcome of the host-microbe interaction?’ This question is simple to raise but difficult to answer, as it is necessary to move our attention from the microbe to the environment.
Environment means, in the case of neonates, mainly the gut, which is to be considered as a battle field in which three armies are engaged: pathogens, commensal bacteria and the immune system. Additional factors are also relevant for the final outcome of the battle: whether the child is born by natural or cesarean section delivery, and the status of the intestinal tissues, mucus, etc.

However, the ultimate weapon of this war is represented by the type of food supplied to troops and this is why complementary feeding can have a relevant role in managing the microbiological risk in infants.

**The Gut as a Bacterial Ecological Niche**

I would like to start presenting some comments on the ‘battle field’, which at the beginning is totally germ-free; however, immediately after birth, a race starts in order to invade the ‘new lands’. These lands are quite easy to be reached as in neonates there are very reduced barriers at the borders:

- Adults tend to eat three meals a day and between these meals gastric pH is low; in newborn babies there is frequent feeding with milk, which is a good buffer [3]. This leads to the pH of the gastric contents being raised for prolonged periods. Under these circumstances, the gastric barrier is not really working and can provide only a limited selective action; this situation closely resembles what happens in adults treated with antiacid compounds.
- In fetus the liver develops from progenitor cells into a well-differentiated organ in which bile secretion can be observed by 12 weeks' gestation. Full maturity takes up to 2 years after birth to be achieved and the real potential of bile salts of neonates in controlling the uptake of orally delivered bacteria is not clear [4]. It should be noted, however, that breast milk does contain some trace of bile salts [5] but it is unclear whether, in addition to the emulsifying activity, there is also an action of selection against sensitive bacteria.
- The intestinal mucosal immune system is fully developed after a full-term birth, but the actual protective function of the gut requires the microbial stimulation of initial bacterial colonization [6]; then it takes some time in between the end of the protective action of antibodies carried by mother's milk and full activation of the neonate's immune mucosal protection.
- The whole load of bacteria in the first days of life is low; in adults the presence of the so-called ‘autochthonous’ (meaning in this case already present) bacteria provides a powerful barrier to the persistence of newcomers; this barrier has been defined as ‘colonization resistance’ and it is possible to measure it in an objective way. Obviously, in neonates this resistance is very low [7]. In addition after a few months of life the gut microbiota is ‘disturbed’ by a revolution in feeding habits: weaning is a
dramatic change for the intestinal microbiota which has to reach a new homeostasis [8].

The First Colonization

As a consequence of this reduced presence of barriers the first bacteria entering the human gut and reproducing in this environment are a mixture of enteric and nonenteric bugs. The first to arrive are not really ‘good guys’ for the host as they belong to Enterobacteriaceae, streptococci, staphylococci [9]. It takes some days for bifidobacteria, lactobacilli and possibly other, still less known anaerobic bacteria to take over from the first bacteria.

Most of the knowledge on microbiota composition of the first part of our life has been obtained by means of classical microbiological techniques such as plate counting, but recent papers have shown, by using a molecular biology approach, that it is probably time to make some changes. Several studies carried out using molecular methods have recently shown the presence of the genus Ruminococcus, described as an important component of the infant’s intestinal microbiota [9, 10]. In fact, the bacterial genus Ruminococcus is recognized to have an important protective effect on the host since it produces ruminococcin A, a bacteriocin that can inhibit the development of many of species of Clostridium [11].

Molecular biology techniques have also allowed to detect the presence of members of the genus Desulfovibrio [12], which have previously only been found in adults. This genus is mainly present in bottle-fed babies but it was also detected in breastfed ones.

By means of both classical and molecular-based techniques it is known that in healthy babies, Escherichia coli or bacteria belonging to Clostridium spp. are the initial colonizers rapidly followed by Bifidobacterium, Bacteroides, Clostridium, Streptococcus, Enterococcus and Actinomyces. Bifidobacterium species appeared already after 5 days in breast-fed babies but with some delay in bottle-fed neonates.

The molecular biology approach now provides, however, a better understanding of the process leading to the formation of the first, stable microbiota in infants. It has been pointed out that, in addition to the bacteria cited above, strictly anaerobic ones are also present in the gut of neonates [12]. The real role of these bacteria in protecting the health of babies is far from being understood. As a consequence, while the concept that microbiota of breastfed babies is the ‘golden standard’ still holds, our feeling that more than 90% of this microbiota is composed of bifidobacteria needs to be revised. Just as an example in several breastfed babies ruminococci are present at the same level as bifidobacteria. However we have to point out that different techniques [denaturing gradient gel electrophoresis (DGGE), fluorescent in situ hybridization (FISH), quantitative polymerase chain reaction (Q-PCR), cloning] resulted
in a determination of the microbiota composition which did not always match. More studies, performed on a large number of subjects to avoid the natural personal variation, are needed and therefore we have to be cautious in drawing conclusions. What seems clear is that molecular ecology of the gut microbiota of infants is rapidly accumulating new data.

**Microbiota Composition and Infant-Specific Pathogens**

Whatever the real composition of gut microbiota is in neonates, it is clear that it is managed by a few tools, when compared to the situation in adults. Moreover, this situation could lead to having an unstable environment where also bacteria with a low pathogenic potential could be harmful and even lethal. Just as an example I will here refer to *Enterobacter sakazakii*, the most recent and ‘fashionable’, infant-specific pathogen.

Pathogenesis of *E. sakazakii* involves bacteremia and/or sepsis, cerebrospinal fluid infection and meningitis, brain abscess or cyst formation, and has been associated with necrotizing enterocolitis [13]. Infant mortality rate for *E. sakazakii* meningitis was reported to be 40–80%. This bacterium seems to be able to breach the blood-brain barrier, as it has a tropism for the central nervous system. The real molecular mechanism of this tropism in neonates and infants, however, remains a mystery. Adhesion potential of this bacterium, a trait necessary for bacterial translocation, has been only recently assessed [14].

It is also surprising to note that, even though the first report of infection caused by this bacterium dates back to the 1960s and the first review of clinical aspects of the reclassified bacterium is dated 1988 [15], the first report on toxin production by *E. sakazakii* appeared only in 2003 [16] and up to now studies showing the real pathogenic features of these bacterium have been very few.

Enterotoxin production as well as adhesion potential have been shown; however these traits are not strong enough to be harmful for the adult, otherwise healthy, population.

We can probably conclude that this bacterium is mainly harmful because its stationary phase cells are remarkably resistant to osmotic and drying stresses compared with other species of the *Enterobacteriaceae* [16]; these technological features are the real ‘pathogenic phenotype’ at least for infants: if these cells arrive in sufficient number in the infant’s gut, they can multiply to such a level to be dangerous. But it seems to be more a problem of poor defense barriers than a high level of toxicity.

**Managing the Microbiota to Reduce Microbiological Risk**

It is then possible to turn the attention to the main section of this review: how can we manage the gut environment of infants in order to reduce the
microbiological risk. What has been outlined before has probably made clear that feeding could have a central role in this effort.

The gut microbiota of an adult is quite a stable environment and several factors contribute to its homeostasis: the selection done by the gastric environment, the lytic action of bile salts, the immune system, the mucus composition, and the colonization resistance. In contrast selection and maintenance of good bacteria in neonates can rely on only two main tools: type of feeding and mucus covering their intestinal tissues [17–19]. Breast milk also provides a strong selective action due to the mother's antibodies, but this action declines with time. I will only deal with the first of these tools, as there is stronger evidence available of the real impact of food on the microbiota composition.

As regards the food as bacterial selection agent it should be noted that the development of the bifidobacteria-dominant microflora has been related to the peculiar composition of human milk. In particular a bifidogenic effect has been ascribed to a low concentration of proteins and phosphorus, the presence of lactoferrin and nucleotides even if oligosaccharides of human milk have the best-documented bifidogenic action. A high percentage of such substances resist digestion in the gastrointestinal tract and reach the colon where they stimulate microbiota development. They are partially excreted with feces, representing the paradigm of the prebiotics [20]. Human milk oligosaccharides are synthesized in the mammary gland through the sequential action of specific glycosyltransferases that add monosaccharide units (galactose, fucose, sialic acid, N-acetyl-glucosamine) to lactose. Among these enzymes, the fucosyltransferases add one or more fucose moieties through specific bonds. Their presence is linked to the expression of the secretor and Lewis genes of the nursing mothers. Depending on the expression of these genes, one or more fucosyltransferases are present and, in turn, they significantly condition both the qualitative and the quantitative composition of fucosyloligosaccharides. Breast milk oligosaccharides have also been found to be analogues for microbial receptors preventing mucosal attachment, the initial step of most infections. As a result, breastfeeding significantly reduces the risk of neonatal septicemia, respiratory tract infections, otitis media, diarrhea and urinary tract infections.

Breast milk is then one of the best example of how potent the diet can be in influencing an ecological, bacterial niche such as the gut and, in turn, how the gut microbiota can have an impact on health and also on physiological functions.

The first defense mechanism to be influenced by gut microbiota is the immune system; data are available pointing out the role played by different microbiota in the early maturation of the gut immune system [21]. In addition, specific bacteria, could enhance different immunoresponses [22, 23]. It was shown [22] that bifidobacteria have species-specific effects on the expression of the dendritic-cell activation marker CD83 and the production of interleukin-10 (IL-10). Whereas CD83 expression was increased and IL-10 production was
induced by Bifidobacterium bifidum, B. longum, and B. pseudocatenulatum, B. infantis failed to produce these effects. It was concluded that B. infantis does not trigger the activation of dendritic cells to the degree necessary to initiate an immune response but that B. bifidum, B. longum, and B. pseudocatenulatum induce a Th2-driven immune response. This observation, even if preliminary, clearly stimulates the need of further, more detailed investigation at the species and subspecies levels, as B. longum is known to be strictly related to B. infantis (fig. 1).

After these remarks on the protective action of breast milk, it is safe to add, however, that this activity is far from being completely understood, as recent data have also shown that breast milk taken from different mothers could have varying potential for selecting bacterial species [19]. The hygienic conditions of parents and, more generally, of the surrounding environment, could also play a role in selecting intestinal biota. It was shown that microbial profiles of babies and their parents could be quite similar, suggesting a vertical transmission determined by genetic and environmental factors [10].

An aberrant composition of gut microbiota between groups of healthy and allergic children has also been reported [24]. Quite interestingly, the serum total IgE concentration correlated directly with E. coli counts in all infants indicating that the presence of these bacteria is associated with the extent of atopic sensitization.

It has been proven by meta-analysis results that prevention of the typical gastrointestinal diseases such as diarrhea is possible by feeding the ‘right’ bacteria to infants; this action does not seem related to a specific bacterium,
as the same positive results can be achieved using different probiotic preparations, containing bacteria or yeast. It seems then that the action could be related to a nonspecific, ecological action more than a strain-specific phenotype [25, 26].

**Preterm Neonates as a ‘Model System’**

To conclude it could be worthwhile to stress that the relevance of the gut environment in managing the selection of ‘good bugs’ is also provided by a negative reference test, the microbiota of preterm neonates. In these subjects the number of bacteria is reduced but also the range of different bacterial species is kept at a minimum [27]. Comparisons between term and preterm neonates in terms of gut microbiota really suggest the relevance of the environment in selecting and sustaining the good bacteria. As an example, it is possible to focus on the need that bacteria have to adhere to intestinal mucus; it has been shown [19] that this feature is age-specific, but we still lack knowledge on the specific interactions between bacteria and mucus of neonates, especially for those delivered preterm and/or very low birth weight subjects.

A low presence of indigenous commensal bacteria could open the door to bacteria causing necrotizing enterocolitis.

Preterm babies have a very low potential in retaining in their gut bifidobacteria [28] but, after one century of investigations, we have now adopted an evidence-based nutritional approach to manage the well-being of infants.

**Intervention on Microbiota Management**

As a first step in this evidence-based nutritional approach we can now deal with intervention studies in which probiotics and/or prebiotics have been fed to neonates in order to mimic, as a final result, the microbiota composition of breastfed babies, which is believed to be the most protective microbiota.

A range of bacteria have been used in different types of formula milk as well as some prebiotic substances, as a mixture or as pure compounds.

It has been shown, in several instances, that probiotic strains, if selected according to scientific criteria, are able to survive, reproduce and persist in the gut of neonates. Colonization seems to be more easy to achieve if the initial numbers of bifidobacteria or lactobacilli are low. However, colonization of preterm neonates seems to be more difficult to achieve but when successful it seems to allow a certain degree of protection against opportunistic infections.

Prebiotics have been shown to be able to manage microbiota composition in both term and preterm neonates; some data on the positive effects of this prebiotic-mediated microbiota on allergy are becoming available. Work in the field is rapidly progressing and results seem promising but I feel it is more
prudent to refer to the cautious approach taken by ESPGHAN in assessing the use of both probiotics and prebiotics [29, 30].

In the end it could be relevant to answer the following ‘old’ question: ‘If we group neonates according to the composition of the microbiota, may we find a correlation with their resistance to disease?’ This is the same question as the one raised by Tissier more than one century ago, ‘Is there any direct link between bacteria in the stool and health?’, but we have to admit that data are still scarce, even if extremely promising.

At the moment this is more an area of research than application, but I am confident that a significant reduction in the microbiological risk for neonates will be provided in the future by an ecological approach, exploiting the protective action of the gut as an ecological niche.

References

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Discussion

Dr. Haschke: Thank you for a fascinating lecture on this neonatal balance between good and bad bacteria. Would you agree that today we can say that vaginal delivery is better than cesarean section in terms of promoting a good bacterial balance early in the gut? There are a lot of data supporting this. If bifidobacteria and lactobacilli are not found in the stool during the first day of life that doesn’t mean that that they are not already present because the baby does not pass stool on the first day, it is meconium. The first day’s stool is what was already in the fetus before birth, it just shows that the fetal gut was sterile. But the stool comes after 24 h, and in some infants after 48 h, and then from the first moment you see the bacteria. So the bacteria are going in by the mouth, probably at birth in most of them, and we have also learned that breast milk contains certain bifidobacteria and lactobacilli. And finally, the ruminococci story is new to me; would it be an advantage for very young infants to have more in the gut to stimulate growth by mechanisms such as the prebiotic approach?

Dr. Morelli: Cesarean delivery is a kind of nightmare for the gut microbiota of a newborn. There are some papers indicating that it took almost 2 years to obtain a normal balance after cesarean section [1, 2]. I have probably underrepresented the
delivery system among the 6 points that are important but I have not tried to go into
detail. I think that this field could be one of the most promising in which to have food
intervention just to promote quicker recovery of the normal microbiota. I am not so
sure that breast milk could be a relevant source of lactobacilli, even though there are
papers showing the presence of bacteria in breast milk [3, 4], I would like to be cau-
tious. It is obvious that there are bacteria in breast milk, this is not new. There are also
good indications that there is a unique and single ecosystem between the mother and
the mouth of the baby. The skin of the mother and the mouth of the baby are exchang-
ing bacteria. The physiological mechanism allowing bacteria to pass into breast milk
has recently been investigated [5]. But the total number of bacteria is estimated to be
low. So this is an argument for the future.

I would like to draw attention to the presence of ruminococci that have been found
in neonates by several groups [6–8]. We know that they have an important anti-
clostridial action, they are Gram-positive and are definitely not pathogenic bacteria.
So it is possible that the ruminococci could play a positive role or nothing, but this is
something new that must be taken into consideration.

Dr. Haschke: We know that the lactobacilli and bifidobacteria might come from the
skin or might be transported into breast milk by a mechanism which we don't yet
understand. But they come from the mother. Where do the ruminococci come from?

Dr. Morelli: From the mother also. There are some studies that have demon-
strated exactly the same bonding pattern between the parents and the baby after the
baby arrived home.

Dr. Giovannini: Compliments, very interesting, but I have a point to clarify: the
problem of probiotics, because sometimes there is a bias between human milk
oligosaccharide and prebiotics. All the papers on prebiotics are only about fecal con-
sistence, and one paper about the lower incidence of atopic dermatitis between 3 and
6 months. We have known for many years and from long experience that probiotics
may be in the food and we know that probiotics will arrive in the gut and are some-
times difficult to replicate. For this reason I think that after the first year of life chil-
dren should perhaps receive probiotics in their food every day. But I am worried about
the problem of prebiotics because sometimes it is not evidence-based and sometimes
prebiotics may be a problem of marketing. We speak too much about fecal culture and
not enough about bacterial DNA which is the best way, a little bit expensive, to detect
a single bacterial strain.

Dr. Morelli: I totally agree with you. Microbiology has a lot of new tools, and as a
science it was born when the microscope, a necessary tool, was invented. Microbiology is not like mathematics in which pencil and paper are used; without a
microscope microbiology wouldn't exist. Now we have the genetic tools to obtain evi-
dence-based information about the real impact of pre- or probiotics. There is now
DNA fingerprinting of each single bacterial strain, so that we are in a position to say
that these bacteria recovered from a fecal sample are exactly the same bacteria as
those fed 2 days earlier, and this is important. It is a tool. I am not a clinician, I have no
explanation for the clinical aspect, the impact and so on. I am in the position to pro-
vide diagnostic tools and information on diagnosis by means of these diagnostic tools.

Dr. Ribeiro: Using your analogy of war strategy and transportation, food, sources,
what is your concern about timing for the use of pro- or prebiotics?

Dr. Morelli: I am not a clinician so I will respond as a microbiologist. The urgency
to intervene, to add pro- or prebiotics, is really relevant in cesarean section or in
babies who have received antibiotics for whatever reason. I feel that in naturally deliv-
ered and breastfed babies it is probably not as necessary in the beginning to support
the microbiota. It is possible that within 2 years my answer will change because we will
most likely have more information, but at this moment I think that babies born by
cesarean section or with a special condition, such as preterm, probably really need support from point zero. Under normal conditions, normal delivery, natural delivery and breastfeeding, we can probably wait.

**Dr. Delgado:** Pathogenic bacteria are presented by lymphocytes and can produce proinflammatory cytokines. With probiotics this mechanism can be changed or modified. Is it possible to produce more anti-inflammatory cytokines with this substance or bacteria?

**Dr. Morelli:** Nestlé has produced a lot of papers about gut-associated lymphoid tissues, and the answer is definitely yes. From my point of view what we are lacking is knowledge about the molecule. In my group we are working on the surface layers, some proteins that are outside the bacteria or the metabolite that are able to cross-talk with intestinal tissue. There are a lot of papers coming out using genomic array, the human genomic array, and there is regulation of the genes involved in this kind of response. So the answer is yes.

**Dr. Agostoni:** You said that we only know 30% of the bacteria present in the gut, and that ruminococci are strong agents against clostridia. Could we speculate that lactobacilli and bifidobacteria and now ruminococci are just proxies for the decrease in clostridia? This is the key point of the probiotic story to take space from clostridia to reduce their numbers and maybe all around the clostridia.

**Dr. Morelli:** The 30% figure was published by a French group for adult microbiota. We do not have exactly the same figures for infants, but we can speculate that more or less we are in the same condition. I have no answer to the second part of your question, but we need more details about the gut microbiota of infants in order to really exploit the good groups in a cleverer way. Probably in the near future we will have prebiotics that will be ruminococci-specific, or whatever other group specific, in order to see their clinical relevance because there is still a kind of open question mark. More than a century ago it was said that bifidobacteria are present in healthy babies, but as far as I know there is not really a lot of clinical data showing that babies with a very high level of bifidobacteria have better health or clinically significant outcome due to the presence of very high levels of bifidobacteria. This is a kind of dogma.

**Dr. Agostoni:** But the reason for my question is that from a practical point of view, as far as I know, the major association for clostridia is gut cancer. An excess amount of meat in the diet is associated with an increased number of clostridia and a supposed predisposition to get cancer.

**Dr. Morelli:** I know what you are trying to suggest but I don’t want to answer very clearly because I am prudent.

**Dr. Haschke:** I assume that the weaning period is more complex. I would not really agree with the conclusion that this good bacteria; might it be that *Lactobacillus* GG or *Bifidobacterium lactis* are contributing to health during the weaning period and later on? There are several data showing that they protect from diarrhea, and they are very important in terms of more rapid recovery from diarrhea, so there are a lot of data there. The bacteria when given either as a supplement or together with a formula have a beneficial effect. So there are health effects, would you agree? In a breastfed infant, how long would you suggest that the bifidogenic gut flora is beneficial, or when does it become trivial because solid foods are introduced anyway and the microbiota is moving towards the adult pattern? Perhaps you can only speculate, but how long would it be beneficial?

**Dr. Morelli:** About the first part of your question, I am not saying that adding selected bacteria will not be useful. I am wondering if a high level of naturally occurring bacteria of the same group of the selected bacteria is really useful or not, because we know that probiotic bacteria have been selected through a very thought-provoking selection scheme in the laboratory so we know that they are able to immune modulate
and so on. This is not true for the naturally occurring bacteria. Then the $10^7$ lacto-
bacilli or $10^9$ bifidobacteria, naturally occurring in the gut, have been selected simply
because they are able to survive in saliva, in the stomach, in the bile, and then they use
the food. This is something clever for them, I am not so sure that it is clever for me. In
the laboratory we have selected bacteria that are able to do something for the human
body. This is not a natural process. We are scientists, we must not presume that the
natural selection scheme is able to select the best bacteria for our health. That is my
point. Frankly, I can only speculate on the second point, so I would prefer to skip it.

Dr. Calçado: As the endosymbiotic theory from the 1920s shows that the mito-
chondria could be a bacterium that was incorporated by us ages ago, we know that
many bacterial genes have important command over our body. In this way the bacte-
rial flora could be called an organ, not a traditional organ like the heart or liver, but a
pool of cells with a genetic expression.

Dr. Morelli: Microbiologists call the human gut microbiota the neglected organ,
and so I really believe that it is an organ. But we have to learn how to exercise this
organ, to train this organ in a better way, not for the organ itself but for the body, and
that is the challenge for the future.

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