Microbiota in health and disease: from pregnancy to childhood

edited by:
Pamela D. Browne
Eric Claassen
Michael D. Cabana
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Part I.
The ‘healthy’ infant gut microbiota
Chapter 1
Infant and child microbiota: current status and directions for future research

P.D. Browne1,2,3*, M.B. Van der Waal2,3 and E. Claassen2
1Developmental Psychology, Behavioural Science Institute, Radboud University, P.O. Box 9104, HE 6500, Nijmegen, the Netherlands; 2Athena Institute, VU University, De Boelelaan 1085, 1081 HV Amsterdam, the Netherlands; 3Clinical Research Rotterdam, Marconistraat 16, 3029 AK Rotterdam, the Netherlands; p.browne@psych.ru.nl

Abstract

A balanced microbial composition is essential in maintaining health during infancy and childhood (0-12 years). Alterations in microbial communities may increase a child’s susceptibility to paediatric disorders. Despite extensive research in recent years and our improved knowledge of the potential functional capacity of the microbiome, causal mechanisms linking dysbiosis to development of paediatric diseases remain speculative. It is still relatively unclear what constitutes a healthy infant and child microbiome in relation to its host. A solid research agenda in paediatric microbiome research is key to elucidate the various roles of the microbiota in health and disease, as well as for developing feasible and therapeutic options. In this chapter, we provide an overview of the potential roles of the microbiota in health and disease in infants and children. Additionally, we summarise the future steps that will be necessary for understanding the functional properties of the microbial communities and for developing preventive and therapeutic strategies to mitigate the impact of dysbiosis on health in infants and children.

Keywords: healthy microbiome, microbial development, early life, future research directions

1.1 Introduction

The aim of this chapter is to provide a general introduction to major concepts in the field of human microbiota research with emphasis on infants (0-2 years of age) and children (2-12 years of age) and to offer directions for future research. This chapter begins with describing the development of gut microbiota during infancy and childhood, challenges we encounter in defining a ‘healthy microbiota’ and the specific diseases that are associated with microbial dysbiosis during the early years of children’s lives. In Sections 1.3 to 1.9, we summarise the future steps proposed by the authors of this book (Chapters 2-17) to push the field of paediatric microbiome research forward. Although the human body harbours various distinct microbiota compositions, the main focus of this book is the human gastrointestinal tract (GIT) microbiota, as it contains the largest and most studied microbial biomass in the paediatric population.
1.2 The infant and child microbiota

The human body harbours trillions of micro-organisms that play an essential role in infant and child health (Greenhalgh, 2016; Walker, 2013). Recent studies suggest that at least the same amount of microorganisms as human cells reside inside us (Bianconi et al., 2013; Sender et al., 2016). The term microbiome refers to the entire collection of these microorganisms, including bacteria, viruses, protozoans, fungi, their genomes and the surrounding environmental conditions. The selection of microorganisms inhabiting a defined environment is called microbiota (Marchesi et al., 2015).

The metabolites and components of microbial communities support proper development and functioning of the major biological systems in the infant’s and child’s body (Rodríguez et al., 2015; Round and Mazmanian, 2009; Walker, 2013; Wopereis et al., 2014). For example, they play an important role in gut maturation and intestinal barrier homeostasis (Putignani et al., 2014; Round and Mazmanian, 2009; Yu, 2012), as well as in immune system development (Gensollen et al., 2016; Gollwitzer and Marsland, 2015; West et al., 2015). There are also indications that intestinal microbiota can influence the developing enteric nervous system (ENS) and central nervous system (CNS), possibly influencing brain function and behaviour (Borre et al., 2014).

Early life microbiota succession starts in utero and from that moment a symbiotic relationship develops between the colonising pioneer bacteria and its host (Walker, 2013). Establishment of complete colonisation at birth, primarily in the gut, and the gradual diversification into a stable microbial ecosystem during infancy, is essential for this symbiotic relationship to develop (Walker, 2013; Wopereis, 2014). At the age of 2-5 years, the gut possesses a microbial profile, which fully resembles ‘adult-like’ microbiota in terms of composition and diversity (Koenig et al., 2011; Rodríguez et al., 2015; Wopereis et al., 2014; Yatsunenko et al., 2012).

Optimal symbiosis may profoundly help to maintain health throughout infancy and childhood (Walker, 2013; Wopereis, 2014). Thus a complete colonisation and development of the microbiota during early life play important roles in maintaining health during infancy and childhood. On the contrary, an aberrant microbiota may increase a child’s susceptibility to paediatric disorders (Walker, 2013). The latter case refers to a state of dysbiosis, which indicates perturbations to the structure or composition of resident commensal communities, relative to the community found in healthy individuals (Petersen and Round, 2014). A state of dysbiosis can be induced by many common practices, such as caesarean section (CS), perinatal antibiotic exposure, lack of breastfeeding or a deviant perinatal environment (Kozyrskyj and Tun, 2017; Walker, 2013). Resulting perturbations of the child’s microbiota may lead to an increased risk for immunological and gastrointestinal disorders during infancy and childhood (Abrahamsson et al., 2014; Dzidic et al., in press; Öhman and Simrén, 2013; Putignani et al., 2014; Scheepers et al., 2015; West et al., 2016). Outside of the intestines, dysbiosis of the respiratory tract and oral microbiota correlates with respiratory tract infections and periodontal diseases, respectively (Hilty et al., 2012; Laufer et al., 2011). Additionally, alterations in the microbiome of the urinary tract could
be involved in development of urinary diseases (Lewis et al., 2013; Peters et al., 2009). Figure 1.1 shows the different body systems that have a highly co-evolved relationship with microbial communities. It also shows the specific diseases that are associated with microbial dysbiosis which are discussed throughout this book.

Fortunately, there are prospects to prevent or treat microbial-related diseases during infancy and childhood. Increasing our understanding of microbial properties and knowledge of pre- and probiotics may help to maintain microbial balance or to develop interventions that may restore aberrant microbiota (McFarland, 2014; Wopereis et al., 2014). Prebiotics are non-digestible food ingredients that may stimulate the growth and/or activity of some bacteria in the colon. Probiotics are defined as ‘living microorganism that, when administered in sufficient amount, confer a health benefit for the host’ (Hill et al., 2014). In attempting to optimise infant and child health by targeting microbiota, it is fundamental to comprehend a ‘baseline’ microbiome during infancy and childhood that is associated with health.

Figure 1.1. The interplay between microbiota, body systems and paediatric diseases. The human microbiota are important for the development and functioning of various physiological systems in the child’s body, including the digestive system, immune system, nervous system, urinary system and respiratory system. Disruption of microbial communities may thus affect the child’s health and behaviour. Paediatric diseases that are associated with dysbiosis and that are discussed in this book include antibiotic associated diarrhoea (AAD), functional gastro-intestinal diseases, constipation, obesity, gastrointestinal infections, necrotizing enterocolitis (NEC), infant colic, atopic disorders, (food) allergies, inflammatory bowel disease (IBD), celiac disease, type 1 diabetes, urinary tract infections, oral infections and upper- and lower respiratory tract infections.
To date, however, it is still unknown what defines a healthy microbiome in the paediatric population. Notably, different body sites harbour different microbial community compositions and therefore in defining an optimal microbiome in infants and children we essentially should define an optimal site-specific baseline microbiome for a distinct body site (Greenhalgh et al., 2016). For all body sites, the microbial compositions significantly evolve during childhood, with most notable changes occurring during infancy. Microbial communities during early life reconfigure their metagenomic composition in response to host-specific physiological and immunological needs at different ages, causing prominent interindividual differences among infants and children, possibly even between genders (Greenhalgh et al., 2016; Matamoros et al., 2013; Quercia et al., 2014; Wallis et al., 2015, 2016; Yatsunenko et al., 2012). Defining an optimal site-specific microbiome is further challenged by the fact that microbiome composition is influenced by its host genetics and epigenetics which shape the host-microbiota interactions (Alenghat and Artis, 2014; Blekhman et al., 2015). Besides specific features of the host, the microbial composition is largely influenced by environmental factors. For example, the geographical location, diet (e.g. breast milk, formula and weaning), household setting (e.g. pets, siblings) and medical interventions (e.g. antibiotics) largely influence its composition (Azad et al., 2013; Mangin et al., 2010; Wopereis et al., 2014). As a result, it is challenging to define what constitutes a healthy well-balanced gastro-intestinal, respiratory and urinary microbial composition during the first twelve years of life.

Nevertheless, despite the vast diversity across environments, culture and geographies of hosts, common patterns can be recognised during infancy and childhood (Scholtens, 2012; Yatsunenko et al., 2012). More specifically, microbial communities at individual body sites someway display distinct distributions of microbial phyla, diversity and relative stability over time. These broad features thus provide a certain indication of an optimal microbial composition in infants and children. Moreover, a state of dysbiosis at specific sites can be linked to particular paediatric diseases providing additional information on what could be defined as a ‘healthy microbiota’ (Greenhalgh et al., 2016).

These insights have been obtained, in part, through analysing large cohorts of paediatric samples and by pooling results from clinical studies. Moreover, great scientific advances in molecular and computational technology in recent years have allowed for accurate classification of the collection and structure, and sometimes functions of microbiota (Rooks and Garrett, 2016). These technical advances have moved the field of microbiome research in the paediatric population forward. However, there are still many questions unanswered. Causal mechanisms linking dysbiosis to development of paediatric diseases remain speculative and it is still relatively unclear what constitutes a healthy microbiome in relation to its host. Moreover, the effects of gut and respiratory modulation approaches are not continuously reproducible. A solid research agenda in microbiome research is key to understanding microbial properties in relation to its host, consequences of dysbiosis and to develop feasible preventive strategies and therapeutic options that mitigate the impact of dysbiosis on health. In the next sections we summarise the
Chapter 1 introduces the infant and child microbiota, summarizing recent insights from pediatric microbiome research and outlining future directions for research in this field.

Chapter 2 reflects on the development of the neonatal microbiota during infancy, identifying possible causes of bacterial dysbiosis within the neonatal gut and associated diseases, such as NEC, neonatal sepsis, and antibiotic-associated diarrhea.

Chapter 3 explores the impact of maternal psychosocial stress and maternal obesity on the infant gut microbiota, reviewing the implications for child health.

Chapter 4 offers examples of the impact of four medical interventions on the infant gut microbiota: caesarean delivery, maternal intrapartum antibiotic prophylaxis, hospitalisation post birth, and postnatal infant antibiotic treatment.

Chapter 5 discusses how the infant microbiome is shaped by breastfeeding and solid foods.

Chapter 6 examines the interaction of the intestinal microbiota and the host immune system during early life.

Chapter 7 investigates the role of the intestinal microbiota in the child's immune system.

Chapter 8 offers perspectives on the role of gut bacteria in the development of the hypothalamic-pituitary-adrenal (HPA) axis, central and enteric nervous systems in early stages of life.

Chapter 9 discusses the role of the gut microbiota in the development of allergic diseases typically found in infants, and reviews the role of pre- and probiotics for prevention and treatment.

Chapter 10 provides an overview of the role of gut microbiota in the development of auto-immune diseases and atopic disorders in children, presenting potential benefits of preventive and therapeutic microbiota-based interventions.

Chapter 11 examines the associations between aberrant gut microbiota and functional gastrointestinal disorders, infectious diseases, and obesity in children, and discussing potential therapeutic strategies to manipulate the microbiota composition.

Chapter 12 outlines the association between intestinal microbiota and infant crying and behaviour, with particular focus on infant colic.

Chapter 13 shares insights into the role of microbial dysbiosis and associated biofilms in acute respiratory tract infections, dental caries, periodontal disease, and oral candidiasis in infants and children.
recommended directions for future paediatric microbiome research described throughout this book (Figures 1.2 and 1.3).

1.3 Factors influencing the gut microbiota development

Perinatal and postnatal environmental factors, including maternal prenatal psychosocial stress (PNS) and maternal obesity (Chapter 3: Browne et al., 2017) may shape the infant microbiota after birth. There are however several substantial knowledge gaps regarding causative mechanisms. For example, the causative pathways on how PNS affects the composition of offspring bacterial microbiota remain elusive. The mechanism by which PNS could alter immune development and may increase risk of allergies in childhood also requires detailed investigation. Regarding the association between maternal obesity during pregnancy and risk of childhood obesity, broader understanding regarding the causative mechanisms that contribute to the increased risk of obesity and diabetes type 2 (DM2) in offspring born to overweight and obese mothers is required, as well as broader understanding on how maternal obesogenic microbes could be transferred to the foetus in utero.

Other examples of perinatal environmental factors that largely affect the developing infant microbiota are pre- and postnatal medical treatments, including caesarean delivery, maternal intrapartum antibiotic prophylaxis and postnatal infant antibiotic treatment (Chapter 4: Kozyrskyj and Tun, 2017). Current studies however cannot provide definitive evidence of causation between these interventions and observed adverse health outcomes in children. For instance, it is still unknown how caesarean sections (CS) can increase the risk for childhood obesity and asthma, and how prenatal maternal and infant antibiotic treatments can have adverse consequences for later health (Kozyrskyj and Tun, 2017; Li et al., 2013; Thavagnanam et al., 2008). A direction for future research could be to explore the ties between pre- and postnatal...
medical treatments, microbial composition and various diseases occurring during infancy and childhood. This research may help suggest potential methods to reduce the impact of these interventions on infant microbiota and consequently improve child health (Kozyrskyj and Tun, 2017).

As one’s diet is one of the major environmental factors impacting the maturation and diversification of the microbiome in early life after birth, it is essential to better understand how feeding practices influence the gut microbiome (Chapter 5: Penders, 2017). For instance, research should be aimed at identifying how the cessation of breastfeeding, the introduction of solid food items or a combination of both determines the maturation of the infant microbiota into an adult-like microbiota. Other suggestions for future research are elucidating the impact of mixed feeding and individual bioactive breast milk components on the infant microbiome. Conducting observational studies that closely monitor the period of solid food introduction and cessation of breastfeeding during infancy would deliver such data. In addition, executing human intervention studies would be useful to measure the impact of individual breast milk components and mixed feeding on infant microbiota. Results from these studies would be useful to improve feeding recommendations during the first stages of life (Penders, 2017).

1.4 Intestinal microbiota and physiological systems and their role in paediatric diseases

The immune system

During early life, the immune system develops in tight interaction with the intestinal microbiota. A balanced gut microbiota is considered necessary for the development of an appropriate innate and adaptive immune response and adequate mucosal barrier function, thus assisting in preventing development of allergic diseases and atopy during infancy and childhood (Honda and Littman, 2016; Chapter 6: Jenmalm and Prescott, 2017). This idea is supported by studies demonstrating associations between gut dysbiosis and the development of allergic diseases, atopy and autoimmune diseases including celiac disease (CD), inflammatory bowel disease (IBD) and type 1 diabetes (DM1) in children (Chapter 9: Szajeweska, 2017; Chapter 10: De Meij, 2017). Still, at this stage it remains elusive what constitutes a healthy gut microbiota that promotes immune tolerance in infants, and how gut dysbiosis may be associated with the development of these diseases (Szajeweska, 2017). More knowledge is also required to better understand which microbial signatures contribute to development of autoimmune-related disorders in children (De Meij, 2017). More insight in the interplay between gut microbiota, immune development and onset of immune-related diseases may help establishing strategies to develop preventive and therapeutic interventions for infants and children who are prone to develop these diseases or who suffer from them (Szajeweska, 2017; De Meij, 2017). Moreover, identifying disease-specific microbial signatures for IBD, CD and DM1, may lead to individualised gut-modifying treatment options (De Meij, 2017).
The infant and child microbiota

**KEY MESSAGE**

1. The priority in research should be to identify what constitutes a ‘healthy, well-balanced’ gastrointestinal, respiratory and urinary microbiota composition during early life and childhood. (Chapter 1)

Maternal and external factors

**KEY MESSAGES**

1. Prenatal maternal factors, such as maternal psychosocial stress and maternal obesity can affect the infant microbial composition, which may adversely impact infant health. (Chapter 3)

2. Upon delivery, diet is one of the major factors impacting the maturation and diversification of the microbiome during early life. (Chapter 5)

Digestive system

**KEY MESSAGES**

1. The first 18 months of life are considered crucial to the development of a healthy neonatal microbiota. (Chapter 2)

2. Proper preliminary colonisation seems necessary to maintain a fine balance between the members of the gut microbiota. Inadequate colonisation may lead to a state of dysbiosis, which can manifest in a number of disease states, including necrotising enterocolitis and neonatal sepsis. (Chapter 2)

3. Microbial metabolites such as short-chain fatty acids or polyamines may influence gastrointestinal functional development during early life. Thus, alterations in the microbial gut composition and activity of microbes may adversely affect gastrointestinal functioning with both short-term and long-term consequences on health. (Chapter 7)

4. Gut dysbiosis may be associated with functional abdominal pain (FAP) and functional constipation (FC). (Chapter 11)

5. The administration of probiotics, either as single treatment or as an adjuvant agent, may provide significant benefits for the treatment of infectious diseases caused by Escherichia coli, Clostridium difficile, Helicobacter Pylori and Shigella. Probiotics may be beneficial for treating functional gastrointestinal disorders and childhood obesity. (Chapter 11)

Immune system

**KEY MESSAGES**

1. The intestinal microbiota likely provides a primary signal for establishment of an adequate mucosal barrier function and the maturation of a balanced postnatal innate and adaptive immune system. (Chapter 6)

2. Early microbial exposures occurring during critical periods of immune maturation seem to have long-term impact on development of immune-mediated diseases, and the maternal microbial environment during pregnancy may also crucially influence immune programming. (Chapter 6)

3. The infant gut microbiota may influence the development of allergic diseases. Still, the role of gut microbiota in allergy has not been fully clarified. (Chapter 9)

4. Modifications of gut microbiota through the administration of probiotics, prebiotics and synbiotics could potentially play a role in the prevention and treatment of allergic diseases and eczema. (Chapter 9 and 10)

5. The intestinal microbiota may harbour potential as a diagnostic biomarker of disease and as a monitor of disease activity in inflammatory bowel disease, coeliac disease and type 1 diabetes in children. (Chapter 10)

Figure 1.3. The key messages by chapter. An overview of the most recent insights into dysbiosis in paediatric diseases, the development of a healthy microbiota during infancy, and the use and safety of pre-, pro- and synbiotics.
1. Introduction to infant and child microbiota

**Nervous system**

1. There exists a compelling case for the role of gut microbiota in mediating CNS development, with downstream consequences on the shaping of behaviour, cognition, and neurodevelopment conditions. (Chapter 8)

2. Signalling along the gut-brain axis, even through single bacterial species, may alter the developmental trajectory of the stress circuitry and functional responses to stress. (Chapter 8)

3. Gut-brain signalling is complex and bidirectional, mediated through multiple candidate pathways that enable this interplay, including the vagus nerve, the immune system, and an array of metabolite mediators. (Chapter 8)

4. Accumulating evidence indicate interplay between gut microbiota, gut inflammation and the gut-brain axis in infants with colic. (Chapter 12)

5. Certain probiotic strains seem effective in treating infant colic in exclusively breastfed infants. (Chapter 12)

**Respiratory system**

1. Changes in local microbiomes have been associated with a growing number of inflammatory and infectious diseases of the respiratory tract and oral cavity during childhood. (Chapter 13)

2. Children that suffer from recurrent respiratory diseases may benefit from an early-stage intervention on a microbiome-level. (Chapter 13)

**Urinary system**

1. The bladder does not function as a sterile storage, instead live bacterial communities are present. (Chapter 14)

2. Evidence in adults suggests that UTIs in general are not caused by one or two pathogens, but rather might be a polymicrobial condition. (Chapter 14)

3. Certain pathogens have the ability to form intracellular bacterial communities with many biofilm-like properties, allowing bacteria to outlast a strong host immune response, and resist detection and antibiotic treatment. (Chapter 14)

**Methodologies**

1. Researchers should broaden their scope in microbiome research: additional focus should be placed on other microorganisms, such as fungi, viruses, and niches outside the gut, including skin and urogenital microbiota. (Chapter 15)

2. The patterns of microbial dysbiosis in affected study subjects are often inconsistent, mainly due to differences in strategies regarding sample harvesting, collection and storage, and microbiota detection techniques. (Chapter 10)

**Pre-, pro- and synbiotics**

1. Pre-, pro- and synbiotics can be used in the prevention and treatment of paediatric diseases, however, much remains unclear regarding optimal use of pre-, pro- and synbiotics. (Chapter 16)

2. The probiotics used in clinical trials to reduce the risk of, prevent, or treat disease during the perinatal period and childhood are considered safe for infants and children. (Chapter 17)

3. In human clinical trials, interventions with placebo products often result in more disease or treatment related side effects compared to treatment with probiotics. (Chapter 17)

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Figure 1.3. The key messages by chapter (continued).
In moving microbial treatment development forward, more detailed animal and human studies focusing on the mechanisms behind the microbiota-immune system development are recommended (Bharwani et al., 2017). Data from large cohort studies may help to identify specific microbial signatures that are present in patients with autoimmune or atopic disorders. This may guide the development of good quality long-term clinical trials to evaluate the effectiveness of (novel) specific pre- and probiotic strains. Results of these trials may also improve understanding on optimal dose, timing and duration of interventions of probiotic application in atopic and autoimmune related disorders for clinical practice (Szajeweska, 2017; De Meij, 2017). In addition, more international collaboration in pooling data from clinical studies is encouraged to increase knowledge on probiotic strain efficacy in preventing and treating autoimmune and atopic disorders (De Meij, 2017).

The digestive system

Optimal colonisation and normal gut microbiota development are necessary to prevent the manifestation of a number of disease states during infancy, including necrotising enterocolitis (NEC), neonatal late-onset sepsis (LOS) and antibiotic-associated diarrhoea (AAD) (Chapter 2: Young et al., 2017). During childhood, gut dysbiosis and physiological temporal variations of the gut microbiota are associated with infectious diseases, such as Clostridium difficile-associated diarrhoea, Helicobacter pylori infection, acute gastroenteritis, childhood obesity and functional gastrointestinal disorders (FGIDs), including functional dyspepsia (FD), irritable bowel syndrome (IBS), abdominal migraine (AM) and functional abdominal pain not otherwise specified (NOS) (Chapter 11: Benninga et al., 2017).

Conducting longitudinal studies and intervention studies may allow us to move from correlation to causality in understanding gastrointestinal gut microbiota-related disorders and will help to develop targeted personalised interventions for disease prevention and management for young patients. Improved understanding of the causal mechanisms may help guide the development of treatments, such as vaginal seeding for NEC or LOS, and pre- and probiotic interventions to protect against NEC, LOS, AAD, infectious diseases, childhood obesity and functional gastrointestinal disorders (Benninga et al., 2017; Young et al., 2017).

The intestinal microbiota play an important role in proper development and functioning of the gastrointestinal system (Chapter 7: Gómez-Gallego and Salminen, 2017). They influence digestive and absorptive functions of nutrients by the gut, intestinal epithelial cell processes and mucosal barrier function. Consequently, alterations in gut microbiota are associated with development of a number of paediatric diseases. However, there are gaps in knowledge regarding these interactions; for example, it is still undetermined to which extent environmental factors guide microbiota development and microbial production of metabolites in infants that shape the gastrointestinal system. In addition, it has been recognised that short-chain fatty acids (SCFA) impact gastrointestinal functioning and development. Therefore, future research should focus on profiling SCFA and on how to modulate these profiles (Gómez-Gallego and Salminen, 2017).
The neurological system

Colonisation events during early life coincide with neurological development and gut-brain axis development in infants. Consequently, disruptions in early-life gastrointestinal colonisation might be linked to gut-brain signalling and central nervous system dysfunction, possibly affecting behaviour in infants and children. Besides, animal studies show us that microbial perturbations during a ‘critical window of development’ during early life can cause structural long-term changes in the brain (Chapter 8: Bharwani et al., 2017; Chapter 12: Sung and Pärty, 2017). Further understanding of the mechanisms underlying gut-brain signalling and the role of gut microbiota in development is critical for: (1) understanding the onset of neurodevelopmental and psychiatric conditions; (2) identifying potentially novel preventative and therapeutic strategies that may mitigate the impact of dysbiosis on neurodevelopment or therapeutic strategies to counteract undesirable stress responses (Bharwani et al., 2017).

Future research should therefore focus on the bidirectional interplay between the CNS, ENS and microbiota during early life. Additional goals are to address how perturbations in the microbiota during early life, either due to stress or antibiotic exposure, may influence long-term behavioural outcomes and alter the risk of neurodevelopmental and psychiatric conditions. Special research focus should also be placed on how specific microbial metabolites and components influence the developing neuroendocrine system and the development of the foetal brain. Additional areas of investigation should aim to identify how the young brain processes microbiota-derived signals, and what neural networks underlie the neurobehavioral effects of certain bacteria. In addition, future research is required to elucidate how the immune system and immunomodulation by the gut microbiota may influence development of the ENS and CNS.

The development of the (prenatal) hypothalamic-pituitary-adrenal (HPA) axis and gut bacteria also overlaps during the perinatal period. Alterations in the gut microbial composition during early life may thus have an impact on HPA axis development and functioning. The subsequent influences on behaviour in infants and children remain elusive. Moreover, it remains to be determined whether a window of vulnerability exists in infants to influence HPA axis programming by the colonisation of gut bacteria (Bharwani et al., 2017).

Future research should therefore focus on how intestinal microbiota during foetal and early life play a role in normal development and appropriate functioning of the HPA axis. Moreover, it should investigate whether dysbiosis can influence neural circuits and behaviour associated with dysfunctional HPA axis or the stress response in infants and children. Additional research should focus on unravelling whether a narrow window of vulnerability to influence HPA axis programming by the colonisation of gut bacteria exists in infants. Hypothetically, this critical window could be a viable target in the prevention and treatment of neurodevelopmental conditions (Bharwani et al., 2017). Future research is encouraged to utilise animal models, in vitro systems and to conduct human intervention studies to gain mechanistic insight in the bidirectional gut-brain signalling on development of the enteric and nervous system in infants. In
addition, prospective (long-term) clinical studies may increase understanding of the relationship between early-life microbiota, metabolomic profiles, behaviour and neurodevelopmental health outcomes in infants and children, and in particular a focus on the long-term impact of perinatal antibiotic therapy (Bharwani et al., 2017).

### 1.5 From bowel to infant behaviour

Recent data suggests that the gut microbiota composition in infants with colic may differ from that in infants without the condition. Moreover, accumulating evidence indicates an interplay between gut microbiota, gut inflammation and the gut-brain axis in infant colic (Chapter 12: Sung and Pärtty, 2017). This concept is, however, currently supported by limited evidence. One of the reasons is that no consistent pattern to differences in microbiota composition between infants with and without colic has been identified. This may be due to the range of different methods that have been used to identify gut microbiota in infants with colic.

Increased understanding of intestinal microbiota in relation to colic forms the basis for examining whether altering gut microbiota, through the use of probiotics, is effective for routine use in preventing or treating infant colic. Hence, future studies should use comprehensive methods to measure gut microbiota composition, including DNA and metabolic analysis of bacterial gut communities. The effectiveness of probiotics should be explored by large, rigorously designed multi-centre trials, using well-defined outcomes and validated outcome measures, with appropriate follow-up duration to confirm the absence of long-term adverse effects (Sung and Pärtty, 2017).

### 1.6 Consequences of dysbiosis outside of the gut

#### The respiratory system

The respiratory and oral microbiomes play a role in the aetiology of respiratory or oral diseases during childhood (Chapter 13: Gerritsen and Younes, 2017). Although advances have been made in understanding the correlation between respiratory tract dysbiosis and adverse health outcomes, significant gaps in knowledge remain. For example, it is uncertain what the paediatric developmental milestones of respiratory microbiota are and how the dynamic homeostasis within the respiratory and oral microbiomes collapses. It is also uncertain which key species within the different respiratory and oral niches play a significant role in respiratory and oral diseases, and to which extent bacterial members of the local microbiome influence the host response to a specific external pathogen. Furthermore, there is limited knowledge of how the total bacterial load or species diversity changes preceding, during or following respiratory diseases and it has yet to be determined whether the local microbiome in the respiratory tract can be manipulated therapeutically to modify exacerbation, frequency, or disease progression. In addition, it is uncertain whether dysbiosis contributes to disease aetiology or whether it is a marker of injury and inflammation (Gerritsen and Younes, 2017).
To improve our understanding of a healthy microbiome homeostasis in oral and respiratory niches, future research should focus on microbial composition as well as activity, combined with measuring differences in host responses (e.g. epithelial gene expression, immune cell activation). It is also warranted to identify mechanisms behind intracellular bacterial community (IBC) and biofilm formation, development and penetration, in relation to respiratory infections. Focus should also be placed on exploring therapies that have the potential to modulate the local ecosystem in order to reselect for beneficial commensals. Addressing these questions in research may provide new insights to develop adjunct or stand-alone therapies that support or target restoration of microbiome homeostasis (e.g. probiotics, prebiotics and synbiotics) to prevent or treat oral and respiratory diseases during childhood (Gerritsen and Younes, 2017).

The urinary tract system

Recent evidence from adult studies indicates that the bladder is not sterile and (commensal) microorganisms present in the bladder are alive. New evidence also suggests that urinary tract infections (UTIs) might be a polymicrobial condition, instead of being caused by one or two pathogens. Additionally, in contrast to previous assumptions, certain pathogens have the ability to form intracellular bacterial communities with many biofilm-like properties, allowing bacteria to outlast a strong host immune response to establish a reservoir of pathogens inside the bladder cells (Chapter 14: Younes and Gerritsen, 2017). However, the majority of these findings result from adult studies and have not been confirmed in children. There is yet insufficient evidence to conclude any relationships between the urinary microbiome and urinary tract symptoms in the paediatric population.

First steps for future research should be the characterisation of the core microbiome of the urinary tract in children and the mechanism of translation into adverse urinary health outcomes, especially for UTIs. Moreover, research focus should be placed on contributing factors to the development of diseases and vulnerable moments across childhood. Such data requires large, longitudinal observational studies across diverse populations, different ages and gender. The resulting data may clarify relationships between the urinary microbiome (UM) and urinary tract symptoms in children, may generate diagnostic information to accurately identify urinary disorders and may guide improving paediatric individual patient management. Future data may also help determining protective factors and behavioural practices to prevent urinary diseases or identifying hidden at-risk individuals. This field should receive special attention in microbiome research as paediatric populations with recurrent UTIs perhaps have the greatest long-term health risks by virtue of their age and physiological immaturity (Youenes and Gerritsen, 2017).

1.7 Assessment of microbiota

Molecular assessments play a central role in elucidating microbiome functionalities associated with paediatric health and disease. Chapter 15 (Radjabzadeh et al., 2017) discusses tools and
molecular analytical methods and provides best-practice recommendations regarding sample collection and handling for paediatric microbiome studies.

Over the past years, microbiome research has mostly been focusing on describing associations between microbiota and traits of health and disease. The logical next step in microbiome research in infants and children is determining causality by initiating more intervention and case-control studies. These studies should profile microbial composition and activity, other microorganisms such as Archaea, viruses and fungi, combined with measuring differences in host responses (e.g. epithelial gene expression, immune cell activation) (Gerritsen and Younes, 2017; Radjabzadeh et al., 2017).

1.8 Manipulating the gut microbiota

Pre-, pro and synbiotics show therapeutic potential of manipulating gut microbiota in the paediatric population (Chapter 16: Vandenplas and Huysentruyt, 2017). Despite a progressive increase in human studies examining probiotic effectiveness in infants and children, more knowledge on the optimal use of pre, pro- and synbiotics for preventing or treating particular diseases is necessary. More information is required on which type of probiotic strain(s), timing, duration of intake and dosage is effective per disease indication. Additionally, in light of the possible ‘critical window of development’ to influence the infant microbiota and thus potentially physiological system functioning, conclusive evidence on safety of probiotic ingestion during infancy is essential (Chapter 17: Van den Nieuwboer et al., 2017). In order to meet these gaps regarding safety and efficacy evidence of probiotics, high quality clinical trials with adequate sample sizes in the paediatric population should be conducted, reporting on efficacy and adverse events related to probiotic intake. International collaboration pooling data from these trials would afterwards help to create efficacy and safety profiles of probiotics per strain, per disease and in different populations. Furthermore, prior to treatment with probiotics, vigorous quality controls for probiotics in the clinical and research setting should be conducted to ensure good quality of products. Common terminology should hereafter be used for these products (VandenPlas and Huysentruyt, 2017; Van den Nieuwboer et al., 2017).

1.9 Conclusions

The refinement of our knowledge of microbial development and function, dysbiosis and related diseases during infancy and childhood may allow reducing the risk of paediatric diseases. Well-designed animal studies and human case-control, intervention and longitudinal studies should be undertaken to understand potential causal mechanisms, possibly creating a basis for developing targeted personalised effective treatments for paediatric diseases. Recommendations for future research presented by the authors in Chapters 2-17 include:

• defining what constitutes a ‘healthy microbiota’ in the different body sites, at different ages;
• identifying which environmental factors (e.g. environment, medication) and to which extent these factors influence microbial compositions;
• using matched controls in human studies to increase understanding of confounders;
• clarifying the role of host dynamics on gut microbiota, including the role of (epi)genetics in microbial development during early life and the possible effect of individual specific physiological characteristics such as gut size, transit time and presence of other gut components such as fungi, Archaea and phages;
• illuminating the complex interactions between the developing infant microbiome and body systems, including the immune, nervous and gastrointestinal system;
• implementing biological system approach to gain insight in the mechanisms behind states of dysbiosis;
• using comparable molecular assessment techniques in research and relevant clinical endpoints in human pre- and probiotic studies;
• evaluating the effectiveness of other possible effective gut modulations therapies, such as faecal transplantations;
• using functional rather than taxonomic analysis of microbiomes as a gold standard.

Employing these concepts and suggestions may facilitate the development of treatments for gut microbiota-related disorders during infancy and childhood, contributing to ensuring optimal health in the next generation.

Conflict of interest

Mark van der Waal declares no conflict of interest. Pamela Browne has participated as a speaker for Winclove Probiotics B.V. Eric Claassen is CEO Vironovative.

References


1. Introduction to infant and child microbiota


Part II.
Factors influencing the gut microbiota development
Chapter 2

Development of the neonatal microbiota

G.R. Young1, S. Zalewski2, S.P. Cummings3 and C. Lanyon1*

1Faculty of Health and Life Sciences, University of Northumbria, Ellison Building, NE1 8ST Newcastle upon Tyne, United Kingdom; 2Newcastle Neonatal Service, Newcastle upon Tyne Hospitals NHS Foundation Trust, Freeman Hospital, Freeman Road, High Heaton, Newcastle upon Tyne NE7 7DN, United Kingdom; 3School of Science and Engineering, Teesside University, Stephenson, Stephenson Street, Tees Valley, TS1 3BA Middlesbrough, United Kingdom; clare.lanyon@northumbria.ac.uk

Abstract

The period immediately following birth is vital to the development of a healthy neonatal intestinal microbiome. As any environment the primary colonisers of the neonatal gastrointestinal tract pave the way for further colonisation. For this reason the first 18 months of life are considered crucial to the development of a healthy neonatal microbiota. When regular preliminary colonisation fails to proceed, the fine balance between the numerous members of the microbiota can be disturbed, which can manifest in a number of disease states, including necrotising enterocolitis and neonatal sepsis. This chapter aims to identify the differences between and possible causes of bacterial symbiosis and dysbiosis within the neonatal gastrointestinal tract. The pathology of associated disease states will also be explained.

Keywords: diseases of infancy, treatment, prevention, symbiosis, dysbiosis, immune response

2.1 Acquisition of initial gastrointestinal microbiota

It has been well reported that the first 18 months of life immediately following birth are the most dynamic period in the development of the gut microbiota (Bergstrom et al., 2014; Koenig et al., 2011). In particular, they are specifically linked with low bacterial diversity and high levels of instability (Tuddenham and Sears, 2015).

The low diversity of bacteria observed in early life can be attributed to the initial scarcity of microbial exposure prior to delivery. Until recently, the neonatal gastro-intestinal (GI) tract was considered sterile, however, DiGiulio et al. (2008), suggested that infants begin gulping the amniotic fluid in the days preceding delivery and primary colonisation of the infant GI tract could be attributable to bacterial species observed in maternal amniotic fluid as evidenced by analysing microbial signatures of the meconium. Nonetheless, the first significant microbial colonisation event occurs during delivery and appears to be the first major determinant of neonatal microbiota composition. Initial colonisation of the neonatal gut is driven by either exposure to the maternal vaginal flora (Lactobacillus, Prevotella dominance), or skin (Staphylococcus, Corynebacterium and Propionibacterium dominance), dependent on whether delivery is vaginal or via caesarean
(Domínguez-Bello et al., 2010). Figure 2.1 describes pre-, peri- and post-natal factors that shape the neonatal gastrointestinal microbiota.

In addition to delivery mode there are other factors which can shape the infant gastrointestinal microbiota following birth. Possibly the second major factor in determining an infant gut community composition is maternal microbiota transfer (Backhed et al., 2015), in a similar manner to transmission of skin microbes during caesarean delivery. It logically follows that high frequency skin to skin contact between the mother and child will ‘seed’ the infant microbiota (Funkhouser and Bordenstein, 2013). On a wider scale Fallani et al. (2010), found that infants born in northern Europe had a different microbial enterotype to those born in southern or Mediterranean Europe, although this could be due to maternal diet affecting the breast milk microbiota composition more than a specific country-specific variation.

Further maternal impact on the infant gut bacterial community stems from the feeding strategy. During the breastfeeding period the majority of infants will have an enterotype characterised by genera such as Bifidobacterium and Lactobacillus (obligate anaerobic Actinobacteria and facultative anaerobic Firmicutes, respectively) (Kashtanova et al., 2016). Two taxa which are abundant in breast milk due to specific milk oligosaccharide metabolising function (Tuddenham

Figure 2.1. Acquisition of the neo-natal microbiome.
2. Development of the neonatal microbiota

and Sears, 2015). In contrast, formula fed infants generally show greater diversity and differing levels of particular taxa, including lower numbers of the keystone protective species Bifidobacteria (Bezirtzoglou et al., 2011; Fallani et al., 2010; Harmsen et al., 2000), but a greater abundance of Bacteroides (Bezirtzoglou et al., 2011; Harmsen et al., 2000), and Atopobium (Bezirtzoglou et al., 2011). Antibiotic administration during the first days of life can significantly alter the abundance of particular taxa within the gut environment (Cheng et al., 2016). Broad-spectrum antibiotic intervention is frequently prescribed immediately following birth to prevent sepsis; however, the evidence to support its efficacy is mainly based upon expert opinion (WHO, 2012). Furthermore, broad-spectrum antibiotics can potentially disrupt the balance of bacterial taxa within the GI tract and cause dysbiosis, resulting in the loss of keystone taxa, reductions in diversity, functional shift and blooms of pathogenic bacteria (for detailed information see Chapter 4: Kozyrskyj and Tun, 2017; Vangay et al., 2015). Subsequently, the microbiota can be seeded by the immediate environment in which the infant resides. For example, preterm neonates kept within the neonatal intensive care unit (NICU), and administered broad-spectrum antibiotics exhibited faecal microbial signatures highly similar to those of swabbed room surfaces (Brooks et al., 2014). In addition, drug-resistant nosocomial bacterial pathogens have been identified in stools of preterm infants held within the NICU (Moles et al., 2015). Colonisation of preterm infants within the NICU with nosocomial species could be a product of invasive therapies required for parenteral feeding, etc. (Petersen et al., 2016).

2.2 Symbiosis and dysbiosis

Symbiosis

A delicate balance is struck between symbiosis and dysbiosis in all environments containing living organisms. The balance in the neonatal gastrointestinal tract is arguably more precarious during the first two years of life than at any other time due to the turbulent nature of the environment and its inhabitants during that period.

Symbiosis in the gastrointestinal tract can be described as a state in which both the luminal environment of the host (infant), and the inhabitants (microbiota), colonising that environment are metabolically and immunologically beneficial to one another. The anoxic environment of the neonatal gastrointestinal tract: colonised by bifidobacteria; is considered an example of this. Bifidobacteria that can metabolise the breast milk oligosaccharides (Klaassens et al., 2009; Penders et al., 2006), modulate the responses of the immature neonatal immune system towards commensal and pathogenic species by promoting immunoglobulin A production (Park et al., 2002). This is mutually beneficial for both host and microbe. As the individual matures from neonate to two year old child, the nature of these symbiotic relationships change in line with the changes in bacterial taxa and gut environment (Backhed et al., 2015) (Figure 2.2). As an example, during weaning – when plant oligosaccharides are introduced to the diet – the microbiota shifts towards a Bacteroides and Firmicutes dominated system, more like that of an adult (Koenig et al., 2011).
Contrary to symbiosis, dysbiosis is the presence of antagonistic relationships between the host and the colonising microbes. Vangay’s mechanisms of dysbiosis (Vangay et al., 2015) are centred round four main types of community evolution, all of which are closely linked:

1. Loss of keystone taxa. Dysbiosis due to loss of keystone taxa arises when population numbers of a major commensal bacterial species diminish. Colonisation resistance is a theory proposed by Lawley and Walker (2013), whereby keystone commensal taxa are responsible for out-competing pathogenic species. In addition, keystone taxa are also involved in immune modulation and liberation/provision of nutrients and vitamins not otherwise biologically available. Should the niche occupied by keystone taxa become vacant, competition between remaining bacterial species within the environment occurs. The vacant niche can be filled by pathogenic species such as *Clostridium difficile* leading to release of virulence factors harmful to the host such as *C. difficile* toxins A and B (Bergogne-Bérézin, 2000), for example in cases of pseudomembranous colitis (Voth and Ballard, 2005). Possible causes for the loss of keystone taxa can be administration of broad-spectrum antibiotics and cessation of probiotic supplementation (Stecher and Hardt, 2008).

**Figure 2.2. Signature of bacterial taxa per infant developmental stage.**

_Dysbiosis_
2. Development of the neonatal microbiota

2. Pathogenic taxa blooms. In the absence of commensal taxa, pathogenic species such as *Clostridium*, *Shigella* and *Staphylococcus*, which may have been unsuited to the environment in which keystone species dominated, can thrive. The detriments to host health stemming from blooms of pathogenic taxa are the production of spores and virulence factors that directly impact host physiology.

3. Reduction in diversity. Diverse microbial communities are associated with health in adult life. A reduction in diversity of bacteria within a community can precede dominance of one particular bacterial species. While dominance of keystone species, such as *Bifidobacterium* is considered healthy in very early life of a breast-fed infant, a reduction in diversity and subsequent spike in non-keystone or invasive species dominance is discordant with health. In the infant gastrointestinal tract reductions in diversity can occur if particular taxa are better suited to the gut environment than others, following a change in the environment due to alterations in feeding strategy or antibiotic treatment.

4. Functional shift. If commensal species are lost from the gastrointestinal tract then the functional genes exclusively associated with those species will also be lost. This constitutes a reduction in the functional capacity of the microbiota, which may or may not be detrimental. For example, at weaning the gastrointestinal microbiota of a child demonstrates a functional shift from breast milk oligosaccharide metabolising bifidobacteria to plant oligosaccharide and fibre metabolising *Bacteroides*. This functional shift is not considered causative of dysbiosis, but is an essential requirement to adapt to a change in diet from milk to solid food.

**Immunology and dysbiosis**

While all neonates are born with an un-educated immune system, full-term infants are supplemented with cross-placental immunoglobulin prior to birth (Simister, 2003), and are immediately able to receive mother’s milk, which contains secretory immunoglobulins (Van de Perre, 2003). Conversely, preterm infants do not receive the same quantity of cross-placental IgG as they are delivered early. Furthermore they are often not ready to breast feed immediately, reducing their exposure to post-natal maternal immunoglobulin (Ig)A and IgM. These factors are frequently cited as key contributing factors to the increased incidence of necrotising enterocolitis (NEC) and sepsis in premature infants (Berrington *et al.*, 2014).

In term vaginal delivery, under physiological settings, the initial microbiota ‘trains’ the immune system. Initial colonisers, such as facultative anaerobes *Enterobactericeae*, *Escherichia* and *Lactobacillus* (Collado *et al.*, 2012), reduce intestinal oxygen to allow colonisation of obligate anaerobes like bifidobacteria. Lipopolysaccharide and teichoic acid, constituents of the bifidobacterial cell wall (Ouwehand *et al.*, 2002), are sampled from the gut lumen by membrane cells in Peyer’s patches: part of the gut associated lymphoid tissue; distributed throughout the gastrointestinal tract. Antigen presenting cells (APCs) then present the lipopolysaccharide to Th0 T cells to promote speciation to Th1 T cells, rather than Th2 cells, by activation of IL-2, IL-12 and IFN-γ pathways. Th1 cells are responsible for educating B cells to produce IgA antibodies, whereas Th2 cells are responsible for B cell production of IgE. IgA is released in
to the gastrointestinal lumen and binds foreign antigens to prevent them crossing the luminal membrane. Importantly, unlike IgE, IgA does not induce an inflammatory response or histamine burst (for more information see Chapter 6: Jenmalm and Prescott, 2017).

In contrast to term infants, preterm infants do not follow similar patterns of immune modulation. Full feeding is delayed because of the under-development of the gastrointestinal tract. This manifests as persistence of *Staphylococcus, Enterobacteriaceae* and other primary colonisers, coupled with retarded *Bifidobacterium* introduction (Arboleya et al., 2012). Absence of *Bifidobacterium* associated immunomodulatory factors predisposes the premature immune system to favour the $T_{H2}$ cell regulated immune response, resulting in histamine burst and pro-inflammatory cytokine release which can lead to disease states.

### 2.3 Disease states and the microbiota

**Necrotising enterocolitis**

NEC is a necro-inflammatory condition of the bowel most often diagnosed in preterm or very low birth weight (<1,500 g) infants, although it has been reported to occur in full term infants. The condition is the main cause of morbidity and mortality in the NICU (Hsueh et al., 2003). Between 20 and 30% of very low birth weight infants who contract NEC die as a direct result (Fitzgibbons et al., 2009).

NEC is inversely correlated with gestational age (Lin and Stoll, 2006) and birth weight (Hsueh et al., 2003), and these two factors appear to be the main predisposing factors to disease onset, however the phenomenon cannot be attributed solely to these factors as it never occurs prenatally (Neu and Walker, 2011), or in germ-free animal models (Morowitz et al., 2010). These observations demonstrate the significant involvement of microbial colonisation of the gastrointestinal tract in the disease. Moreover, outbreaks of NEC within wards showing a common bacterial species have been reported (Boccia et al., 2001; Caplan and Jilling, 2001). In spite of this, no one bacterial aetiological pathogen has been identified across studies, with abundances of *Clostridium* (Alfa et al., 2002; Cassir et al., 2015), *Escherichia* (Stewart et al., 2013; Zhou et al., 2015), and *Enterobacteriaceae* (Morrow et al., 2013; Stewart et al., 2012), all correlating with NEC onset. Evidence for the role of microbial dysbiosis in the onset of NEC has been found via positive correlation of the disease with early empirical antibiotic use (Alexander et al., 2011; Cotten et al., 2009), and a negative correlation with breast milk (Meinzen-Derr et al., 2009) and probiotic consumption (Alfaleh and Bassler, 2008; Alfaleh et al., 2010).

While links between the microbiota and NEC are correlational rather than causative they fit extremely well with Vangay’s mechanisms of microbial dysbiosis discussed earlier. Imbalance of primary colonising species can be exacerbated by administration of antibiotics, which results in a reduction in diversity, pathogenic taxa blooms and a loss of protective keystone taxa. Furthermore, the protective effect of probiotic supplementation against NEC suggests that
replenishment of microbial keystone taxa can down-regulate NEC onset through promotion a symbiotic relationship of the microbiota with the host, although the exact role of these keystone taxa is yet to be elucidated.

Treatment of NEC should be focused on reducing the immunological stress within the gastrointestinal tract. Early identification and intervention is preferable to late-stage surgical intervention due to the fragile nature of the patient, who may struggle to recover from bowel resection surgery. Surgical NEC has been linked with neurological complications in later life (Petty and Ziegler, 2005). Ideally, treatment strategies for NEC are aimed at prevention and protection and include standardised slow enteral feeding (Viswanathan et al., 2015), maternal commensal transfer via kangaroo care (Seidman et al., 2015), probiotic supplementation and lactoferrin (an iron-binding glycoprotein) administration (Pammi and Abrams, 2011; Sharma and Shastri, 2016), although further research and clinical trials may be required to confirm this.

Following disease onset and diagnosis, cessation of feeding and administration of specific antibiotics is recommended in an attempt to clear the specific bacterial agent causing the gastrointestinal inflammatory response and prevent re-colonisation of the gut environment with pathogenic species. During this period parenteral feeding is required and is usually achieved through the installation of a central venous catheter. Following antibiotic administration, probiotic re-colonisation may be required, as described under section ‘management strategies’.

**Neonatal sepsis**

Neonatal sepsis can be classed as early or late onset. Early onset sepsis is mostly associated with invasive pathogenic bacteria accession during delivery: either from the maternal gastrointestinal tract or nosocomial; and is diagnosed as a positive blood culture observed within the 72 h following delivery (Stoll et al., 2002). Late onset sepsis is associated with systemic invasion of pathogenic species during the post-natal period and is diagnosed by positive blood culture observation between 4 and 90 days following delivery (Rubin et al., 2002).

Similarly to NEC, severely premature and low birth weight infants are at greater risk of developing neonatal sepsis. This risk can be linked with the immaturity of cellular immunity associated with preterm infants or with an improperly formed intestinal mucosal barrier, permitting ingested pathogens to invade surrounding tissues and the circulating blood. Neonates who develop NEC demonstrate an even greater propensity to neonatal sepsis development on account of the intestinal perfusion injuries resulting from *pneumatosis intestinalis*. Further risk factors include the use of indwelling medical devices (Donlan, 2001; Li et al., 2015), such as parenteral feeding tubes and endotracheal catheters in NEC patients, which provide a potential environment for pathogen biofilm formation (Stewart and Costerton, 2001).

The most effective treatment of neonatal sepsis is administration of intravenous antibiotics directed specifically at the causative bacterial agent. Taxa most often associated with neonatal
sepsis include group B streptococci (Schrag et al., 2000), coagulase negative staphylococci (Klingenberg et al., 2005), and Escherichia (Stoll et al., 2011). Haemophilus influenza (Hershckowitz et al., 2004), is regularly observed in early onset sepsis while Staphylococcus aureus (Healy et al., 2004), is more often observed in late onset sepsis.

**Antibiotic-associated diarrhoea**

Antibiotic-associated diarrhoea (AAD) is observed in 5-30% of hospitalised patients receiving antibiotics, dependant on antibiotic type (Bartlett, 2002; Wistrom et al., 2001). Onset can be very early during treatment or up to several weeks following cessation of the antibiotic course and can include a range of symptoms from nuisance diarrhoea (self-limiting watery stools which can resolve within days), to fulminant pseudomembranous colitis with fever, leucocytosis and even hypoalbuminemia. Risk factors increasing the likelihood of antibiotic associated diarrhoea include immune-compromisation (specifically a lack of IgG adaptive immunity (Kyne et al., 2000)), prolonged hospitalisation and antibiotic usage (Bartlett, 2002). This combination of risk factors predisposes neonates held within hospital for various complications to significantly increased risk of AAD than other demographics.

Antibiotics associated with increased gastric motility, such as amoxicillin-clavulanate and the motilin receptor agonist erythromycin, can lead to antibiotic associated diarrhoea by promoting small bowel and gastric emptying. In addition, antibiotics targeting anaerobes have been associated with greater prevalence of AAD due to reduced carbohydrate metabolism which manifests in osmotic diarrhoea (Chassany et al., 2000). The pathogenesis of the condition can be linked to all four of Vangay’s mechanisms of dysbiosis. In cases of AAD non-specific biocidal agents disturb the colonisation resistance provided by keystone anaerobic taxa within the environment which precedes a functional shift to a community less capable of metabolising carbohydrates. The loss of these keystone anaerobes constitutes a reduction in overall bacterial diversity and enables pathogenic taxa to bloom within the recently vacated niche.

In cases of suspected AAD the first duty of a clinician is to discontinue administration of the antibiotic associated with the condition and identify the causative organism. *C. difficile* is the most often identified causative pathogen in 10-20% of AAD and is most often linked with more serious cases. However, as previously mentioned many cases may be self-resolving following removal of the associated antibiotic agent. These cases are generally linked with other causative organisms including Clostridium perfringens, S. aureus and possibly Candida sp. For these reasons most laboratories offer a *C. difficile* toxin enzyme immunoassay. Due to a false negative rate of 10-20% in toxin enzyme immunoassay a toxin targeted tissue culture assay is the gold standard for *C. difficile* AAD diagnosis (Mylonakis et al., 2001), however is less often used due to the timescale for results (24-48 h). Immunoassays that target both *C. difficile* toxins A and B are preferential to ones targeting solely toxin A as strains of *C. difficile* expressing only toxin B have been implicated in AAD (Johnson et al., 2001). Further indications of *C. difficile* AAD
include histologic identification of pseudomembranous colitis which is not ubiquitous but specific for *C. diff* involvement.

The Infectious Diseases Society of America recommend the use of oral metronidazole at 500 mg 3 times or 250 mg 4 times daily for 10 days as treatment of AAD (Bartlett, 2002). Vancomycin has also been proved an effective antibiotic, however this should be saved for metronidazole resistant infections, to reduce emergence of vancomycin resistance. The use of anti-peristaltic agents has been advised against as it promotes toxin retention (Guerrant et al., 2001), however the use of probiotics to antagonise *C. difficile* has been suggested. Administration of probiotic *Lactobacillus* or *Saccharomyces* has been employed (D’Souza et al., 2002) to replenish carbohydrate metabolising function and antagonise *C. difficile*. While the limited results of meta-analyses so far suggest that probiotics can be protective against AAD more mechanistic studies are required to elucidate the reasons for this protective effect. Furthermore, probiotic administration of *Saccharomyces* has been associated with fungemia, therefore the use of this probiotic in the immunocompromised neonate is advised against.

### 2.4 Management strategies and microbiota associated disease

**Vaginal microbial transfer in infants born by caesarean delivery**

The differences in microbial colonisation of infants born by caesarean delivery compared to vaginally delivered infants are well described (Dominguez-Bello et al., 2010). However, more important is the association of caesarean delivery with non-communicable diseases, such as obesity (Chapter 4: Kozyrskyj and Tun, 2017; Kuhle et al., 2015), diabetes (Cardwell et al., 2008; Kozyrskyj and Tun, 2017), and asthma (Chapter 9: Szajewska, 2017; Chapter 10: De Meij, 2017; Magnus et al., 2011), later in life. This has led to new practices to try to ameliorate the effect of caesarean delivery on microbiota for example, vaginal seeding where vaginal fluids are swabbed after caesarean delivery, and then transferred to the infant’s mouth, face and skin. Despite a high level of interest in the non-scientific literature, a relative lack of scientific analysis remains. Vaginal seeding has recently been shown to alter the microbiota of caesarean born infants to resemble a community more like that of the vaginally delivered (Dominguez-Bello et al., 2016). However, although differences appear on the microbial level there remains a need to prove this has an effect on health outcomes, and further high quality research is needed.

There have been some concerns regarding the safety of this practice (Cunnington et al., 2016). Potential transfer of pathogenic organisms (such as Group B *Streptococci*), via vaginal seeding increases the risk of early onset sepsis and further concerns remain regarding transmission of viruses like HIV and Herpes simplex. However valid these concerns may be, it is unlikely that it provides a significantly higher risk than if the infant were to be born through vaginal delivery. Until more data is available it is difficult to say with any certainty. When assessing infants after delivery, it will be important for clear documentation of vaginal seeding to ensure paediatricians are aware of exposure to vaginal organisms.
Early administration of colostrum and promotion of breastfeeding

After exposure to vaginal taxa, breastfeeding appears to be the next major source of microbes for the developing gut. Breast milk itself is not sterile, with lactic acid bacteria identified in milk, areola and skin of nursing mothers (Martin et al., 2003). Although initially felt to be secondary to transfer of microbes from the infants’ mouth recent studies have shown a potential endogenous route of microbial transfer into mothers’ milk with increased bacterial DNA found in peripheral mononuclear cells of breast feeding mothers (Perez et al., 2007). It is likely that any deviation from the normal process of breast feeding, such as milk expression and nasogastric tube feeding alters the normal microbial colonisation process. Promotion of breastfeeding remains a vital role for health professionals in facilitating the transfer of protective maternal microbes and immunogenic molecules to the developing neonate as protection against NEC and sepsis.

Colostrum appears particularly important as it interacts with gastrointestinal associated lymphoid tissue found in the oropharynx and gut, and has been shown to stimulate immune maturation. This may be particularly important in extremely preterm infants who are unable to breast feed in the normal way. This can result in a greater TH2:TH1 T cell ratio, manifesting a stronger IgE mediated inflammatory immune response and increasing propensity to NEC. Indeed, a double blinded randomised controlled trial (RCT) of oropharyngeal colostrum administration in preterm infants (Lee et al., 2015) showed increased levels of secretory IgA and lactoferrin in urine and decreased pro-inflammatory cytokines in preterms who received oropharyngeal colostrum as well as reducing the risk of clinical sepsis.

Oligosaccharides as prebiotics

Breast milk has long been identified as a major protective factor in the development on NEC and late-onset sepsis (LOS) (Maayan-Metzger et al., 2012). Human milk oligosaccharides (HMO): unconjugated glycans present in breast milk; have been shown to have a prebiotic effect. HMOs are associated therefore with increased bifidobacterial colonisation (Asakuma et al., 2011) and decreased risk of colonisation with pathogenic bacteria (Yu et al., 2013), resulting in reduced likelihood of NEC, sepsis or AAD. The HMO Disialyllacto-N-tetraose has been shown to decrease the risk of NEC in the neonatal rat model (Jantscher-Krenn et al., 2012). Recent work on 2’-fucosyllactose has identified one potential mechanism for this protective effect. Administration of 2’-fucosyllactose was associated with an increase in intestinal mucosal perfusion secondary to increases in nitric oxide synthase (Good et al., 2016). In clinical practice it is therefore imperative to encourage mothers who are able, to feed their infant breast milk. This is particularly important in Preterm infants and resources should be allocated to support mothers in this practice as long as they can.

Although it is impossible to replicate the complex structure of an HMO, the formula milk industry has tried to reproduce their efficacy by adding oligosaccharides into formula milk in an attempt to replicate the protective effects of maternally expressed breast milk. The use of
2. Development of the neonatal microbiota

Fructo-oligosaccharide enriched formula has been shown to increase bifidobacterial colonisation in preterm infants (Kapiki et al., 2007), and has since been replicated in a formula using galacto-oligosaccharides in healthy term infants (Matsuki et al., 2016). There remains a niche to replicate the protective effects of HMOs from maternally expressed breast milk against NEC/sepsis in formula milk.

**Lactoferrin supplementation**

Lactoferrin is one of many bio-active glycoproteins found in milk and appears to have significant effects on microbial colonisation. Potential effects of lactoferrin on the host include immune regulation as well as iron sequestration, disruption of the microbial cell membrane and competitive inhibition of host receptors like glycosaminoglycans (Embleton et al., 2013). Supplementation with bovine lactoferrin has been shown to reduce the risk of late onset sepsis (Manzoni et al., 2009), and NEC (Manzoni et al., 2014) in preterm infants. Large scale RCTs of lactoferrin supplementation are underway (ELFIN, 2013), to identify the effect of lactoferrin in clinical practice as well as on exploring its mechanistic action on microbes and host immune response (Embleton, 2016).

**Probiotics for prevention of NEC and late onset sepsis**

Whether probiotics reduce the risk of NEC and LOS in preterm infants is a controversial topic, however many NICUs across the world are routinely using them for this purpose. Clear mechanisms of action can be difficult to unpick due to complex crossover of risk factors implicated in the development of disease in preterm infants (Embleton et al., 2016). Toll-like receptor (TLR)-4 appears to be an important signalling molecule in the NEC pathway and some evidence has shown activation of TLR-9 by probiotic bacterial DNA to be protective in non-human models (Good et al., 2014).

Probiotic use reduces the risk of stage 2 NEC (risk ratio (RR) = 0.45; 95% confidence interval (CI) 0.33-0.56) and mortality (RR=0.65; 95%CI 0.52-0.81) in this group of patients. However, there is marked heterogeneity between studies within the meta-analysis and the largest RCT to date. Costeloe et al. (2016), studying the effect of *Bifidobacterium breve* BBG-001, failed to find a positive effect. Probiotic supplementation did not lead to colonisation in all the infants in the intervention group and, interestingly, 37% of the placebo group were colonised by 2 weeks of age. This showed the need for mechanistic work within probiotic trials in the future. Taxa specific effects may be important, and further research is required to identify specific strains, such as those suggested by Warner et al. (2016): *Bifidobacteria, Bacteroidetes or Propionibacteria*; that may be of benefit however functional rather than taxonomic analysis of ‘healthy’ microbiomes should be considered the gold standard due to the high evolutionary rate within bacterial systems.
A meta-analysis has also supported the use of probiotics for reducing the risk of LOS (Rao et al., 2016), in preterm infants (RR=0.86; 95%CI 0.78-0.94). This significant reduction in risk was not found in the subgroup analysis of infants less than 28 weeks and/or 1000 g. One potential explanation for this could be due to the high proportion of line-related coagulase-negative staphylococci (CONS) sepsis in this subgroup. It is unlikely that probiotics would have a significant effect in reducing the rate of these.

Concerns remain about safety of probiotic administration in this very vulnerable population. For example, there are case reports of probiotic bacteria positive blood cultures (Bertelli et al., 2015). While probiotics are classed as food products they need not go through the same vigorous quality control as licenced pharmaceuticals and this has further exacerbated concerns (for more information on safety of probiotic administration, see Chapter 16: Vandenplas and Huysentruyt, 2017).

2.5 Future research

The authors would suggest the following categories to direct future research towards:

- assessment of the legitimacy and safety of vaginal seeding with regard to introduction of ‘healthy’ vaginal-delivery-like microbiota following caesarean delivery to prevent dysbiotic states such as NEC and LOS;
- investigation in to mechanisms of probiotic protection from infantile diseases such as NEC and AAD;
- assessment of efficacy of lactoferrin administration as protection against NEC onset;
- expansion of the mechanistic understanding of what a ‘typical’ microbiome is beyond the taxonomic level to allow comparison of healthy and unhealthy communities. Knowledge at this level will facilitate the development of treatments, such as vaginal seeding, probiotic and prebiotic administration to protect against NEC, LOS and AAD.

Conflict of interest

The authors confirm that there are no conflicts of interest.

References

2. Development of the neonatal microbiota


2. Development of the neonatal microbiota


Chapter 3

Impact of maternal prenatal psychosocial stress and maternal obesity on infant microbiota

P.D. Browne¹,²*, E. Van den Berg³ and C. De Weerth¹
¹Developmental Psychology, Behavioural Science Institute, Radboud University, P.O. Box 9104, HE 6500 Nijmegen, the Netherlands; ²Athena Institute, VU University, De Boelelaan 1085, 1081 HV Amsterdam, the Netherlands; ³Amsterdam University College (AUC), Science Park 113, 1098 XG Amsterdam, the Netherlands; p.browne@psych.ru.nl

Abstract

The prenatal period is a critical window of development for all major physiological systems in the human body. During pregnancy, maternal prenatal psychosocial stress (PNS) and maternal obesity are identified as risk factors for infant and child health. Several possible mechanisms have been suggested to explain this link, including the transfer of maternal microbiota to offspring. PNS and maternal obesity may negatively affect maternal microbiota during pregnancy. By means of maternal microbial transfer in utero and at birth, PNS and maternal obesity could negatively affect the offspring’s gut microbial colonisation. During delivery infants will come in contact with maternal vaginal and gut microbiota, and a spurt in infant gut colonisation will commence. Appropriate colonisation and gut microbiota development in new-borns are important for gut health, and therefore for child health and development. Results from animal and human studies show that PNS can affect the infant microbial composition. These findings need to be confirmed in large prospective cohort studies. In addition, a number of human studies indicate that maternal obesity may alter maternal gut microbiota and through vertical transfer of obesogenic maternal microbes may consequently predispose offspring to obesity. The relevance of these maternal obesogenic microbes in the infant’s gut for weight trajectory over the life course requires further evaluation. In this chapter, we will review the current understanding of how PNS and maternal pregnancy obesity may affect maternal gut microbiota and consequently infant microbiota and health. We will also discuss recent findings concerning proposed mechanisms of action. Finally, we will offer recommendations for clinical practice and future research.

Keywords: early life programming, maternal prenatal psychosocial stress, maternal microbiota-obesity link, (infant) microbiota, health implications

3.1 Introduction

It has become increasingly clear that the intestinal microbiota in new-borns is important for gut health and therefore child health and development (Mueller et al., 2014). Maternal intestinal and vaginal microbiota are the primary source of the infant gut microbiota (Rautava et al., 2012). An infant’s gut is thought to start to become colonised with maternal microbiota while in the
womb, and to show the largest spurt in colonisation after birth (Aagard et al., 2014; Collado et al., 2016). In vivo and observational studies suggest that appropriate microbial colonisation of an offspring’s gut during pregnancy and at birth is essential for the maturation of physiological functions, including the immune system and organ systems (Mueller et al., 2014; Rautava, 2016). Conversely, an aberrant maternal microbial composition that is transferred to an offspring could potentially increase the risk of infections and development of autoimmune conditions, including allergies and asthma in the young child (Gomez de Agüero et al., 2016; Johnson and Ownby, 2017; Round and Mazmanian, 2009).

There are several prenatal risk factors that may negatively affect maternal microbiota (e.g. vaginal and gut microbiota) during pregnancy. In this chapter we will limit ourselves to two factors that have only recently been studied in this context, namely maternal prenatal psychosocial stress (PNS) and obesity. In addition, since most human data are available on the association between PNS, maternal obesity and maternal gut microbiota, this chapter will focus on the gut (Isolauri et al., 2016; Zijlmans et al., 2015). Therefore, the influence of PNS and maternal obesity on vaginal microbiota composition falls beyond the scope of this chapter.

PNS and maternal obesity may both induce unfavourable changes of the maternal gut microbiota composition (Garcia-Mantrana and Collado, 2016; Jašarević et al., 2015; Soderborg et al., 2016). In the following sections we will review the current understanding of how PNS and maternal obesity may affect maternal gut microbiota and consequently infant microbiota and health. We start by describing the concept of ‘early life programming’ in light of the complex interactions between microbes and the host. We then continue by discussing recent evidence on the relation between these important maternal prenatal risk factors and the offspring’s gut microbiota. Next, we outline proposed mechanisms of action for both factors. Finally, we outline proposed mechanisms of action for both factors and offer recommendations for clinical practice and future research.

### 3.2 Early life programming

The prenatal period is a sensitive window of development for all major physiological systems in the infant body, with an adverse early life environment leading to an increased risk for neuropsychiatric conditions, immunological and metabolic disorders, as well as gastrointestinal dysfunction in later life (Golubeva et al., 2015; Rodriguez et al., 2015). The manner in which the early life environment, especially during pregnancy and infancy, influences the development of the child and later adult is known as ‘early life or developmental programming’ (Galley et al., 2014; Gohir et al., 2015; Reynolds et al., 2013; Rodriguez et al., 2015; Zijlmans et al., 2015). For example, during pregnancy, the developing physiological systems of the foetus are dependent on the mother, and refinements of these functions seem to be influenced by maternal factors, such as maternal diet, metabolic diseases, and possibly maternal microbiota (Johnson and Ownby, 2017; Sanz, 2011). Thus, in the context of developmental programming, early life exposures to PNS and
maternal obesity may represent mechanisms by which developmentally programmed phenotypes may manifest later in life. In the following sections we will address both these factors separately.

3.3 Maternal prenatal psychosocial stress

Maternal prenatal psychosocial stress has been defined as a combination of stressful situations or stressors (e.g. stressful life events, racial discrimination), the perception and appraisal thereof, and subsequent stress responses as subjective experienced and expressed emotions (e.g. depressive or anxiety symptoms) (Beijers et al., 2014; Christian, 2012). A more specific component of psychosocial stress is pregnancy-specific anxiety, characterised by anxieties about the pregnancy itself, such as the fear of bearing a handicapped child or the fear of the delivery itself (Beijers et al., 2014). PNS has unique implications for infant and child health. For example, it has been associated with an increased risk for neurodevelopmental, cardiovascular, and metabolic disorders in infants and children (Beijers et al., 2014; Golubeva et al., 2015). Moreover, PNS may result in preterm delivery and low birth weight, in turn leading to a wide range of complications (Loomans et al., 2013). Pregnancy-specific anxiety usually accompanies other types of maternal psychosocial stress, but interestingly, it has also been found to be a powerful independent predictor of pregnancy outcome and infant health (Alderdice et al., 2012; Tollenaar et al., 2011).

Given that PNS is a broad concept, comprising different types of stressors and anxieties, the prevalence of PNS in the pregnant population is not easily determined. Nevertheless, taking into account the high proportion of pregnant women experiencing anxiety and depression during pregnancy (between 10-20% worldwide), it is conceivable that even a greater proportion of mothers experience PNS during pregnancy (Cardwell, 2013; Heron, 2004; Marcus, 2003). Physiologically, the stress response is mediated by the hypothalamic-pituitary-adrenal-axis (HPA axis). The HPA axis is a complex network of hormonal interactions between three endocrine glands: the hypothalamus, the pituitary gland, and the adrenal glands (Ulrich-Lai and Herman, 2009). In reaction to stress there is a quick release of (nor)adrenergic hormones and peptides, such as corticosteroid releasing hormone (CRH) by the hypothalamus, inducing release of adrenocorticotropic hormone (ACTH) by the pituitary gland, consequently activating a slow release of cortisol from the adrenal glands into blood (Tollenaar et al., 2011). The HPA axis is activated in response to stressors, but is also involved in various other bodily processes, such as metabolic and immune regulatory processes, and even in mood and emotions (Lupien, 2009).

**Impact of maternal prenatal psychosocial stress on infant microbiota**

The gut microbiota may play a role in the relation between PNS and adverse infant microbial composition and health outcomes (Bailey et al., 2004; Zijlmans et al., 2015). For example, Bailey and colleagues (2004) showed that maternal prenatal stress in monkeys may alter the offspring’s intestinal microbiota by reducing the overall numbers of intestinal bifidobacteria and lactobacilli (Bailey et al., 2004). Similar results were found in a first study in humans; Zijlmans et al. (2015)
prospectively investigated 56 vaginally born healthy Dutch babies for the first 110 days after birth. In late pregnancy, mothers filled in questionnaires and collected saliva samples for cortisol determination. For each infant, five faecal samples were analysed using a high-throughput phylogenetic microarray to determine microbial composition. Both elevated levels of maternal self-reported stress, as well as increased cortisol concentrations, had strong associations with the composition of the infant microbiota (Zijlmans et al., 2015). Furthermore, the results showed an increased abundance of Proteobacteria and a decreased abundance of lactobacilli (lactic acid bacteria) in the subset of infants born from mothers experiencing ‘high prenatal stress’ (high on both reported and cortisol) compared to those from mothers with ‘low prenatal stress’ (low on both) (Figure 3.1). Moreover, these elevated levels of PNS were also associated with an increase in reported gastrointestinal symptoms and allergic reactions in the young infants.

**Health implications**

The bacterial signatures found in infants from high stress mothers may have multiple effects on a child’s health status. Proteobacteria are gram-negative organisms and may play a role in infection by producing lipopolysaccharides (LPS), which are inflammatory mediators in metabolic diseases (Cani et al., 2012). Additionally, *Escherichia/Shigella*, genera within Proteobacteria, have been shown to increase risk for allergies and atopic diseases (for more information on allergic diseases related to microbiota, see Chapter 9: Szajewska, 2017; Chapter 10: De Meij, 2017; Azad

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**Figure 3.1.** The average relative abundance of Bacilli, Proteobacteria, Bacteroidetes, Clostridia and Actinobacteria in infants with (A) exposure to low levels of maternal prenatal reported stress/anxiety as well as low levels of pregnancy cortisol, and (B) exposure to high levels of maternal prenatal reported stress/anxiety as well as high levels of pregnancy cortisol (Zijlmans et al., 2015; Figure is reproduced with permission from Elsevier).
3. Maternal prenatal factors influencing infant microbiota

and Kozyrskyj, 2012; Ling et al., 2014). Moreover, a decrease in lactobacilli and bifidobacteria, possibly as a result of PNS, has been related to increased crying behaviour in infants (for more information see Chapter 12: Sung and Pärtty, 2017; De Weerth et al., 2013; Pärtty et al., 2012).

Aetiology

Several physiological mechanisms are proposed explaining how maternal psychological stress may alter her gut bacteria, and consequently, that of the offspring. These mechanisms may act independently or in concert:

- PNS leads to elevations in maternal stress hormones level, such as cortisol. Cortisol regulates bile acid production in the liver, altering and controlling levels of cholesterol and bile acid. High levels of cortisol can therefore directly influence the maternal gut microbiota composition by increasing bile acid production (Zijlmans et al., 2015).
- Elevated cortisol concentrations may alter the acidity of gut secretions and gut motility, causing shifts in the maternal microbiota composition (Deloose, 2016; Koren et al., 2012).
- Elevated levels of cortisol may disrupt the gut barrier function and change the permeability of the gut (Li et al., 2013; Vanuytsel et al., 2014), potentially influencing the maternal gut microbiota (Dinan and Cryan, 2012).
- Elevated levels of PNS may increase bicarbonate production by the liver and inhibit gastric acid production in the stomach, resulting in an environment that is less favourable for beneficial bacteria (e.g. lactic acid bacteria) in the maternal gut (Dinan and Cryan, 2012; Knowles et al., 2008; Koren et al., 2012).
- Corticotropin-releasing hormone (CRH) plays an important role in the psychological stress response and recent evidence suggests that CRH may act both centrally (central nervous system) and peripherally (intestines) through CRH receptors. In the gut, CRH induces alterations of intestinal motility and impairment of the intestinal barrier function (Keita et al., 2010; Rodiño-Janeiro et al., 2015; Söderholm and Perdue, 2001; Tache and Perdue, 2004; Vanuytsel et al., 2014; Wallon et al., 2008). For example, a study in humans demonstrated that acute psychological stress, proven by elevated salivary cortisol levels, can increase small intestinal permeability with CRH as one of the key mediators (Vanuytsel et al., 2014). Finally, peripheral CRH activates CRH receptors resulting in the release of various mediators, such as proteases and tumour necrosis factor alpha (TNF-α), which in turn can alter intestinal permeability (Keita et al., 2010). Changes in intestinal permeability and motility have been related to an altered gut microbiota composition (Dinan and Cryan, 2012).
- Chronic exposure to stress hormones, such as cortisol, promote an inflammatory response and influence the immune system, which in turn can affect maternal intestinal microbial composition and function (Marques et al., 2015; Palm et al., 2015).

Other mechanisms would act directly in the foetus:

- Maternal cortisol crossing the placenta may increase foetal concentrations of cortisol, affecting the development of the foetal HPA axis, and in turn resulting in higher infant basal cortisol levels (Duthie and Reynolds, 2013; Tollenaar et al., 2011). As mentioned
above, elevated cortisol levels can influence the natural development of the gut microbiota by affecting gut secretions, motility, permeability and barrier function (Deloose and Tack, 2016; Dinan and Cryan, 2012; Koren et al., 2012).

- During pregnancy foetuses are partially protected from high cortisol by activity of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) present in the placenta. HSD11B2 activity may be compromised when the mother is stressed or anxious, allowing greater transfer of cortisol from mother to foetus (Duthie and Reynolds, 2013; O’Donnell et al., 2012), possibly interfering in the natural development of the infant gut microbiota.
- Interestingly, recent data suggest that PNS can directly influence the developing foetal immune system. It is yet to be determined whether altered development of the infant’s immune system affects normal development of the infant gut microbiota (Andersson et al., 2016).

**Recommendations for clinical practice**

Given the impact of PNS on maternal and child health, identifying PNS in the prenatal setting is of importance, and if identified, stress/anxiety management interventions could be offered. Until now, no universally effective evidence-based therapy for PNS has been identified (Lever Taylor et al., 2016; Marc et al., 2011). Yet, there are some therapies that may have positive effects. For example, there is some evidence that meditation, mindfulness-based and relaxing interventions, yoga and muscle relaxation training may help relieve perceived stress or anxiety in pregnancy (Bastani et al., 2005; Beddoe and Lee, 2008; Field et al., 1999; Lever Taylor et al. 2016; Richter et al., 2012; Satyapriya et al., 2013; Teixeira et al., 2005; Ventura et al., 2012). More clinical trials need to be conducted to confirm the effects of these therapies in reducing PNS.

**Recommendations for future studies**

Currently, human evidence for the (casual) relationship between PNS, an altered infant microbiota composition and adverse child health outcomes remains limited. More observational studies would offer opportunities for replicating and extending current findings by Zijlmans et al. (2015). Moreover, interventional human studies using probiotic bacterial or stress/anxiety management interventions are recommended to determine causal relations between PNS and adverse infant outcomes possibly mediated by the maternal/infant gut microbiota. Importantly, these observational and intervention studies should have appropriate sample sizes for drawing accurate conclusions. Finally, future experimental studies could provide further mechanistic insight into the correlation between PNS, an altered maternal gut bacteria and adverse infant health outcomes.

### 3.4 Maternal pregnancy obesity

Maternal obesity is a prenatal risk factor for obesity in offspring (Galley et al., 2014; Mustila et al., 2013; Power and Schulkin, 2012; Soderborg et al., 2016). The prevalence of maternal
obesity enormously increased in the modern human population (Power and Schulkin, 2012). Currently, 34% of women who are of childbearing age in the United States are obese and 59.5% are overweight (Morea et al., 2013). In Europe, the prevalence of obesity during pregnancy ranges between 6.2 and 36.5%, depending on different geographical variations (Morea et al., 2013).

Obesity in children has severe consequences for child health (Gurnani et al., 2015). For example, childhood obesity results in increased risk for hypertension, dyslipidaemia, glucose intolerance, non-alcoholic fatty liver disease (NAFLD), polycystic ovarian syndrome (PCOS), obstructive sleep apnoea (OSA) and cardiovascular abnormalities during childhood (Challier et al., 2008; Daniels et al., 2005; Soderborg et al., 2016; Steinberger and Daniels, 2003). In addition, obesity and diabetes often coexist during pregnancy (Murphy et al., 2011). Gestational diabetes is also identified, together with genetic predisposition, as a prenatal risk factor for obesity in offspring (Allnutt et al., 2015; Chico et al., 2016). However, given that most available human data is on the impact of maternal obesity on infant microbiota and related adverse infant health outcomes, the central focus of this review is maternal obesity (Soderborg et al., 2016).

**Impact of maternal pregnancy obesity on infant microbiota**

The positive association between maternal obesity and risk of obesity in offspring is driven largely by shared familial risk factors for weight gain, including genetic and environmental factors (Lawlor et al., 2011). However, a large longitudinal European cohort study found that both shared familial factors as well as intrauterine environmental factors explain the positive association between maternal overweight/obesity and increased weight gain in the offspring at 18 years (Lawlor et al., 2011). The gut microbiota may play a meaningful role, as it strictly regulates metabolism by managing fat storage and energy balance (Ley et al., 2005; Nieuwdorp et al., 2014; Turnbaugh et al., 2009). Possible mechanisms are the suggested in utero and during delivery transfer of obesogenic maternal microbes to the foetus/infant, predisposing the child to obesity (Santacruz et al., 2010). Thus, the bacterial signatures found in obese mothers, the maternal obesogenic signature, may affect infant microbial development, which in turn may affect weight gain in children. Here, we present evidence that maternal obesity may cause shifts in offspring microbiota composition (Collado et al., 2010; Soderborg et al., 2016).

Collado et al. (2010) found associations between increased maternal weight during pregnancy and infant microbiota development during the first six months of life. In their study they examined the relation between weight gain, overweight during pregnancy and pre-pregnancy weight on infant's microbiota composition. Results showed that infants at the age of six months of women with excessive weight gain during pregnancy (>16 and >11.5 kg for normal-weight and overweight mothers, respectively) had lower concentrations of bifidobacteria and higher Staphylococcus aureus concentrations, compared to pregnant women with normal weight gain (Collado et al., 2010). They also found that being overweight during pregnancy (body mass index (BMI) ≥25 kg/m²) was related to the infant's microbial composition during the first six months of life. Infants born to overweight mothers had lower concentrations of Bacteroides-Prevotella
species at one month of age and higher concentrations of *Clostridium*, *Staphylococcus* and *Akkermansia* at six months of age, compared to infants of normal-weight mothers. Moreover, *Bifidobacterium* counts were lower in infants from overweight mothers at 6 months of age compared to infants of mother with normal-weight during pregnancy. These results were similar for infants with mothers who were already overweight before pregnancy, defined as pre-pregnancy overweight (BMI ≥25 kg/m²). Pre-pregnancy overweight was related to higher concentrations of *Staphylococcus* and *Clostridium histolyticum*, and lower concentrations of *Bifidobacterium* in infants at six months of age, compared to infants whose mothers had normal pre-pregnancy weights (Collado *et al.*, 2010). Another study observed higher concentrations of *Akkermansia* in obese/overweight women and consequently higher concentrations of the same genus in their six month old infants (Collado *et al.*, 2012). In sum, there are indications that maternal obesity may alter maternal microbiota composition, which may be transferred from mother to foetus/infant during the prenatal period (Gohir *et al.*, 2015).

**Health implications**

The preceding text triggers questions about the relevance of the observed shifts in infant gut microbes for child weight trajectories over the life course. A recent review on the infant gut microbiota in relation to obesity risk reported that early elevations in *Bacteroides fragilis* species and lower levels of *Bifidobacterium* correlated with weight gain and obesity during childhood (Koleva *et al.*, 2015). However, evidence from prospective interventional human studies is limited and these studies show conflicting results (Laitinen *et al.*, 2009; Luoto *et al.*, 2010a; Mustila *et al.*, 2012, 2013). For example, a large long-term longitudinal intervention study by Luoto *et al.* (2010a) evaluated the impact of a perinatal probiotic intervention on childhood overweight during a 10-year follow-up. The results showed that a perinatal probiotic intervention could moderate excessive weight gain during the period of birth until 2 years of age. However, it was unrelated to excessive weight gain after two years of age (Luoto *et al.*, 2010a). Two other interventional studies assessed the effects of maternal probiotic-supplemented dietary counselling on perinatal growth in infants. Prenatal diet/probiotic intake diminished the risk of larger size at birth, but did not show a significant effect on growth and weight in the offspring (Laitinen *et al.* 2009; Luoto *et al.*, 2010b). In other interventional studies, antenatal dietary and physical activity counselling showed both positive and no effect on infant weight gain outcomes during the first years of life (Mustila *et al.*, 2012, 2013).

In sum, the extent to which maternal obesogenic microbes impact infants’ weight trajectories over the life course remains speculative (Galley *et al.*, 2014). Nonetheless, the positive association observed between maternal probiotic/dietary interventions and offspring birth size and weight gain during the first two years of life, indicate that a potential relationship exists between specific maternal gut bacteria and offspring metabolic health status (Luoto *et al.*, 2010b).
Aetiology

As mentioned above, a proposed hypothesis to explain the relationship between maternal pregnancy obesity and child obesity, is the vertical transfer of obesogenic maternal microbes to the offspring, as presented in Figure 3.2 (Gohir et al., 2015; Ollberding et al., 2015; Scheepers et al., 2015; Serino et al., 2016). In considering the vertical route of transfer, we will address three questions: (1) what is the theoretical basis for the maternal microbiota-obesity link; (2) through which routes can maternal obesogenic microbes potentially be transferred to the foetus/infant during the perinatal period; (3) what are the potential mechanisms for microbiota-induced obesity in children?

![Figure 3.2](image)

Figure 3.2. A theoretical model supported by empirical studies on microbial changes in the gut during pregnancy in lean versus obese women, and the consequences of maternal obesity on metabolic outcomes in offspring. The gut microbiota changes throughout pregnancy. Lean women, who are thought to possess a healthy microbial composition, maintain the same number of Firmicutes (green) and Proteobacteria (red) during pregnancy, paired with an increase in Bacteroidetes (blue) and Actinobacteria (yellow). Obese women with an aberrant gut microbiota, namely increased numbers of Firmicutes and Actinobacteria and decreased Bacteroidetes, display additional unfavourable modifications during pregnancy. Actinobacteria and Firmicutes further increase, whereas Proteobacteria and Bacteroidetes stay the same. The abnormal composition confers a disrupted intrauterine environment potentially leading to intestinal dysbiosis in infants with an obese mother, compared to the infants of lean mothers, consequently predisposing these children to adverse metabolic outcomes (Gohir et al., 2015; Ollberding et al., 2015; Scheepers et al., 2015; Serino et al., 2016). (Figure adapted from Gohir et al. (2015); reproduced with permission from Elsevier).
Theoretical basis for the maternal microbiota-obesity link

During pregnancy, obesity has been associated with shifts in the gut microbial composition, such that microbiota from obese women significantly differ from lean women. For example, Santacruz et al. (2010) reported increased abundances of Staphylococcus and Escherichia coli (resp. belonging to the Firmicutes and Proteobacteria), and a decrease in Bifidobacterium and Bacteroides (resp. belonging to Actinobacteria and Bacteroidetes) in over-weight pregnant women, as compared to lean pregnant women in the second trimester (Santacruz et al., 2010). These observations suggest that there is a ‘maternal gut microbiota-obesity link’ present. Several possible mechanisms have been suggested to explain this link (Collado et al., 2008):

- Diet may promote a specific microbiota composition in obese mothers (Luoto et al., 2010b). Data from non-pregnant subjects shows that diet can induce a shift in gut microbiota that enables increased energy harvest from food (Jumpertz et al., 2011).
- The gut microbiota may affect weight gain during pregnancy through increased absorption of glucose and fatty acids (Collado et al., 2008).
- Specific microbiota compositions may induce catabolic pathways, which consequently may increase storage of energy from food (Bäckhed et al., 2007).
- Gut microbes may modulate fasting-induced adipocyte factor, a regulator of lipid metabolism and adiposity (Collado et al., 2008; Mandard et al., 2006).
- In addition to food intake and storage, the gut microbiota have inflammatory properties that may contribute to overweight and obesity during pregnancy (Collado et al., 2008). Maternal obesity is associated low-grade inflammation (Basu et al., 2011; Madan et al., 2009) and recent studies suggest a role for gut microbiota in driving this state of inflammation (Koren et al., 2012). For example, high concentrations of S. aureus during pregnancy may contribute to inflammatory processes and increased fat storage (Collado et al., 2008). Conversely, high numbers of bifidobacteria in the gut may help with the normalisation of inflammatory processes that are associated with maternal obesity (Cani et al., 2007; Collado et al., 2008).
- Certain gut microbes produce short-chain fatty acids (SCFAs). SCFAs are ligands for G-protein-coupled receptors (GPCRs) that have essential functions in the intestine and pancreas, including gut motility, nutrient absorption, and maternal metabolism (Priyadarshini et al., 2014; Samuel et al., 2008). Moreover, SCFAs may play a role in inducing satiety by influencing satiety hormones (Park et al., 2015). Additionally, SCFAs may shift immunoregulatory cells toward an anti-inflammatory phenotype (Chang et al., 2014; Park et al., 2015), thereby possibly reducing the state of low-grade inflammation that is associated with maternal obesity (Basu et al., 2011; Madan et al., 2009). Obese pregnant women may have less SCFAs-producing bacteria in their intestines.

Transfer routes for maternal obesogenic microbes to the foetus/infant

Three routes for the transfer of maternal obesogenic gut microbiota to the offspring are proposed: in the womb (Santacruz et al., 2010), during delivery, and through lactation (Garcia-Mantrana and Collado, 2016).
In animal models, *in utero* transmission of maternal gut bacteria to the foetal gut has been demonstrated (Jiménez *et al.*, 2008). In humans, evidence for *in utero* transfer comes from Hu and colleagues (2013). They found that the meconium of new-borns of mothers diagnosed with DM2, compared to mothers with neither diabetes nor gestational diabetes mellitus, contained a bacterial colonisation in which especially the *Bacteroides*, *Parabacteroides*, and *Lachnospiraceae* were more prevalent. This microbial signature is similar to that of adult DM2 patients (Hu *et al.*, 2013). In addition, Penders *et al.* (2006) showed that maternal nutritional habits may change the gut microbial composition during pregnancy, which appears to influence the microbial composition in the meconium at birth (Penders *et al.*, 2006). Although these observational studies indicate the possibility of a maternal obesogenic microbiota transfer *in utero*, it is still unknown how these obesogenic microbes are exactly transferred to the foetus (Collado *et al.*, 2010).

Another route for transfer of obesogenic maternal microbiota is during vaginal parturition. Vaginally delivered new-borns are colonised with bacterial strains from the mother’s vaginal- and gastrointestinal tract (Neu and Rushing, 2011). However, a large cohort study showed that mode of delivery (vaginal delivery or caesarean section (CS)) in overweight and obese women during pregnancy was not significantly associated with an increased risk of childhood overweight at 7 years of age (Ajslev *et al.*, 2011). Instead, delivery by CS showed tendency towards an increased risk of overweight in boys. Notably, the authors did find a strong association between maternal pre-pregnancy overweight and the risk of childhood overweight.

The final proposed route for transfer of obesogenic microbiota is through lactation (Cabrera-Rubio *et al.*, 2012). Recent studies indicate that maternal weight status may influence the composition of human milk. For example, lower microbial diversity, higher *Staphylococcus* and *Lactobacillus* and lower *Bifidobacterium* counts were found in breast milk of obese mothers compared to normal weight mothers over the first six months of breastfeeding (Cabrera-Rubio *et al.*, 2012). Nonetheless, exclusive breastfeeding is associated with a reduced risk of childhood obesity in infants (Horta *et al.*, 2015; Modrek *et al.*, 2016) whose mothers are overweight or obese (Yeung *et al.*, 2016). Hence, these results merely indicate that it would be desirable to better understand the mechanisms by which maternal obesity may influence the milk microbial composition. Broader understanding of these mechanisms may help in the development of strategies that may optimise the quality of breast milk in obese women (Garcia-Mantrana and Collado, 2016).

In sum, several routes may be involved in the transfer of maternal obesogenic microbiota to offspring (Garcia-Mantrana and Collado, 2016; Santacruz *et al.*, 2010). To date, it remains under debate which route plays the most important role for the transfer of maternal obesogenic microbiota to offspring.
Potential mechanisms for microbiota-induced obesity in children

There are several mechanisms by which gut microbiota can potentially induce obesity in children. For example, it is suggested that lower amounts of bifidobacteria can affect weight gain in infants through mucosal host-microbe crosstalk, immune regulation and inflammatory control (Kalliomäki et al., 2008; LeBouder et al., 2006). Additionally, higher concentrations of Bacteroides, Clostridium, and Staphylococcus potentially predispose to greater energy extraction from food and subsequent storage, paired with lessened control of inflammation, both typical in obesity (Collado et al., 2010). Importantly, these microbial shifts were observed during the first six months of life in infants of overweight mothers (Collado et al., 2010). Microbial changes during the first six months of life are of special interest since rapid weight gain during this period is associated with an increased risk of obesity during childhood, even more than weight status at birth (Stettler et al., 2002; Taveras et al., 2009).

Furthermore, Collado et al. (2012) observed higher concentrations of Akkermansia in stool of obese/overweight women and their offspring (Collado et al., 2012). The role of Akkermansia muciniphila in relation to obesity remains ambiguous. This bacterium is commonly found in the intestines where it degrades mucin and is known to protect against inflammation (Derrien et al., 2016). It is also associated with healthier metabolic status in overweight and obese adults (Dao et al., 2016). Whether A. muciniphila exerts a possible protective effect in overweight mothers and their infants remains elusive and requires further investigation.

It is important to highlight that there are multiple factors acting together to form the basis of a potentially aberrant foetal and infant gut microbiota, predisposing offspring to obesity. Aberrant infant microbial composition will be the result of a complex interaction of many factors next to maternal microbial composition, including genetic risk, epigenetic transmission, maternal diet, maternal lifestyle, familial environment (e.g. siblings, hygiene), maternal health, treatment with antibiotics during pregnancy and delivery, infant antibiotic exposure, the duration of breastfeeding and complementary diet, mode of delivery, ethnicity, geographical location, and sexual dimorphism, as indicated in Figure 3.3 (Ajslev et al., 2011; Garcia-Mantrana and Collado, 2016; Kozyrskyj et al., 2016; Laursen et al., 2016; Soderborg et al., 2016). Each of these factors is critical in regulating appetite, metabolism, and inflammation, as they all play a role in immune signalling, toxin release, and metabolic signalling and functioning (Soderborg et al., 2016).

Recommendations for clinical practice

Regular exercise during pregnancy has proven an effective method of controlling weight gain during pregnancy (Muktabhant, 2012) and could potentially enhance the gut microbiota composition (Clarke et al., 2014). For example, physical activity has been shown to increase microbial diversity, increase the proportion of A. muciniphila and decrease inflammatory metabolic markers (Clarke et al., 2014). The effect of regular exercise during pregnancy on maternal microbial composition and metabolic outcomes should be evaluated in future studies.
3. Maternal prenatal factors influencing infant microbiota

Furthermore, a balanced and healthy diet during pregnancy may be recommended to help optimise beneficial microbial composition during the prenatal period in mothers and their offspring. Diet is put forward as an important factor influencing the gut microbiota and energy uptake in the gut (Clarke et al., 2014; Jumpertz et al., 2011). Food intake high in fibre and low in refined sugar and saturated fat helps to support a healthy gut environment for beneficial bacteria to thrive (Chan, 2013). In addition, diet during pregnancy may also affect infant microbiota (Penders et al., 2006). Penders et al. (2006) found in their study that infants born to mothers who consumed an organic or biodynamic diet during pregnancy had slightly lower numbers of E. coli compared to infants whose mothers consumed a regular diet.

Finally, although the effects of probiotics during pregnancy has shown limited success on reducing maternal weight during pregnancy (Ilmonen et al., 2011; Luoto et al., 2010b), there is some evidence that probiotics may be beneficial in improving the metabolic status of pregnant mothers. Certain bacterial strains ingested during pregnancy may improve maternal glucose metabolism and insulin sensitivity, and reduce the incidence of gestational diabetes (Laitinen, 2009; VandeVusse, 2013). Gestational diabetes is, together with genetic and epigenetic factors, associated with obesity in offspring (Allnutt et al., 2015; Chico et al., 2016). Thus, probiotics during pregnancy may indirectly improve infant metabolic health and help prevent child obesity.
Recommendations for future research

Although data from human studies suggest the vertical transmission of maternal obesogenic microbiota in obese and overweight women predisposing offspring to obesity, it remains largely unclear what causal pathways are involved (Galley et al., 2014). Most available studies are observational where it is impossible to control for confounders (e.g. genetics, behavioural, and diet) that contribute to developing childhood obesity (Galley et al., 2014).

Better understanding how maternal obesity affects the development of both maternal and infant gut microbiota may help to identify targeted interventions during the prenatal period. Applying effective interventions could potentially reduce the transmission of obesity risk to the future generation (Soderborg et al., 2016). Future animal and human studies should be conducted to obtain mechanistic insights into the vertical transfer of maternal obesogenic microbes to offspring. For example, studies should evaluate the alterations that occur in the maternal gut microbiota and milk composition due to maternal overweight and the subsequent effect on gut microbial composition in meconium and the infant’s gut. Moreover, it is recommended to investigate whether supplementation of probiotics, diet and/or exercise could improve maternal gut microbiota and milk composition and subsequently the bacterial composition of the neonatal gut. Additionally, large long-term human interventional cohort studies would be highly valuable. These would help to identify to what extent maternal obesogenic microbiota can potentially induce obesity in offspring and to what extent the observed effects persist through childhood (Galley et al., 2014).

3.5 Conclusions

Several maternal prenatal risk factors acting in concert potentially form the basis of an aberrant infant gut microbiota with potential substantial implications for child health. PNS and maternal obesity have been identified as prenatal risk factors. Several pathways are proposed through which PNS may act directly or indirectly on infant microbial composition and function. Maternal obesity could potentially lead to a ‘maternal obesogenic microbial signature’, which could be vertically transferred from mother to foetus/infant. This could possibly predispose offspring to childhood obesity. Results are promising but more research is needed before strong conclusions may be drawn. Incorporating psychological as well as physiological maternal predictors and child outcomes in future studies is deemed recommendable. Finally, research on the effectiveness of pre- and probiotics on PNS and maternal obesity may provide interesting causal insights on how to obtain (long-term) health benefits for pregnant women and their offspring.

Conflict of interest

C. De Weerth and E. Van den Berg declare no conflict of interest. P.D. Browne has participated as a clinical investigator for Clinical Research Rotterdam B.V. and as a speaker for Winclowe Probiotics B.V.
3. Maternal prenatal factors influencing infant microbiota

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3. Maternal prenatal factors influencing infant microbiota


Chapter 4
The impact of birth and postnatal medical interventions on infant gut microbiota

A.L. Kozyrskyj1*, S.L. Bridgman1 and M.H. Tun2
1Department of Pediatrics, University of Alberta, 3-527 Edmonton Clinic Health Academy, 11405 87th Avenue, Edmonton, AB T6G IC9, Canada; 2School of Public Health, University of Alberta, 3-300 Edmonton Clinic Health Academy, 11405 87th Avenue Edmonton, AB T6G IC9, Canada; kozyrsky@ualberta.ca

Abstract

Established during infancy, the initial colonisation and development of the complex gut microbial community of our gastrointestinal tract can be shaped by common medical interventions, such as caesarean section and antibiotic use. This chapter provides evidence on the gut microbial impact of four medical interventions: (1) caesarean delivery, (2) maternal intrapartum antibiotic prophylaxis (IAP), (3) hospitalisation post birth, and (4) postnatal infant antibiotic treatment. Reductions in bifidobacteria and members of the Bacteroidaceae family (e.g. Bacteroides fragilis) are by far the most common perturbations in microbial composition following each of these interventions, especially after elective or emergency caesarean section. On the other hand, genus Clostridium and the Enterbacteriaceae (e.g. Klebsiella, Escherichia coli) are likely to become more abundant in infants delivered by caesarean, exposed to maternal antibiotics, hospitalised post birth and treated with antibiotics. Often, the enterococci and staphylococci also become more abundant. Differential impact on gut microbiota is observed by type of caesarean delivery and antibiotic administered to the mother or infant. IAP with penicillin or cefazolin, or newborn treatment with intravenous penicillin (plus gentamicin) is associated with higher abundance of Enterococcus and Staphylococcus aureus. Klebsiella emerge after newborn intravenous ampicillin (plus gentamicin) treatment. The Veillonella become more abundant in the infant gut after emergency (but not elective caesarean), whereas they are found to be depleted two months after newborn treatment with oral cephalexin. Of note, dysbiosis from perinatal medical interventions also occurs in the early breastfed infant and is enhanced by prematurity.

Keywords: gut dysbiosis, caesarean delivery, intrapartum antibiotic prophylaxis, hospitalisation post birth, postnatal antibiotic treatment
4.1 Introduction

Women who undergo caesarean section delivery uniformly report a greater number of medical interventions, less mother-infant contact after birth and suboptimal breastfeeding practices (Chalmers et al., 2009). As unsatisfying as they are for women and their families, they also derail the normal development of the gut microbiome during infancy. This development starts with pioneer microbes, the enterobacteria, which prepare the gut for anaerobic bacteria like bifidobacteria, Clostridia and other Firmicutes, and members of the Bacteroidetes. For a concise and comprehensive overview of all factors that affect this process, see Van Best et al. (2015). The focus of this chapter is on medical interventions during birth and the postnatal period, and how they affect the infant gut microbiome.

Four medical interventions will be reviewed: (1) caesarean section delivery (CS); (2) maternal intrapartum antibiotic prophylaxis (IAP); (3) hospitalisation post birth (home and hospital birth); and (4) postnatal infant antibiotic treatment. Appendix 4.1 provides details on most of the studies cited in this chapter, grouped according to taxon perturbation within each of the medical interventions. By way of background, high-throughput sequenced-based studies typically report on taxon-specific microbial abundance relative to the total abundance of other microbes present in the biological sample. Culture and qPCR (polymerase chain reaction assay) studies enumerate absolute quantities of microbes, which are not reported in relative terms since quantification of all microbial species present in a sample is rarely possible with these methods. On the other hand, culture and qPCR methods enable greater reporting of microbes at the species level. All methods enable determinations of the percent colonisation with a microbe.
4.2 Caesarean section delivery

The WHO recommendation that population rates of CS not exceed 15% has been in effect for almost 30 years (WHO, 1985). This benchmark remains appropriate to protect the woman and foetus from life-threatening complications, and also to prevent excess maternal and neonatal morbidity and mortality (Althabe and Belizan, 2006; Karlstrom et al., 2013). Currently in North America and Europe, 24 and 19% of births, respectively, are by CS; at 29%, Latin America has the highest global rates (Betran et al., 2016). CS rates are rising despite a lack of evidence for increasing prevalence of obstetric emergencies or medical risk (Bailit et al., 2004; MacDorman et al., 2008). The convenience of scheduling birth and fear of pain are contributing factors (Holmgren et al., 2012; Miesnik and Reale, 2007). Also, contrary to WHO guidelines, less than 30% of infants in middle-income countries are exclusively breastfed for the first 5 months of life (Victora et al., 2016), and less so if they are born by CS (Al-Sahab et al., 2010). The consequences of CS birth should not be underestimated. Women undergoing CS are at greater risk for haemorrhage and uterine rupture (Armson, 2007; Liu et al., 2007), and their newborns have higher rates of respiratory distress and infection (Karlstrom et al., 2013; Magnus et al., 2011). Beyond maternal and neonatal risks, a number of risks to child health have been linked to CS birth, including food allergy (Sanchez-Valverde et al., 2009), asthma (Huang et al., 2015) and overweight (Li et al., 2013).

Impact on infant gut microbiota: summary of evidence

No less than 7 cohort studies around the world have documented reduced faecal abundance of genus *Bacteroides* or reduced diversity of the phylum *Bacteroidetes* in infants up to 4 months following caesarean delivery. This evidence comes from 5 European (n=1,853) and 2 North American cohorts (n=300) (Backhed et al., 2015; Hesla et al., 2014; Jakobsson et al., 2014; Madan et al., 2016; Penders et al., 2006, 2013). Bifidobacteria are also less abundant and less diverse in the newborn gut within one week after CS (Backhed et al., 2015; Dogra et al., 2015; Penders et al., 2006), while the staphylococci are more plentiful (Backhed et al., 2015; Madan et al., 2016; Stokholm et al., 2016). Maternal skin has been identified as the source of staphylococci at birth in caesarean-delivered infants (Dominguez-Bello et al., 2010). Finally, *Clostridium difficile* has also been found to a greater extent in the stools of caesarean versus vaginally-delivered infants within one month after birth (Penders et al., 2006). Colonisation of the infant gut with *C. difficile* is becoming increasingly more prevalent than it was in the 1980s (Adlerberth and Wold, 2009), especially following CS (Adlerberth et al., 2014). While microbial changes have been detected up to 7 years following CS (Salminen et al., 2004), *C. difficile* colonisation as soon as 1 month after birth has been found to mediate the association between caesarean-induced gut dysbiosis and atopic disease (Penders et al., 2013; Van Nimwegen et al., 2011).
Differences between elective and emergency caesarean

Emergency and elective caesarean differ by indication, with foetal distress a common feature in the former. As well, many emergency caesareans are performed after a trial of labour, exposing newborns to maternal vaginal microbes. Unfortunately, most published studies do not separately report on elective versus emergency caesarean or even the percentage contribution for each to the study population. Among the few publications to date (Azad et al., 2016; Stokholm et al., 2016), gut microbiota profiles before three months of age in infants delivered by emergency CS were found to differ from those following elective CS; in the Canadian Healthy Infant Longitudinal Development (CHILD) cohort, this difference was independent of breastfeeding status (Azad et al., 2016). Based on culture methods, a higher percentage of neonates were colonised with Citrobacter freundii and S. aureus one week after emergency than elective CS (or vaginal birth) in the COPSAC2010 cohort; by one month of age, Citrobacter colonisation rates were equivalent between elective and emergency caesarean (Stokholm et al., 2016). Relative to vaginal birth in two Swedish cohorts, the faecal abundance of Veillonella species was higher at age 1-3 weeks in mixed samples of infants delivered by elective and emergency CS (Hesla et al., 2014; Jakobsson et al., 2014). Among infants in the CHILD cohort, the Veillonella were more abundant in faecal samples at three months following emergency CS than vaginally delivery; these differences were not seen for elective CS (Azad et al., 2016).

Common findings for elective and emergency caesarean

Certainly, varying indications for CS across studies may contribute to conflicting findings. In addition, culture-based studies are less effective in detecting strict anaerobes, such as the Bacteroides species (Stokholm et al., 2016). Notwithstanding these differences, the relative abundance of infant faecal Bacteroidetes (from the CHILD sequence-based study) was substantially reduced at 3 months of infant age following elective and emergency CS (Azad et al., 2016). Faecal enterobacteria were also more abundant in both types of this surgical intervention (Azad et al., 2016; Backhed et al., 2015; Stokholm et al., 2016). Interestingly, Stokholm et al. (2016) observed opposing results for individual species of cultured enterobacteria that were common to emergency and elective CS; a higher proportion of infants were colonised with Klebsiella within one month after delivery, while Escherichia coli were detected less often in faecal samples (Stokholm et al., 2016). Klebsiella species were also found to be more abundant in the newborn gut following CS in the GUSTO cohort (Dogra et al., 2015). Finally, Enterococcus becomes more abundant in the infant gut at one or three months following emergency and elective CS (Azad et al., 2016; Stokholm et al., 2016); Stokholm et al. (2016) also reported greater colonisation of the infant hypopharyngeal microbiome with Enterococcus faecalis three months after CS delivery.
Conclusions

Against a backdrop of climbing rates of CS, evidence is accumulating on the impact of CS on the infant gut microbiome. While the mechanism for how CS increases the risk of disease is not well understood, perturbations in infant gut microbial ecology, such as lowered abundance of the genus *Bacteroides*, likely play a role. Beyond its direct impact on microbial exposure, CS normally requires antibiotic prophylaxis, may follow microbial colonisation from prelabour rupture of membranes (DiGiulio, 2012) and can delay onset of breastfeeding (Brown and Jordan, 2013; McDonald *et al.*, 2012). Noteworthy is that before three months of age, the impact of CS on gut microbial composition has been found to be stronger than breastfeeding (Madan *et al.*, 2016) and to be independent of breastfeeding status (Azad *et al.*, 2016).

4.3 Maternal intrapartum antibiotics

Less well-studied is the impact of maternal intrapartum antibiotic prophylaxis (IAP) on infant gut microbiota, despite the fact that almost 40% of newborn infants are indirectly exposed to antibiotics administered during vaginal delivery (Fairlie *et al.*, 2013; Persaud *et al.*, 2015; Stokholm *et al.*, 2013). In North America, IAP constitutes a standard of care to prevent early-onset neonatal *Group B Streptococcus* (GBS) sepsis and maternal infection post CS (Van Schalkwyk, 2010). It is also becoming a routine part of the birthing process subsequent to climbing rates of CS delivery and GBS colonisation in pregnancy (Johri *et al.*, 2006). However, IAP is not routinely practised across the globe. While North American guidelines recommend IAP following universal vaginal screening for GBS, those from the UK and Australia advocate for risk-based management approaches (Homer *et al.*, 2014). The adoption of a non-culture risk factor approach for GBS in Denmark has reduced IAP to 13% of vaginal deliveries (Stokholm *et al.*, 2013). In contrast, 27% of vaginal deliveries or more than 85% of GBS-culture positive pregnancies result in IAP in Canada and the US (Fairlie *et al.*, 2013; Persaud *et al.*, 2015).

Consequences for offspring health

Effective in preventing early-onset neonatal sepsis, IAP for GBS has been linked to amoxicillin-resistant late-onset *E. coli* infections in hospitalised infants up to 90 days after birth (Didier *et al.*, 2012). In the long-term, infant antibiotic treatment is associated with childhood asthma and obesity (Azad *et al.*, 2014; Penders *et al.*, 2011), conditions also linked to gut dysbiosis in early life (Penders *et al.*, 2007; Vael *et al.*, 2011). While this evidence originates from studies of postnatal antibiotic use, IAP disruption of initial gut colonisation of the newborn has greater potential to alter the natural succession of microbiota throughout infancy.

Impact on infant gut microbiota: summary of evidence

Findings on IAP in vaginal birth provide the main evidence for IAP independence from CS since, with few exceptions, for example in Norway (Opoien *et al.*, 2007), all women undergoing
elective CS receive IAP. In a study of 84 full-term Italian newborns born vaginally, Aloisio et al. (2014) found lower faecal bifidobacterial counts seven days after maternal IAP with intravenous ampicillin for GBS than in its absence. In a follow-up study of these infants, bifidobacteria were no longer reduced in number at 30 days post IAP. No differences were seen in faecal concentrations of lactobacilli or Bacteroides fragilis at seven or 30 days (Corvaglia et al., 2016). In a smaller scale comparison by Arboleya et al. (2015), exclusively-breastfed full-term infants exposed to IAP (single dose of ampicillin) and hospitalised for three days exhibited an IAP effect between two and 90 days after birth, namely reduced abundance of Bifidobacteriaceae and Bacteroidaceae relative to controls.

Recently published from the CHILD cohort at three months of age, Azad et al. (2016) reported lowered abundance of Bacteroidaceae in faecal samples of full-term infants delivered vaginally after maternal IAP with penicillin (n=40) versus no IAP (n=96). Statistical significance was also found at the Bacteroides or Parabacteroides species level, but it did not survive correction for multiple testing. The family Clostridiaceae but not individual Clostridium species were more abundant following vaginal IAP. Compared to the infant gut dysbiosis seen following elective and emergency caesarean, there were fewer taxon differences at the genus level with IAP. Stokholm et al. (2016) also observed fewer changes with vaginal birth IAP than CS in their term infants at age one month, manifested as greater colonisation with Klebsiella and Staphylococcus species.

**Interactive effects with gestational age and breastfeeding status**

While studies of full-term infants indicate that IAP has a less profound effect on gut microbes than CS delivery, the impact of IAP may vary by gestational maturity. In the preterm context (mean gestational age of 30 weeks, primarily CS birth and hospitalisation for 50 days), Arboleya et al. (2015) observed several changes to gut microbial composition following maternal IAP. To begin with, all preterm infants had a much lower abundance of Bacteroidetes than term infants throughout the 3-month study. A large IAP effect was not evident at two days after birth, when only Leuconostaceae were more abundant in preterm infants not exposed to either IAP or postnatal antibiotics (e.g. ampicillin/gentamicin). Rather it emerged 30 days later, when significant clustering by IAP status was observed; infants exposed to IAP exhibited a lower relative abundance of Bifidobacteriaceae, Lactobacillales and Streptococcaceae, and a higher abundance of Enterobacteriaceae relative to non-exposed infants. At this postnatal age, no differences were observed between IAP-exposed and non-exposed infant with respect to gestational age, birth weight or length of hospital stay. The IAP impact was greater than direct administration of antibiotics to the infant.

As with CS, the impact of IAP in vaginal delivery on gut microbiota is also evident in the early breastfed infant. Azad et al. (2016) found genus Clostridium to be more abundant in IAP-exposed than non-exposed vaginally-born term infants who were exclusively breastfed at three months; the Clostridia did not vary by IAP status in partially breastfed infants. Findings from Corvaglia et al. (2016) show that IAP of vaginal birth mitigates faecal enrichment with bifidobacteria that
is normally seen with exclusive breastfeeding at 7 days after birth; increases to lactobacilli with breastfeeding were unaffected at seven or 30 days after birth in their study.

4.5 Hospitalisation post birth

Anywhere between 11% and 27% of infants, born by emergency and elective CS respectively, are hospitalised beyond one day in Western Europe (Penders et al., 2006). In Canada (and the US), almost 100% of these infants stay for at least two days (Tun, 2016). Up to 62% of newborns born vaginally remain in hospital for longer than one day.

The isolation of pathogenic bacteria from the hands and clothing of hospital staff suggests that patients, as well as hospital staff, are in close proximity to antibiotic-resistant strains of bacteria namely: methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), *Pseudomonas*, *Acinetobacter* and *Clostridium* (Weber et al., 2013). While many have published on hospital-based antibiotic resistance, impact on the gut microbial composition of the newborn is a new field of inquiry. Furthermore, unlike in the adult, pathogens like *C. difficile* have higher colonisation rates in infants in the absence of obvious gastrointestinal adverse effects (Adlerberth et al., 2014); a variety of maternal and household factors have promoted colonisation with this organism, such as birth mode, extent of breastfeeding, maternal parity and household pets (Azad et al., 2013; Bridgman et al., 2016).

**Home birth: impact on infant gut microbiota**

Vaginal birth at home relative to the hospital setting has been shown to strikingly reduce the risk of asthma at age seven and to food sensitisation at age one in offspring of parents with allergic disease (Van Nimwegen et al., 2011). In the same study from the KOALA cohort, the presence of *C. difficile* in the gut one month after birth was found to mediate the association between birth site (home versus hospital) and childhood asthma. In fact, findings from the Swedish ALADDIN cohort indicate that relative to a home birth, being born vaginally in a hospital alters the composition of the whole gut microbial community six months later (Hesla et al., 2014).

**Hospital birth: impact on infant gut microbiota**

Extended hospitalisation post birth to two days or longer further alters gut microbial composition of infants. In the KOALA cohort, the two largest changes to one-month microbial composition after extended hospitalisation were increases to the colonisation rates and microbial counts for *C. difficile*, and reductions in the colonisation and counts for *Bifidobacteriaceae* (Penders et al., 2006). Among infants hospitalised for 2-3 days versus same day discharge, *E. coli* were more common in faecal samples one month later. When hospital length-of-stay was longer at 4-6 days, the *B. fragilis* were reduced in number. Duration of hospitalisation remained a significant determinant for detecting *C. difficile* in infant faecal samples, independent of maternal antibiotic use, birth mode, infant feeding and other home environment factors. Each additional day of
hospitalisation after birth increased the likelihood of *C. difficile* colonisation by 1.13 (95% CI: 1.01-1.25) (Penders et al., 2006).

More recently, in the CHILD cohort study we reported depletion of specific gut microbiota at three months according hospitalisation post birth and this depended on birth mode (Tun, 2016). The relative abundance of *Bacteriodetes* was inversely proportional to the duration of hospital stay for each birth type. Only following elective caesarean delivery was the correlation between abundance of *Bacteroidaceae* or *Bacteroides* and hospital length-of-stay statistically significant (*r*=-0.27, *P*<0.01). Put together, these observations suggest a role for hospital-acquired microbes such as *C. difficile*, and reduced colonisation resistance, in modifying community gut composition (Rousseau et al., 2011).

### 4.6 Infant postnatal antibiotics

After breast milk and other dietary supplements, antibiotics are the next most commonly ingested substances by infants. Antibiotics affect colonisation of the intestine by suppressing commensal bacteria and causing the emergence of resistant pathogens. Despite a plethora of published articles relating infant antibiotic treatment to development of allergic disease (Penders et al., 2011), and more recently overweight in children (Azad et al., 2014), evidence for the impact of infant antibiotic treatment on gut microbial composition is less plentiful.

**Antibiotic treatment of infant: impact on gut microbiota**

Oral amoxicillin is the drug of choice for acute otitis media and other respiratory infections in infants. When given to older infants between the ages of 1-2 years, Mangin et al. (2010) found a reduction in the number of different bifidobacterial species in faecal samples immediately at the end of the antibiotic course compared to baseline. Total bifidobacterial concentrations were unchanged and other gut microbiota were not assessed. In contrast, intravenous (IV) treatment of 6-month old infants for five days with ceftriaxone (an antibiotic excreted in bile) for pneumonia (Savino et al., 2011), resulted in a significant reduction in total faecal bacterial counts at the end of treatment. Faecal concentrations of the *Enterobacteriaceae* and enterococci in these full-term infants were also reduced compared to baseline. After five days of ceftriaxone therapy, lactobacilli were no longer detected in infant faecal samples (Savino et al., 2011).

Among young infants at 1-3 months of age, most oral antibiotics (penicillins, cephalosporins, macrolides) cause gut microbial changes 3-5 days after the cessation of treatment (Bennet et al., 2002). These changes are evident in vaginally-born, breastfed infants; reductions to bifidobacteria, lactobacilli and *Bacteroides* are the most profound. Similarly, in the KOALA cohort of predominantly vaginally-born and exclusively breastfed infants at age one month, Penders et al. (2006) observed lower faecal counts of bifidobacteria and *B. fragilis* as determined by qPCR in those previously treated with oral antibiotics. Within a European consortium of birth cohorts, of which three-quarters of infants were delivered vaginally but only 50% had been
4. Medical interventions impact infant gut microbes

Exclusively breastfed, Fallani et al. (2010) reported greater abundance of faecal enterobacteria among infants receiving antibiotics by six weeks of age. No gut dysbiosis was observed in these infants in the period after breastfeeding cessation.

Intravenous antibiotic treatment of the neonate for suspected sepsis immediately after birth can significantly and persistently alter gut microbial composition. Using sequenced-based methods, Fouhy et al. (2012) reported that a 48 hour course of IV ampicillin and gentamicin in full-term newborns significantly elevated the abundance of the Proteobacteria phylum compared to controls, and lowered proportions of genus Bifidobacterium and Lactobacillus four weeks after the cessation of treatment. Bacteroidetes were also detected less often in antibiotic-treated infants. By week eight, the faecal abundance of Bifidobacterium and Lactobacillus returned to levels comparable to control infants. Yet, levels of Proteobacteria remained significantly higher and the number of different Bifidobacterium species remained low in antibiotic-treated infants. A reduction in gut microbial diversity was also observed in the Tanaka et al. (2009) study of Japanese full-term newborns hospitalised for one week and supplemented with formula. Unlike the typical IV regimen, neonates received oral cephalaxin for four days after birth. Gut microbial diversity was reduced on the third day of treatment and remained lowered two months later. These community changes were accompanied by the appearance of Enterococcus faecium, and reductions in genus Bifidobacterium and Veillonella, and in members of the Bacteroidaceae family; the latter two microbial taxa were not detected at two months of age.

Using a cross-over design of rectal cultures, Parm et al. (2010) nicely demonstrated that antibiotic type determines the extent of colonisation by individual species. Treatment of preterm newborns with an IV penicillin G and gentamicin regimen resulted in colonisation of the gut with S. aureus and Enterococcus species 5-7 days after treatment, while combined treatment with ampicillin and gentamicin favoured colonisation by other staphylococci. Moreover, the ampicillin regimen resulted in a greater duration of colonisation with Klebsiella pneumonia and ampicillin-resistant Serratia (both members of the Proteobacteria). Similarly, Lindberg et al. (2011) reported a negative association between antibiotic treatment (with broad spectrum beta-lactams) and S. aureus colonisation rate in six month old Italian infants. This correlation was not seen in Swedish infants who were mainly treated with the narrow spectrum penicillin V, without activity against S. aureus.

Indirect exposure from breast milk

Maternal postnatal antibiotics are an understudied source of antibiotic exposure to infants. Although most beta-lactams are considered safe during breastfeeding, the presence of even small quantities of antibiotics in breast milk can potentially alter infant gut microbiota (Soto et al., 2014). In the CHILD cohort, where postnatal antibiotics were mostly administered to women after an emergency CS, a higher abundance of genus Clostridium at three months of age after emergency CS was observed in exclusively breastfed but not among infants supplemented with formula (Azad et al., 2016).
4.7 Conclusions

Clearly, emergency CS is medically indicated, antibiotics are needed to treat and prevent infection, and breastfeeding has a multitude of benefits. In this review, we presented evidence that gut dysbiosis in the developing infant occurs after common medical interventions in the perinatal time period. Reductions in bifidobacteria and members of the Bacteroidaceae family (e.g. B. fragilis) are by far the most common perturbations in microbial composition following exposure to IAP, hospitalisation post birth and treatment with antibiotics, and especially after elective or emergency caesarean delivery. On the other hand, genus Clostridium and Enterbacteriaceae (e.g. Klebsiella, E. coli) are likely to become more abundant in infants following these exposures. Often, the enterococci and staphylococci also become more abundant. Differential impact on gut microbiota is observed by type of caesarean delivery and antibiotic administered to the mother or infant. IAP with penicillin or cefazolin, or newborn treatment with IV penicillin (plus gentamicin) is associated with higher abundance of Enterococcus and S. aureus. Klebsiella emerge after newborn IV ampicillin (plus gentamicin) treatment. The Veillonella become more abundant after emergency but not elective caesarean, whereas they are found to be depleted two months after newborn treatment with oral cephalaxin. Of note, dysbiosis from perinatal medical interventions also occurs in the early breastfed infant and is enhanced by prematurity.

While findings from the CHILD cohort suggest that early breastfeeding may modify IAP and CS-associated dysbiosis of the gut microbiome later in infancy (Azad et al., 2016), not all IAP-induced dysbiosis can be ‘restored’ with breastfeeding (Corvaglia et al., 2016). Reported reversals to dysbiosis, for example to Enterobacteriaceae, after the discontinuation of antibiotic treatment (Jakobsson et al., 2014), may also be subsequent to emerging resistant strains and not continued breastfeeding. Indeed, higher abundance of these gram-negative microbiota at six months of age has been associated with higher adiposity among toddlers (Dogra et al., 2015). Higher ratios in the abundance of the Enterobacteriaceae to Bacteroidaceae have also been reported to predict food sensitisation in infants (Azad et al., 2015). Finally, readers should bear in mind that gut microbial alterations, which occur during critical windows in the development of the immune system, even minor perturbations, may have long-term consequences (Cox et al., 2014).

Conflict of interest

The authors confirm that there are no conflicts of interest.

References


4. Medical interventions impact infant gut microbes


4. Medical interventions impact infant gut microbes


### Appendix 4.1
Study details of infant gut microbial dysbiosis by medical intervention type and taxon category.

<table>
<thead>
<tr>
<th>Microbial group</th>
<th>Study details, methods and findings</th>
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<tbody>
<tr>
<td><strong>Caesarean section (CS)</strong></td>
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<tr>
<td><em>Bacteroidetes</em> (phylum), <em>Bacteroidaceae</em> (family), <em>Bacteroides</em> (genus)</td>
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<tr>
<td>Jakobsson et al., 2014</td>
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<tr>
<td>Observed microbial change at infant age</td>
<td>• ↓ <em>Bacteroidetes</em> diversity and % <em>Bacteroides</em> colonisation at 1 week, 3 and 12 months in all CS (n=9) vs all vaginal birth (n=15)</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• Vaginal (n=15) no IAP (87%), elective (n=6)/emergency CS (n=3)</td>
</tr>
<tr>
<td>Infant population</td>
<td>• Placebo arm of probiotics trial, full term Swedish; 83% exclusively breastfed until 3 months; CS antibiotics given after cord clamping, no postnatal antibiotics</td>
</tr>
<tr>
<td>Microbial profiling method</td>
<td>• Roche 454 Genome Sequencer at V3/V4</td>
</tr>
<tr>
<td></td>
<td>• FDR correction</td>
</tr>
<tr>
<td>Azad et al., 2016 (a); Azad et al., 2013 (b)</td>
<td></td>
</tr>
<tr>
<td>Observed microbial change at infant age</td>
<td>• ↓ <em>Bacteroidetes</em>, <em>Bacteroidaceae</em>, <em>Bacteroides</em> and <em>Parabacteroides</em> abundance at 3 months in each CS type (or combined) vs vaginal birth no IAP (vaginal overall)</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• Vaginal no IAP (n=96), elective CS (n=17), emergency CS (n=23)</td>
</tr>
<tr>
<td>Infant population</td>
<td>• Vaginal (n=18), elective/emergency CS (n=6)</td>
</tr>
<tr>
<td></td>
<td>• Cohort, full term, Canadian; 52% exclusively breastfed until 3 months; same results if exclusive or no exclusive breastfeeding; postnatal antibiotics in 11%; IV cefazolin IAP for CS</td>
</tr>
<tr>
<td>Microbial profiling method</td>
<td>• Illumina MiSeq at V4a</td>
</tr>
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<td></td>
<td>• SiSeq at V5-V7b</td>
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<td></td>
<td>• FDR correction</td>
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<tr>
<td>Hesla et al., 2014</td>
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<tr>
<td>Observed microbial change at infant age</td>
<td>• ↓ <em>Bacteroides</em> abundance at 1 week, 3 weeks, 2 months in CS (n=18) vs vaginal birth (n=95)</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• Vaginal ± IAP (n=95) including home birth (n=24), elective/emergency CS (n=18); 55% of CS were emergency in the original cohort</td>
</tr>
<tr>
<td>Infant population</td>
<td>• Cohort, full term Swedish with anthroposophic sample, IAP in 10% of all births; 83% exclusively breastfed until 2 months; postnatal antibiotics in 3% of original cohort</td>
</tr>
<tr>
<td>Microbial profiling method</td>
<td>• Roche 454 Genome sequencer at V3/V4</td>
</tr>
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<td></td>
<td>• FDR correction</td>
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<tr>
<td>Microbial group</td>
<td>Study details, methods and findings</td>
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<tr>
<td><strong>Citation</strong></td>
<td><strong>Study groups and number of infants</strong></td>
</tr>
<tr>
<td>Backhed et al., 2015</td>
<td>• Vaginal (n=98) no IAP (90%), emergency CS (n=15)</td>
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<tr>
<td>Observed microbial change at infant age</td>
<td>• Bacteroides and Parabacteroides abundance at birth and 4 months of age in emergency CS versus vaginal birth</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• Cohort, full term, Swedish, antibiotic prophylaxis for GBS before delivery not intrapartum; 67% CS antibiotics given after cord clamp; 69% exclusively breastfed until 4 months; postnatal antibiotics in 5% of vaginal births</td>
</tr>
<tr>
<td>Infant population</td>
<td>• HiSeq of genome contigs</td>
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<tr>
<td>Penders et al., 2006</td>
<td>• Vaginal birth at home (n=480) vs CS (n=108)</td>
</tr>
<tr>
<td>Observed microbial change at infant age</td>
<td>• ↓ Bacteroides fragilis counts and % colonisation at 1 month in CS vs vaginal birth at home</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• Cohort, full term, Dutch with anthroposophic sample; 68% exclusively breastfed until 1 month; postnatal antibiotics in 3%</td>
</tr>
<tr>
<td>Infant population</td>
<td>• qPCR assay for bifidobacteria, Escherichia coli, Clostridium difficile, B. fragilis, lactobacilli of 16S rDNA gene sequences</td>
</tr>
<tr>
<td>Microbial profiling method</td>
<td>• FDR correction</td>
</tr>
<tr>
<td>Penders et al., 2013</td>
<td>• Vaginal (n=391), elective/emergency CS (n=144); assisted vaginal births not in comparison</td>
</tr>
<tr>
<td>Observed microbial change at infant age</td>
<td>• ↓ % B. fragilis colonisation (and most counts) at 5 weeks, 13 weeks, 1 month in CS vs spontaneous vaginal birth</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• Bacterial lysate randomised trial of high (atopic) risk infants, full term, Dutch; 89% breastfed until 1 month; no postnatal antibiotics</td>
</tr>
<tr>
<td>Infant population</td>
<td>• qPCR assay for bifidobacteria, E. coli, C. difficile, B. fragilis, lactobacilli of 16S rDNA gene sequences</td>
</tr>
<tr>
<td>Microbial profiling method</td>
<td>• FDR correction</td>
</tr>
<tr>
<td>Madan et al., 2016</td>
<td>• Vaginal ± IAP (n=70), elective/emergency CS (n=32)</td>
</tr>
<tr>
<td>Observed microbial change at infant age</td>
<td>• ↓ Bacteroides abundance at 6 weeks in CS vs vaginal birth, independent of breastfeeding status</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• Cohort, full term, US, 69% exclusively breastfed at 6 weeks; no postnatal antibiotics</td>
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<tr>
<td>Infant population</td>
<td>• Illumina MiSeq at V4/V5</td>
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<tr>
<td>Microbial profiling method</td>
<td>• FDR correction</td>
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<table>
<thead>
<tr>
<th>Microbial group</th>
<th>Citation</th>
<th>Study details, methods and findings</th>
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</table>
| Bifidobacteria  | Dogra et al., 2015 | • ↓ Bifidobacterium abundance on day 3 after birth in CS vs vaginal birth  
• Vaginal (n=57) no IAP (67%), CS (n=18)  
• Cohort, late preterm and full-term, Singapore, 20% exclusively breastfed on day 3  
• V1-3 and V5-7 Illumina sequencing  
• FDR correction |
|                 | Backhed et al., 2015 | • ↓ Bifidobacterium species richness at birth and 4 months in CS vs vaginal birth  
• Vaginal (n=98) no IAP (90%), emergency CS (n=15)  
• Cohort, full term, Swedish, antibiotic prophylaxis for GBS before delivery not intrapartum; 67% CS antibiotics given after cord clamp; 69% exclusively breastfed until 4 months; postnatal antibiotics in 5% of vaginal births  
• HiSeq of genome contigs |
| Penders et al. 2006 | • ↓ bifidobacterial counts and % colonisation at 1 month in CS vs vaginal birth at home  
• Vaginal birth at home (n=480) vs CS (n=108)  
• Cohort, full term, Dutch with anthroposophic sample; 68% exclusively breastfed until 1 month; postnatal antibiotics in 3%  
• qPCR assay for bifidobacteria, E. coli, C. difficile, B. fragilis, lactobacilli of 16S rDNA gene sequences  
• FDR correction |
| Staphylococcus   | Dominguez-Bello et al., 2010 | • ↑ proportion of Staphylococcus in rectum at birth in CS vs vaginal birth  
• Vaginal no IAP (n=4), elective CS (n=5)  
• Cohort, full term, Venezuela  
• Roche 454 Genome Sequencer at V2 |
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<td><strong>Microbiota in health and disease: from pregnancy to childhood</strong></td>
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<td><strong>A.L. Kozyrskyj, S.L. Bridgman and M.H. Tun</strong></td>
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<td><strong>Citation</strong></td>
<td><strong>Study details, methods and findings</strong></td>
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</table>
| Backhed *et al.*, 2015 | • ↑ *Staphylococcus* abundance at birth in CS vs vaginal birth  
• Vaginal (n=98) no IAP (90%), emergency CS (n=15)  
• Cohort, full term, Swedish, antibiotic prophylaxis for GBS before delivery not intrapartum; 67% CS antibiotics given after cord clamp; 69% exclusively breastfed until 4 months; postnatal antibiotics in 5% of vaginal births  
• HiSeq of genome contigs |
| Madan *et al.*, 2016 | **Observed microbial change at infant age**  
• ↑ *Staphylococcus* abundance at 6 weeks in CS vs vaginal birth, independent of breastfeeding status  
**Study groups and number of infants**  
• Vaginal +/- IAP (n=70), elective/emergency CS (n=32)  
**Infant population**  
• Cohort, full term, US, 69% exclusively breastfed at 6 weeks; no postnatal antibiotics  
**Microbial profiling method**  
• Illumina MiSeq at V4/V5  
• FDR correction |
| Stokholm *et al.*, 2016 | **Observed microbial change at infant age**  
• ↑ *Staphylococcus aureus* at 1 week in emergency and elective CS vs vaginal birth, independent of breastfeeding status  
**Study groups and number of infants**  
• Vaginal (n=549) no IAP (87%), emergency CS (n=85), elective CS (n=66)  
**Infant population**  
• Cohort, full term, Danish; exclusive breastfeeding for 3.4 months on average; postnatal antibiotics in 3%  
**Microbial profiling method**  
• Bacterial culture on selected media; unable to culture *Bacteroides* |
| **Clostridiales, Clostridium and C. difficile** | | |
| Penders *et al.*, 2006 | **Observed microbial change at infant age**  
• ↑ *C. difficile* counts and % colonisation at 1 month in CS vs vaginal birth at home  
**Study groups and number of infants**  
• Vaginal birth at home (n=480) vs CS (n=108)  
**Infant population**  
• Cohort, full term, Dutch with anthroposophic sample; 68% exclusively breastfed until 1 month; postnatal antibiotics in 3%  
**Microbial profiling method**  
• qPCR assay for bifidobacteria, *E. coli, C. difficile, B. fragilis*, lactobacilli of 16S rDNA gene sequences  
• FDR correction |
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<thead>
<tr>
<th>Microbial group</th>
<th>Citation</th>
<th>Study details, methods and findings</th>
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</table>
| **Clostridiales** | Azad et al., 2016 | Observed microbial change at infant age • ↑ *Clostridiales* abundance at 3 months in each CS type vs vaginal no IAP; ↑ *Clostridium* in emergency CS
Study groups and number of infants • Vaginal no IAP (n=96), elective CS (n=17), emergency CS (n=23)
Infant population • Cohort, full term, Canadian, 52% exclusively breastfed until 3 months; same results if exclusive or no exclusive breastfeeding; postnatal antibiotics in 11%; IV cefazolin IAP for CS
Microbial profiling method • Illumina MiSeq at V4 • FDR correction |

**Enterococcus species**

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<thead>
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<th>Citation</th>
<th>Study details, methods and findings</th>
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</table>
| Azad et al., 2016 | Observed microbial change at infant age • ↑ *Enterococcus* abundance at 3 months in emergency CS vs vaginal no IAP; higher abundance in elective CS did not survive FDR correction
Study groups and number of infants • Vaginal no IAP (n=96), elective CS (n=17), emergency CS (n=23)
Infant population • Cohort, full term, Canadian, 52% exclusively breastfed until 3 months; same results if exclusive or no exclusive breastfeeding; postnatal antibiotics in 11%; IV cefazolin IAP for CS
Microbial profiling method • Illumina MiSeq at V4 • FDR correction |

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<tr>
<th>Citation</th>
<th>Study details, methods and findings</th>
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</table>
| Stokholm et al., 2016 | Observed microbial change at infant age • ↑ *Enterococcus faecalis* colonisation at 1 week in CS (n=151) vs vaginal birth
Study groups and number of infants • Vaginal (n=549) no IAP (87%), elective CS (n=66), emergency CS (n=85)
Infant population • Cohort, full term, Danish; exclusive breastfeeding for 3.4 months on average; postnatal antibiotics in 3%
Microbial profiling method • Bacterial culture on selected media; unable to culture *Bacteroides*

**Proteobacteria, Enterobacteriaceae, Klebsiella**

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<tr>
<th>Citation</th>
<th>Study details, methods and findings</th>
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</table>
| Stokholm et al., 2016 | Observed microbial change at infant age • ↑ *Klebsiella* species colonisation at 1 week, 1 month in CS (n=151) vs vaginal birth
Study groups and number of infants • Vaginal (n=549) no IAP (87%), elective CS (n=66), emergency CS (n=85)
Infant population • Cohort, full term, Danish; exclusive breastfeeding for 3.4 months on average; postnatal antibiotics in 3%
Microbial profiling method • Bacterial culture on selected media; unable to culture *Bacteroides* |
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<tr>
<th>Microbial group</th>
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<th>Study details, methods and findings</th>
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<tbody>
<tr>
<td><strong>Backhed et al., 2015</strong></td>
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<tr>
<td>Observed microbial change at infant age</td>
<td></td>
<td>• ↑ Enterobacter abundance at 4 months in emergency CS vs vaginal birth</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td></td>
<td>• Vaginal (n=98) no IAP (90%), emergency CS (n=15)</td>
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<tr>
<td>Infant population</td>
<td></td>
<td>• Cohort, full term, Swedish, antibiotic prophylaxis for GBS before delivery not intrapartum; 67% CS antibiotics given after cord clamp; 69% exclusively breastfed until 4 months; postnatal antibiotics in 5% of vaginal births</td>
</tr>
<tr>
<td>Microbial profiling method</td>
<td></td>
<td>• HiSeq of genome contigs</td>
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<tr>
<td><strong>Azad et al., 2016</strong></td>
<td></td>
<td></td>
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<tr>
<td>Observed microbial change at infant age</td>
<td></td>
<td>• ↑ Enterobacteriaceae abundance at 3 months in emergency CS vs vaginal no IAP</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td></td>
<td>• Vaginal no IAP (n=96), elective CS (n=17), emergency CS (n=23)</td>
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<tr>
<td>Infant population</td>
<td></td>
<td>• Cohort, full term, Canadian, 52% exclusively breastfed until 3 months; same results if exclusive or no exclusive breastfeeding; postnatal antibiotics in 11%; IV cefazolin IAP for CS</td>
</tr>
<tr>
<td>Microbial profiling method</td>
<td></td>
<td>• Illumina MiSeq at V4 • FDR correction</td>
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**Intrapartum antibiotic prophylaxis (IAP) for GBS**

**Bacteroidetes and genus Bacteroides**

Corvaglia et al., 2016

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<tbody>
<tr>
<td>Observed microbial change at infant age</td>
<td></td>
<td>• ↔ counts of B. fragilis counts at 7 or 30 days relative to controls</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td></td>
<td>• Vaginal no IAP (n=35), vaginal IAP with IV ampicillin (n=49)</td>
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<tr>
<td>Infant population</td>
<td></td>
<td>• Full term Italian infants born vaginally to mothers +/- GBS, exclusively breastfed</td>
</tr>
<tr>
<td>Microbial profiling method</td>
<td></td>
<td>• Ion Torrent Sequencer at V2-8</td>
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Azad et al., 2016

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<tbody>
<tr>
<td>Observed microbial change at infant age</td>
<td></td>
<td>• ↓ Bacteroidetes abundance at 3 months relative to controls; trend at genus level</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td></td>
<td>• Vaginal no IAP (n=96), vaginal IAP (n=40)</td>
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<tr>
<td>Infant population</td>
<td></td>
<td>• Cohort, full term, Canadian, 52% exclusively breastfed until 3 months; same results if exclusive or no exclusive breastfeeding; postnatal antibiotics in 11%; IV penicillin G for IAP or clindamycin if penicillin allergy</td>
</tr>
<tr>
<td>Microbial profiling method</td>
<td></td>
<td>• Illumina MiSeq at V4 • FDR correction</td>
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<table>
<thead>
<tr>
<th>Microbial group</th>
<th>Citation</th>
<th>Study details, methods and findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bifidobacteria</strong></td>
<td>Aloisio <em>et al.</em>, 2016 (a); Corvaglia <em>et al.</em>, 2016 (b)</td>
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</tr>
<tr>
<td>Observed microbial change at infant age</td>
<td>• ↓ bifidobacterial diversity at 7 days</td>
<td></td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• ↔ bifidobacterial counts at 30 days relative to controls</td>
<td></td>
</tr>
<tr>
<td>Infants</td>
<td>• Vaginal no IAP (n=10), vaginal IAP with IV ampicillin (n=10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Vaginal no IAP (n=35), vaginal IAP with IV ampicillin (n=49)</td>
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<tr>
<td>Infant population</td>
<td>• Full term Italian infants born vaginally to mothers +/- GBS, exclusively breastfed</td>
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<tr>
<td>Microbial profiling method</td>
<td>• Ion Torrent Sequencer at V2-8</td>
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<tr>
<td><strong>Arboleya <em>et al.</em>, 2015</strong></td>
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<tr>
<td>Observed microbial change at infant age</td>
<td>• ↓ <em>Bifidobacteriaceae</em> and <em>Bacteroidaceae</em> abundance between 2-90 days after birth relative to controls</td>
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</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• Vaginal no IAP (n=10), vaginal IAP with single dose of ampicillin (n=3)</td>
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<tr>
<td>Infant population</td>
<td>• Full-term infants exclusively-breastfed and hospitalised for 3 days</td>
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</tr>
<tr>
<td>Microbial profiling method</td>
<td>• Ion Torrent Sequencer</td>
<td></td>
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<td></td>
<td>• FDR correction</td>
<td></td>
</tr>
<tr>
<td><strong>Clostridium, Enterococcus, Staphylococcus, Klebsiella</strong></td>
<td>Azad <em>et al.</em>, 2016</td>
<td></td>
</tr>
<tr>
<td>Observed microbial change at infant age</td>
<td>• ↑ <em>Clostridium</em> (P&lt;0.01) and <em>Enterococcus</em> (P=0.02) abundance at 3 months relative to controls; FDR P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td>• Vaginal no IAP (n=96), vaginal IAP (n=40)</td>
<td></td>
</tr>
<tr>
<td>Infant population</td>
<td>• Cohort, full term, Canadian, 52% exclusively breastfed until 3 months; same results if exclusive or no exclusive breastfeeding; postnatal antibiotics in 11%; IV penicillin G for IAP or clindamycin if penicillin allergy</td>
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</tr>
<tr>
<td>Microbial profiling method</td>
<td>• Illumina MiSeq at V4</td>
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<td>• FDR correction</td>
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<td><strong>Stokholm <em>et al.</em>, 2016</strong></td>
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<tr>
<td>Observed microbial change at infant age</td>
<td>• ↑ % colonisation by <em>Klebsiella, Staphylococcus haemolyticus</em> at 7 days relative to controls</td>
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<tr>
<td>Study groups and number of infants</td>
<td>• Vaginal no IAP (n=477), vaginal IAP (n=72)</td>
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<tr>
<td>Infant population</td>
<td>• Cohort, full term, Danish; exclusive breastfeeding for 3.4 months on average; postnatal antibiotics in 3%</td>
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<tr>
<td>Microbial profiling method</td>
<td>• Bacterial culture on selected media; unable to culture <em>Bacteroides</em></td>
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<tr>
<td>Microbial group</td>
<td>Citation</td>
<td>Study details, methods and findings</td>
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<tr>
<td><strong>Bacteroidetes and B. fragilis</strong></td>
<td>Tun, 2016</td>
<td>• Inverse correlation between hospital length-of-stay (LOS) and <em>Bacteroidetes</em> abundance (phylum, family, genus level) at 3 months; statistically significant in elective CS only</td>
</tr>
<tr>
<td>Observed microbial change at infant age</td>
<td></td>
<td>• Vaginal no IAP (n=379), vaginal IAP (n=194), elective CS (83), emergency CS (121)</td>
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<tr>
<td>Study groups and number of infants</td>
<td></td>
<td>• Cohort, full term, Canadian, 52% exclusively breastfed until 3 months</td>
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<tr>
<td>Infant population</td>
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<td>• Illumina MiSeq at V4</td>
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<tr>
<td>Microbial profiling method</td>
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<td>• FDR correction</td>
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<tr>
<td>Penders <em>et al.</em>, 2006</td>
<td></td>
<td>• ↓ <em>B. fragilis</em> counts and % colonisation at 1 month if LOS 4-6 days vs none; no longer significant after adjustment for covariates</td>
</tr>
<tr>
<td>Observed microbial change at infant age</td>
<td></td>
<td>• No hospitalisation (737), LOS 4-6 days (73)</td>
</tr>
<tr>
<td>Study groups and number of infants</td>
<td></td>
<td>• Cohort, full term, Dutch with anthroposophic sample; 68% exclusively breastfed until 1 month; postnatal antibiotics in 3%</td>
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<tr>
<td>Infant population</td>
<td></td>
<td>• qPCR assay for bifidobacteria, <em>E. coli</em>, <em>C. difficile</em>, <em>B. fragilis</em>, lactobacilli of 16S rDNA gene sequences</td>
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<td>Microbial profiling method</td>
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<td>• FDR correction</td>
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**Hospitalisation beyond 1 day after birth**

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<th>Study details, methods and findings</th>
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<tbody>
<tr>
<td><strong>Bifidobacteria</strong></td>
<td>Penders <em>et al.</em>, 2006</td>
<td>• ↓ counts and % colonisation at 1 month if LOS &gt;3 days vs none; no longer significant after adjustment for covariates</td>
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<tr>
<td>Observed microbial change at infant age</td>
<td></td>
<td>• No hospitalisation (737), LOS &gt;3 days (97)</td>
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<tr>
<td>Study groups and number of infants</td>
<td></td>
<td>• Cohort, full term, Dutch with anthroposophic sample; 68% exclusively breastfed until 1 month; postnatal antibiotics in 3%</td>
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<tr>
<td>Infant population</td>
<td></td>
<td>• qPCR assay for bifidobacteria, <em>E. coli</em>, <em>C. difficile</em>, <em>B. fragilis</em>, lactobacilli of 16S rDNA gene sequences</td>
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<td>Microbial profiling method</td>
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<td>• FDR correction</td>
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### 4. Medical interventions impact infant gut microbes

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<tr>
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<tr>
<td><strong>C. difficile</strong></td>
<td>Penders <em>et al.</em>, 2006</td>
<td>- ↑ <em>C. difficile</em> counts and % colonisation at 1 month if LOS &gt; 1 day. Risk of colonisation at 1 month of 1.13 (95%CI: 1.01-1.25) for each additional hospitalisation day, independent of covariates</td>
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<td></td>
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<td>- No hospitalisation (737), LOS &gt; 1 day (183)</td>
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<td>- Cohort, full term, Dutch with anthroposophic sample; 68% exclusively breastfed until 1 month; postnatal antibiotics in 3%</td>
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<td>- qPCR assay for bifidobacteria, <em>E. coli</em>, <em>C. difficile</em>, <em>B. fragilis</em>, lactobacilli of 16S rDNA gene sequences</td>
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<td>- FDR correction</td>
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#### Postnatal infant antibiotics at birth or during infancy

**Bifidobacteria, lactobacilli, phylum Bacteroidetes, family Bacteroidaceae and genus Bacteroides**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Bennet <em>et al.</em>, 2002</td>
<td>- ↓ counts of bifidobacteria and <em>B. fragilis</em> 1 week after treatment</td>
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<tr>
<td></td>
<td>- Oral cefaclor or cefadroxil (n=7), no antibiotics (n=18)</td>
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<tr>
<td></td>
<td>- Post antibiotics with controls, all Swedish infants vaginally born and breastfed at 1-3 months of age</td>
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<td>- Bacterial culture on specific media</td>
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<tr>
<td>Penders <em>et al.</em>, 2006</td>
<td>- ↓ counts of bifidobacteria and <em>B. fragilis</em> at 1 month independent of covariates</td>
</tr>
<tr>
<td></td>
<td>- Oral antibiotics before 1 month (n=28), no antibiotics (n=98)</td>
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<tr>
<td></td>
<td>- Cohort, full term, Dutch with anthroposophic sample; 68% exclusively breastfed until 1 month; postnatal antibiotics in 3%</td>
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<td></td>
<td>- qPCR assay for bifidobacteria, <em>E. coli</em>, <em>C. difficile</em>, <em>B. fragilis</em>, lactobacilli of 16S rDNA gene sequences</td>
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<tr>
<td></td>
<td>- FDR correction</td>
</tr>
<tr>
<td>Fouhy <em>et al.</em>, 2012</td>
<td>- ↓ abundance of bifidobacteria, lactobacilli and Bacteroidetes/Bacteroidaceae/Bacteroides at 4 but not 8 weeks after treatment; no change in counts of bifidobacteria</td>
</tr>
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<td></td>
<td>- At birth, course of IV ampicillin/gentamicin (n=9); no antibiotics (n=9)</td>
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<td></td>
<td>- Post antibiotics with controls, full term, 72% vaginal birth; 44% breastfed</td>
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<td>- 16S rRNA and rpoB-specific primers; quantitative PCR</td>
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<td>Microbial group</td>
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<td>Enterococcus and Clostridium</td>
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<td>Tanaka et al., 2009</td>
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<td>Study groups and number of infants</td>
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<td>Infant population</td>
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<td>Microbial profiling method</td>
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<td>Fouhy et al., 2012</td>
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<td>Microbial profiling method</td>
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<td>Parm et al., 2010</td>
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<td>Study groups and number of infants</td>
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### Microbiota in health and disease: from pregnancy to childhood

#### 4. Medical interventions impact infant gut microbes

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<tr>
<td><strong>Staphylococcus</strong></td>
<td>Tanaka et al., 2009</td>
<td><strong>Observed microbial change at infant age</strong> • ↑ % colonisation by <em>Staphylococcus</em> at end of treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Study groups and number of infants</strong> • At birth, oral cephaalexin for 4 days (n=5); no antibiotics (n=18) • Post antibiotics with controls, full term infants vaginally born and partially breastfed</td>
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<td><strong>Infant population</strong> • V2 by DGGE</td>
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<tr>
<td><strong>Parv</strong> et al., 2010</td>
<td></td>
<td><strong>Observed microbial change at infant age</strong> • ↑ % colonisation <em>Staphylococcus aureus</em> 5-7 days after treatment in penicillin vs ampicillin regimen</td>
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<td><strong>Study groups and number of infants</strong> • At birth, course of IV penicillin/gentamicin (n=71); ampicillin/gentamicin (n=68)</td>
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<td><strong>Infant population</strong> • Cross-over design, preterm infants, Estonia</td>
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<td><strong>Microbial profiling method</strong> • Bacterial culture on specific media</td>
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</table>

**Proteobacteria (phylum), Enterobacteriaceae (family) and Klebsiella (genus)**

| **Fouhy** et al., 2012 |          | **Observed microbial change at infant age** • ↑ *Proteobacteria, Enterobacteriaceae* at 4 and 8 weeks post treatment |
|                       |          | **Study groups and number of infants** • At birth, course of IV ampicillin/gentamicin (n=9); no antibiotics (n=9) |
|                       |          | **Infant population** • Post antibiotics with controls, full term, 72% vaginal birth; 44% breastfed |
|                       |          | **Microbial profiling method** • 16S rRNA and rpoB-specific primers; quantitative PCR |

| **Tanaka** et al., 2009 |          | **Observed microbial change at infant age** • ↑ % colonisation by *Enterobacteria* 3 weeks after treatment |
|                       |          | **Study groups and number of infants** • At birth, oral cephaalexin for 4 days (n=5); no antibiotics (n=18) |
|                       |          | **Infant population** • Post antibiotics with controls, full term infants vaginally born and partially breastfed |
|                       |          | **Microbial profiling method** • V2 by DGGE |

<p>| <strong>Bennet</strong> et al., 2002 |          | <strong>Observed microbial change at infant age</strong> • ↑ counts of <em>Klebsiella</em> 3-5 days after treatment |
|                       |          | <strong>Study groups and number of infants</strong> • Oral amoxil (n=9), no antibiotics (n=18) |
|                       |          | <strong>Infant population</strong> • Post antibiotics with controls, all Swedish infants vaginally born and breastfed at 1-3 months of age |
|                       |          | <strong>Microbial profiling method</strong> • Bacterial culture on specific media |</p>
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<tr>
<td>Parm et al., 2010</td>
<td><em>↑ % colonisation</em> <em>Klebsiella</em> 5-7 days after treatment in ampicillin vs penicillin regimen</td>
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<tr>
<td>Observed microbial change at infant age</td>
<td>• At birth, course of IV ampicillin/gentamicin (n=68); penicillin/gentamicin (n=71)</td>
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¹ DGGE = denaturing gradient gel electrophoresis; FDR = false discovery rate; IAP = intrapartum antibiotic prophylaxis; LOS = length of stay.
Chapter 5
Early diet and the infant gut microbiome: how breastfeeding and solid foods shape the microbiome

J. Penders
Department of Medical Microbiology, School of Translational Research in Metabolism (NUTRIM) and School for Public Health and Primary Care (Caphri), Maastricht University Medical Center+, P.O. Box 5800, 6202 AZ Maastricht, the Netherlands; j.penders@maastrichtuniversity.nl

Abstract

Upon delivery, diet is one of the major factors impacting the maturation and diversification of the microbiome in early life. Both the type of milk given to the suckling infant and the subsequent introduction of solid foods appear to be involved in shaping the microbiome. In contrast to infant formulas, breast milk contains a wide variety of bioactive factors that support the development and maturation of the infant gut. Amongst others a complex mixture of human milk oligosaccharides, lactoferrin, lysozyme, immunoglobulins and lipids all contribute to an intestinal microbiome in breastfed infants that is clearly distinct from that of formula-fed infants. Moreover, breast milk contains its own microbiome and may as such directly seed the infant gut with bacteria. The intestinal microbiome of breastfed children is less diverse and characterised by a high abundance of bifidobacteria and lactobacilli. The indigenous microbiome of formula-fed children is, on the other hand, characterised by a higher diversity, with increased numbers of bacteroides, clostridia and Enterobacteriaceae including opportunistic pathogens such as Clostridium difficile and Escherichia coli. The introduction of solid foods exposes the infant gut microbiota to a whole new range of nutrients. Consequently, the microorganisms capable of degrading complex sugars and starch flourish, intra-individual variations in the microbiota become less pronounced and the composition starts to resemble the adult microbiome. It is however still debated whether the major driving force in the development of an adult-like microbiome is the actual timing of the introduction of these new food substrates, the cessation of breastfeeding or a combination of both.

Keywords: human milk oligosaccharides, breast milk microbiome, formula, lysozyme, lactoferrin, IgA, fibres

5.1 Introduction

The intestinal microbiota is extremely variable in the first years of life, and diet appears to be one of the major factors impacting shifts in the infant microbiome. Colonisation of the intestinal tract may already start before delivery by microbial transfer through the placental barrier as indicated by the presence of a wide variety of microbial taxa in the human placenta, amniotic fluid, umbilical cord blood and meconium (Aagaard et al., 2014; DiGiulio, 2012; Funkhouser...
and Bordenstein, 2013; Jimenez et al., 2008b). Subsequently, an inoculum of maternal and environmental bacteria, of which the composition is strongly affected by both mode and place of delivery (Dominguez-Bello et al., 2010; Penders et al., 2006b, 2013), rapidly seeds the new-born’s intestines. Generally, facultative anaerobes such as Escherichia, Streptococcus, Staphylococcus and Enterococcus spp., are predominant at first due to the oxygen-rich environment (Avershina et al., 2014; Backhed et al., 2015). These initial colonisers are succeeded and soon outnumbered by anaerobic bacteria, mainly belonging to the phyla Actinobacteria and Firmicutes. This transition can be attributed to the introduction of breast milk or infant formula, highlighting the first diet-driven colonisation event (Voreades et al., 2014).

5.2 The intestinal microbiome in breastfed and formula-fed infants

The World Health Organisation (WHO) and the United Nations International Children’s Emergency Fund (UNICEF) recommend exclusive breastfeeding for the first six months of life. Thereafter, to meet their evolving nutritional requirements, infants should receive age appropriate, nutritionally adequate and safe complementary foods while breastfeeding continues for up to two years and beyond (WHO, 2014).

Breastfeeding is an unequalled way of providing the optimal food for healthy growth and development in infants. Next to nutrients, breast milk contains a wide variety of bioactive factors that support the development and maturation of the infant gut, systemic metabolism and the innate and adaptive immune system (O’Sullivan et al., 2015). Moreover, it plays a crucial role in the establishment of the infant’s indigenous microbiome. The reduced incidence of infections as well as several non-communicable diseases among breastfed infants could at least in part be attributed to the beneficial effects of breast milk on the infant microbiome.

Exclusively breast-fed infants harbour an indigenous microbiota that is characterised by a low microbial diversity as a result of the predominance of bifidobacteria, which can account for up to 70-80% of the total bacterial numbers in the infants’ faeces (Goldsmith et al., 2015).

It is however a misconception that bifidobacteria are the hallmark of breastfed, but not bottle-fed, infants (Adlerberth and Wold, 2009). Although in some studies a lower abundance of bifidobacteria in exclusively formula-fed infants has been reported (Bezirtzoglou et al., 2011; Yoshioka et al., 1983), bifidobacterial counts in infants receiving formula feeding tend to be as high as in breastfed infants in the vast majority of studies (Adlerberth and Wold, 2009; Benno et al., 1984; Fallani et al., 2010; Harmsen et al., 2000; Mitsuoka and Kaneuchi, 1977; Penders et al., 2005, 2006b). Despite the similar total bifidobacterial counts, the *Bifidobacterium* composition at the species level has been shown to differ according to the type of infant feeding. *Bifidobacterium longum* subsp. *infantis* (B. *infantis*), *B. longum* subsp. *longum* (B. *longum*) and *Bifidobacterium breve* are the most predominant species in breastfed infants. On the other hand, infants fed traditional formulas contain relatively more *Bifidobacterium catenulatum* and *Bifidobacterium adolescentis*, species that are also commonly found in adults. However, supplementation of
formulas with the prebiotic fibres galacto-oligosaccharides (GOS) and fructo-oligosaccarides (FOS), appears to induce a faecal microbiota with bifidobacterial species that closely resemble the microbiota of breastfed infants (Haarman and Knol, 2005).

Infant formulas have been modified and fortified throughout the past decades, and consequently the exact composition may differ considerably between studies. Nevertheless, the microbiome of formula-fed infants has consistently been characterised by a higher diversity, with increased numbers of members of *Bacteroides*, *Clostridium* and *Enterobacteriaceae* including opportunistic pathogens such as *Clostridium difficile* and *Escherichia coli* (Azad et al., 2013; Backhed et al., 2015; Bezirtzoglou et al., 2011; Fallani et al., 2010; Penders et al., 2006a). Moreover, as demonstrated in a study in which the faecal microbiome throughout the first year of life was comprehensively assessed by metagenomic sequencing, formula-fed infants display enhanced populations of *Ganulicatella adiacens*, *Citrobacter* spp., *Enterobacter cloacae* and *Bilophila wadsworthia* whereas breast-fed infants have increased numbers of several *Lactobacillus* species that are being used as probiotics (Backhed et al., 2015). Even up to the age of 12 months, bifidobacteria and lactobacilli still dominated the microbiota in infants that continued to receive breast milk.

Altogether formula feeding has been shown to direct the gut microbiome more rapidly into an adult-like composition (Backhed et al., 2015; Praveen et al., 2015), to increase the number of opportunistic pathogens and pro-inflammatory bacterial taxa (Azad et al., 2013; Backhed et al., 2015; Penders et al., 2006b, 2013) and to decrease the number of specific short chain fatty acids (O’Sullivan et al., 2015; Pourcyrous et al., 2014; Siigur et al., 1993). Along with the complexity of breast milk, the specific components in human milk that favour the support of a specific microbiota are also numerous.

**Human milk oligosaccharides**

After lactose and lipids, human milk oligosaccharides (HMOs) are the third most abundant component of human milk with concentrations ranging from ~20 g/l in colostrum to 5-10 g/l in mature milk (Kunz et al., 2000).

There are over 200 different types of oligosaccharides known to be present in human milk. In most cases HMOs consist of lactose at the reducing end, a backbone of alternating N-acetyl glucosamine and galactose, sporadic branching by N-acetyl glucosamineβ1,6-galactose linkages, and often terminal additions of sialic acid, fucose or both (Newburg and Grave, 2014).

Moreover, HMOs can be bound to other components in milk, such as lipids and proteins, referred to as human milk glycans. In contrast, cow’s milk, the basis of most infant formulas, contains only trace amounts of oligosaccharides (Martin-Sosa et al., 2003). Although, many infant formulas have now been fortified to compensate for this deficit, they only include one or few more simple oligosaccharides such as GOS, FOS or inulin (O’Sullivan et al., 2015).
HMOs and their related glycoconjugates are indigestible by infants and function as prebiotics, encouraging the growth of beneficial bacteria, such as bifidobacteria, that are able to utilise these components as nutrients (Andreas et al., 2015). B. infantis in particular is equipped to consume HMOs (Asakuma et al., 2011; LoCascio et al., 2007). In the presence of HMOs, B. infantis upregulates the expression of a variety of glycoside hydrolases and intestinal membrane transporters that are essential for the breakdown of HMOs (Garrido et al., 2011; Kim et al., 2013; Yoshida et al., 2012), suggesting complete hydrolysis of HMOs to monosaccharides in the cytosol of this bacterium (Garrido et al., 2011). B. infantis furthermore induces increased expression of anti-inflammatory cytokines by intestinal epithelial cells when grown in the presence of HMOs as compared to lactose (Chichlowski et al., 2012).

Besides bifidobacteria, members of the genus Bacteroides are also capable of consuming some types of HMOs (Yu et al., 2013). Both Bifidobacterium and Bacteroides species are involved in the production of short-chain fatty acids and lactate, thereby lowering the intestinal pH, further modulating the microbiota, and exerting systemic effects (Lewis et al., 2015).

Despite the distinct microbiome composition of breastfed as compared to formula-fed infants, there are still considerable inter-individual variations in the indigenous microbiome of breastfed children. One of the reasons might be the variations in the HMO profiles of their mothers as the quantity and composition of HMOs not only varies over the course of the lactation period, but also between lactating women (Bode, 2012; Cabrera-Rubio et al., 2012). Indeed, preliminary data show that maternal HMO profiles could predict several bacterial genera detected in infant faeces (Wang et al., 2015).

Maternal genotype, in particular the secretor status, is one of the major drivers of variations in HMO profiles. The structure and concentration of fucosylated HMOs is determined by the secretor (FUT2) status and Lewis (FUT3) blood type genes encoding fucosyltransferase enzymes. Non-secretors lack a functional FUT2 enzyme and, in contrast to secretors, the breast milk of non-secretors does not contain α1-2-fucosylated HMOs (Bode, 2012). A functional FUT3 gene encodes the enzyme α1,3/4-fucosyltransferase, which adds even more complexity to the HMO profiles (Goldsmith et al., 2015). Infants fed by non-secretor mothers have a delayed establishment of a bifidobacteria-dominated microbiota (Lewis et al., 2015). This delay may be due to difficulties in the infant acquiring the bifidobacterial species able to consume the specific milk oligosaccharides delivered by the mother.

Next to the growth enhancing effect of beneficial bacteria, HMOs have also a protective function by preventing gastrointestinal infections. Due to the analogue shape to carbohydrates on the cell surface of intestinal epithelial cells, HMOs can act as receptor decoys, sequestering pathogenic bacteria and preventing their cell adhesion to epithelial cells and subsequent infection (Andreas et al., 2015). The genetic variation in HMO profiles also impacts the protective effect of breast milk. Higher levels of 2-linked fucosylglycans in maternal milk are associated with lower risk of moderate-to-severe diarrhoea in breast-fed infants (Morrow et al., 2005). The milk of non-
secretor mothers lacks 2'-fucosyllactose and related fucosylglycans, and is therefore less able to protect against important enteropathogens that bind to α1-2-fucose, such as Campylobacter, enterotoxigenic E. coli and major strains of calciviruses (Newburg and Morelli, 2015).

**Proteins: lysozyme, lactoferrin and immunoglobulins**

Breast milk contains hundreds of different proteins with a variety of functions, including providing nutrients, antimicrobial properties and/or immune-modulating activities. Glycosylated breast milk proteins, such as lactoferrin, lysozyme and immunoglobulins have important protective properties against infectious microorganisms and avoid their attachment to the intestinal mucosa, assisting the development of the commensal bacteria. Both lactoferrin and lysozyme are important antimicrobial proteins that are present at high levels in human milk but not in the milk of dairy animals (Maga et al., 2013).

Lactoferrin is an iron binding protein. By sequestering iron away from (opportunistic) pathogenic bacteria, lactoferrin preserves this important nutrient for the enterocytes. Lysozyme hydrolyses the peptidoglycan layer of (gram-positive) bacteria, eventually resulting in lysis of bacterial cells. Human studies dissecting the role of these individual milk components on the infant microbiome are currently lacking. However, in a pig model it was shown that variations in milk lysozyme levels alone are sufficient to alter the microbiome. Piglets fed milk from transgenic goats, expressing human lysozyme at similar concentrations as found in human milk, had levels of **Firmicutes** that declined and levels of **Bacteroidetes** that increased over time and that were significantly different from levels in control-fed animals (Maga et al., 2012, 2013). On a lower taxonomic level, the abundance of beneficial bacteria including **Bifidobacteriaceae** and **Lactobacillaceae** increased and the abundance of bacterial families associated with disease (Mycobacteriaceae, Streptococcaceae, Campylobacteriales) decreased with consumption of lysozyme milk (Maga et al., 2012).

The production of endogenous IgA in humans starts several months after birth and subsequently increases during the first years of life. As such, maternally secretory IgA (sIgA) provided by breastfeeding is the sole source of sIgA in new-borns (Pabst et al., 2016). IgA deficiency is the most common primary immune deficiency in humans, but so far neither the effect of IgA deficient lactating mothers on the infant microbiome nor the direct impact of IgA deficiency on ones microbiome has been systematically investigated. However, in weanling mice receiving maternal sIgA in breast milk, the gut microbiota was significantly different as compared to mice not receiving sIgA. Moreover, these differences were magnified when the mice reached adulthood, suggesting that passive sIgA shape the composition of the microbiome (Rogier et al., 2014).
**Lipids**

Lipids, the largest energy source of breast milk, mainly consist of triacylglycerides (~98% of the lipid fraction). The remaining lipid fraction predominantly consists of diacyl- and monacylglycerides, free fatty acids, phospholipids and cholesterol (Andreas et al., 2015).

Breast milk has also been shown to contain a lipid-dependent antimicrobial activity. Lipases in the infant gut convert milk triglycerides into fatty acids and monoglycerides. In particular medium-chain saturated and long-chain unsaturated fatty acids and their respective monoglycerides appear to exert antimicrobial properties (Isaacs, 2001).

Lipids added to milk and formula have been shown to inactivate a number of pathogens including respiratory syncytial virus (RSV), herpes simplex virus type 1 (HSV-1), *Haemophilus influenzae*, and Group B *Streptococcus* (Isaacs et al., 1995).

A major difference in lipid hydrolysis products formed in the infant gut after feeding breast milk compared with infant formula are the relatively high concentrations of medium-chain fatty acids. *In vitro* fermentation of faecal microbial communities from 2-5 month-old infants in the presence of varying concentrations medium-chain fatty acids, demonstrated that increasing levels of these lipid hydrolysis products caused a significant increase in the relative abundance of *Lactobacillus* and *Bifidobacterium* spp., including *B. breve*, *B. adolescentis* and *Bifidobacterium pseudocatenulatum*. This suggests that the high concentration of medium-chained fatty acids in breast milk might have functional effects on the establishment of the gut microbiota in early life (Cabrera-Rubio et al., 2012).

**The breast milk microbiome**

Breast milk has also a direct impact on the infant microbiome by providing an inoculum of viable commensal bacteria at concentrations of $10^2$-$10^4$ per ml breast milk. As such, an exclusively breastfed infant will consume the significant amount of about $10^5$-$10^7$ commensal bacteria while suckling (Heikkila and Saris, 2003).

The presence of viable bacteria in breast milk, as detected by traditional plate counting techniques, was already noted in studies conducted in the 1950s and 1970s (Davidson et al., 1979; Dorr and Sittel, 1953; Newburg and Morelli, 2015). These and subsequent culture-dependent studies indicated that facultative anaerobic skin bacteria, such as members of the genera *Staphylococcus* and *Streptococcus* often predominated in breast milk (Heikkila and Saris, 2003; Jost et al., 2013, 2015). At lower concentrations lactic acid bacteria, such as *Lactobacillus* and *Enterococcus*, members of the gram-negative family *Enterobacteriaceae*, as well as obligate anaerobes including *Bifidobacterium* spp. have been cultured from breast milk (Heikkila and Saris, 2003; Jimenez et al., 2008a; Jost et al., 2013; Solis et al., 2010).
5. The impact of infant feeding and weaning on the gut microbiome

With the introduction of culture-independent molecular techniques, a microbial diversity in human milk beyond expectancy has been unravelled, including major gut-associated obligate anaerobes such as *Bacteroides* and several members of *Clostridia*, including butyrate-producers (e.g. *Faecalibacterium* and *Roseburia*), which are important for colonic health (Jost et al., 2013). A study on variations in the breast milk microbiome over the course of lactation indicated that staphylococci, streptococci, and lactobacilli derived from the milk ducts and skin predominated in colostrum, whereas after six months a higher abundance of oral taxa, including *Veillonella* and *Prevotella*, were found in breast milk (Cabrera-Rubio et al., 2012). The latter taxa may originate from the infant oral cavity and end up in breast milk through retrograde flow back into the mammary ducts during suckling (Goldsmith et al., 2015).

Other studies have supported the hypothesis of an entero-mammary pathway, postulating that maternal gut bacteria could translocate through the epithelial barrier, migrate to the mammary glands and subsequently colonise the breastfed neonate (Fernandez et al., 2013). Most convincing support for this entero-mammary pathway comes from a study in which a combination of culture-dependent and culture-independent tools were used to examine whether viable strains of gut-associated obligate anaerobes were shared between the maternal and neonatal gut microbiome via breastfeeding. Next to facultative anaerobes, obligate anaerobic genera, including *Bifidobacterium*, *Bacteroides* and *Clostridia* were detected in maternal faeces, breast milk and neonatal faeces. Moreover, using pulse-field gel electrophoreses of *B. breve* isolates from maternal faeces, breast milk and neonatal faeces of a single mother-child pair revealed identical restriction profiles, suggesting transfer of this strain from the maternal to the neonatal gut via breast milk (Jost et al., 2014).

Breast milk may thus be directly contributing in seeding the infant gut microbiome with pioneer bacteria that can subsequently further shape the microbiome, although a conclusive understanding of the origin and importance of the breast milk microbes remains to be determined.

5.3 Introduction of solid foods and weaning

Weaning is considered to be the next critical period in microbiota development as the infant switches from a completely milk-based diet towards a variety of food components thereby exposing the colonic microbiota to a wide variety of plant and animal-derived glycans. The start of weaning has often been linked to an increased diversity of the intestinal microbiota, in which bifidobacterial species are complemented and gradually replaced by more complex ecosystems that contain specialists that are capable of degrading and fermenting these novel carbohydrates. In this respect, the bacteria capable of degrading plant-borne fibres, such as *Ruminococcus bromii*, play a unique ecological role as these keystone species release a plethora of simple carbohydrates that consequently support the growth of numerous other fermentative microorganisms (Dore and Blottiere, 2015; Xe et al., 2013).
Indeed various studies comparing the microbiota of geographic distinct populations have indicated that a diet rich in plant fibres has a significant impact on the indigenous microbiome (Claesson et al., 2011; De Filippo et al., 2010; Yatsunenko et al., 2012).

The microbiota in non-Western populations, known for their high consumption of plant-derived carbohydrates, has consistently been shown to be more diverse, enriched in *Prevotella* spp. and depleted in *Bacteroides* spp. as compared to Western populations consuming a diet high in sugar, starch and animal protein and fat (Salonen and de Vos, 2014).

Interesting, a study comparing the microbiome of children living in Europe and rural Africa revealed pronounced differences, including the above mentioned *Prevotella-Bacteroides* trade-off, between the two populations (De Filippo et al., 2010). However, these differences between groups were not yet apparent in children still being breastfed, highlighting that both cessation of breastfeeding and the subsequent dietary pattern upon weaning drive the geographical differences in the indigenous microbiome.

In a study that intensively monitored the development of the microbiota of 14 infants throughout the first year of life, it was shown that the microbial community profiles of babies became more similar to each other and to adult stool samples as they became older. Although no common developmental patterns could be identified, dramatic shifts rather than gradual changes in the microbial population structures, often following the introduction of solid foods, were likely responsible for this conversion toward a generalised ‘adult-microbiota’ (Palmer et al., 2007).

A study on the impact of weaning on the faecal microbiota composition of infants from five European countries (Sweden, Scotland, Germany, Italy and Spain) showed that geographical differences in the infant microbiota as well as the effects of birth mode and the initial type of infant feeding were still apparent post-weaning. Moreover, the introduction of solid foods was associated with a significant decrease in the proportions of bifidobacteria, enterobacteria, *C. difficile* and *Clostridium perfringens* species, while proportions of bacteria belonging to the *Clostridium cocoides* and *Clostridium leptum* groups significantly increased (Fallani et al., 2011).

Intensive sampling of the microbiota of 7 exclusively breastfed and 7 exclusively formula-fed infants from 1 month post-partum up to the age of 18 months, showed that the gradual process of weaning of infants onto solid foods is accompanied with a change in the complexity of the faecal microbiota and that weaning and the cessation of breastfeeding both affected the convergence of the infant microbiota (Roger and McCartney, 2010).

The majority of these studies included only a low number of children and/or collected a limited number of samples per child (Roger and McCartney, 2010). Consequently, the effect of the introduction of solids and the cessation of breastfeeding on bacterial succession could often not be completely disentangled in these studies. Indeed cessation of breastfeeding might be as, or even more, important as the introduction of solids. This is supported by a double-blind...
controlled trial comparing the effect of fish oil to sunflower oil supplementation to examine the impact of n-3 polyunsaturated fatty acids on the faecal microbiota in 9 to 18 month old infants (Andersen et al., 2011). Fish oil supplementation affected changes in large bacterial groups, but only among children who were no longer breastfed at the start of the intervention.

Metagenomic analysis of the microbiome of 98 Swedish infants revealed distinct energy source utilisation in the infant gut throughout the first year of life. The microorganisms of new-borns and 4-month-old infants were enriched in genes required for degradation of sugars from the breast milk, whereas the 12-month microbiome was enriched in genes involved in degradation of complex sugars and starch associated with the older infant diet (Backhed et al., 2015). However, the compositional changes as well as the increased capacity to degrade complex carbohydrates as a result of the introduction of solids did not become apparent until the infants stopped breast-feeding. This led the authors to conclude that cessation of breastfeeding rather than introduction of solid foods is the major driver in the development of an adult microbiota (Backhed et al., 2015).

5.4 Practical recommendations and future perspectives

It is evident that, also with respect to the establishing microbiome, breast milk is the best nutritional choice for infants. A wide variety of bioactive molecules and HMO’s contribute to a microbiota in which bifidobacteria dominate and opportunistic pathogens are suppressed. However, most of what is currently known about the role of infant feeding on the establishing microbiome comes from studies comparing exclusively formula-fed and exclusively breastfed infants. In reality many children receive mixed feeding after the first few months of life and little is known how mixed feeding influences the gut microbiome (O’Sullivan et al., 2015). In addition, it is still unclear whether it is the cessation of breastfeeding, the introduction of solid food items or a combination of both that is being determinative for maturation into an adult-like microbiota.

To disentangle these effects, studies in which the development of the infant microbiome is being monitored more closely during periods of solid food introduction and cessation of breastfeeding are urgently needed.

Moreover, human studies examining the impact of individual bioactive breast milk components on the infant microbiome are scarce. Techniques such as 1H NMR and mass spectrometry are capable of measuring metabolites in biofluids in extremely low concentrations (Andreas et al., 2015). These metabonomic approaches may assist in unravelling how the variation in breast milk composition between individuals and over time is linked to its effect on the infant microbiome.

Conflict of interest

The author declares no conflict of interest.
References


5. The impact of infant feeding and weaning on the gut microbiome


5. The impact of infant feeding and weaning on the gut microbiome


Part III.
Human microbiota and physiological systems
Chapter 6
The intestinal microbiota and the child’s immune system

M.C. Jenmalm¹,²* and S.L. Prescott²,³
¹Division of Neuro and Inflammation Sciences, Department of Clinical & Experimental Medicine / AIR pl 10, Faculty of Medicine and Health Sciences, Linköping University, 581 85 Linköping, Sweden; ²International Inflammation (in-FLAME) network of the World Universities Network; ³School of Paediatrics and Child Health, University of Western Australia and Princess Margaret Hospital for Children, G.P.O. Box D184, Perth, Western Australia 6840, Australia; maria.jenmalm@liu.se

Abstract

The microbiota provides crucial signals for the development and function of the immune system. The intestinal microbiota in mammals represents the ecological site on Earth with the highest density of bacteria, and this complex ecosystem seems to provide a primary signal for establishment of an adequate mucosal barrier function and the maturation of a balanced postnatal innate and adaptive immune system. Our modern lifestyle and the declining biodiversity diminish the exposure to microbes with which we have co-evolved, and this is considered a major factor driving abnormal postnatal immune development. Early microbial exposures occurring during critical periods of immune maturation seem to have long-term impact on development of immune mediated diseases, and the maternal microbial environment during pregnancy may also crucially influence immune programming. In this chapter we explore the interaction of the intestinal microbiota and the host immune system in early life, with focus on studies in humans. We propose that future detailed studies on the complex interactions between the developing infant microbiome and immune system are important for establishing strategies to promote childhood health and prevent development of immune mediated diseases.

Keywords: intestinal microbiota, immune maturation, mucosal immunity, adaptive immunity, innate immunity, mucosal barrier function, pregnancy, childhood

6.1 Introduction

The microbiome profoundly influences the physiology of the host and modern global ecological changes may have perturbed also our internal microbial ecosystems (Gillings et al., 2015; Segata, 2015; Von Hertzen et al., 2015). Reduced intensity and diversity of microbial exposure is considered a major factor driving abnormal postnatal immune development and the increasing prevalence of immune mediated diseases in affluent societies (Gollwitzer and Marsland, 2015; Wesemann and Nagler, 2016; West et al., 2015b). The intestinal microbiota is quantitatively the most important source of microbial stimulation and may provide a primary signal for the maturation of a balanced postnatal innate and adaptive immune system (Gensollen et al., 2016; Gollwitzer and Marsland, 2015; Houghteling and Walker, 2015; Wesemann and Nagler, 2016;
In this chapter we explore the interaction of the intestinal microbiota and the host immune system in children, with focus on studies in humans.

### 6.2 Co-evolutionary interdependency of the microbiome and the immune system

No animal has evolved independently of microbial symbionts (Gilbert et al., 2015; McFall-Ngai et al., 2013; Rosenberg and Zilber-Rosenberg, 2016). Microbes were the only life form on Earth for most of its history, and multicellular life arose in a world dominated by prokaryotes (Gilbert et al., 2015; McFall-Ngai et al., 2013; Rosenberg and Zilber-Rosenberg, 2016). Intimate, complex and dynamic interactions between animal hosts and microbes have profoundly influenced animal evolution, and still do (Gilbert et al., 2015; McFall-Ngai et al., 2013; Rosenberg and Zilber-Rosenberg, 2016). Nearly half a billion years of co-evolution with vertebrates has reciprocally shaped the repertoires of the microbial symbionts and the immune system (Gilbert et al., 2015; Maynard et al., 2012). Our modern and affluent lifestyle and the declining biodiversity diminish the exposure to microbes with which we have co-evolved, however, leading to establishment of microbial communities differing substantially from those of our ancestors (Blaser and Falkow, 2009; Gillings et al., 2015; Segata, 2015; Ursell et al., 2013).

The immune system shapes homeostasis between the host and the microbial symbionts, allowing a mutualistic partnership, where the microbiota provides digestive and protective advantages to the host in a sheltered environment with abundant nutrition, while pathogenic and invasive behaviour of microbes evokes eliminatory measures (Gilbert et al., 2015; Maynard et al., 2012). It is likely that our immune system has evolved as much to preserve and protect beneficial microbes as to fend off pathogens (Gilbert et al., 2015; Maynard et al., 2012; McFall-Ngai, 2007; Travis, 2009). In vertebrates, the evolution of an adaptive immune system, with the capacity to recognise and remember beneficial as well as pathogenic microbes and with the ability to both suppress and promote innate inflammatory mechanisms, provided opportunities for more sophisticated relationships with increasingly complex microbial communities (Gilbert et al., 2015; Kawamoto et al., 2014; Maynard et al., 2012; McFall-Ngai, 2007; Sutherland et al., 2016; Travis, 2009). The mammalian intestine is the ecological site on Earth with the highest density of bacteria (Ley et al., 2006). Encounters between microbes from this complex ecosystem and immune cells may have profound effects on immune maturation when occurring during critical time windows of developmental programming (Gensollen et al., 2016; Gollwitzer and Marsland, 2015; Wesemann and Nagler, 2016; West et al., 2015b, 2016).

### 6.3 The first microbial encounters

**Do the first microbial encounters occur in utero?**

Contrary to previous assumptions of a ‘sterile womb’ paradigm, in which the first acquisition of bacteria occurs at birth, new data suggest that the first interactions between the microbiota
and the host may be initiated in utero (Aagaard et al., 2014; Collado et al., 2016; Funkhouser and Bordenstein, 2013). Intrauterine infections may cause preterm deliveries (Stout et al., 2013) and any microbial presence in utero was assumed a danger for the foetus. However, intracellular bacteria have been histologically demonstrated in the basal plate (the peripheral region of the placenta on the maternal side in contact with the uterine wall) at a similar rate in preterm and term pregnancies without overt infection (Stout et al., 2013). Furthermore, bacterial DNA has been detected in in placenta (Collado et al., 2016; Rautava et al., 2012; Satokari et al., 2009), amniotic fluid (Collado et al., 2016; Rautava et al., 2012), umbilical cord (Jimenez et al., 2005) and meconium (Collado et al., 2016; Gosalbes et al., 2013; Jimenez et al., 2008) after ‘sterile’ term elective caesarean section (CS) deliveries. Moreover, extensive deep sequencing demonstrated a low abundance but metabolically rich placental microbiome, primarily composed of non-pathogenic commensal microbiota from the *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* phyla, in normal healthy pregnancies at term (Aagaard et al., 2014). Importantly, data obtained by 16S rRNA gene sequencing only demonstrates the presence of microbial DNA, without direct evidence of viable bacteria. Nonetheless, the presence of microbial DNA in the intrauterine compartment suggests that the foetus may be in direct contact with microbial components during gestation (Abrahamsson et al., 2015). Direct presentation of maternal bacterial components to the foetus has been recognised as a potential route for immune imprinting (Abrahamsson et al., 2015; Gollwitzer and Marsland, 2015; West et al., 2016), which may in some way prepare for the much larger inoculum transferred during vaginal delivery (Adlerberth et al., 2007; Azad et al., 2016; Bäckhed et al., 2015; Bokulich et al., 2016; Dogra et al., 2015; Funkhouser and Bordenstein, 2013; Jakobsson et al., 2014; Penders et al., 2013; van Nimwegen et al., 2011; Yassour et al., 2016) and breastfeeding (Abrahamsson et al., 2009; Azad et al., 2016; Funkhouser and Bordenstein, 2013; Mastromarino et al., 2015). In line with this suggestion, exposure of germ free mice to bacteria only during pregnancy increased the numbers of group 3 innate lymphoid cells and monocytes in the intestines of the offspring (Gomez de Aguero et al., 2016). The transient colonisation (from day 4 to 15 of the 21-day long gestation period) was made possible by using a genetically engineered *Escherichia coli* strain that can persist in the intestine only for three days unless continuously supplemented. Interestingly, presence of live bacteria in the foetus was not required for the immunomodulatory effects in this model, and maternal antibodies seemed to facilitate transfer of microbial molecules to the offspring during gestation (Gomez de Aguero et al., 2016).

**Do prenatal microbial exposures affect immune programming?**

It would be highly interesting to elucidate whether prenatal exposures to maternal microbial component exert immunomodulatory effects also in humans. This would be in line with allergy intervention studies showing that a combined prenatal and postnatal supplementation seems to be important for the preventive effect of probiotics on infant eczema (Abrahamsson et al., 2015; Jenmalm and Duchén, 2013; West et al., 2016). Furthermore, maternal exposure to a traditional farm environment during pregnancy confers stronger protection against allergic sensitisation and disease than postnatal exposure alone (Von Mutius, 2016). Speculatively, more pronounced
effects of prenatal microbial exposures on adaptive immune responses in humans as compared with mice may be envisioned, as rodents exhibit a slower rate of immunological maturation than humans prior to birth (Kuper et al., 2016; Mold and McCune, 2012). Thus, while mature T cells can be found in the peripheral tissues of the human foetus as early as 10-12 gestational weeks (Haynes et al., 1988) and circulate in significant numbers by the end of the second trimester (Papiernik, 1970), they do not fully populate the periphery in mice until after birth (Friedberg and Weissman, 1974; Mold and McCune, 2012). Developing human lymph nodes contain B and T lymphocytes by mid-gestation, with follicles present by the late second trimester and germinal centres noted before birth (Mold and McCune, 2012; Spencer et al., 1986). In contrast, mouse lymph nodes are poorly organised at birth, with follicle development not occurring until the early postnatal period (Cupedo et al., 2004; Friedberg and Weissman, 1974; Mold and McCune, 2012). Furthermore, B and T lymphocytes appear in Peyer’s patches of developing intestinal tissue at gestational day 18.5 in mice (Adachi et al., 1997), much later than in humans (Mold and McCune, 2012), where intestinal B cells are present from approximately 14 weeks of gestation (Jones et al., 2001; Spencer et al., 1986) and intestinal T cells can be identified from 12-14 weeks of gestation (Jones et al., 2001; Spencer et al., 1986). Functionally active regulatory T cells have also been identified in human mesenteric lymph nodes at this age (Cupedo et al., 2005; Michaëlsson et al., 2006). Interestingly, at birth, up to 5-10% of neonatal T cells are differentiated into either memory or effector cells, suggesting activation in utero (Zhang et al., 2014), possibly at least partially due to foetal immune priming with maternal microbial products (Abrahamsson et al., 2015; Gollwitzer and Marsland, 2015; West et al., 2016). Furthermore, somatic hypermutations have been observed in the B cell receptor repertoire at gestational week 22 (Rechavi et al., 2015).

Potential sources of the placental microbiome

When the placental microbiome composition from the extensive deep sequencing study was compared with the oral, skin, nasal, vaginal, and gut microbiomes from non-pregnant controls, it was found to be most closely related to the oral microbiome (Aagaard et al., 2014). While microbiota sampling and characterisation at multiple sites from the same pregnant women would give important information to address this further, this has led to speculation that the placental microbiome is partially established by haematogenous spread of oral microbiota (Aagaard et al., 2014; Abrahamsson et al., 2015). Another hypothesis is that maternal bacteria may reach the placenta via the bloodstream after dendritic cell facilitated translocation over the gut epithelium (Abrahamsson et al., 2015; Funkhouser and Bordenstein, 2013). An experimental mouse study using labelled Enterococcus faecium demonstrated transfer of maternal bacteria to foetuses in utero via the gastrointestinal tract (Jimenez et al., 2005), and enhanced translocation of gut bacteria to mesenteric lymph nodes has been demonstrated during pregnancy and lactation (Abrahamsson et al., 2015; Perez et al., 2007). In support of an entero-mammary-pathway, maternal intestinal microbes have been detected in immune cells circulating in peripheral blood and in breast milk in both lactating mice and humans (Perez et al., 2007).
6. Gut microbiota and childhood immunity

**Microbial transmission from mother to offspring during delivery**

Vertical transmission of maternal vaginal and gut microbes to the neonate occurs during vaginal delivery (Adlerberth et al., 2007; Azad et al., 2016; Bäckhed et al., 2015; Bokulich et al., 2016; Dogra et al., 2015; Funkhouser and Bordenstein, 2013; Jakobsson et al., 2014; Penders et al., 2013; Van Nimwegen et al., 2011; Yassour et al., 2016). CS delivery, which is performed with increasing rates worldwide and may increase the risk for development of allergy and other immune mediated diseases (West et al., 2015a), thus disrupts the opportunities for the microbiota to be transferred from a mother to her baby (Adlerberth et al., 2007; Azad et al., 2016; Bäckhed et al., 2015; Dogra et al., 2015; Funkhouser and Bordenstein, 2013; Jakobsson et al., 2014; Penders et al., 2013; Van Nimwegen et al., 2011), to provide a genetically tailored microbiota and optimal mutualism (Blaser and Falkow, 2009; Funkhouser and Bordenstein, 2013). The potential for vertical transmission of ancestral microorganisms to the next generation is decreased as well, particularly when horizontal transmission is also diminished by antibiotics, antibacterial agents, decreased family size, etc. as described in Chapter 2 by Young et al. (2017) (Blaser and Falkow, 2009; Gillings et al., 2015; Segata, 2015; Ursell et al., 2013). Vaginally delivered infants, but not infants born by CS, share a significantly higher proportion of gut microbiota 16S rRNA gene sequences with their own mother than with other mothers during the first year of life (Bäckhed et al., 2015; Bokulich et al., 2016; Jakobsson et al., 2014). The importance of maternal gut derived bacteria in early infant gut colonisation is also supported by the findings of a recent one month-follow up study, where CS delivered neonates were inoculated with maternal vaginal microbes (Dominguez-Bello et al., 2016). Thus, the gut microbiota of the infants was not influenced by the ‘vaginal seeding’ to the same extent as their skin and oral microbiota, as maternal gut derived bacteria, which are specialised to thrive in this niche, expanded in the stool samples of vaginally delivered but not inoculated CS delivered neonates (Dominguez-Bello et al., 2016). Delivery by CS has been associated with persistent changes in the gut microbiota of children followed for up to one (Adlerberth et al., 2007; Azad et al., 2016; Bäckhed et al., 2015) and two (Bokulich et al., 2016; Jakobsson et al., 2014) years, and minor differences could be detected in one study even at seven years of age (Salminen et al., 2004). The disruptions in infant gut microbial ecology caused by CS delivery include a reduced abundance of the immunomodulatory genus Bacteroides (Azad et al., 2016; Bäckhed et al., 2015; Bokulich et al., 2016; Dogra et al., 2015; Faith et al., 2014; Jakobsson et al., 2014; Penders et al., 2013; Yassour et al., 2016) and a decreased diversity of the Bacteroidetes phylum (Jakobsson et al., 2014). Increased colonisation with the opportunistic pathogen Clostridium difficile, that expands when gut microbiota niches are vacant (Costello et al., 2012), has also been reported among infants born by CS in several studies (Adlerberth et al., 2007; Penders et al., 2013; Van Nimwegen et al., 2011).
6.4 Interactions between intestinal microbes and the immune system during infancy

**Postnatal immune maturation**

The effector functions of the innate and adaptive immune system are not yet fully developed at birth (Dowling and Levy, 2014). The capacity to initiate $T_H^1$ responses is particularly limited in neonates (Abrahamsson et al., 2011; Dowling and Levy, 2014; Gollwitzer and Marsland, 2015; Krumbiegel et al., 2007; White et al., 2002; Wilson et al., 1986), and this $T_H^2$-skewed state is likely a consequence of the intrauterine immune milieu during pregnancy (Jenmalm, 2011). Initially, infants are partially protected by placental transfer of maternal IgG during the third trimester of pregnancy (Kachikis and Englund, 2016), and by secretory IgA in breast milk if breastfed (Pabst et al., 2016). The infant's own adaptive immune capabilities then gradually matures during infancy (Abrahamsson et al., 2011; Dowling and Levy, 2014; Gollwitzer and Marsland, 2015), concurrent with the establishment of a gut microbiota of increasing complexity (Lim et al., 2016; Rodriguez et al., 2015) (Figure 6.1). The intestinal microbiota is quantitatively the most important source of microbial stimulation and may provide a primary signal for the maturation of a balanced postnatal innate and adaptive immune system (Gensollen et al., 2016; Gollwitzer and Marsland, 2015; Houghteling and Walker, 2015; West et al., 2015a,b). The mucosal immune system plays a central role in this developmental process by directly interacting with microbes and innocuous environmental antigens, while providing protection from the external environment and pathogens (Gensollen et al., 2016; Gollwitzer and Marsland, 2015; Houghteling and Walker, 2015; Pabst et al., 2016; West et al., 2015a,b). Naïve and T regulatory ($T_{reg}$) cells populate the lymphoid and mucosal tissues during infancy, while differentiation of functional memory T cells occurs in certain mucosal sites such as the lung and intestines (Thome et al., 2016).

**Postnatal maturation of the mucosal immune system**

The intense microbial colonisation after birth requires efficient establishment of mucosal barriers. Tight junctions connecting adjacent intestinal epithelial cells, production of an apical mucous layer by mucin producing goblet cells, secretion of broadly targeted antimicrobial proteins by Paneth cells and transcytosis of secretory IgA antibodies contribute to intestinal epithelial barrier function (Pabst et al., 2016; Peterson and Artis, 2014; Wesemann and Nagler, 2016). The permeability of the gastrointestinal mucosa decreased during the first month of life in human neonates (Catassi et al., 1995), and the intestinal barrier function may then continue to increase during infancy (Kalach et al., 2001). While the regulation of mucin and antimicrobial peptide production during infancy needs to be characterised, mucins (Rokhsefat et al., 2016), β-defensins and the cathelicidin-derived microbial peptide LL-37 (Kai-Larsen et al., 2014) are expressed already in the foetal human gut. Establishment of the intestinal barrier in early life has mostly been studied in animal models (Fulde and Hornef, 2014). The neonatal murine intestinal epithelial architecture and barrier function are relatively immature at birth.
compared to the neonatal human intestine, however, and continues to mature over the first three weeks of life (Fulde and Hornef, 2014). Thus, rodent intestines in the second week of life are thought to represent the maturity of early third trimester human intestines (Fulde and Hornef, 2014). While human babies are born at term with a mature-like crypt-villus structure and differentiated Paneth cells, the intestinal epithelium of the murine neonate is immature and flat, with intestinal crypts, protruding villi and Paneth cells developing as late as 10 days after birth (Fulde and Hornef, 2014). These caveats are thus important to consider when translating findings from rodent studies to the human situation. Despite these limitations, microbial postnatal colonisation has been demonstrated in mice to be important both for establishing and regulating an appropriate epithelial barrier function (Peterson and Artis, 2014; Rooks and Garrett, 2016; Wesemann and Nagler, 2016). These barrier regulating effects could be both directly on the epithelium, via microbial pattern recognition receptors and via microbial metabolites, such as short chain fatty acids, and by driving the development of the mucosal immune system.
Important factors inducing IgA isotype switching include the T regulatory cell derived cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β, while microbial derived Toll-like receptor (TLR) signals stimulate T cell independent IgA production via induction of APRIL and BAFF, CD40L and TNF related cytokines, from gut epithelial cells and dendritic cells (Gutzeit et al., 2014; Peterson and Artis, 2014). Vitamin A-derived retinoic acid can also promote IgA production, and additionally increases the expression of the gut-homing markers CCR9 and α4β7 integrin on the B cells (Gutzeit et al., 2014).

**Immune development in germ-free mice**

Germ-free mice born and raised under sterile conditions show extensive defects in the development of gut-associated lymphoid tissues, with fewer and smaller Peyer's patches and mesenteric lymph nodes than animals housed under specific pathogen free conditions, as well as impaired development and maturation of isolated lymphoid follicles and very few IgA secreting plasma cells in the lamina propria (Belkaid and Hand, 2014; Gensollen et al., 2016; Rooks and Garrett, 2016; Wesemann and Nagler, 2016). Systemic immunological disturbances are also observed in germ free mice, including fewer germinal centres in the spleen (Kuhn and Stappenbeck, 2013), decreased numbers of splenic T helper cells and overproduction of Th2 cytokines (Belkaid and Hand, 2014; Gensollen et al., 2016; Kuhn and Stappenbeck, 2013; Mazmanian et al., 2005; Rooks and Garrett, 2016). How these findings from rodent studies translate to the situation in human infants is not clear.

**Postnatal microbial colonisation and secretory IgA production**

Recovery of intestinal lymphoid structures and mesenteric lymph node size and cellularity is observed after microbial colonisation of the germ free animals (Gensollen et al., 2016; Pabst et al., 2016; Peterson and Artis, 2014; Rooks and Garrett, 2016; Wesemann and Nagler, 2016). Secretory IgA production is also normalised (Gensollen et al., 2016; Pabst et al., 2016; Peterson and Artis, 2014; Rooks and Garrett, 2016; Wesemann and Nagler, 2016). While similar studies are not possible to perform in humans, postnatal microbial colonisation seem important in driving maturation of mucosal IgA responses (Fagerås et al., 2011; Houghteling and Walker, 2015; Sjögren et al., 2009b). Thus, the appearance of IgA positive cells in the lamina propria (Rognum et al., 1992) and the rapid increase in secretory IgA levels after birth (Fagerås et al., 2011; Pabst et al., 2016) are most likely primarily the result of colonisation of mucosal surfaces by commensal bacteria. In the human gut, low numbers of IgA+ plasma cells correlated with poor expression of the IgA inducing cytokine APRIL until one month of age, when both IgA+ plasma cells and APRIL appeared (Gustafson et al., 2014). Generation of intestinal IgA+ plasma cells approached but did not attain adult levels at two years of age (Gustafson et al., 2014). Gut mucosal IgA responses to various bacterial taxa change during infancy (Dzidic et al., 2016; Planer et al., 2016). In infants, perinatal administration of a prebiotic and probiotic combination for 6
months increased faecal IgA levels (Kukkonen et al., 2010). Interestingly, increased diversity of *Bifidobacterium* species in faeces samples collected 1 week, 1 month and 2 months after birth associated with increased salivary secretory IgA levels at 6 months of age (Sjögren et al., 2009b). Moreover, a slower maturation of the salivary secretory IgA system was observed in Swedish as compared with Estonian infants at the end of the last century (Fagerås et al., 2011). At this point of time, lactobacilli and eubacteria were more commonly detected in the gut microbiota of Estonian than Swedish infants, whereas the reverse was true for *C. difficile* (Sepp et al., 1997). As *C. difficile* expands when gut microbiota niches are vacant (Costello et al., 2012), this may indicate a disturbed intestinal microbiota ecology in the Swedish infants, which also were at higher risk of developing allergy as compared with the Estonian infants (Fagerås et al., 2011; Voor et al., 2005). In line with the importance of secretory IgA for an adequate mucosal barrier function (Pabst et al., 2016; Peterson and Artis, 2014; Wesemann and Nagler, 2016), low levels of salivary and intestinal secretory IgA are associated with an increased risk for allergic manifestations during early life (Fagerås et al., 2011; Kukkonen et al., 2010; Sandin et al., 2011), and aberrant IgA responses to the gut microbiota during infancy were recently observed to precede asthma and allergy development during the first seven years of life (Dzidic et al., 2016).

**Microbial strain-dependent mucosal immunomodulation**

In mice, certain bacterial strains have potent immunomodulatory effects. Mono-colonisation of germ free mice with *Clostridium*-related Gram positive segmented filamentous bacteria, adhering tightly to the surface of epithelial cells in the terminal ileum, induces development of intestinal isolated lymphoid follicles and tertiary lymphoid tissue (Lécuyer et al., 2014) and profoundly enhances the number of T_h17 cells in the lamina propria (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Schnupf et al., 2013). Moreover, whereas induction of T_h17 cells was not observed after transfer of rat or human microbiota to germ free mice, addition of murine segmented filamentous bacteria expanded T_h17 cells (Chung et al., 2012). While the rat microbiota contained segmented filamentous bacteria, they failed to colonise mice, demonstrating the importance of host specificity (Chung et al., 2012). The presence of segmented filamentous bacteria in the human microbiota still remains to be proven (Schnupf et al., 2013), and little is known on the development of mucosal T_h17 responses in children (Følsgaard et al., 2013; Mubarak et al., 2016).

Colonic regulatory T cell numbers and function are enhanced in germ free mice after colonisation with murine microbiota (Atarashi et al., 2011; Obata et al., 2014), altered Schaedler flora (Geuking et al., 2011), a consortium of 46 murine (Atarashi et al., 2011) or 17 human (Atarashi et al., 2013; Ohnmacht et al., 2015) *Clostridia* strains, as well as various strains from *Bacteroides* (Faith et al., 2014) or several different taxa (Sefik et al., 2015). The gut microbiota-derived short chain fatty acids butyrate and propionate can also stimulate peripheral regulatory T cell generation via epigenetically mediated histone deacetylase inhibition, enhancing acetylation of the murine Foxp3 locus (Arpaia et al., 2013; Tanoue et al., 2016). Vitamin A-derived retinoic acid can also support regulatory T cell differentiation in combination with TGF-β (Ohnmacht et al., 2015).
In addition, retinoic acid increases the expression of the gut-homing markers CCR9 and α4β7 integrin on the regulatory T cells (Tanoue et al., 2016). Interestingly, mono-colonisation of germ-free animals with polysaccharide A (PSA) producing *Bacteroides fragilis* increases the suppressive and IL-10 producing capacity of colonic regulatory T cells and induces IL-10 production (Round and Mazmanian, 2010). The zwitterionic PSA molecule has an unusual combination of positive and negative charges within each repeating unit and signals through TLR2 directly on Foxp3+ regulatory T cells to promote immune tolerance (Round et al., 2011).

The neonatal immune system has thus evolved to require diverse developmental signals from microbes to mature normally (Gensollen et al., 2016; Gollwitzer and Marsland, 2015; Houghteling and Walker, 2015; Wesemann and Nagler, 2016; West et al., 2015a). An appropriate timing of these microbial developmental signals seems to be required for an optimal development (Gensollen et al., 2016; Gollwitzer and Marsland, 2015; Houghteling and Walker, 2015; Wesemann and Nagler, 2016; West et al., 2015a). Thus, several immunological abnormalities observed in germ free mice can only be reversed if the animals are colonised with symbiotic microbes during the perinatal period, whereas later colonisation fails to reverse homeostasis. Normalisation of excessive allergen induced Th2 and IgE responses in germ free mice seems to require a critical colonisation time window, both after oral (Cahenzli et al., 2013; Sudo et al., 1997) and airway (Olszak et al., 2012) exposure to allergens. Furthermore, reversal of Th2 cytokine dependent oxazolone-induced colitis via regulation of iNKT cell homeostasis is only obtained after neonatal (Olszak et al., 2012) or even maternal colonisation throughout gestation (An et al., 2014), with similar findings observed for intestinal proinflammatory responses (El Aidy et al., 2013; Yamamoto et al., 2012) and LPS-induced proinflammatory responses in mesenteric lymph node cells (Hansen et al., 2012).

**Early microbial exposures and development of immune-mediated diseases**

While it is not clear if a similar critical colonisation time window exists in humans, early life events occurring during critical periods of immune maturation can have long-term impact on development of immune mediated diseases (Gollwitzer and Marsland, 2015; Jenmalm, 2011; Jenmalm and Duchén, 2013; West et al., 2015a, 2016). In support of this hypothesis, children who later develop allergic disease show differences in the composition and diversity of their gut microbiota during the first months of life compared with those who do not (Abrahamsson et al., 2012, 2014; Arrieta et al., 2015; Azad et al., 2015; Bisgaard et al., 2011; Björkstén et al., 2001; Ismail et al., 2012; Kalliomäki et al., 2001; Penders et al., 2007, 2013; Sjögren et al., 2009a; West et al., 2015a). Early establishment of a diverse gut microbiota, with repeated exposure to new bacterial antigens, may be more important than the distribution of specific microbial species in shaping a normal immune mucosal and systemic maturation (West et al., 2015a). Even prenatal exposures may shape immune developmental trajectories. Thus, maternal exposure to a traditional farm environment during pregnancy confers stronger protection against allergic sensitisation and disease than postnatal exposure alone (Von Mutius, 2016). Importantly, allergy intervention
studies also indicate that a combined prenatal and postnatal supplementation is important for the preventive effect of probiotics on infant eczema (Abrahamsson et al., 2015; Jenmalm and Duchén, 2013; West et al., 2016). To understand the complex interactions between the maternal and offspring microbiome and immunity in humans, further studies are required, however. To this end, the continuously decreasing cost/throughput ratio of current sequencing platforms will allow microbiome analysis in larger paediatric cohorts with detailed immunological and clinical outcomes. Multidisciplinary collaborations in large research consortia would aid in accomplishing this goal.

6.5 Conclusions

The intestinal microbiota likely provides a primary signal for establishment of an adequate mucosal barrier function and the maturation of a balanced postnatal innate and adaptive immune system. Future detailed studies on the complex interactions between the developing infant microbiome and immune system are important for establishing strategies to promote childhood health and prevent development of immune mediated diseases.

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6. Gut microbiota and childhood immunity


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Chapter 7
Microbiota and the gastro-intestinal system in children

C. Gómez-Gallego* and S. Salminen
1Functional Foods Forum, Faculty of Medicine, University of Turku, Itäinen Pitkäkatu 4A, 20014 Turku, Finland; cargom@utu.fi

Abstract

A healthy microbiota cannot be defined in general, as individual and interindividual variations are considerable. In the future, it may be possible to characterize a healthy microbiota on an individual level related to geographical location, diet and genetic factors. Deviations in microbiota are nevertheless often easier to identify, especially when they relate to particular disease risks, for example obesity and metabolic diseases or allergies. Microbiota development commences earlier than previously thought and may be initiated at fertilization and continue through pregnancy. The establishment of an intestinal microbial community appears to be a stepwise controlled process covering the first year of life. Factors affecting its compositional development are associated with genetic and environmental factors; mother’s health status, lifestyle and microbiota; geographic location and antibiotic treatments among others. Differences in microbiota composition in the early stages of life can be correlated with various microbial functional properties and differences in the production of microbial metabolites such as short-chain fatty acids or polyamines. Several studies show that these differences have an impact on gastrointestinal development, with consequences for later health. Alterations in the compositional development and activity of microbes may be partially responsible for the programming of the infant immune system and later health. Vaginal delivery, breastfeeding and limiting the perinatal influence to the use of antibiotics should be recommended for the promotion of normal microbiota development.

Keywords: bacterial metabolites, short-chain fatty acids, polyamines, intestinal development, infant, lactation, programming

7.1 Introduction

Exposure to microbes commences before the sperm cell and egg have met and both semen and vaginal microbiota composition and activity may promote or prohibit fertilization (Maendar et al., 2015). Thereafter exposure to microbes is continuous and the colonization of the human intestinal tract begins during pregnancy (Figure 7.1). The presence of microbes in the healthy human placenta, umbilical cord and meconium suggests that foetal microbial contact is a physiological phenomenon (Collado et al., 2016; Satokari et al., 2009). However, the origin and the significance of intrauterine microbes for infant gut colonization remain to be elucidated. Both epidemiological studies and experimental data indicate that foetal microbial contact is
causally related to both health and disease risk. The maternal microbial environment is thus important and depends on the exposure of the mother to microbes, nutrition during pregnancy, the mother’s health status and use of medicines (for more information see Chapters 3 and 4) (Figure 7.1).

The development of the intestinal microbiota is an essential process in human biology. However, the temporal, perhaps stepwise progression of this process is not well understood. In addition, the sources of specific microbes forming the ecosystem which influences human health, development of disease and general well-being are still only partly known.

Figure 7.1. Microbiota maturation during the first year of life: basis for later programming. The microbial colonisation pattern during the first year of life is modulated by a number of factors, including among other mother’s health status, lifestyle and microbiota; father’s microbiota; genetic background and geographical origin; environmental factors and antibiotic treatments. Between all of these factors, those related to the mother seem to exert a great impact on the infant microbiota. Differences in microbiota and their metabolites could produce a different physiological response during the development of the immune system, gastrointestinal system and associated organs such as liver or pancreas, which could have an effect on health status even in adult life.
7.2 Microbial colonisation in early life

The types of microbes frequently found in the infant gut vary according to genetic background, geographic area, environment, feeding practices and possibly intrauterine environment (Figure 7.1). A recent study reported Proteobacteria to be the most prevalent phylum in the amniotic fluid with particularly high abundance of species belonging to the Enterobacteriaceae family (Collado et al., 2016). Enterobacter and Escherichia/Shigella were present in colostrum and meconium and infant faeces. Propionibacterium was the second most common genus present in the amniotic fluid and placenta, but also detectable in the meconium. Streptococcus and Staphylococcus have been found in the meconium and infant faeces, and Lactobacillus in amniotic fluid, placenta, colostrum and meconium. Bifidobacteria appear in the new-born most likely consequent upon breastfeeding, as human milk always contains bifidobacteria, commonly the Bifidobacterium longum species. It is of note that viable microbes, including staphylococci and propionibacteria, are found in the placenta and amniotic fluid as well as in infant faeces and human milk. These bacteria form the basis of intestinal colonisation, which is reinforced by the mother’s microbiota and environmental exposure to bacteria at birth. Thereafter, the human milk microbiota composition (Cabrera-Rubio et al., 2012; Hunt et al., 2011) and human milk oligosaccharides have a major impact on the breastfed infant’s microbiota, rendering it significantly different from the microbiota of a formula-fed infant. In contrast to previous conceptions, human milk and oligosaccharides also promote bacterial groups such as staphylococci, probably facilitating the development of the gut barrier and immune system (Hunt et al., 2012; Salminen et al., 2016).

7.3 Host-microbe homeostasis in the gut

The intestinal epithelium acts as a mediator which actively participates in the homeostatic relationships in the host-microbe interplay (Duerr and Hornef, 2012). This homeostasis is reciprocal and the gut microbiota influences the structural maturation of the epithelium, modulating growth and morphological and functional development together with other agents such as genetic factors and feeding practices (Cummins and Thompson, 2002; Tomas et al., 2013). Analysis of the faeces of healthy infants shows that the greater part of the microbiota comprises mainly bifidobacteria, followed by the lactobacilli/enterococci group, clostridia and Bacteroides spp. (Ouwehand et al., 2004). An important consideration here, however, is not only what species and strains are present, but also what is their function in the intestinal tract, particularly in respect of microbiota-host interaction at the level of microbiota-derived molecules (Donia and Fischbach, 2015).

During the postnatal period extensive changes take place in the gastrointestinal tract morphology and hydrolytic and absorptive functions (Le Huerou-Luron et al., 2010). Interestingly, breast- and formula-fed children, with different intestinal microbiota compositions, show differences in the morphology of their mucosa, epithelial permeability, the activity of the brush-border enzymes, the absorption rates of nutrients, and the pancreatic endocrine and exocrine function.
(Le Huerou-Luron et al., 2010). In addition, formula fed children show a high microbial diversity with higher levels of *Bacteroides* spp., *Clostridium* spp. and *Escherichia coli* (Van Best et al., 2015).

It has been proposed that the microbiota constitutes a highly active metabolic organ and is able to perform functions we could not have executed on our own (Backhed et al., 2004), complementing the metabolic traits encoded in our own genome (Verbeke et al., 2015). For example, the intestinal environment allows specific microbes to ferment food components, resulting in the generation of a wide array of biologically active metabolites (Guzman et al., 2013). Moreover, it is able to process digestible and indigestible components from both our diet and many host-derived components (Russell et al., 2013; Xu et al., 2007). Among the metabolites produced by this process are essential vitamins, short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA), amines and polyamines (PAs), ammonia, several gases, phenolic compounds and some unique molecules not found in other habitats. Other metabolic activities include the activation or inactivation of bioactive food components such as isoflavonoids, flavonoids and plant lignans and the transformation of bile acids and xenobiotics (Verbeke et al., 2015). Based on our as yet relatively limited knowledge on the metabolites the following statements can be made (Donia and Fischbach, 2015; Verbeke et al., 2015):

- Some metabolite molecules are produced by the gut microbiota at high levels, sometimes exceeding tens of milligrams each day.
- Some bacterial metabolites are generally permeable and accessible to the host’s cells, such as amino acid metabolites, short chain fatty acids or polyamines.
- Distinct gut bacterial communities can produce different metabolite profiles, and the range of rare metabolites requires further studies to assess their potential effect on human health.

To act as intestinal barrier is one of the most important functions of the intestinal epithelium. It must act as a common barrier providing the fluid exchange while selectively discriminating in the passage of solutes (Turner, 2009), preventing the entrance of pathogens and harmful elements and allowing the absorption of nutrients (Di Mauro et al., 2013). Intestinal mucosal permeability is adaptable and may be regulated in response to extracellular stimuli, such as diet and bacteria (Turner, 2009). It is now clear that the production of bacterial metabolites in the gastrointestinal tract affects many systemic host processes, such as intestinal permeability and immune system development (see this Chapter and Chapter 6: Jenmalm and Prescott, 2017), metabolic regulation and vascular health. Although most of these effects have been well documented in laboratory animals, demonstrations in human subjects are scant.

The intestinal barrier also promotes passage of molecules and information between gut lumen and the components of the endocinral, neuronal, immunological routes implicated in regulation of intestinal homeostasis (Di Mauro et al., 2013). Alterations in the microbial colonisation during first months of life is correlated with an altered postnatal maturation of epithelial cell barrier functions with a consequential altered permeability that might facilitate the entrance of pathogens and antigens (Di Mauro et al., 2013).
Since microbiota transfer is already initiated in uterus, the role of both the mother’s but also the father’s microbiota is important (Maendar et al., 2015). However, little is known as to how bacteria transfer from the gut to other tissues, including the placenta, amniotic fluid and breast tissue and breast milk (Carmen Collado et al., 2016).

7.4 Molecules of bacterial origin and their influence on gastrointestinal tract development

**Short-chain fatty acids**

Many carbohydrates present in plant-derived foods are not digested or slowly digested in the small intestine, making them available for microbial fermentation in the large intestine (Russell et al., 2013). Hexose and pentose sugars are fermented by intestinal bacteria with the formation of saturated aliphatic organic acids, such as acetate, succinate, propionate, butyrate and formate, in a proportion depending on the strains and species present in the bacterial community (Russell et al., 2013). These SCFA are rapidly absorbed by the colonocytes (Verbeke et al., 2015), being important in maintaining intestinal homeostasis and epithelial integrity (Guzman et al., 2013; Thorburn et al., 2015).

Due to the inaccessibility of the human proximal colon to direct investigation and the rapid absorption of SCFA from the colonic lumen, it is extremely difficult to quantify SCFA production rates and define what is normal in their ‘healthy’ production (Verbeke et al., 2015). However, it is known that SCFA production (quality and quantity) has a major impact on intestinal health (Figure 7.2).

SCFA functions are regarded as beneficial for the host, depending of the nature of the fatty acids involved (Havenaar, 2011; Kanauchi et al., 2013; Kasubuchi et al., 2015; Verbeke et al., 2015). These functions include, amongst others: (1) acting as an energy source for colonocytes; (2) regulation of lipogenesis and cholesterol synthesis; (3) suppression of appetite directly via the hypothalamus; (4) release of satiety hormones; (5) potential anti-cancer effects; (6) improvement in gut barrier function; (7) modulation of bowel motor function; (8) modulation of carbohydrate metabolism.

In early infancy, the reported predominant SCFA are acetate and lactate in breast-fed; and acetate, propionate and butyrate in formula-fed infants (Le Huerou-Luron et al., 2010; Verbeke et al., 2015). However, if infant formulas are supplemented with specific oligosaccharides, faecal SCFA are reportedly more similar to those in breast-fed infants (Verbeke et al., 2015). Although no adverse effects of SCFA are described for adults or children, it has been suggested that extremely high levels of butyrate should be avoided in infants as they may be related to necrotising enterocolitis (NEC) (Havenaar, 2011). Dietary effects on SCFA production appear to be more critical in preterm infants, where breast-fed infants sometimes have higher concentrations of SCFAs than those fed with a fortified formula for preterm infants (Pourcyrous et al., 2014).
The rate and amount of SCFA produced depends on the composition and density of the colon microbiota in combination with the type of dietary fibres available for microbial fermentation (Havenaar, 2011). Different SCFA have different physiological roles. For example, acetate is used by the liver as precursor to the synthesis of cholesterol and long-chain fatty acids, while propionate reduces lipogenesis and inhibits cholesterol synthesis (Verbeke et al., 2015). Thus differences in microbiota populations are able to produce different SCFA with similar diets, with different physiological consequences.

**Polyamines**

Polyamines, including putrescine, spermidine, and spermine, are small organic polycations common in prokaryotic and eukaryotic cells. They are involved in the synthesis and stabilisation of DNA, RNA, cell membranes and protein molecules, as well as in cell proliferation and differentiation, and in the regulation of enzymatic activity. Many studies report a relationship between PAs and intestinal barrier function (Matsumoto and Kurihara, 2011), which includes...
the maturation of postnatal intestinal tissue, promotion of the intestinal mucus, recovery of the intestinal mucosa, and induction of the synthesis and stability of tight junction (Figure 7.2). Studies in animals demonstrate how PAs play a significant role in the growth and development of the digestive system of neonates, including associated organs such as liver and pancreas and the immune system (Gómez-Gallego et al., 2014; Larque et al., 2007; Perez-Cano et al., 2010; Sabater-Molina et al., 2009). However, although similar processes are assumed in humans, information in this area is scant.

Although almost all of our cells are able to produce polyamines from amino acids, some studies have demonstrated the importance of exogenous PAs, dietary PAs (absorbed mainly in the upper intestine) and microbiota-produced PAs (absorbed in the lower intestine), mainly for enterocytes and during high-growth rate stages of cells (Loser, 2000; Matsumoto and Kurihara, 2011).

Some bacteria which produce PAs secrete them into the extracellular environment, as they use PAs as extracellular signals in biofilm formation, cellular differentiation and cell-to-cell communication (Matsumoto and Kurihara, 2011).

When polyamine-producing bacteria (Bacteroides spp., Fusobacterium spp. and some probiotics strains of Lactobacillus spp. and Bifidobacterium spp.) are predominant in the intestine, the intestinal luminal PAs concentration is high; however, when PA-absorbing bacteria are predominant in the gut, this concentration is low (Matsumoto and Kurihara, 2011). This could have a substantial impact on the immature gastrointestinal system. Moreover, it has been indicated that dietary components, such as arginine, can modulate PAs absorption and release for intestinal bacteria (Kibe et al., 2014). Consequently, differences in intestinal microbiota and dietary polyamine precursors could affect the availability of intestinal polyamines for enterocytes, which may impact on gastrointestinal development.

### 7.5 Conclusions

Gut microbiota exerts an important role in the structural maturation of the intestinal epithelium during lactation. This effect is mainly mediated by bacterial metabolites and, as different bacteria produces different metabolites; different microbiota profiles may influence gastrointestinal development in an unusual way with both short-term and long-term consequences.

Recent changes in lifestyle and clinical practices affecting the perinatal period have altered the way the intestinal microbiota develops, and such changes may influence gastrointestinal functional development. Antibiotic treatments should be carefully considered, not only with regard to their therapeutic necessity but also for their impact on long-term microbiota development.

Short-chain fatty acids and polyamines are bacterial metabolites with reported effects in gastrointestinal tract development. Further studies are required to attain a fuller understanding of microbial produced metabolites and their impact on health.
7.6 Pressing gaps in knowledge

- No universal definition exists for the healthy intestinal microbiota. Genetic and environmental factors modify the outcome and the same bacterial groups could have different influences depending on individual idiosyncrasy.
- It is not well known how mother’s health and nutrition and other environmental factors such as mode of delivery or perinatal use of antibiotics during first year of life guide microbiota development and microbial production of metabolites.
- It is important to improve the data on SCFA profiles and their impact on gastrointestinal tract during lactation with information on how to modulate these profiles.
- More detailed studies are required on the role of polyamines during lactation in humans and not just in animal models or in vitro.

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7. Microbiota and the gastro-intestinal system in children


Chapter 8
The interplay between the microbiota and the central nervous system during neurodevelopment

A. Bharwani1,2,3, J. Bienenstock1,3 and P. Forsythe3,4*
1Department of Pathology & Molecular Medicine, McMaster University, 1280 Main Street West, HSC-2N16, ON L8S 4K1 Hamilton, Canada; 2Michael G. DeGroote School of Medicine, McMaster University, 1280 Main Street W, ON L8S 4K1 Hamilton, Canada; 3McMaster Brain-Body Institute, St. Joseph’s Healthcare, T3302, 50 Charlton Avenue East, ON L8N 4A6 Hamilton, Canada; 4Department of Medicine, McMaster University, Health Sciences Centre, 3W10, 1200 Main Street West, ON L8N 3Z5 Hamilton, Canada; forsytp@mcmaster.ca

Abstract

Recent advances in technology and research have led to tremendous strides in understanding the critical role of gut bacteria in neurodevelopment. Both the presence and composition of this intestinal community influences various aspects of central and enteric nervous systems physiology, thus shaping behaviour and neural function. Furthermore, signalling along the gut-brain axis, even through a single bacterial species, can alter the developmental trajectory of the stress circuitry and functional responses to stress. Gut-brain signalling is complex and bidirectional, mediated through multiple candidate pathways that enable this interplay, including the vagus nerve, the immune system, and an array of metabolite mediators. Given the sensitivity of the early developmental period to environmental perturbations, and the possible long-term consequences on the onset of neurodevelopmental and psychiatric conditions, further understanding of the mechanisms underlying gut-brain signalling and its role in development is critical to gaining insight into such conditions and identifying potentially novel therapeutic strategies.

Keywords: gut-brain signalling, brain, behaviour, vagus, stress, enteric nervous system

8.1 Introduction

The recent years have observed a transformative shift in our perspective on neurodevelopment and function. No longer is the brain considered a privileged organ, contained and operating within itself; rather, there is a growing appreciation in the literature of peripheral pathways that modulate the enteric and central nervous systems, and the corollary of such a relationship on the development of neural function and behaviour. Notably, due to leaps of innovation in high-throughput methods, particularly in sequencing technology, the intestinal microbiota has garnered keen interest for its impact on the physiological and mental well-being of the host, especially due to its influence during the development of the brain early in life. These pathways of signalling, often collectively referred to as the ‘gut-brain axis’, present an exciting opportunity
to better understand the development of neurological systems and their associated functions, including the stress response and emotional behaviours, and the consequences of early life modulation of these systems during adulthood (Figure 8.1).

8.2 Central nervous system development

During the initial years of exploratory work in a field that still remains in its infancy, the conventionalisation of germ-free (GF) models – animals that lack a microbiota – with a ‘normal’ bacteria consortium proved to be a tremendously useful tool in elucidating the critical role of gut bacteria in neurodevelopment. Some of the earliest work in the literature described observations regarding altered development of social and anxiety-like behaviours in GF mice, along with changes in the associated neural correlates – deficits that were reversed upon early-life exposure to bacteria (Desbonnet et al., 2014; Heijtz et al., 2011; Neufeld et al., 2011). Since then, the literature has grown replete with observations of the microbiota’s role in various facets of central nervous system (CNS) development, including that of the central serotonergic system, baseline amygdala function, and hippocampal neurogenesis, often resulting in changes that are retained into adulthood (Clarke et al., 2012; Dinan et al., 2015; Ogbonnaya et al., 2015). Moreover, these changes are often sex-specific, which remains a severely underexplored aspect of the role of the microbiota during development.

There also exist intriguing parallels between the central and peripheral consequences of host-microbiota interactions. For instance, the gut microbiota modulates the blood-brain and intestinal epithelial barriers – both of which play a critical role in regulating the passage of cells and molecules at the interface of different environments (Braniste et al., 2014; Kelly et al., 2015). These particular interactions are mediated by the release of microbial-derived short chain fatty acids (SCFAs) such as butyrate that alter the expression of tight junction proteins and barrier-coordinating transcription factors. Similarly, the indigenous microbes of the intestinal community also influence the development of central and peripheral glial cells, regulating their homeostasis throughout life. Gut microbes are critical to the development, migration, and colonisation of mucosal enteric glial cells, which partake in the mucosal immune response and maintain the intestinal epithelial barrier (Kabouridis et al., 2015). Similarly, the morphology, activation, and functional phenotype of microglia, which serve as the resident tissue phagocytic cells of the brain, are also regulated through metabolite signals derived from these bacteria and the diversity of the intestinal community (Erny et al., 2015). Given the critical role of these immune cells in the pruning and maintenance of synapses and neuronal connectivity throughout maturation, such changes have important implications on the development of behaviour and the onset of neurodevelopmental disorders (Zhan et al., 2014). Moreover, albeit through alternative pathways, microbial metabolites and dysbiosis of the gut have been observed to influence autism-like behaviours and comorbid gut barrier defects in a mouse model of autism spectrum disorders (ASD) – many of which were successfully corrected upon treatment with Bacteroides fragilis, a beneficial strain of bacteria (Hsiao et al., 2013).
Figure 8.1. Potential routes of communication along the gut-brain axis that enable bidirectional signalling between the microbiota and the brain. Bacteria in the gut can synthesise a variety array of neuroactive mediators – neurotransmitters, short chain fatty acids – that can alter behaviour and neural function. These bacteria also recruit signals from the mucosal and systemic immune systems, influencing immune regulation and cytokine signals that can communicate with the nervous systems. Such pathways enable bidirectional interplay between the microbiota and the central nervous system, resulting in changes in behaviour, neural function, and the stress circuitry (adapted with permission from Forsythe et al., 2010).
More recently, a study demonstrated the impact of circulating maternal microbial products on the development of the foetal brain and postnatal behaviour (Humann et al., 2016). Circulating bacterial cell wall released from the maternal microbiome during antibiotic treatment or infection – also found in the bloodstream during healthy circumstances – can cross the placenta and interact with immune ligand receptors to induce neuronal proliferation, resulting in long-term postnatal behavioural changes in learning and memory tasks. Similarly, perturbation of the maternal microbiota during pregnancy through the administration of non-absorbable antibiotics also impacts the composition of the offspring microbiota and has downstream ramifications on exploratory and locomotor behaviour (Tochitani et al., 2016).

It ought to be noted that although this chapter emphasises evidence stemming from rodent models, in particular for their relevance to translational applications, there are extensive data in the literature demonstrating the evolutionarily conserved nature of interactions between the microbiota and host, with functional consequences on behavioural development. This includes congregation behaviour in locusts, mating preference in fruit flies, and aggregation of the German cockroach, all of which rely upon the presence of gut bacteria and their production of metabolites and volatile compounds, discussion of which lies beyond the scope of this chapter (Dillon et al., 2002; Sharon et al., 2011; Venu et al., 2014; Wada-Katsumata et al., 2015).

### 8.3 Stress-response programming and early life stress

The neuroendocrine network responsible for coordinating the body’s response to stress, the hypothalamus-pituitary-adrenal (HPA) axis, is highly sensitive to stimuli during early-life. Indeed, in some of the earliest and oft-cited works in the field, GF mice were demonstrated to possess higher baseline levels of corticosterone and a more exaggerated response to acute stress, resulting in a pronounced release of stress hormones (Crumeyrolle-Arias et al., 2014; Neufeld et al., 2011b; Sudo et al., 2004). This is a likely corollary to an observed decrease in the expression of central glucocorticoid receptors, given their role in negative feedback regulation of the HPA axis response. Intriguingly, colonisation of these mice during a temporally specific postnatal developmental window completely corrects and reverses these changes, elegantly highlighting the sensitivity of HPA axis programming to the colonisation of gut bacteria. However, whether such a narrow window of vulnerability to the influence of gut bacteria exists in humans remains unknown. Such interactions during stress also influence aspects of cognition and memory, as demonstrated in a non-invasive Citrobacter rodentium model: infected mice exhibited non-spatial and working memory dysfunction, but only after exposure to a psychological stressor. This was observable even after pathogen clearance but was prevented upon pre-treatment with Lactobacillus bacteria, thus indicative of effects beyond merely those associated with sickness behaviour (Gareau et al., 2011).

Signalling along the gut-brain axis is bidirectional, and thus in addition to the bottom-up influence of the microbiota on HPA axis development, there also exists evidence of top-down effects of stress and HPA axis activity on the community. Through detection of serotonin or
stress-associated release of epinephrine and norepinephrine via a bacterial receptor for host adrenergic signalling, bacteria species can alter their virulence and the release of toxins, as well as changes in the growth of the community (Clarke et al., 2006; Knecht et al., 2016; Lyte and Arulanandam, 1996; Lyte and Ernst, 1993). Stress also alters the composition and profile of the microbiota, resulting in reduced diversity and richness, and nuanced changes in the abundance of specific bacterial species (Bharwani et al., 2016; O’Mahony et al., 2009). The complexity of host-microbes interactions during stress was encompassed by a study demonstrating that changes in gut bacteria are necessary but not sufficient for early life stress-induced behavioural deficits in the host (De Palma et al., 2015). Furthermore, there is a growing body of both human and non-human animal research demonstrating that the infant microbiota can also be impacted by stimuli during the prenatal period, including maternal stress, which may later predispose the developing offspring to gastrointestinal disorders and behavioural changes, perhaps through region- and sex-specific changes in the availability of metabolites in the brain (Jašarević et al., 2015a,b; O’Mahony et al., 2009; Zijlmans et al., 2015). Together, these data indicate the sensitivity of the developing neonate brain to microbial colonisation of the gut and disruptions thereof, and highlight this critical window as a viable target in the prevention and treatment of neurodevelopmental conditions.

8.4 Enteric nervous system development

Given its ability to function independently of the CNS (Bayliss and Starling, 1899), and its position at the interface between intestinal bacteria and the CNS, the influence of the microbiota on the development of the enteric nervous system (ENS) bears mentioning, as well as its implications in periphery-CNS communication. Absence of gut bacteria during development is characterised by reduced nerve density and number, and lower frequency and amplitude of muscle contractions in the jejunum and ileum (Collins et al., 2014). The microbiota is also critical to the excitability and function of intrinsic sensory neurons of the ENS, which relay microbial-derived signals to ascending vagal fibres that project to the brain, indicating their importance in gut-brain signalling (McVey Neufeld et al., 2013, 2015; Perez-Burgos et al., 2014). Furthermore, the activity of these sensory neurons can also be modulated by a single strain of neuroactive commensals, such as B. fragilis or a previously characterised Lactobacillus rhamnosus (Kunze et al., 2009; Mao et al., 2013) – observations that suggest the influence of these bacteria on the brain and behaviour may be indirectly mediated through signals carried forth by the enteric nervous system.

8.5 Signalling mechanisms underpinning gut-brain communication

Vagus nerve

The vagus is the tenth cranial nerve, extending between the gut and the dorsal vagal complex (DVC) in the brainstem, innervating various viscera along the way. It extends bidirectionally, thus carrying both afferent and efferent signals to and from the brain respectively. Given
its well-established role in sickness behaviour and in transmitting signals from the GI tract that collectively control ingestive behaviour and satiety (Maier et al., 1998), the vagus has consequentially been investigated in numerous studies for its role in mediating microbial-brain signalling. Early evidence demonstrated that infection with an enteric pathogen – Campylobacter jejuni – elicits markers of neuronal activation in the nuclei of the DVC and in the forebrain, independently of immune signalling (Gaykema et al., 2004; Goehler et al., 2005). Similarly, subclinical bacterial infection can elicit anxiety-like behaviour in the absence of overt inflammation, further implicating the role of the vagus (Lyte et al., 1998).

Compelling evidence from murine models has demonstrated that chronic administration of a neuroactive Lactobacillus strain reduces anxiety-like behaviour and alters the expression of GABA receptors in the brain (Bravo et al., 2011). The integrity of the vagus nerve is necessary for these effects, as demonstrated by their abolition following a subdiaphragmatic vagotomy. This association between vagal signalling and behavioural changes is further fuelled by evidence from human studies on Vagal Nerve Stimulation (VNS), a US Federal Drug Administration-approved therapy for intractable forms of Major Depressive Disorder (Cristancho et al., 2011; Groves and Brown, 2005). In investigations to elucidate the mechanistic underpinnings of this form of gut-brain communication, electrophysiology studies in ex vivo tissue demonstrated that application of L. rhamnosus bacteria causes rapid firing of the vagus in the absence of bacteria translocation across the mucosal epithelium (Perez-Burgos et al., 2013). Moreover, the majority of these signals are transmitted to the vagus indirectly via intrinsic primary afferent neurons that exhibit increased excitability following exposure to this bacteria strain (Mao et al., 2013; Perez-Burgos et al., 2014). However, that a vagotomy does not prevent bacteria-induced behavioural changes in all bacteria models indicates that vagal-mediated signals may be strain-specific and not the sole route of gut-brain signalling (Bercik et al., 2010).

**Immune system**

Consideration of its malleability to the influence of the microbiota throughout life, and its role in maintaining and responding to changes in the microbiome community suggests that the immune system is an important mediator at the interface between the host and gut bacteria. Ubiquitously located immune receptors on host cells – including on neuronal and glial cells of the CNS – enable recognition of and response to conserved microbial-associated molecular patterns (Forsythe and Kunze, 2013). An ever-present and diverse microbiota is necessary for the development of peripheral and central components of the immune system (Erny et al., 2015; Umesaki et al., 1995). Through regulated passage of bacteria across the host epithelium, the mucosal immune system samples the microbial community and elicits downstream pathways – IgA production, cytokine release, T cell proliferation – in an effort to both regulate the intestinal community as well as mediate bacteria-host communication (Forsythe and Bienenstock, 2010). This bears particular relevance for neural function, given evidence of the adaptive immune system's role in learning and cognition, and the influence of peripheral cytokines in behavioural changes (Brynskikh et al., 2008; Dantzer, 2001). Corroborating these results are numerous studies
demonstrating the influence of selective bacteria species on immune system function in healthy and pathological states, as well as downstream immune-mediated effects of changes in the microbiota community on aspects of cognition and memory, including neurogenesis (Desbonnet et al., 2010; Mohle et al., 2016; Ogbonnaya et al., 2015). It thus appears that local microbial-immune interactions at the level of the intestinal epithelium can mediate systemic changes in the host, thus contributing to CNS function during homeostasis or states of disturbance.

Metabolites and soluble mediators

Microbial signals to the brain can also be transmitted through soluble mediators, either systemically released to directly influence the CNS, or locally to influence the aforementioned signalling pathways. Specific bacteria can exert their influence through the synthesis and release of important neurotransmitters and neuromodulators: GABA, serotonin, acetylcholine, and norepinephrine (Lytte, 2011, 2013; Wikoff et al., 2009). Moreover, the presence of these bacteria is necessary for the development of the host’s serotonergic system, more than 90% of which is synthesised in the gut (Clarke et al., 2012; Erspamer, 1966; Yano et al., 2015). This influence is critical to the development of the brain, given that gut bacteria influence hippocampal serotonin levels, and that placental serotonin serves as an exogenous source that contributes to the development of the foetal forebrain prior to the recruitment of endogenous serotonin from dorsal raphe neurons (Bonnin et al., 2011; Clarke et al., 2012).

Another class of molecules that has garnered increasing attention for their potential role in gut-brain signalling is SCFAs – the by-product of dietary carbohydrate fermentation by gut bacteria. Several of these molecules, including butyrate and propanoate, exhibit neuroactive characteristics. Butyrate possesses histone deacetylase inhibition properties, administration of which induces antidepressant-like effects through altering levels of neurotrophic factors in the brain (Schroeder et al., 2007). Signalling through SCFAs in the periphery has also been implicated in the development and functional regulation of microglia, the brain’s resident phagocytic immune cells (Erny et al., 2015). These molecules from the periphery can cross the BBB and accumulate at detectable levels in the CNS, thus perhaps positioning them to serve as mediators of gut-derived signals (Frost et al., 2014; Wyss et al., 2011).

Although the vast array of signals recruited by bacteria for inter-kingdom communication cannot possibly be entirely enumerated within this work, recent work has shed light on the diversity of such pathways. microRNA (miRNA) released by host epithelial cells can enter bacteria to regulate gene expression, function, and growth, enabling regulation of the gut microbiota community (Liu et al., 2016). The effect of specific bacterial species on the immune and enteric nervous systems can also be recapitulated through application of its isolated proteins and polysaccharides, as well as through microvesicles shed by the microorganism that contain material from the parent bacteria (Al-Nedawi et al., 2014; Mao et al., 2013). Whether such signals are sufficient or necessary to mediate the effect of neuroactive bacteria on neural function and behaviour is an area of research that remains under investigation.
8.6 Conclusions

The literature is growing with evidence of the bidirectional interplay between the CNS and the microbiota during developmental, thus further expanding the possible sources of influence during early life that determine the developmental trajectory of the brain. There exists a compelling case for the role of these bacteria in mediating CNS development, with downstream consequences on the shaping of behaviour, cognition, and neurodevelopment conditions, lending tremendous potential to a field within which investigations have traditionally been restricted to the brain. However, there exist large gaps in our fundamental understanding of such interactions: what are the microbiota-derived signals that recruit the ENS, vagus nerve, and immune system for bottom-up, bacteria-induced signalling? How are these signals processed by the brain, and what neural networks underlie the neurobehavioural effects of certain bacteria? Furthermore, there is a growing emphasis on investigating the regulatory role of the bidirectional gut-brain signalling on neurodevelopment, in an effort to understand how perturbations in the microbiota, either due to stress or antibiotic exposure, during a period of increased sensitivity may influence long-term behavioural outcomes and alter the risk of neurodevelopmental and psychiatric conditions. In order to progress this field we need prospective clinical studies relating early-life microbiota and metabolomic profiles to neurodevelopmental health outcomes and in particular a focus on the long-term impact of perinatal antibiotic therapy.

Animal models and in vitro systems should be utilised to address major questions related to mechanism. Such questions include: How do specific microbial metabolites and components influences the developing neuroendocrine and system immune? How do the immune system, and immunomodulation by the microbiota, influence development of enteric and central nervous systems?

With such knowledge it may be possible to develop microbe- or gut-brain axis-based preventative strategies that mitigate the impact of dysbiosis on neurodevelopment.

Conflict of interest

The authors declare no conflict of interest.

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8. Gut-brain signalling during neurodevelopment


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Part IV.
Gut microbiota: it’s role in paediatric diseases
Chapter 9
The role of intestinal microbiota in infant allergic diseases

H. Szajewska
Department of Paediatrics, The Medical University of Warsaw, Zwirki i Wigury 63A, 02-091 Warsaw, Poland; hania@ipgate.pl

Abstract

In many countries, particularly in populations with a Western lifestyle, allergic diseases are on the rise. As genetic factors have not changed, environmental factors must be playing a role. Recently, attention has been focused on the role of the gut microbiota. However, despite many years of extensive research, the link between the gut microbiota and allergic diseases still has not been fully clarified. The primary objective of this chapter is to provide an overview of the role of the gut microbiota in the development of allergic diseases. The role of probiotics and/or prebiotics as microbiota modifiers for the prevention and treatment of allergic diseases is reviewed. The focus is on allergic diseases typically found in infants.

Keywords: children, allergy, atopy, eczema, food allergy, randomised controlled trial, meta-analysis

9.1 Introduction

Allergic diseases can occur at almost any age (Boyce et al., 2011; Muraro et al., 2014a). However, some allergic manifestations are most likely to develop for the first time in particular age groups. For example, in infants and children younger than 3 years of age, allergy to food (especially milk, eggs, wheat, nuts) is the most common, affecting up to 8% of children (Sicherer, 2011). The pathophysiology is multifactorial. Allergy is triggered by environmental factors in individuals with genetic susceptibility. The majority of affected infants and children have one or more symptom(s) involving one or more organ system(s), mainly the gastrointestinal tract and/or skin. After the age of 3 years, allergy to inhalants becomes the predominant allergy. Thus, later in life, asthma, allergic rhinitis, and hay fever become common. For diagnosing allergy, obtaining a detailed clinical history is critical. The gold standard for the diagnosis of a potential food allergen is the double-blind, placebo-controlled challenge. Strict avoidance of the offending allergen is the main therapeutic option.

The rising number of children and adults with allergic disorders in many countries, particularly in populations with a Western lifestyle, is a major public health concern (Pawankar et al., 2014). The origins of this increase are still not well understood. As genetic factors have not changed, environmental factors are thought to be playing a role. Recent evidence has demonstrated that, among other factors, disturbances in gut microbiota, defined as dysbiosis, may be relevant (Figure 9.1). This chapter summarises available information on the role of the gut microbiota.
in the development of allergic diseases, with focus on allergic diseases typically found in infants and young children. Moreover, the role of probiotics and/or prebiotics for the prevention and treatment of allergic diseases is reviewed. Finally, this chapter presents suggestions for future clinical research.

### 9.2 Gut microbiota & allergy

Results from experimental studies suggest that early exposure to microbial antigens plays an important role in the development of the immune system and the establishment of a balance between T helper (T\(_{h1}\)) 1/T\(_{h2}\) cell responses. Thus, among other processes (Arrieta et al., 2014), contact with microbes appears to be essential for the development of oral tolerance (Sansonetti and Medzhitov, 2009).

Originally, the so-called ‘hygiene hypothesis’ suggested that improved hygiene and reduced exposure of the immune system to the microbial stimulus (‘too clean’ of an environment) during infancy and early childhood predispose to impaired immune regulation in later life, leading to either T\(_{h2}\) diseases (such as allergy) or T\(_{h1}\) diseases (such as type 1 diabetes) (Bach and Chatenoud, 2012; Prescott, 2003; Strachan, 1989). More recently, the hygiene hypothesis has
been challenged (Hahtela et al., 2015; Hanski et al., 2012; Renz et al., 2011). While exposure to some pathogens protects against atopy, other exposures promote allergic diseases. Factors such as the timing of exposure and the properties of the infectious agent, host genetic susceptibility, and other environmental factors also may be important (Fishbein and Fuleihan, 2012; Guarner et al., 2006). Nevertheless, hypotheses suggesting that gut microbiota alterations could lead to the development of allergy are commonly considered. Factors that may change gut microbiota (Figure 9.1) and the subsequent effects on allergy risk are discussed below.

**Delivery mode**

There is consistent evidence that the mode of delivery affects gut microbiota (Adlerberth and Wold, 2009; Azad et al., 2013; Dominguez-Bello et al., 2010; Salminen et al., 2004). Also, recent data using culture-independent methods have confirmed that colonisation patterns differ between infants born by caesarean section compared with infants born vaginally. Infants delivered by caesarean section had lower total microbiota diversity, as well as lower abundance and diversity of *Bacteroidetes* phylum, during the first 2 years of life. Moreover, reduced levels of T1/1-associated chemokines, with a shift of the T1/1/T1/2 balance towards a more allergic T1/2 response, were documented in infants delivered by caesarean section compared with those born vaginally (Jakobsson et al., 2013). One recent study concluded that mode of delivery was one the key factors (together with cessation of breastfeeding) driving the assembly of an adult-like gut microbiota (Bäckhed et al., 2015). Finally, another recent study showed that differences in gut microbiota composition between infants born naturally and infants delivered by caesarean section were greater than differences due to feeding methods (breast milk versus formula) (Madan et al., 2016).

It has been hypothesised that these differences in gut microbiota between infants born via caesarean section versus those born vaginally may contribute to the risk of allergic diseases and asthma. However, data are conflicting. One systematic review found that caesarean section does appear to moderately increase the risk for allergic rhinitis, asthma, hospitalisation for asthma, and food allergy/food atopy but not the risk of inhalant atopy or atopic dermatitis (AD) (Bager et al., 2008). Also, another meta-analysis found that caesarean section increases the risk of asthma in children by 20% (Thavagnanam et al., 2008). However, the association between the mode of delivery and some allergic manifestations has not been confirmed in some subsequent studies (Pyrhönen et al., 2013).

**Feeding after delivery**

Multiple observations have repeatedly shown that the mode of feeding after birth (breastfeeding versus formula) influences the composition of the gut microbiota (Bezirtzoglou et al., 2011; Le Huërou-Luron et al., 2010; Penders et al., 2006). This is mainly due to the fact that human milk contains human milk oligosaccharides that can stimulate the growth and/or activity of beneficial bacteria such as *Bifidobacterium* (Zivkovic et al., 2011). Term infants who were
born vaginally at home and were breastfed exclusively seemed to have the most ‘beneficial’ gut microbiota (highest numbers of bifidobacteria and lowest numbers of *Clostridium difficile* and *Escherichia coli*). Compared with breast-fed infants, exclusively formula-fed infants were more often colonised with *E. coli*, *C. difficile*, *Bacteroides*, and lactobacilli (Penders et al., 2006). The USA infant twin cohort study also detected differences between the breast-fed and formula-fed infants (Yatsunenko et al., 2012). Finally, one recent study showed that mixed feeding (breast milk and formula), which despite recommendations from scientific societies and WHO is a common practice in many settings, resulted in intestinal microbiota communities similar to those found in exclusively formula-fed infants (Madan et al., 2016).

While the effect of infant feeding on gut microbiota is clear, conflicting data exist on the relationship between breastfeeding and allergic disease risk. Recently, a Lancet review concluded that in children who are breastfed, ‘there is no clear evidence of protection against allergic disorders: no association with eczema or food allergies and some evidence of protection against allergic rhinitis in children younger than 5 years’ (Lodge et al., 2015; Victora et al., 2016). A variety of methodological problems are likely to have contributed to these inconsistent results (including an inability to randomise and blind; the retrospective design of many studies addressing the association between breastfeeding and allergic disease; parental recall bias; and reverse causality).

**Early-life antibiotic use**

Data have consistently shown that antibiotic exposure has an effect on gut microbiota (Fouhy et al., 2012; Hällström et al., 2004; Penders et al., 2006). Some data show that not only antibiotic use by the infant (Penders et al., 2006), but also maternal antibiotic intake during birth, alters the microbiota of new-borns (Arboleya et al., 2015). Interestingly, one recent retrospective study suggests that early-life antibiotic use may diminish breastfeeding benefits in childhood (Korpela et al., 2016). Compared with infants who did not receive antibiotics during breastfeeding, infants who received antibiotics during breastfeeding and up to 4 months after weaning had a higher likelihood of developing excess weight gain and infections during childhood (for more information see Chapter 7: Gómez-Gallego and Salminen, 2017). Today, it remains unclear whether there is a similar link for allergic disorders. Evidence on the effects of early-life antibiotic use and subsequent development of allergic diseases such as asthma, allergic rhinitis, eczema, and food allergy remains inconsistent (Karpa et al., 2012; Koplin et al., 2012; McBride et al., 2012).

**Early-life farm exposure**

It has been hypothesised that early-life farm exposure, reduced cleanliness, and subsequent increased microbial exposure would lead to a more diverse intestinal microbiota. Intriguingly, a 2007 study carried out in several European countries found that compared to non-farming children, children from farming backgrounds had less gut microbial diversity (Dicksved et al., 2007).
Studies on early-life farming exposure and subsequent allergy risk have yielded inconsistent results. Earlier, a protective association between early-life farm exposure and respiratory symptoms and allergy in children was reported in developed countries (Von Mutius and Vercelli, 2010). A 2012 study confirmed such an effect in affluent countries, but it found that exposure to farm animals during pregnancy and during the first year of life was associated with increased symptoms of asthma, rhinoconjunctivitis, and eczema in children living in non-affluent countries (Brunekreef et al., 2012). Several studies, including the PARSIFAL (Prevention of Allergy-Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle) study and the GABRIELA (Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community [GABRIEL] Advanced Study), showed a strong association between early-life farm exposure and lower prevalence of asthma and atopic sensitisation (Ege et al., 2011). In contrast, there is no evidence to formulate a conclusion as to whether exposure to a farming environment affects food allergy risk.

Other factors

Other important determinants of the gut microbiota composition in infants include the country of origin (birth in an industrialised country was associated with reduced gut microbiota diversity) (Yatsunenko et al., 2012), infant hospitalisation (hospitalisation and prematurity were associated with a higher prevalence and counts of C. difficile) (Penders et al., 2006), and time of weaning (as stated earlier, cessation of breastfeeding rather than introduction of solid food was more likely to contribute to the maturation of the infant’s gut microbiota) (Bäckhed et al., 2015).

Differences in the microbiota in allergic and non-allergic individuals

In humans, it has been suggested that the composition of the gut microbiota during early life may predict the subsequent development of allergic disorders (Arrieta et al., 2015; Bjorksten et al., 2001). Atopic subjects have more clostridia and tend to have fewer bifidobacteria than non-atopic subjects (Kalliomaki et al., 2001). Reduced diversity of gut microbiota is associated with an increased risk of atopic eczema (Abrahamsson et al., 2014; Forno et al., 2008; Penders et al., 2007; Wang et al., 2008; West et al., 2015). Several studies have also demonstrated a link between infant gut microbiota composition and wheeze and asthma (Abrahamsson et al., 2014; Van Nimwegen et al., 2011).

9.3 Gut microbiota manipulation

Key observations that gut microbiota may play a role in the pathogenesis of allergic diseases have provided a strong basis for developing strategies aimed at gut microbiota normalisation. Among others, these strategies include the administration of probiotics and/or prebiotics.
9.4 Probiotics

Probiotics are defined as ‘live microorganisms that, when administered in adequate amounts, confer a health benefit on the host’ (Hill et al., 2014). In humans, by far, the most commonly used probiotics are bacteria from the genus *Lactobacillus* or *Bifidobacterium* and a non-pathogenic yeast, *Saccharomyces boulardii*. The exact mechanisms by which probiotics mediate protection against allergic diseases are not known. However, strengthening of gut mucosal barrier function, activation of TH2 counter-regulatory immune responses, and maintenance of the gut microbial balance may play a role.

**Prevention**

A 2015 systematic review (Cuello-Garcia et al., 2015) reviewed the role of probiotics for the prevention of allergies. The reviewers concluded that there are significant benefits of probiotic supplements in reducing the risk of eczema when administered to women during the last trimester of pregnancy (14 randomised controlled trials (RCTs), n=3,109, relative risk (RR) = 0.71, 95% confidence interval (CI) = 0.60-0.84) or during breastfeeding (10 RCTs, n=1,595, RR=0.61, 95%CI=0.50-0.74); however, no such effect was observed when probiotics were used exclusively during breastfeeding (1 RCT, n=88, RR=0.57, 95%CI=0.29-1.11). Probiotics given to infants also reduced the risk of eczema (15 RCTs, n=3,447, RR=0.81, 95%CI=0.7-0.94). In contrast to the effect on eczema, probiotics compared with no probiotics had no effect on the risk of other allergies such as asthma/wheezing, food allergy, and allergic rhinitis as well as no effect on the nutritional status or incidence of adverse effects. Overall, the quality of evidence was low or very low due to the risk of bias, inconsistency and imprecision of the results, and the indirectness of available research.

In 2015, the World Allergy Organization (WAO) developed recommendations about the use of probiotics in the prevention of allergy based on the findings from the systematic review discussed above (Table 9.1) (Fiocchi et al., 2015). One important limitation of the WAO guidelines is the lack of answers to the most important practical questions. Which probiotic(s) should be used to reduce the risk of eczema? When should one start the administration of probiotics with proven efficacy? When should one stop? What is the dose of an effective probiotic? Of note, 2014 recommendations developed by the European Academy of Allergy and Clinical Immunology (EAACI), based on the results of a systematic review of RCTs (De Silva et al. 2014), concluded that there is no evidence to support the use of probiotics (also prebiotics) for food allergy prevention (Muraro et al., 2014b). In summary, at the present time, there is insufficient evidence that any specific probiotic plays a significant role in the prevention of atopic disease in the infant.

**Treatment**

The role of probiotics in the treatment of AD/eczema remains questionable. The most recent systematic review with a meta-analysis identified 25 RCTs involving 1599 participants (Kim et
9. Microbiota in infant allergic diseases

Compared with placebo, the use of probiotics (in some trials together with prebiotics) significantly reduced Scoring of Atopic Dermatitis (SCORAD) values overall (weighted mean difference (WMD) -4.51, 95%CI = -6.78 to -2.24), in adults (WMD -8.26, 95%CI = -13.28 to -3.25), and in children 1 to 18 years of age (WMD -5.74, 95%CI = -7.27 to -4.20), but not in infants younger than 1 year (WMD 0.52, 95%CI = -1.59 to 2.63) (Kim et al., 2014).

Data regarding whether probiotics may be effective in the management of cow’s milk allergy (CMA) are mixed, with encouraging results in more recent studies. A 2008, double-blind, placebo-controlled RCT performed in 119 children infants with CMA found that *Lactobacillus casei* CRL431 and *Bifidobacterium lactis* Bb12 added to extensively hydrolysed formula did not significantly affect clinical tolerance to cow’s milk after 6 and 12 months of treatment. At 12 months, the cumulative tolerance to cow’s milk was 81% in the placebo group and 77% in the probiotics group (odds ratio (OR) = 1.1, 95%CI = 0.6 to 1.9) (Hol et al., 2008). Results from a more recent study suggest that the choice of probiotics and infant formula selection influence the rate of acquisition of tolerance in children with CMA. One RCT randomly allocated infants

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Table 9.1. Current recommendations for the use of probiotics and prebiotics for allergy prevention.

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<th>Probiotics</th>
<th>Prevention of allergy</th>
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<td></td>
<td>• Evidence does not indicate that probiotic supplementation reduces the risk of developing allergy in children.</td>
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<td>• There is a likely net benefit from using probiotics. The WAO guideline panel suggests using probiotics in:</td>
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<td>− pregnant women at high risk(^1) for having an allergic child;</td>
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<td>− women who breastfeed infants at high risk(^1) of developing allergy;</td>
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<td>− infants at high risk(^1) of developing allergy.</td>
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<th>Prebiotics</th>
<th>The WAO guideline panel suggests:</th>
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<tr>
<td></td>
<td>• using prebiotic supplementation in not-exclusively breastfed infants, both at high(^1) and at low risk for developing allergy</td>
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<tr>
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<td>• not using prebiotic supplementation in exclusively breastfed infants.</td>
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<td>(conditional recommendations; very low quality evidence).</td>
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<td></td>
<td>No recommendation about prebiotic supplementation during pregnancy or in breastfeeding mothers.</td>
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\(^1\) High risk was defined as the presence of a biologic parent or sibling with asthma, allergic rhinitis, eczema, or food allergy.
with CMA (while still receiving intact protein formula) to either a group that received extensively hydrolysed casein formula or a group that received the same extensively hydrolysed formula containing *Lactobacillus rhamnosus* GG. After 6 months of an exclusion diet, a double-blind, placebo-controlled, milk challenge was performed in 55 patients, and evidence of tolerance was seen in 21.4 and 59.3% of infants, respectively. However, the difference in acquisition of immunotolerance was significant only for those children with non-IgE-mediated CMA ($P=0.017$) (Berni-Canani *et al.*, 2012). Another open-label, non-RCT evaluated the acquisition of tolerance in a total of 260 infants aged 1 to 12 months with confirmed CMA fed 5 different formulas. The rate of oral tolerance after 1 year of treatment, as determined by a food challenge, was significantly higher in the groups that received extensively hydrolysed casein formula, particularly with *Lactobacillus* GG (78.9%), but also without (43.6%), compared with the other groups that received hydrolysed rice formula (32.6%), soy formula (23.6%), and amino acid-based formula (18.2%) (Berni-Canani *et al.*, 2013).

Together, while these data are promising, larger RCTs are needed to confirm these findings, to define the mechanisms of action, and to evaluate the potential factors influencing the response in subjects with CMA.

### 9.5 Prebiotics

A 2015 expert definition defines a prebiotic as ‘a non-digestible compound that, through its metabolisation by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host’ (Bindels *et al.*, 2015). In humans, non-digestible carbohydrates, such as inulin, oligofructose, fructooligosaccharides (FOS), and galactooligosaccharides (GOS), are the most intensively studied and commonly used prebiotics. Many studies have shown that they increase the faecal counts of bacteria thought to be beneficial such as bifidobacteria or certain butyrate producers.

**Prevention**

See Table 9.1 for a summary of the 2014 EAACI (Muraro *et al.*, 2014a,b) and 2016 WAO (Cuello-Garcia *et al.*, 2016) guidelines for the use of prebiotics in the prevention of allergy. The latter recommendations are based on findings from 18 RCTs. All studies were carried out in non-breastfed infants. Supplementation with prebiotics compared with no supplementation resulted in a reduced risk of developing asthma or recurrent wheezing (RR=0.37, 95%CI = 0.17 to 0.8), a reduced risk of developing food allergy (RR=0.28, 95%CI = 0.08 to 1.00), and a similar risk of developing eczema (RR=0.57, 95%CI = 0.3 to 1.08). As with probiotics, the effects of different prebiotics are not equivalent. It remains unclear which prebiotic(s) to use; however, in 15 studies, a mixture of FOS/GOS was used.

In contrast to the above findings, a 2016 double-blind, placebo-controlled RCT found no effect of using a partially hydrolysed whey formula containing FOS/GOS (0.8 g/100 ml) compared
with un-supplemented formula on the risk of eczema in a high-risk population by 12 months (n=863, OR=0.99, 95%CI = 0.71 to 1.37) (Boyle et al., 2016).

**Treatment**

One small RCT (n=29) found that the administration of kestose, a fructo-oligosaccharide, at a daily dose of 2 g for 12 weeks, compared with placebo reduced the SCORAD score at week 6 (25.3 vs 36.4, respectively; \( P=0.004 \)) and week 12 (19.5 vs 37.5, respectively; \( P<0.001 \)). The mechanism as to how kestose improves the symptoms of AD remains unclear (Shibata et al., 2009).

**9.6 Synbiotics**

The term ‘synbiotic’ refers to a product that contains both a probiotic and a prebiotic (Schrezenmeir et al., 2001).

**Prevention**

A 2016 meta-analysis identified 2 RCTs (n=1,320) that evaluated the effects of synbiotics in the prevention of AD. Compared with placebo, the administration of synbiotics had no effect on the risk of AD (RR=0.44, 95%CI = 0.11 to 1.83) (Chang et al., 2016).

**Treatment**

The same meta-analysis identified 6 therapeutic RCTs (369 children enrolled; aged 0 months to 14 years). Compared with placebo, the use of synbiotics significantly reduced SCORAD values at 8 weeks (WMD -6.56, 95%CI = -11.43 to -1.68). Pre-planned subgroup analysis showed that the beneficial effect was significant only when mixed strains of bacteria were used (WMD -7.32, 95%CI = -13.98 to -0.66) and only when synbiotics were used in children aged 1 year or older (WMD -7.37, 95%CI = -14.66 to -0.07).

**9.7 Conclusions**

- Evidence suggests that the gut microbiota play a critical role in the regulation of the immune system and may influence the development of allergic diseases. Still, the role of gut microbiota in allergy has not been fully clarified.
- The understanding of factors modulating gut microbiota early in life may have implications for allergy prevention.
- Modifications of gut microbiota through the administration of probiotics, prebiotics, and synbiotics are employed to prevent and treat allergic diseases.
- According to WAO, currently, there is no evidence to support the administration of probiotics to reduce the risk of allergic diseases. However, there is growing evidence that specific
probiotics may be beneficial for preventing eczema. Further research is needed to clarify which probiotic strain(s) and dosages should be used.

- The WAO suggests using prebiotic supplementation in not-exclusively breastfed infants, both at high and at low risk for developing allergy. However, it remains unclear which prebiotic(s) to use.
- Preventive measures need to be safe. As the optimal composition of the gut microbiota, if one exists, remains unclear, caution is needed in cases of gut microbiota modification that are expected to have long-lasting effects.

9.8 Recommendations for future clinical research

- To optimise infant health, a better understanding is needed regarding what constitutes a healthy gut microbiota that promotes immune tolerance, as well as how effectively gut microbiota modifications influence infant health.
- Considering that the pooled results showed potential benefits of using probiotics/prebiotics for allergy/eczema prevention, further research with adequately powered long-term RCTs is needed to evaluate the effectiveness of specific strains of probiotics and/or prebiotics. Guidance is required regarding the optimal dose, timing, and duration of intervention.
- Novel probiotics/prebiotics for preventing and treating allergy should be studied.

Conflict of interest

The author declares no conflict of interest.

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Microbiota in health and disease: from pregnancy to childhood


H. Szajewska


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H. Szajewska


Chapter 10
The intestinal microbiota and allergic and auto-immune disorders in children

T.G.J. De Meij
VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands; t.demeij@vumc.nl

Abstract

There is increasing evidence that the aetiology of atopic disorders and auto-immune diseases is associated with intestinal dysbiosis. The intestinal microbiota may therefore be considered to harbour tremendous potentials as a diagnostic biomarker of disease, and as a monitor of disease activity. Microbial alterations seem to occur prior to disease onset, strengthening the hypothesis that gut microbiota may play an etiologic role in their development. Furthermore, the onset and the natural course of disease may be influenced by preventive therapeutic strategies manipulating the microbiota, such as dietary advice, prebiotics, probiotics and faecal transplantation. Despite promising results in experimental studies, mainly in animal models, manipulation of the intestinal microbiota in children with atopic disorders and auto-immune diseases is currently disappointing in terms of treatment outcome; it is hampered by insufficient insight in disease-specific microbial signatures. Furthermore, the patterns of microbial dysbiosis in affected study subjects are inconsistent, mainly due to differences in strategies regarding sample harvesting, collection and storage, and microbiota detection techniques. Expanding knowledge may provide opportunities to further explore the options to develop individualised preventive and therapeutic interventions in paediatric atopic and auto-immune diseases, including the use of prebiotics and probiotics and faecal transplantation. In this chapter, an overview is given of the available evidence on the role of intestinal microbiota in aetiology of auto-immune diseases and atopic disorders in childhood. In addition, potential benefits of preventive and therapeutic microbiota-based interventions are discussed.

Keywords: microbiota, atopic disorders, auto-immune diseases, prebiotics, probiotics, faecal transplantation

10.1 Introduction

Many atopic disorders and auto-immune diseases, including inflammatory bowel disease, coeliac disease and type 1 diabetes, have been linked to microbial disturbances occurring in (early) childhood. The intestinal microbiota, therefore, may be considered to harbour tremendous potentials as a diagnostic biomarker of disease and as a monitor of disease activity. Furthermore, the onset and the natural course of disease may be influenced by therapeutic strategies manipulating the microbiota composition. Over the past years, studies are emerging
using microbiota-based interventions in children. In this chapter an overview is given of the role the intestinal microbiota might play in the aetiology of paediatric auto-immune diseases and atopic disorders in childhood. Moreover, it presents the available evidence regarding preventive and therapeutic microbiota-based interventions, including dietary advice, prebiotics, probiotics and faecal transplantation.

10.2 Coeliac disease

Diagnostics

Coeliac disease is an autoimmune, inflammatory disease of the small bowel in genetically susceptible persons, triggered by the ingestion of gluten (Husby et al., 2012). The prevalence of coeliac disease is 1 to 3% in the general population and about 10% among first-degree family members of patients with coeliac disease (Catassi et al., 2014; Myleus et al., 2009). The diagnosis is established with a combination of gluten-dependent symptoms, high antibody levels, the presence of human leukocyte antigen (HLA)-DQ2 or HLA-DQ8, and characteristic histological findings in duodenal biopsies. The latter requires upper endoscopy under general anaesthesia. Current guidelines state that in children with typical clinical symptoms, the combination of high antitransglutaminase type 2 antibody levels (at least 10 times the upper limit of normal) with antiendomysium antibodies and HLA type DQ2 or DQ8, is enough for the diagnosis, thus circumventing the need for endoscopy (Husby et al., 2012). Over 95% of patients have the DQ2 heterodimer, while most of the remaining patients have the HLA-DQ8 heterodimer or half of the DQ2 heterodimer (DQB1*02). On the other hand, up to 40% of the general population have HLA type DQ2 or DQ8, indicating that other environmental factors than gluten are involved as well in the development of coeliac disease (Greco et al., 2002).

Microbiota

Over the past years, the pathogenesis of coeliac disease has increasingly been linked to the gut microbiota, based on observations that neonatal infections and recurrent rotavirus infections are associated with an increased prevalence (Pavone et al., 2007; Stene et al., 2006). Furthermore, two studies found an inverse association between coeliac disease and *Helicobacter pylori* infection (Lasa et al., 2015; Lebwohl et al., 2013). The commensal gut microbiota plays an important role in the maintenance of the intestinal barrier function, by stimulating gut immunity and the proliferation of epithelial cells, and by competing with pathogens (Ashida et al., 2012; Wells et al., 2011). The interaction between gut microbiota and enteric mucosa is mediated by the same epithelial receptors which activate innate immunity, provoking activation of various intracellular cascades. In this interplay Toll-like receptors (TLRs) have an important role; they enable the detection and binding of specific bacterial antigens of commensal microbiota and potential pathogens (Round and Mazmanian, 2009). In coeliac disease, this reaction could also be directed against specific prolamine peptide fragments (Marasco et al., 2016). Another mechanism by which microbial changes may contribute to the pathogenesis of coeliac disease
is the increased transport of dietary gluten peptides to the subepithelial lymphoid tissue as a result of microbiota-induced mucosal barrier impairment (Cenit et al., 2015). Following transmucosal transport, binding of gluten fragments to HLA-DQ2 or HLA-DQ8 molecules may trigger an adaptive immune response involving T helper 1 (T_{H1}), T_{H2} and T_{H17} cells, leading to production of pro-inflammatory cytokines and of coeliac disease related antibodies. In a recent in vivo study, gut bacteria have been described to manipulate immunogenicity of gluten peptide by exhibiting distinct gluten metabolic patterns, thereby modulating the risk of autoimmunity (Caminero et al., 2016).

Several studies have focused on the identification of (a combination of) bacterial species, in duodenal mucosa and faeces, potentially involved in the pathophysiology of coeliac disease. Majority of these studies reported compositional differences, like increased abundance of *Bacteroides* and *Proteobacteria* spp. in affected subjects (Collado et al., 2009; Nadal et al., 2007; Sanchez et al., 2013), while in other studies no significant differences were detected (Cheng et al., 2013; De Meij et al., 2013). The described patterns of microbial dysbiosis in affected subjects vary widely between studies, possibly due to differences in sample harvesting, age of subjects (children versus adults), collection, storage and microbiota detection techniques. As the HLA system influences both commensal and potentially pathogenic bacteria, gut microbiota composition may already be altered prior to the clinical onset of coeliac disease. In healthy infants with the HLA-DQ2 or HLA-DQ8 heterodimer and at least one first-degree relative with coeliac disease, increased abundance of *Firmicutes* and *Proteobacteria* and lower numbers of *Actinobacteria* and *Bifidobacterium* species were observed compared to low-risk children (Olivares et al., 2015).

Microbiota may also influence the phenotype of coeliac disease. Different microbial communities have been observed in patients with predominantly gastrointestinal symptoms, compared to patients with, e.g. dermatitis herpetiformis (Wacklin et al., 2013). Moreover, a subgroup of adult patients with persistent symptoms after at least three years of gluten-free diet had a different microbial signature compared to diet responders. The authors speculated that this specific subgroup might benefit from gut microbiota manipulation with probiotics, antibiotics, or faecal transplantation (Wacklin et al., 2013).

**Microbial management**

The apparent differences in microbiota composition between coeliac disease patients and controls have elicited research on microbiota-targeted strategies for disease onset and course, mainly involving prebiotics and probiotics. Most studies concerned in vitro and animal models, demonstrating that the administration of probiotics, especially *bifidobacteria* and *lactobacilli*, might defer disease onset and improve clinical symptoms in a subset of patients (Marasco et al., 2016). These benefits could result from either immune response modulation, decrease of intestinal permeability, or stimulation of pre-digestion of dietary gluten. However, current evidence regarding the effects of probiotics in adult and paediatric cases with coeliac disease is as yet insufficient to recommend its application in clinical practice. The cornerstone of
treatment in established coeliac disease consists of lifelong adherence to a strict gluten-free diet, it is not yet to be expected that this treatment will significantly change by application of microbiota-based strategies. Since pathogenesis of coeliac disease seems to be associated with microbial disturbance, decrease of disease burden in coeliac could in particular be expected from strategies aimed at prevention/delay of disease onset. Therefore, future studies should focus on development of preventive interventions to modify microbiota-related pathways involved in disease pathogenesis. These studies should preferably include subjects with a positive family history of coeliac diseases and with coeliac disease-associated HLA alleles.

10.3 Inflammatory bowel disease

Inflammatory bowel disease (IBD), with the main subtypes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic relapsing inflammatory condition of the intestinal tract. IBD manifests itself in 7 to 20% of cases already in childhood. Over the last decades, the incidence of paediatric IBD is on the rise, while age at presentation shows a downward trend (Malaty et al., 2010). Flexible endoscopy of upper and lower gastrointestinal tract, with mucosal biopsies for histologic confirmation, remains the gold standard for the initial diagnosis and follow-up management of children with suspected or established paediatric IBD.

Aetiology and microbiota

The aetiology of IBD is considered to be a complex interplay between genetic risk factors, over 160 susceptibility loci have been described (Jostins et al., 2012), and environmental factors, including the intestinal microbiota as important component. The impact of gut microbiota on the development of IBD has been appreciated by observations that germ-free animals develop colitis only after colonisation with gut bacteria, and remain free of colitis when raised in a germ-free environment (Sartor, 2008). In patients with CD, diversion of the faecal stream, thus lowering the burden of intestinal microbiota, resulted in reduction of inflammation in the excluded bowel segments, while relapses occurred after restoration of gut continuity (d’Haens et al., 1998). More recently, studies on probiotics and faecal microbiota transplantation as therapeutic strategies in IBD have substantiated the role of the gut microbiota in IBD aetiology (Wasilewski et al., 2015).

Various pathophysiological mechanisms have been proposed to explain the role of microbiota in IBD pathogenesis. It has been suggested that IBD patients exhibit an excessive response to gut microbiota components. Overgrowth of pathogens may increase mucosal permeability and induce pathogenic immune responses. This stimulates pathogenic innate and T-cell immune responses, provoking an inflammatory cascade that could end in the development of IBD (Abraham and Medzhitov, 2011) (Figure 10.1). Decreased production of secretory IgA also contributes to bacterial overgrowth and could provoke this inflammatory cascade. Ineffective down-regulation leads to overproduction of cytokines by antigen-presenting and epithelial cells, resulting in TH1 and TH17 differentiation and ultimately in inflammation (Shim, 2013). Defective regulatory (T_{reg}) cells cause decreased secretion of interleukin (IL)-10 and transforming
10. Microbiota and allergic and auto-immune disorders

Pediatric IBD, it remains unclear whether microbial changes precede or follow IBD onset.

Figure 10.1. Mechanisms of host defence and tolerance towards intestinal microbes. The intestinal environment modulates cellular differentiation in the immune system to control defence against pathogens and tolerance. (A) Defence mechanisms: intestinal epithelial cells provide a physical barrier between the luminal microbes and the underlying intestinal tissues to control defence and tolerance. Specialised epithelial cells produce a mucus layer and secrete antimicrobial proteins that limit bacterial exposure to the epithelial cells. Production of large amounts of IgA provides additional protection from luminal microbiota. Innate microbial sensing by epithelial cells, dendritic cells (DCs), and macrophages is mediated through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs). Activation of PRRs on innate cells induces various pathways that mediate microbial killing and activate adaptive cells. DCs present antigens to naïve CD4 T cells in secondary lymphoid organs (Peyer’s patches, mesenteric lymph nodes) where factors such as the phenotype of the antigen presenting cells and cytokine milieu modulate differentiation of CD4 T-cell subsets (Th1, Th2, Th17, Treg) with characteristic cytokine and intestinal homing profiles. (B) Tolerance mechanisms: defence mechanisms that limit microbial entry into intestinal tissues also serve as a mechanism of tolerance. Activation of PRRs on the unique populations of macrophages and DCs in the intestinal lamina propria does not result in secretion of proinflammatory cytokines, in contrast to similar activation of systemic innate cells. DC present antigen to T cells in the Peyer’s patches and mesenteric lymph nodes, which can lead to differentiation of Treg populations, regulated by interleukin (IL)-10, transforming growth factor (TGF), and retinoic acid. Thymic stromal lymphopoietin (TSLP) and other factors secreted by epithelial cells in the intestinal environment can contribute to tolerance of intestinal immune cells (Abraham, 2011; Figure is reproduced with permission from Elsevier).

growth factor (TGF)-β, and decreased immune tolerance to bacterial antigens (Aldhous, 2012). These immunologic pathways are not likely to be caused by single pathogens, but rather by a combination of keystone microbes which may disturb the precarious balance of the overall gut microbiota (Hajishengallis et al., 2012). Despite increased knowledge and understanding of interactions between microbiota and intestinal immune system and the etiologic role in paediatric IBD, it remains unclear whether microbial changes precede or follow IBD onset.
10.4 IBD and microbiota composition

Several reports have characterised microbiota composition in faecal samples and colonic biopsies of \textit{de novo} paediatric IBD patients. Shared observations include decreased bacterial diversity, an increased intestinal mucosal-adhesive microbes and alterations of specific bacterial communities. The largest study so far on microbiota profiling in paediatric CD showed that microbial communities from 447 children with \textit{de novo} CD could accurately be differentiated from controls (AUC 0.85) (Gevers et al., 2014). This ‘CD’ microbial signature was best observed in ileal mucosa, but could not be detected from faecal samples. In the same study it was shown that both rectal and ileal mucosa-associated microbiota correlated well with CD phenotype. The most prevalent bacterial communities differentiating affected subjects from controls included \textit{Enterobacteriaceae}, \textit{Pasteurellaceae} (\textit{Haemophilus} spp.), \textit{Veillonellaceae}, \textit{Neisseriaceae} and \textit{Fusobacteriaceae}. Exposure to antibiotics strongly influenced microbiota composition and generally amplified the observed microbial differences (Gevers et al., 2014). In several studies CD-associated dysbiosis was characterised by a decreased abundance of \textit{Faecalibacterium prausnitzii}, which exerts anti-inflammatory effects, and is therefore considered a biomarker of healthy state (Quévrain et al., 2016; Schwertz et al., 2010; Sokol et al., 2008). However, the protective role of \textit{F. prausnitzii} could be debated as a decreased abundance of \textit{F. prausnitzii} is associated with improvement of disease activity in paediatric CD (Gerasimidis et al., 2014; Hansen et al., 2012). Similar to CD, significant microbiota differences have been described between paediatric UC and controls. The magnitude of these differences was larger in severe, corticosteroid-refractory cases, indicating a correlation between disease severity and the extent of microbial alterations (Michail et al., 2012). In a recent study comparing microbial signatures of 26 UC and 36 CD children, CD patients had a significantly increased abundance of \textit{Bacteroides fragilis}, \textit{Clostridium ramosum} and \textit{Eubacterium cylindroides}, while UC was associated with increased levels of \textit{F. prausnitzii} and \textit{Sutterella wadsworthia} (Kolho et al., 2015). In this study, a negative correlation was observed between intestinal inflammation, indicated by faecal calprotectin levels and microbial diversity. The abundance of a specific set of bacteria seemed to be of value to predict the response to anti-tumour necrosis factor (TNF)-\(\alpha\). These findings illustrate the potential of microbiota profiling for early diagnosis and follow-up of disease activity in IBD, and even to predict therapeutic response.

10.5 Microbiota-targeted therapies

The differences in composition of intestinal microbiota might lead to an aberrant immune response to these microbes; consequently, these findings have induced an ever increasing number of studies on the potential of microbiota-targeted therapeutic interventions in IBD, using dietary interventions, probiotics, prebiotics, and faecal microbiota transplantation.
**Exclusive enteral nutrition**

The preferred induction treatment in (European) children with paediatric CD consists of the administration of exclusive enteral nutrition (EEN) for a six to eight week period. EEN has been shown a successful therapeutic strategy in paediatric CD, and although the mechanism of its efficacy has yet to be elucidated, it is clear that it has an extensive impact on gut microbiota composition and faecal metabolic activity (Gerasimidis et al., 2014). Until now, however, it has not been possible to infer a causative association between EEN induced specific microbial alterations and improvement of disease activity. Significant differences in methodology hamper reliable comparison between study results. In the largest study so far exploring associations between the gut microbiota and colonic inflammation during EEN, the microbiota of 23 CD patients had a broader functional capacity compared to healthy controls, while, unexpectedly, microbial diversity decreased during EEN (Quince et al., 2015). Increased knowledge of underlying mechanisms of induction by EEN may allow for improvements in composition and application of EEN, for example in the prevention of relapses. Furthermore, given the possible relationship between changes in microbiota composition and the efficacy of EEN, which carries a high burden on children, knowledge of the microbiota profile at diagnosis could enable the selection of children who might benefit from EEN and those who should receive medication rather than EEN.

**Probiotics**

A variety of probiotic products has been studied in IBD; studies on single agents mainly focused on non-pathogenic *Escherichia coli* Nissle, *Lactobacillus reuteri* ATCC 55730, *Lactobacillus rhamnosus* GG, and *Saccharomyces boulardii*. Studies evaluating the efficacy of multi-species formula mainly used VSL#3, containing four strains of lactobacilli, three strains of bifidobacteria and 1 strain of streptococcus. In UC, a meta-analysis of 12 clinical trials, one of them in children, showed that VSL#3 contributes to the induction and maintenance of remission (Shen et al., 2014). In the paediatric study, 29 de novo UC patients were randomised to either VSL#3 (doses ranging from 450-1,800 billion bacteria per day, depending on weight) or placebo, next to standard induction and maintenance therapy, with a follow up of one year. Children with adjuvant VSL#3 showed significant better remission rates (92.8% with VSL#3, 36.4% treated with placebo) and significantly lower number of relapses (Miele et al., 2009). In another study, including 31 paediatric patients with mild to moderate UC, rectally introduced *L. reuteri* ATCC 55730 lead to endoscopic, histological, and immunologic improvement of disease activity of distal UC (Oliva et al., 2012). Future studies are needed to assess which strains and dosages, could be used in different clinical settings. Adult studies suggest efficacy for VSL#3 in pouchitis, but there is currently no paediatric evidence for this indication. So far, none of the probiotics studied has been shown effective in the treatment of CD and their use for this indication is not recommended in children.
**Prebiotics**

Despite several promising results in animal studies, there is only very limited information on the efficacy of prebiotics in humans with IBD, and no data are available for paediatric IBD. Considering some positive effects in the studies available in adults, it would appear that prebiotics may have potential in IBD treatment, but more evidence is needed (Guandalini, 2014).

**Faecal microbiota transplantation**

Similar to probiotics, faecal microbiota transplantation (FMT) has been considered a relatively novel microbiota-targeted strategy in the therapy for IBD, which, hypothetically, could restore microbial homeostasis. In both adults and children, FMT has been successfully applied in treatment of chronic *Clostridium difficile* infection. Controlled trials are lacking for paediatric IBD, but four small-scaled observational studies, including 17 children with UC and 9 with CD, have shown that FMT seems to be well tolerated in active paediatric IBD (Kellermayer *et al.*, 2015; Kunde *et al.*, 2013; Suskind *et al.*, 2015a,b). However, the reports on efficacy of FMT are contradictory. In one study with 10 children with mild-to-moderate UC, disease activity scores significantly improved after FMT (Kunde *et al.*, 2013), while in another study with four children with moderate UC, no clinical and biochemical benefit was observed (Suskind *et al.*, 2015a). Obviously, randomised controlled trials are necessary to assess the value of FMT in paediatric IBD. Future, preferably longitudinal, studies should focus on the role of the microbiota in provoking disease onset and to determine whether microbiota composition may predict risk of flares and response to therapy. A multifaceted approach should include assessment of microbial functional activity and immune responses. Increased knowledge on host-microbe interactions may lead to development of novel diagnostic and therapeutic approaches in IBD therapy.

**10.6 Type 1 diabetes**

Type 1 diabetes (T1D) is an autoimmune disease caused by cell-mediated destruction of insulin-secreting pancreatic beta cells, leading to deficient insulin production (Eisenbarth, 1986). T1D incidence in Europe increases about 3-4% per year with the steepest increase observed in children below five years of age (Harjutsalo *et al.*, 2008). T1D is the most common type of diabetes in children and adolescents, although type 2 diabetes is increasingly diagnosed in this age group.

**Aetiology**

T1D is considered to result from a combination of genetic predisposition and, largely unknown, environmental factors (Bluestone *et al.*, 2010). The rising incidence of T1D has coincided with an increase in socioeconomic and hygienic standards and, therefore, aetiology has been linked to changes in microbiota colonisation. Day care attendance, having siblings and sharing bedrooms, and indoor dog exposure during the first year of life have all been associated with a decreased
risk of T1D (Cardwell et al., 2008; D’Angeli et al., 2010; Virtanen et al., 2014), while frequent exposure to antibiotics in early life seems to increase the risk for T1D (Kilkkinen et al., 2006). Although the underlying mechanisms remain largely unclear, it is suggested that microbiota alterations in pre-T1D subjects may induce increased intestinal permeability, resulting in an aberrant immune response leading to destruction of pancreatic beta cells (Vaarala et al., 2008). Furthermore, T1D children also have an altered intestinal microbiota from a functional point of view; affected subjects have a higher abundance of butyrate-producing and mucin-degrading bacteria (Brown et al., 2011). Microbiota-induced butyrate production seems to be involved in regulation of intestinal permeability. Butyrate stimulates function of tight junctions and consequently protects against autoimmunity including development of T1D. Several studies have described microbiota composition in children with T1D (De Goffau et al., 2013; Giongo et al., 2011; Mejía-León et al., 2014; Murri et al., 2013; Soyucen et al., 2013). A shared observation in affected patients was reduced microbial diversity, dominance for Bacteroides and reduced Firmicutes, compared to controls. However, a T1D-specific microbial signature has not been identified so far.

**Microbiota and therapy**

Despite the increasingly recognised role of the microbiota in T1D aetiology, robust data on microbiota-based interventions in humans aiming at manipulation of disease onset and course, is lacking so far. In young T1D-prone rodents, experimental microbiota manipulation provided protection from islet autoimmunity, suggesting that microbiota-based therapeutic strategies have potential as preventive intervention in individuals with increased genetic risk (Dunne et al., 2014; Markle et al., 2013). Focus of future studies should be on the functionality of microbial communities associated with T1D risk and prevention, including analyses of the host- and microbiota related metabolome. Unravelment of functional pathways involved in T1D onset may allow for development of rationale-based microbiota-related therapeutic strategies to manipulate T1D onset and its course (Dunne et al., 2014).

**10.7 Atopic disorders**

**Allergic march**

Atopy is a genetically, usually IgE-mediated predisposition to develop allergic hypersensitivity reactions, including asthma, eczema, and allergic rhinoconjunctivitis. From early infancy to childhood, a typical succession of atopic phenotypes has been described, called the allergic march (Spergel, 2010). This term concerns the predominance in early childhood of atopic dermatitis and concomitant sensitisation to food allergens and aeroallergens, moving towards the focus of this section; the development of asthma and allergic rhinitis later in childhood.
Aetiology and microbiota

Recent epidemiological and experimental studies have supported the hypothesis that, next to genetic factors, microbial stimulation of the immune system alters the development of tolerance to allergens (Legatzki et al., 2014). In particular the intestinal microbiota is considered to play a pivotal role because of its involvement in maintenance of the precarious balance between activation of $T_H1$ and $T_H2$ cells, essential for the development of oral tolerance. Improper exposure to commensals in infancy may disturb this balance and provoke a $T_H2$-predominated response and consequently the production of cytokines IL-4 and IL-5 (Muir et al., 2016). The intestinal microbiota may also manipulate epithelial TLRs signalling pathways, which are involved in detection of intraluminal bacterial communities. Genetic variations, such as mutations in TLR2 and TLR4 co-receptor CD14, may predispose for the development of allergic reactions through inducing an aberrant immune response (Muir et al., 2016). There are several experimental studies suggesting that gut microbial colonisation, next to local influences, also has systemic immune-regulatory effects (Arnold et al., 2011; Olszak et al., 2012; Zhang et al., 2012). These studies, mostly in mouse models, indicate the existence of a gut-lung axis mediated by the immune system, although the exact mechanism by which gut microbes induce experimental allergic asthma has to be elucidated (Olszak et al., 2012; Trompette et al., 2014).

Microbiota and environmental factors

Atopic diseases have reached epidemic proportions during the past decades, predominantly in westernised countries. This steep rise in incidence may, at least partly, be explained by the hygiene hypothesis, indicating that excessive cleanliness of an infant’s environment reduces the number of infectious stimuli, negatively influencing development of the immune system. Several studies have illustrated the influence of specific extrinsic factors, such as way of delivery, diet, exposure to antibiotics, exposure to animals, and surrounding environment, on the risk of atopic disorders.

In several studies, caesarean section has been associated with an increased risk for asthma and allergic rhinitis, through disturbance of intestinal colonisation, mainly reflected by decreased gut microbiota diversity in the first two years of life, delayed Bacteroidetes colonisation, and reduced serum $T_H1$ chemokine levels (Jakobsson et al., 2014; Pistiner et al., 2008; Thavagnanam et al., 2008). However, an association between birth by caesarean section and occurrence of allergic symptoms in children aged 1-4 years was not observed in another study (Pyrhonen et al., 2013).

Neonatal feeding patterns have unambiguously been shown to influence early microbiota composition. Breastfeeding has been described to protect against early childhood wheezing and eczema, but there is no evidence that it protects against the development of food or respiratory allergy and allergic dermatitis in childhood (Prince et al., 2015; Snijders et al., 2006). Since the increased prevalence of asthma over the last decades coincided with a sharp increase in antibiotic use, a possible causal relationship was considered. In several studies the association between the use of broad-spectrum antibiotics early life and development of atopic disorders...
and asthma in childhood has been evaluated. Although many studies reported a positive association, conclusions are inconsistent and current evidence to support the relationship is weak (Kuo et al., 2013). It has been suggested that observed associations between early use of antibiotics and development of asthma in childhood in most of these studies might be attributed to confounding factors, such as respiratory tract infections (Lapin et al., 2014). Studies investigating the association between exposure early in life to farm animals, cats and dogs, and development of asthma and allergic rhinoconjunctivitis later in childhood, reported controversial results. In a recent nationwide cohort study in Sweden exposure to dogs and farm animals during the first year of life seemed to reduce the risk for asthma in children at age 6 years (Fall et al., 2016). Opposite findings were reported in a world-wide epidemiological study, including 206,332 children aged 6-7 years and 329,494 adolescents aged 13-14 years. Here, a positive relationship was found between the presence of a cat at home in the first year of life and symptoms of asthma, allergic rhinoconjunctivitis, and eczema at age 6-7 years. A similar positive association was found between these symptoms in adulthood and exposure at that time to dogs, and to both cats and dogs (Brunekreef et al., 2012a). Furthermore, exposure to farm animals during pregnancy but also in the first year of life increased the risk of symptoms of asthma, rhinoconjunctivitis and eczema in children aged 6-7 years, but only in those living in non-affluent countries (Brunekreef et al., 2012b).

**Microbiota composition in atopy**

Several studies have characterised microbial signatures in atopy. Reduced abundance of the genera *Faecalibacterium*, *Lachnospira*, *Veillonella* and *Rothia* within the first 100 days of life has been linked to the development of allergic diseases (Arrieta et al., 2015), while absence of *Bifidobacterium* in neonatal faeces seemed to increase the risk of atopy within the first five years of life (Sjogren et al., 2009). In previous studies, using culture-based techniques, high abundance of *E. coli*, *C. difficile* was associated with allergic disorders in childhood (Kalliomaki et al., 2001; Penders et al., 2006). In summary, these studies suggest that disturbance of neonatal colonisation may lay the foundation for the development of allergic disease and asthma later in childhood.

**Microbiota manipulation**

Numerous prebiotics and probiotics have been tested to manipulate intestinal microbiota in children at risk for atopic diseases. Although supplementation with probiotics seems to have potential as preventive strategy, the available literature remains inconclusive. Therefore, no evidence-based recommendations on its use in childhood can be given at this point (for detailed information see Chapters 9 (Szajewska, 2017) and 16 (Vandenplas and Huysentruyt, 2017)). Future studies using different probiotic mixtures aiming at manipulation of onset and natural course of atopic diseases in childhood are needed to develop evidence-based guidelines on microbiota modifiers (Legatzki et al., 2014). In addition, several environmental factors (e.g. way of delivery, dietary habits, use of antibiotics and presence of pets) have been linked to microbial imbalance and could play a role in the aetiology of atopic diseases. However, more knowledge...
on the association between these environmental factors and development of atopic disease is needed. This could help to develop recommendations for evidence-based life-style interventions in the future.

Conflict of interest

The author declares that there is no conflict of interest regarding the publication of this chapter.

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10. Microbiota and allergic and auto-immune disorders


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10. Microbiota and allergic and auto-immune disorders


Chapter 11
Intestinal microbiota and its role in the development of paediatric gastrointestinal disorders

M.A. Benninga1*, M. Vink2 and L.M.A. Akkermans3,4
1Department of Paediatric Gastroenterology and Nutrition, Emma Children’s Hospital/Academic Medical Centre, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands; 2Amsterdam University College (AUC), Science Park 113, 1098 XG Amsterdam, the Netherlands; 3Amsterdam University College (AMC), Science Park 113, 1098 XG Amsterdam, the Netherlands; 4Prof. Em., University Medical Centre Utrecht, Department of Surgery, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands; m.a.benninga@amc.uva.nl

Abstract

The human gut microbiota play an essential role in health and disease because of its influence on metabolism, digestion, nutrient absorption, immune system modulation and prevention of enteric pathogen colonisation. In this chapter, the role and interaction of the gut microbiota and probiotics in infectious diseases, functional gastrointestinal disorders, such as functional abdominal pain (FAP) and functional constipation (FC), and obesity amongst children are discussed. Infectious diseases caused by Escherichia coli, Clostridium difficile, Helicobacter pylori, and Shigella, pose a great disease burden in both developed and underdeveloped countries. The administration of probiotics, either as single treatment or as an adjuvant agent, provides significant benefits, yet, additional studies determining optimal dose and strains per disease are required. The role of gut microbiota in FAP is less clear, although alterations of the gut microbiota are suggested to negatively influence symptom generation. Probiotics appear to improve treatment success, mainly in children with irritable bowel syndrome. Dysbiosis of the gut is assumed to play a role in FC as well. Current evidence, however is insufficient to support the use of specific strains as a treatment in children with constipation. Children with obesity have a distinct gut microbial composition in comparison to lean children. Studies that specifically look at the effect of perinatal probiotics on childhood overweight and obesity are rather disappointing with regard to long-term effectiveness. The positive short-term effects on gut microbiota modulation and consequently weight of perinatal probiotic supplementation, however, indicate that it is relevant to examine the effects of continuous supplementation. Probiotics are suggested to be beneficial for infectious diseases, functional gastrointestinal disorders, and obesity. There is, however, a definite need to determine which species, specific strains and combinations of strains of probiotics are most efficacious.

Keywords: children, gastrointestinal infections, obesity, abdominal pain, constipation, prebiotics, probiotics
11.1 Introduction

Composition and physiological temporal variations of the human gut microbiota play an essential role in health and disease as it plays a critical role in metabolism, digestion, nutrient absorption, as well as in immune system modulation and prevention of enteric pathogen colonisation (Sonnenburg et al., 2004). Disease phenotypes are a result of complex interactions between bacteria, viruses, and eukaryotes (Clemente et al., 2012).

Until recently, it has been assumed that at the age of approximately 3 years, the gut microbiota composition has converged towards a relatively stable, adult-like pattern (Yatsunenko et al., 2012) However, various recent studies demonstrate substantial compositional differences between children of different ages and adults suggesting that children cannot be regarded as miniature adults (Agans et al., 2011). A recent study on the short-term and long-term stability of the intestinal microbiota, in healthy children from 12 to 18 years of age, showed that the stability is an individual characteristic varying per phylum at both short-term and long-term intervals (De Meij et al., 2016). Moreover, age-related dietary and physiological factors can alter the capacity of the gut which can lead to an increased susceptibility to gastrointestinal infections (Hopkins et al., 2001). For example, children between the age of 16 months and 7 years appear to have a significant greater proportion of Enterobacteria compared to adults between the age of 21 and 34 years (Hopkins et al., 2001). Apart from age-dependent differences in microbiota composition and susceptibility for infections, it was recently also suggested that there exist sex-dependent differences (Singh and Manning, 2016). Examination of how immunological factors cause divergent manifestations of infections in children, as compared with adults, may provide new insights for therapeutic modification or prevention of acute and/or persistent infections and its complications (Harris et al., 2013).

In this chapter the role and interaction of the gut microbiota and probiotics in infectious diseases, functional gastrointestinal disorders, such as functional abdominal pain and functional constipation, and obesity will be discussed.

11.2 Intestinal microbiota and gastrointestinal infections in children (2-12 years)

Escherichia coli infection

Enterotoxigenic Escherichia coli (EREC) is a major cause of diarrhoea in infants and in travellers from developed countries to underdeveloped countries, especially in regions of poor sanitation. In adults, it may not always be necessary to consider antidiarrheal treatment because of the self-limiting character of the disease. For infants and toddlers an early, evidence-based treatment is important to improve the quality of life (both of the infant and the parents) and may even save lives (Henker et al., 2008) There is accumulating evidence that administrating probiotics can prevent or cure some forms of enterotoxigenic E. coli diarrhoea (Davodabadi et al., 2015; Henker
et al., 2008; Szajewska et al., 2001). A recent study on individual-specific changes in human gut microbiota after challenge with enterotoxigenic *E. coli* and subsequent ciprofloxacin treatment provides the first indication of microbial taxa that may prevent the colonisation of the human intestinal tract by enterotoxigenic *E. coli* (Pop et al., 2016)

**Clostridium difficile infection**

*Clostridium difficile* is a Gram-positive, anaerobic spore-forming bacillus, which can exist as both toxigenic and non-toxigenic forms. It is an important nosocomial pathogen in adults and it appears to be increasingly prevalent in paediatric patients. Asymptomatic carriage in healthy new-borns of up to 70% has been reported. In these children, there is a decreasing trend in carriage rate with increasing age: with colonisation falling to adult levels of around 5% by the age of 2 years (Lees et al., 2016). The exact role of *C. difficile* in the paediatric gut remains unclear. Further investigations on the serological and local host responses to *C. difficile* carriage may shed light on disease mechanism (Lees et al., 2016). Concern about *C. difficile* disease exists for children with higher rates of infections and specific groups of children with haematological malignancies, inflammatory bowel disease and cystic fibrosis following lung transplantation (Enoch et al., 2011). The significance of the relative presence of *C. difficile* in children on the microbiota in later life has yet to be determined (Lees et al., 2016). Defining *C. difficile* disease in paediatric infections is complicated due to the lack of a standardised scoring system, making it more difficult to quantify disease burden and treat infected children. In children over 3 years of age it is advised that testing should be performed in the same criteria circumstances as it would be in adults, i.e. acute diarrhoea and recent history of antibiotic use (Schutze and Willoughby et al., 2013).

Given the consensus that children who do have *C. difficile* infection run a much milder disease than adults, it is appropriate to tailor treatment as such, with the first steps being supportive care (rehydration) and discontinuation of unnecessary antibiotics, or at least narrowing spectrum and reviewing course length, prior to considering active treatment with metronidazole/vancomycin (Lees et al., 2016). Recent literature proposed that probiotics are effective in children and adults at preventing *C. difficile*-associated diarrhoea, however, additional studies are required to determine the optimal dose and strains of probiotics (Lau and Chaimberlain, 2016). There is also increasing evidence that faecal microbiota transplant using healthy, related screened donor stool to treat recurrent *C. difficile* infections had lasting clinical improvement of gastrointestinal symptoms during and after treatment with antibiotics in children (Russell et al., 2014).

**Helicobacter pylori infection**

*Helicobacter pylori* colonises the human stomach, typically during early childhood, and persists for decades, if not for lifetime in the host (Salama et al., 2013). Not only in low-income but also in developed counties *H. pylori* is acquired predominantly in early childhood before 5 years of age. *H. pylori* infections cause chronic gastritis, which is asymptomatic in the majority of carriers
in children. Nonetheless, it is considered a major risk factor for the development of gastric and duodenal ulcers and gastric malignancies. There exists a reduced gastric inflammation in *H. pylori*-infected children compared with infected adults and this is strongly associated with enhanced mucosal regulatory T cells (T\(_{\text{reg}}\)) activity in children (Harris *et al.*, 2013).

During *H. pylori* infection, the relative abundance of bacterial communities in the human stomach appears to change, reflected in the increase in non-*Helicobacter* Proteobacteria, *Spirochetes*, and *Acinetobacter* accompanied by a decrease in *Acinobacteria*, *Bacteroidetes*, and Firmicutes. However, the impact of these changes in microbiota on the pathogenesis of *H. pylori*-triggered inflammation in children and adults is not known (Harris *et al.*, 2013; Maldonado-Contreras *et al.*, 2011). Recent studies show that probiotics could play a beneficial role in the management of *H. pylori* infection. For example, ingestion of probiotics may suppress the *H. pylori* load and may modify immune response and intestinal microbiota in infected children. However, at this moment it cannot be recommended as a single therapeutic agent (Francavilla *et al.*, 2014). Yet, there is agreement in the literature that probiotics may improve eradication rates and may reduce treatment-associated side effects when added to standard treatment (Emara *et al.*, 2015).

**Shigellosis**

Among the various enteric pathogens, *Shigella* spp. are some of the most common and deadly bacterial pathogens in the world, not only in low-income but also in developed countries, especially in travellers to less industrialised countries. Antibiotics can be used to treat shigellosis, however resistance has emerged (Gu *et al.*, 2012). Therefore, alternative approaches for reducing the incidence and severity of shigellosis are urgently needed. To date, the management of acute gastroenteritis has been based on the option of ‘doing least’: oral rehydration-solution administration, early feeding, no testing, and no unnecessary drugs. The European Society of Paediatric Infectious Diseases guidelines make strong recommendations for use of probiotics in the management of acute gastroenteritis, particularly those with documented efficacy such as *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri*, and *Saccharomyces boulardii* (Ciccarelli *et al.*, 2013).

### 11.3 Functional abdominal pain disorders in children

Children with ‘functional abdominal pain disorders (FAPs),’ diagnosed according to Rome IV criteria, have chronic or recurrent abdominal pain, which after appropriate medical evaluation cannot be attributed to another medical condition (Hyams *et al.*, 2016). FAPs affect approximately 20% of children worldwide and include functional dyspepsia (FD), irritable bowel syndrome (IBS), abdominal migraine (AM) and functional abdominal pain not otherwise specified (NOS) (Table 11.1, Figure 11.1) (Korterink *et al.*, 2015a). These disorders have great impact on patients’ quality of life, daily activities and school absenteeism and can have long-term psychological consequences (Chiou and Nurko, 2010). Standard medical care may consist of reassurance, education, dietary, pharmacologic, psychosocial, and complementary/
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Table 11.1 Rome IV criteria for functional abdominal pain disorders (Hyams et al., 2016).

**Functional dyspepsia**

Must include 1 or more of the following bothersome symptoms at least 4 days per month for at least 2 months:
1. Postprandial fullness.
2. Early satiation.
3. Epigastric pain or burning not associated with defecation.
4. After appropriate evaluation, the symptoms cannot be fully explained by another medical condition.
Within FD, the following subtypes are adopted:
1. Postprandial distress syndrome includes bothersome postprandial fullness or early satiation that prevents finishing a regular meal. Supportive features include upper abdominal bloating, postprandial nausea, or excessive belching.
2. Epigastric pain syndrome, which includes all of the following: bothersome (severe enough to interfere with normal activities) pain or burning localised to the epigastrium. The pain is not generalised or localised to other abdominal or chest regions and is not relieved by defecation or passage of flatus. Supportive criteria can include (a) burning quality of the pain but without a retrosternal component and (b) the pain commonly induced or relieved by ingestion of a meal but may occur while fasting.

**Irritable bowel syndrome**

Must include all of the following for at least 2 months, for at least 6 months before diagnosis:
1. Abdominal pain at least 4 days per month associated with one or more of the following:
   a. related to defecation;
   b. a change in frequency of stool;
   c. a change in form (appearance) of stool.
2. In children with constipation, the pain does not resolve with resolution of the constipation (children in whom the pain resolves have functional constipation, not irritable bowel syndrome).
3. After appropriate evaluation, the symptoms cannot be fully explained by another medical condition.

**Abdominal migraine**

1. Paroxysmal episodes of intense, acute periumbilical, midline or diffuse abdominal pain lasting 1 hour or more (should be the most severe and distressing symptom).
2. Episodes are separated by weeks to months.
3. The pain is incapacitating and interferes with normal activities.
4. Stereotypical pattern and symptoms in the individual patient.
5. The pain is associated with 2 or more of the following:
   a. anorexia;
   b. nausea;
   c. vomiting;
   d. headache;
   e. photophobia;
   f. pallor.
6. After appropriate evaluation, the symptoms cannot be fully explained by another medical condition.
alternative medicine interventions (Korterink et al., 2015b). Despite ongoing efforts to identify causal and contributing factors in FAPs, successful management is complicated by incomplete pathophysiological understanding. Altered gut motility, visceral hypersensitivity, abnormal brain-gut interaction, psychosocial disturbance and immune activation have been suggested as possible explanation for the symptoms (Figure 11.2; Koloski et al., 2012; Korterink et al., 2015a; Simrén et al., 2013). Moreover, psychological symptoms, low socioeconomic status, parental gastrointestinal complaints and single parent and immigrant households are associated with chronic abdominal pain in children (Chitkara et al., 2005; Hotopf et al., 1998; Hyams et al., 1996).

**Microbiome and FAPs**

Recent insights generated by non-culture based analysis of the intestinal microbiota, indicate that the composition of intestinal microbiota may be of importance in the pathogenesis of FAPs, especially IBS. Changes in the microbiome may contribute to symptoms in these patients through the interaction with host factors, such as age, diet, transit and genetic constitution. These interactions may be related to alterations in gut neuromotor-sensory function, barrier function of the gut and/or the brain-gut-axis (Ohman and Simren, 2013).
Indeed it has been noted that children with IBS had a greater proportion of the phylum *Proteobacteria*, and genera such as *Dorea* (a member of *Firmicutes*) and *Haemophilus* (a member of *Proteobacteria*); in addition, it was also noted that species such as *H. parainfluenzae* and *Ruminococcus* were more abundant and *Bacteroides* were markedly less abundant in children with IBS than healthy individuals as controls (Saulnier *et al.*, 2011). More evidence that gut microbiota may play a role in the pathogenesis of FAPs is supported by the fact that in a subset of IBS and FD-patients, both in children and adults, onset of symptoms follows an enteric infection (post-infectious IBS) (Futagami *et al.*, 2015; Saps *et al.*, 2008; Schwille-Kiuntke *et al.*, 2015). A study comparing the faecal microbiota of healthy children and paediatric patients with diarrhoea-predominant IBS noted that levels of *Veillonella, Prevotella, Lactobacillus* and *Parasporobacterium* were increased in patients with IBS, whereas a reduction in levels of *Bifidobacterium* and *Verrucomicrobi um* was reported (Rigsbee *et al.*, 2012). Although further studies are needed to clarify and clearly identify the exact changes in the gut microbiota of

Figure 11.2. Mechanisms underlying the irritable bowel syndrome (IBS) (Korterink *et al.*, 2015).
children with FAPs, these research efforts provide some insight to the possibility of alteration of the microbiota leading to symptom generation.

Treatment

Few high-quality randomised clinical trials (RCTs) evaluating the effect of probiotics in children with FAPs are available. A meta-analysis of five paediatric RCTs reported a significantly higher treatment success of *L. rhamnosus* GG, *L. reuteri* DSM 17938 and VSL#3 compared with placebo (pooled RR 1.50; 95% CI 1.22-1.84). Subgroup analysis showed results being mainly applicable for IBS (pooled RR 1.62; 95% CI 1.27-2.06) (Korterink et al., 2014). Another study evaluating the effect of *L. reuteri* DSM 17938, compared with placebo, confirmed earlier findings and reported significantly reduced frequency and intensity of abdominal pain in 101 children with FAPs (Weizman et al., 2016). More recently, a study in children with IBS, showed that a mixture of probiotics, *Bifidobacterium infantis* M-631, *Bifidobacterium breve* M-16V1 and *Bifidobacterium longum* BB5361 or is safe and is associated with better AP control and improved quality of life when compared to placebo (Giannetti et al., 2016).

11.4 Functional constipation in childhood

Functional constipation (FC) is a common paediatric healthcare problem worldwide, with reported prevalence ranging between 0.7% and 29.6% and a mean female-to-male ratio of 2.1:1 (Mugie et al., 2011a). FC is characterised by infrequent bowel movements, hard and/or large stools, painful defecation, faecal incontinence, and is often accompanied by abdominal pain (Tabbers et al., 2014). These symptoms can have a significant impact on a child’s well-being and health-related quality of life (Wald et al., 2011). It is estimated that constipation in children accounts for 3% of visits to a general paediatrician and up to 25% of visits to a paediatric gastroenterologist in the USA (Tabbers et al., 2014). Healthcare costs for children with constipation are higher than those for control subjects, mostly because of ambulatory care costs and, to a lesser degree, costs related to hospitalisations and emergency room visits (Choung et al., 2011). To define FC and other functional defecation disorders in children, the Rome IV criteria were recently updated in 2016 (Benninga et al., 2016; Hyams et al., 2016). The Rome IV criteria for FC in children differentiate between children up to 4 years of age and children aged >4 years (Table 11.2).

Pathophysiology

In approximately 95% of children with constipation, no organic cause can be identified (Tabbers et al., 2014). In the remainder of cases, constipation has an organic cause, such as a metabolic or endocrine disorder, anorectal anomalies, neuromuscular diseases, or Hirschsprung’s disease. The pathophysiology of FC is still incompletely understood but is likely to be multifactorial. One important etiological factor, especially in young children, is withholding behaviour, frequently occurring after a negative experience – e.g. a hard, painful, and/or frightening bowel movement
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Functional constipation often has a negative impact on children’s lives. Stool-withholding behaviour can lead to faecal impaction – the presence of a large faecal mass in either the rectum or the abdomen. As a result, faecal impaction often leads to overflow faecal incontinence, which is involuntary loss of soft stools that pass an obstructing faecal mass. Psychosocial factors, such as major life events, may play an important role in the pathophysiology of FC as well. Behavioural disorders, such as autism spectrum disorders and attention deficit/hyperactivity disorder, are associated with a higher risk of childhood constipation (McKeown et al., 2013; Peeters et al., 2013). Lastly, factors such as socioeconomic status, educational level, sexual and physical abuse and parental child-rearing attitudes have been designated as influencing factors in the pathophysiology of FC in children (Van Dijk et al., 2014).

11.5 Gut microbiota

The development of new culture-independent techniques has enabled the identification of bacterial species that were previously not detected by culture-based methods, leading to the definition of the human microbiome (Johnson et al., 2012). The gut harbours the vast majority of our resident microbes and the gut microbiota has therefore been a subject of many metagenomics
studies. A recent study in 61 healthy Dutch children, 2 to 18 years of age, showed that the microbial composition stability is individualised and varied per phylum at both short-term and long-term intervals (De Meij et al., 2016). This was consistent throughout all age cohorts of youth. Diversity and stability for all bacterial phyla were correlated: diversity of the phylum *Bacteroidetes* was highest and this phylum showed the highest stability compared with the phyla *Proteobacteria* and FAFV (*Firmicutes, Actinobacteria, Fusobacteria* and *Verrucomicrobia*). The presence of a shared microbial core, consisting of a limited number of species, was present in the majority of healthy children (De Meij et al., 2016).

Gut microbiota changes have been found in a wide variety of diseases, including gastrointestinal and non-gastrointestinal diseases (Johnson et al., 2012). Dysbiosis, defined as a state of disordered microbial ecology that causes disease, has been assumed to play a role in functional constipation as well. Studies, however regarding the intestinal microbiota composition in chronic constipation are scarce. In children, only one study, using conventional culturing techniques, has compared gut microbiota of children with functional constipation with those of healthy controls (Zoppi et al., 1998). In this study, a statistically significant increase in colonic clostridia and bifidobacteria strains were observed as compared to controls. However, since the vast majority of bacterial gut communities is not cultivable, this method is inadequate for monitoring complex intestinal microbiota communities. A more recent study assessed gut microbiota in obese children, 8 with constipation and 14 non-constipated controls. This study showed a significant decrease in abundance of the *Prevotella* genus (phylum: *Bacteroidetes*) and an increased representation of several genera of *Firmicutes* in constipated patients compared with controls (Zhu et al., 2014). One of the possible mechanisms in which the gut microbiota is considered to influence gut motility is by the production of methane as a consequence of anaerobic fermentation of carbohydrates and the production of hydrogen in the gut (Triantafyllou et al., 2014). There is strong evidence from animal studies that methane delays intestinal transit, possibly acting as a neuromuscular transmitter, and methane production has been associated with constipation in adults (Triantafyllou et al., 2014).

**Diagnosis and treatment**

Evaluation of childhood constipation is primarily based on a detailed medical history and a thorough physical examination (Tabbers et al., 2014). In general, laboratory investigation of constipated children rarely uncovers an underlying disease, such as hypothyroidism or celiac disease. Treatment of childhood constipation consists of a four-step approach involving education (including information about the prevalence and pathophysiology of constipation and the necessity of long-term treatment), disimpaction, prevention of re-accumulation of faeces and behavioural therapy. Long-term follow-up also seems to be critically important for treatment success (Tabbers et al., 2014).
Probiotics

It has been suggested that children with constipation may benefit from probiotics, prebiotics and/or synbiotics. The use of probiotics in the treatment of constipation has been investigated in both children and adults, however the studies are highly heterogeneous, using different probiotic agents, definitions and outcome measures. Therefore, caution is warranted when interpreting these results. In adults, a recently published systematic review cautiously suggested a possible beneficial effect of probiotics on whole gut transit time, stool frequency, and stool consistency (Dimidi et al., 2014). The most commonly studied bacterial species were Bifidobacterium lactis and Lactobacillus casei Shirota. For B. lactis a statistically significant effect on stool frequency and stool consistency was found, whereas this was not the case for L. casei Shirota. In children, few randomised control trials (RCT) on the use of probiotics in constipation have been published and the results from these studies are difficult to interpret due to significant heterogeneity. A recently published systematic review reported that probiotic species used in paediatric studies for the treatment of constipation include L. rhamnosus, L. reuteri, Lactobacillus casei, Bifidobacterium lactis and B. longum (Tabbers, 2014). This systematic review concluded that current evidence is insufficient to support the use of probiotics in the treatment of constipation in children. Adequately powered, detailed studies are still required to provide evidence for probiotic efficacy in treating constipation and to detail the specific properties of different species and strains so that a more tailored approach in the use of probiotics for constipation can be pursued.

11.6 Dysbiosis of the gut and childhood obesity (2-12 years)

Aetiology/pathology

The fundamental cause of obesity is an energy imbalance in calorie consumption and energy expenditure (WHO, 2015). There is a growing body of literature that recognises the importance of the gut microbiota in the pathogenesis of obesity because of its ability to influence insulin regulation of the host, for example via short chain fatty acid (SCFA) production (Vrieze et al., 2010).

Although no concise composition of an obesogenic gut microbiota profile can be determined, comparative research indicates that the gut microbiota of obese children is distinct from normal weight children. Several studies have compared the gut microbial composition of obese and lean children between the ages of 2-12. A Swedish study evaluated the bacterial composition of overweight/obese and lean children aged 4-5, and found a different overall composition between both categories (Karlsson et al., 2012). A similar study counted the gut bacteria in 51 children aged 5, and showed no significant difference. However, anaerobic gram-positive bacteria, including Bifidobacterium and Eubacteria, were correlated with BMI when adjusted for sex and age (Sepp et al., 2013). In contrast, a study following 49 children up to seven years old showed a higher abundance of Bifidobacterium in children who remained normal weight (Kalliomäki et al., 2008). A study in children and adolescents aged 6-16 indicated a significant link between
the abundance of *Lactobacillus* species and *Bifidobacterium fragilis* and obesity (Ignacio *et al.*, 2016). Payne *et al.* (2011) compared the gut microbiota of obese children and children of normal weight aged 8-14 and found, however, no significant quantitative differences in gut microbiota communities, which might be due to the high degree of variation between the groups and within the groups (Payne *et al.*, 2011). Furthermore, they did not control for dietary influences. A cross-sectional study evaluating the faecal samples of obese and overweight children and lean children aged 6-16 did find a significant elevated level of *Firmicutes:Bacteroidetes* ratio in obese children. More specifically, they found relatively low proportions of *Bacteroides vulgatus* and high concentrations of *Lactobacillus* species in obese children. *Staphylococcus* species were furthermore positively associated with energy intake (Bervoets *et al.*, 2013). Likewise, a study in Kazakhstan showed a higher *Firmicutes:Bacteroidetes* ratio in obese children aged 7-13. This finding was significant amongst girls, but not in boys when gender-stratified (Xu *et al.*, 2012). A study by Balamurugan *et al.* (2010) found no significant difference in the gut microbial count of *Bacteroides-Prevotella*, *Bifidobacterium*, *Lactobacillus acidophilus* group or *Eubacterium rectale* in 28 obese and lean Indian children aged 11-14. They did, however, find a significantly higher amount of *Faecalibacterium prausnitzii* bacteria (Balamurugan *et al.*, 2010).

To date, few studies have measured gut microbiota composition in infants and evaluated weight gain over time. Ollberding *et al.* (2015) found that a higher abundance of *Firmicutes* during infancy is associated with an increased weight-for-length in early life (Ollberding *et al.*, 2015). The KOALA cohort study assessed the weight of children for 10 years after analysing their gut microbial composition at age 1, and found that a higher prevalence of *B. fragilis* was linked to weight gain (Scheepers *et al.*, 2015). Evidently, findings still show great inconsistencies. This highlights a definite need for more longitudinal research to elucidate the effect of the infant gut microbiota and specific species on the development of childhood obesity.

**Diagnostics**

Obesity is defined as an abnormal or excessive fat accumulation with potential negative health effects (WHO, 2015). Adult overweight and obesity are measured by Body Mass Index (BMI): the weight in kilograms divided by the square height in meters (kg/m²), with a BMI≥30 indicating obesity (WHO, 2015). Measuring childhood overweight and obesity is more complicated, because children and adolescents undergo numerous physiological changes as they constantly grow. For children aged 2-12, BMI is calculated and expressed as a percentile, which can be obtained from a sex- and age-adjusted graph. A BMI≥the 95th percentile indicates obesity (Centers for Disease Control and Prevention, 2015).
Diet

Whether nutrition and prebiotic supplementation are effective methods to modulate obese-related gut microbiota in children remains elusive. Few studies have investigated the impact of diet on the gut microbiota and obesity in children. La-Ongkham et al. (2015) compared 60 healthy children aged 8-11 from central Thailand and North-East (NE) Thailand (La-Ongkham et al., 2015). Children from central Thailand had a significantly different eating pattern, preferring rice, breakfast cereal, and cow milk. NE Thailand children favoured a high consumption of meat, and a wide variety of carbohydrates, protein sources, and fruit and vegetables. The study demonstrated a higher abundance of Bacteroidetes in children from NE Thailand, which suggests that a diet rich in varieties of protein sources and carbohydrates, including fruit and vegetables, promotes a high abundance of Bacteroidetes.

Another study compared the gut microbial composition of children aged 1-6 from Florence, Italy, and a rural African village in Burkina Faso (BF). In BF, the children’s diet was low in fat and animal protein, rich in starch, fibre and plant polysaccharides, and mostly vegetarian. Whereas the children from Florence ate a typical Western diet high in animal protein, sugar, starch, and fat, and low in fibre. The microbial composition was significantly different between the two groups, indicating a high Bacteroidetes:Firmicutes ratio in the children from BF. Furthermore, a unique abundance of Prevotella and Xylanibacter bacteria was detected, which was completely absent in the European group. This is an important indication, as the species present in the gut of the children from BF acquired a SCFA producing ability (De Fillipo et al., 2010). The studies by La-Ongkham et al. (2015) and De Fillipo et al. (2010) demonstrate co-evolution of the gut microbiota according to dietary habits, which is of influence on the risk of obesity. They furthermore indicate that a diet high in fibre should be promoted to decrease the risk of childhood obesity.

Prebiotics

Prebiotics are suggested to modulate obese-related microbiota, yet further research tackling the underlying mechanisms in relation to obesity is warranted. Oligofructose has prebiotic properties, and is repeatedly studied in research assessing its effect on overweight and obesity. A study conducted by Liber and Szajewska (2014) evaluated the effects of oligofructose in comparison to placebo (maltodextrin) in children aged 7-18 over 12 weeks and found no significant difference in BMI-for-age between both groups (Liber and Szajewska, 2014). A similar study on the other hand, did find a decrease of caloric intake in the intervention group, but provides no possible explanation related to the gut microbiota (Hume et al., 2015). A subsequent study, based on these findings, evaluated the differences in gut microbial composition and showed a significant increase of Bifidobacteria after 16 weeks of supplementation. A significant reduction in trunk body fat was found as well, suggesting that prebiotic fibre shows potential to be an inexpensive and non-invasive treatment to reduce body fat, possibly because of its positive
effects on the gut microbiota colonisation (Nicolucci et al., 2015). Furthermore, in vitro research by Barczynska et al. (2015), who collected faecal samples from obese children aged 5-15 and added dietary fibre preparations from potato starch, showed an increase in Bacteroidetes and an inhibition of Firmicutes strains (Barczynska et al., 2015). Moreover, they found an increase in SCFA, which suggests that these preparations may be useful in the prevention or treatment of obesity (Barczynska et al., 2015).

Probiotics

Perinatal probiotic supplementation may affect the gut microbial composition and obesity during the early years of life, however, it does not appear to prevent childhood obesity at a later age. A 10-year follow-up study evaluated supplementation of mothers with L. rhamnosus GG for 4 weeks preceding expected delivery until 6 months after delivery. Supplementation with probiotics caused a reduction in weight gain in the first 24-48 months of life. By the time they had reached the age of 4, the experimental group tended to have a lower BMI in comparison to controls, yet at the age of 7 and 10 no significant difference was detected (Luoto et al., 2010). A similar follow-up study assessed the effects of supplementing the infant with Lactobacillus paracasei during weaning. Body composition and metabolic markers did not seem to differ between the experimental and the control group at the age of 8-9 (Karlsson et al., 2015).

These studies are not consistent, emphasising the inadequacy of current knowledge. Taking into account that distinct strains of probiotics affect obesity in a different manner, various strains need to be investigated. Moreover, existing research suggests that perinatal probiotics cause short-term modification of the gut microbiota and consequently weight. It might therefore be relevant to examine the effects of continuous supplementation or a gradual increase of supplementation with probiotics during childhood, in order to evaluate its potential for long term protection of childhood obesity.

11.7 Conclusions

Clearly, future research needs to determine which species, specific strains and combinations of strains of probiotics are most efficacious in infectious diseases, functional gastrointestinal disorders such as functional abdominal pain and functional constipation, and obesity or whether probiotic treatment should be adapted to the disturbances in the gut microbiota of the individual patient.

11.8 Recommendations for future research

- Probiotics represent a promising source of molecules for the development of novel anti-infectious therapy in children. However, more and larger well-conducted studies are needed to investigate optimal strain, dosage, bioavailability, duration of treatment and safety of
probiotics on prevention of traveller’s diarrhoea, *C. difficile*-associated diarrhoea, side effects of triple therapy in *H. pylori* eradication and acute diarrhoea.

- Large well-designed randomised controlled multi-centre trials are necessary to evaluate which strain or combination of strains, in what dosage and for what duration are beneficial in children with functional gastrointestinal disorders.

- Future research should focus on the longitudinal effects of the infant gut microbiota and specific species on the development of childhood obesity. As perinatal probiotic supplementation has beneficial short-term effects on childhood obesity, it would be interesting to examine continuous maternal and infant probiotic supplementation and to evaluate its long term effectiveness on reducing or preventing childhood obesity.

**Conflicts of interest**

M.A. Benninga is consultant for Shire, Sucampo, Norgine, Coloplast, Danone, Sensus, FrieslandCampina and Novolac. L.M.A. Akkermans and M. Vink have no conflicts of interest to declare.

**References**


11. Intestinal microbiota and paediatric GI disorders


11. Intestinal microbiota and paediatric GI disorders

Part V.
From bowel to infant behaviour
Chapter 12
The association between intestinal microbiota and infant crying and behaviour

V. Sung¹ and A. Pärtyt²
¹Murdoch Childrens Research Institute, The Royal Children’s Hospital and The University of Melbourne, Flemington Road, Parkville, Victoria, 3052, Australia; ²Department of Pediatrics and Adolescent Medicine, Turku University Hospital and University of Turku, Kiinamyllynkatu 4-8, 20520 Turku, Finland; valerie.sung@rch.org.au

Abstract

This chapter describes the emerging evidence suggesting the association between intestinal microbiota, use of probiotics and infant crying and behaviour, with particular focus on infant colic. It first describes the challenges in defining and measuring infant colic, its epidemiology and burden, and its association with later mental health problems. The chapter discusses the aetiological theories of infant colic, from the traditional hypotheses to the more recent evidence of the role of gut microbiota, inflammation and the gut-brain axis. The chapter then outlines the evidence on the role of probiotics in treating and preventing infant colic. It proposes recommendations on managing infant colic, and finally concludes with suggestions for future clinical research.

Keywords: infant colic, infant behaviour, probiotics, Lactobacillus reuteri, Lactobacillus, coliforms

12.1 Introduction

Definitions of infant colic

Infant colic is excessive crying of unknown cause in young infants, usually resolving by three months of age. The most widely accepted definition was penned by Wessel in 1954 as ‘paroxysms of irritability, fussing or crying lasting for a total of more than three hours a day and occurring on more than three days in any one week’ in an otherwise healthy and thriving infant. Most definitions of infant colic are based on crying duration (St James-Roberts, 2012), whilst some are based on parental perception of crying as a problem (Barr and Geertsma, 2003).

Variability in the definition of colic has been paralleled by non-uniformity in measuring it (Steutel et al., 2014). Some research studies use subjective measures of crying duration, such as parental interviews or non-validated questionnaires capturing parental perception of crying duration and the extent to which this is a problem for parents. Other studies use the more objective and validated Baby Day Diary (Barr et al., 1988), which asks parents to record infant crying or fussing prospectively.
Most authors agree that infant colic is a diagnosis of exclusion. It is estimated from clinical samples that less than 5% of infants with excessive crying have an underlying medical cause (Barr, 2000; Heine, 2008).

**Burden and epidemiology of infant colic**

The reported prevalence of infant colic has varied widely, due to the variations in the definitions and measurement of infant colic. A systematic review on the occurrence of infant colic in the community reported its prevalence to be between 10 and 40% (Lucassen et al., 2001).

Although infant colic is usually considered a self-limiting condition, it imposes significant immediate burden to affected families. It is the most common proximal risk factor for child abuse, or the Shaken Baby Syndrome (Barr et al., 2006; Lee et al., 2007; Talvik et al., 2008). Infants with colic are two to four times more likely than infants without colic to have mothers with symptoms of postnatal depression (Josefsson et al., 2001; McMahon et al., 2001; Smart and Hiscock, 2007; Vik et al., 2009). Infant colic leads to parental feelings of guilt, stress, exhaustion and reduced parental interactions with their infants (Kurth et al., 2011). It is associated with marital dissatisfaction and conflicts and disruptions to family life (Long and Johnson, 2001; Meijer and Van den Wittenboer, 2007). Infants with colic have shorter sleep (Kirjavainen et al., 2001), more feeding difficulties (Miller-Loncar et al., 2004) and are more likely to stop breastfeeding prematurely (Li et al., 2008) than those without colic. Infant colic costs the United Kingdom around 65 million pounds per year (Morris et al., 2001).

**Infant colic and long-term effects including mental health**

The long-term effects of infant colic – which, by definition, is a self-limiting condition – are not comprehensively studied thus far. However, the fact that the crying resolves should not necessarily imply a lack of lasting effects. A handful of studies have reported no long-term associations with future cognitive, allergic, maternal mental health or family functioning problems (Castro-Rodriguez et al., 2001; Clifford et al., 2002b; Rao et al., 2004). However, many studies have suggested the opposite (Kalliomaki et al., 2001b; Munck et al., 2008; Pärty et al., 2013a; Savino et al., 2005b), with other studies additionally reporting associations with long-term behavioural and sleep problems (Becker et al., 2004; Canivet et al., 2000; Lehtonen et al., 1994a; Savino et al., 2005b). Most of these studies, however, fail to differentiate between infants with colic and infants with persistent crying beyond three months (Becker et al., 2004; Canivet et al., 2000; Savino et al., 2005b). In addition, interpretation across studies is limited by different definitions and methods of measuring colic, selected populations of infants, unbalanced infant characteristics between cases and controls, and recall bias in defining colic (Canivet et al., 2000; Kalliomaki et al., 2001b; Munck et al., 2008; Savino et al., 2005b). Indeed, whether infant colic is associated with significant long-term effects remains controversial.
12.2 Aetiological factors and the possible role of intestinal microbiota in infant colic

The underlying aetiology of infant colic remains elusive, contributing to the controversies in its definition and inhibiting the development of successful management strategies. The following section describes the different aetiological theories that have evolved over time.

‘Traditional’ gastrointestinal, neurobiological and psychosocial hypotheses

Gastrointestinal dysfunction leading to discomfort or pain has traditionally been considered to underlie infant colic. Colonic hyperperistalsis, spasms or increased rectal pressure could underlie colic based on the evidence that dicyclomine hydrochloride, which can relax colonic smooth muscle and reduce spasms, is effective in treating colic. This drug, however, has significant, potentially life-threatening side effects and therefore is no longer recommended for use in infants. Excessive intragastrointestinal air, carbohydrate malabsorption, lactose overload and the role of gut hormones motilin and ghrelin are theories that have not been supported by evidence, with gas-reducing agents such as simethicone being conclusively ineffective.

Cow’s milk protein allergy may play a role in infant colic, based on early evidence of a high prevalence of irritability in older infants with cow’s milk protein allergy. Many trials have demonstrated the use of extensively hydrolysed formulae, completely hydrolysed formulae, or maternal dietary cow’s milk elimination to be effective in treating some infants with colic. However, such studies have methodological limitations and none are population based (Heine, 2013). From a practical point of view, maternal hypoallergenic diets are difficult to follow and maintain. Moreover, only a minority of infants with colic respond to cow’s milk protein elimination (Heine, 2013), and cow’s milk protein allergy probably accounts for less than 5% of cases of colic (Barr and Geertsma, 2003). Crying infants are often labelled to have gastro-oesophageal reflux disease (GORD). However, studies have failed to demonstrate any association between GOR/GORD and crying in infants (Heine et al., 2006), and four randomised trials and a systematic review have consistently concluded that anti-reflux medications are ineffective for crying (Gieruszczak-Bialek et al., 2015; Jordan et al., 2006; Loots et al., 2014; Moore et al., 2003; Orenstein et al., 2009).

Psychosocial theories of infant colic include delayed or immature neuromaturation, difficulties in transitioning through stages of neurobehavioural organisation, infant temperament, problems with the mother-infant interaction, maternal stress and depression. However, none have been proven to be causal.

Gut microbiota differences between infants with and without colic

Most recently, gut microbiota, inflammation and the gut-brain axis have all been proposed to play a part in the pathophysiological mechanisms of colic. As demonstrated in Figure 12.1, the
occurrence of infant colic parallels the most remarkable changes in gut microbiota colonisation during the first weeks of life.

A handful of studies in the last decade have indicated a possible role of gut microbiota in infant colic. These are summarised in Table 12.1.

The first study that examined gut microbiota composition of infants with colic with culture methods in 1994 found no significant differences in gut microbiota between infants with and without colic, except for *Clostridium difficile* which more frequently colonised infants with colic during the time of peak crying when compared to controls (Lehtonen *et al.*, 1994b). However, in 2004 an Italian group demonstrated that breastfed infants with colic were less frequently colonised by *Lactobacillus* species than those without colic (Savino *et al.*, 2004). The following year, the Italian group indicated breastfed infants with and without colic had similar overall colonisation rates but different patterns of *Lactobacillus* species – *Lactobacillus brevis* and *Lactobacillus lactis subsp. lactis* colonised only those with colic, while *Lactobacillus*...
Table 12.1. Studies investigating the role of gut microbiota in infant colic.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study type</th>
<th>Sample size/population</th>
<th>Microbial methods¹</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lehtonen et al., 1994b</td>
<td>Case-control</td>
<td>Infants with colic at peak crying (n=55) and 3 months (n=46) vs age-matched controls (n=49, n=45)</td>
<td>Gas-liquid chromatography, quantitative culture</td>
<td><em>Clostridium difficile</em> more frequently colonised cases during peak crying time.</td>
</tr>
<tr>
<td>Savino et al., 2004</td>
<td>Case-control</td>
<td>Breastfed infants with colic (n=42) vs controls (n=29)</td>
<td>Quantitative culture</td>
<td><em>Lactobacillus</em> species less and gram-negative bacteria more frequently colonised cases.</td>
</tr>
<tr>
<td>Savino et al., 2005a</td>
<td>Case-control</td>
<td>Breastfed infants with colic (n=30) vs controls (n=26)</td>
<td>Quantitative culture</td>
<td><em>Lactobacillus brevis</em> and <em>Lactobacillus lactis lactis</em> colonised cases only, <em>Lactobacillus acidophilus</em> colonised controls only.</td>
</tr>
<tr>
<td>Mentula et al., 2008</td>
<td>Case-control</td>
<td>Infants with colic (n=9) vs controls (n=9). 5 infants with colic received a probiotic, 4 received a placebo</td>
<td>Quantitative culture, cellular fatty acid analysis, gas and short chain fatty acid production</td>
<td>Indole-producing coliforms (<em>Escherichia, Klebsiella</em>) more frequently colonised cases.</td>
</tr>
<tr>
<td>Rhoads et al., 2009</td>
<td>Case-control</td>
<td>Infants with colic (n=19) vs controls (n=17)</td>
<td>Electrophoresis/sequencing, chromatography, ELISA</td>
<td>Faecal calprotectin levels doubled, <em>Klebsiella</em> species more frequent and diversity of total bacteria lower in cases; <em>Enterobacter</em> and <em>Pantoea</em> species found only in controls.</td>
</tr>
<tr>
<td>Savino et al., 2009</td>
<td>Case-control</td>
<td>Breastfed infants with colic (n=41) vs controls (n=39)</td>
<td>Quantitative culture, PCR</td>
<td><em>Escherichia coli</em> more frequently colonised cases.</td>
</tr>
<tr>
<td>Savino et al., 2011</td>
<td>Case-control</td>
<td>Breastfed infants with colic (n=45) vs controls (n=42)</td>
<td>Quantitative culture, PCR</td>
<td>Total coliforms (<em>E. coli, Klebsiella, Enterobacter, Enterococcus</em>) higher in cases.</td>
</tr>
<tr>
<td>Ali, 2012</td>
<td>Case-control</td>
<td>Infants with colic (n=55) vs controls <em>Helicobacter pylori</em> stool (n=30)</td>
<td>Higher proportion of cases tested positive to <em>H. pylori</em> antigen.</td>
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</table>
**L. acidophilus** colonised only those without colic (Savino et al., 2005a). A 2013 Iranian study of 70 breastfed infants with and without colic also showed *L. acidophilus* to be present in 20% of infants without colic, but absent in those with colic (Akbarian-Rad et al., 2013). A 2012 Finnish study of 89 healthy infants without colic who were at risk of developing allergies suggested *Bifidobacterium* and *Lactobacillus* species to be protective against crying and fussing in the first three months of life (Pärtty et al., 2012). Similarly, a 2013 study of infants with and without colic from the Netherlands showed that crying infants had reduced numbers of *Lactobacillus* and *Bifidobacterium* (De Weerth et al., 2013b). It is worth noting that the infants with colic in the

<table>
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<tr>
<td>Pärtty et al., 2012</td>
<td>Cross-sectional</td>
<td>Infants without colic with family</td>
<td>Quantitative PCR, FISH assay</td>
<td><em>Bifidobacterium</em> and <em>Lactobacillus</em> species protective against cry/fuss.</td>
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<td></td>
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<td>history of atopy (n=89)</td>
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<tr>
<td>Akbarian-Rad et al., 2013</td>
<td>Case-control</td>
<td>Breastfed infants with colic (n=35) vs</td>
<td>Quantitative culture</td>
<td><em>L. acidophilus</em> more frequently colonised controls.</td>
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<td></td>
<td></td>
<td>controls (n=35)</td>
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<tr>
<td>Ali and Borei, 2013</td>
<td>Case-control</td>
<td>Infants with colic (n=50) vs controls</td>
<td>Quantitative culture</td>
<td>Higher proportion of cases tested positive to <em>H. pylori</em> antigen.</td>
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<tr>
<td></td>
<td></td>
<td>(n=50)</td>
<td><em>H. pylori</em> stool antigen test</td>
<td></td>
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<tr>
<td>De Weerth et al., 2013b</td>
<td>Case-control</td>
<td>Infants with colic (n=12) vs controls</td>
<td>Microbial DNA analysis by HITChip</td>
<td>Cases more frequently colonised by <em>Escherichia</em> and <em>Klebsiella</em>, less frequently colonised by bifidobacteria and lactobacilli. The diversity and stability of microbiota lower in cases.</td>
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<td></td>
<td></td>
<td>(n=12)</td>
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<tr>
<td>Roos et al., 2013</td>
<td>Randomised trial of</td>
<td>Infants with colic (probiotic n=15,</td>
<td>DNA pyrosequencing of 16S rRNA genes</td>
<td>Responders more frequently colonised by <em>Bacteroides</em> species. No difference in microbial composition.</td>
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<tr>
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<td><em>Lactobacillus reuteri</em></td>
<td>controls n=14)</td>
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<tr>
<td>Pärtty et al., 2015b</td>
<td>Case-control</td>
<td>Infants with colic (n=30) vs controls</td>
<td>Quantitative PCR</td>
<td>The number of <em>Bifidobacterium</em> higher in controls.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=11)</td>
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</table>

1 ELISA = enzyme-linked immunosorbent assay; FISH = fluorescent in situ hybridisation; HITChip = Human Intestinal Tract Chip; PCR = polymerase chain reaction.
2005 Italian study and all infants in the 2012 Finnish study were at risk of developing allergies, with a positive family history of atopy (Pärty et al., 2012; Savino et al., 2005a). This could be a significant confounder, considering that a 2010 study suggested that infants with cow’s milk protein allergy have higher levels of anaerobic and Lactobacillus species, and lower levels of Bifidobacteria, when compared with non-allergic controls (Thompson-Chagoyan et al., 2010).

Altogether, six studies have indicated that infants with colic are more frequently colonised with gram-negative bacteria, especially coliforms such as Escherichia and Klebsiella, than infants without colic. This further supports the notion that infants with colic may have more gas-forming coliform species that contribute to gaseous distension and subsequent distress (De Weerth et al., 2013b; Mentula et al., 2008; Rhoads et al., 2009; Savino et al., 2004, 2009, 2011). Interestingly, a 2011 Italian study examined the protective mechanisms of Lactobacillus species against gas-forming coliforms. The study demonstrated that two out of 27 Lactobacillus strains tested had an antimicrobial effect against six gas-forming coliform species isolated from breastfed infants with colic (Savino et al., 2011).

Two recent studies have implicated Helicobacter pylori in infant colic. The studies of infants with and without colic from Saudi Arabia (n=85) and Egypt (n=100) found higher proportions of infants with colic had positive H. pylori stool antigen compared to those without colic (Ali, 2012; Ali and Borei, 2013). These findings are interesting considering H. pylori is an organism known to cause chronic gastric inflammation (Ruggiero, 2010). It may be associated with short-term abdominal pain lasting less than three months, but not chronic recurrent abdominal pain in children (Spee et al., 2010). However, the prevalence of H. pylori vary by geography and is generally low in children. In Chile, its prevalence is as low as 1% at three months of age, rapidly increasing after 15 months of age to 20% at 24 months of age – an age by which colic has resolved (O’Ryan et al., 2013).

Recent studies have used more advanced technology to measure faecal composition in infants with and without colic. A 2013 study in the Netherlands using microbial DNA analysis of faecal composition of 24 infants with and without colic demonstrated that the diversity and stability of the microbiota were significantly lower in infants with colic than in controls (De Weerth et al., 2013b). Interestingly, the differences in gut microbiota composition between the colic and control groups disappeared by three to four months of age, indicating alterations to the gut microbiota may be temporary rather than permanent (De Weerth et al., 2013a). This parallels the natural improvement in crying seen around this age.

In contrast, another 2013 study that re-analysed faecal samples from an early probiotic supplementation trial of Lactobacillus reuteri in Italian infants with colic by pyrosequencing found no effect of the probiotic on gut microbial diversity (Roos et al., 2013). Similarly, a Finnish probiotic intervention study found no difference in gut microbiota composition by qPCR between infants with colic receiving Lactobacillus rhamnosus GG (LGG) or placebo (Pärty et al., 2015b).
To conclude, there is accumulating evidence to indicate that the gut microbiota composition in infants with colic may differ from that in infants without colic, although there is no consistent pattern to these differences. One of the reasons for this may be the range of different methods used to identify gut microbiota. Most of the older studies used traditional culturing methods and focused on specific bacteria, so that the findings depended entirely on which specific microbial species were examined. Recent years have seen the development of more comprehensive methods to measure gut microbiota composition, including DNA analysis of gut microbial diversity and these methods should be used in future studies.

**Possible mechanisms linking intestinal microbiota and infant crying**

The early gut colonisation process begins in utero and proceeds in a stepwise manner during birth and infancy (Rautava et al., 2012). This process is substantially influenced by a number of factors, including maternal gut and vaginal microbiota, maternal health status, maternal stress, diet and probiotic use during pregnancy, prenatal and postnatal antibiotic use, mode of delivery, type of feeding, and environmental factors in hospital and at home (Rautava et al., 2012). Consequently, an aberration in any of these components might hold the answer to gut microbiota alterations underlying colic. However, many of the separate factors such as mode of delivery and type of feeding have already been widely studied, and there is evidence to indicate that these factors, at least separately, are not associated with the development of colic (Clifford et al., 2002a; Lucassen et al., 2001; Mentula et al., 2008; Rhoads et al., 2009; Yalcin et al., 2010). The effect of maternal health status, diet, stress, the use of probiotics and antibiotics during pregnancy and their joint effect on gut microbiota development in infant colic has not been widely investigated and should be addressed in the future.

Altered gut microbiota has been strongly implicated in inflammatory disorders in the last few years (Comito and Romano, 2012; Kang et al., 2010; Martinez-Medina et al., 2006; Nemoto et al., 2012; Papa et al., 2012). An association between altered gut microbiota and cow’s milk protein allergy has been found in a handful of studies (Berni Canani and Di Costanzo, 2013; Thompson-Chagoyan et al., 2010, 2011). Differences in neonatal gut microbiota have also been suggested to precede the development of later allergic disorders (Bisgaard et al., 2011; Kalliomaki et al., 2001a). There is, however, no consensus on which microbial species may play a part in gut inflammation.

Therefore, it would not be surprising if gut microbiota, food allergy, gut inflammation and infant colic proved to be closely linked. In fact, a recent study of 36 American term infants reported faecal calprotectin levels to be twice as high in infants with colic than those without (Rhoads et al., 2009). Calprotectin, a calcium-binding protein expressed predominantly by neutrophils, is a marker of gut inflammation. It is elevated in faecal and serum samples of children and adults with inflammatory bowel disease (Konikoff and Denson, 2006; Kostakis et al., 2013) and other paediatric inflammatory conditions such as cow’s milk protein allergy (Baldassarre et al., 2010), necrotising enterocolitis, coeliac disease and intestinal cystic fibrosis (Vaos et al.,
2013). However, the link between calprotectin and colic is far from conclusive, being directly contradicted by the only other study to examine this association. This Norwegian study of 76 infants with colic did not find a difference in faecal calprotectin levels between infants with and without colic (Olafsdottir et al., 2002). This could be partially explained by the huge variability in faecal calprotectin levels found in normal healthy infants, and generally higher levels found in neonates than older children and adults (Campeotto et al., 2004, 2009; Nissen et al., 2004; Rugtveit et al., 2002; Savino et al., 2010). Indeed, there is a lack of consensus for ‘normal cut-point’ levels for faecal calprotectin in infants.

Data from an unpublished Finnish study indicate that infants with colic have increased concentrations of pro-inflammatory chemokines, such as IL-8, MCP-1 and MIB-1β in serum as compared with infants without colic (Pärtyt, unpublished data). In addition this study showed that faecal levels of *Clostridium leptum* and *Clostridium coccoides* were negatively associated with pro-inflammatory biomarkers. These findings give further support to the hypothesis that infant colic may be an inflammatory gastrointestinal condition related to intestinal dysbiosis. However, it remains to be established whether the microbiota alterations in colic are causes or consequences of an inflammatory response.

In addition to the proposed interplay of interactions between gut microbiota and gut inflammation in colic, a concept of a microbiota-gut-brain axis is emerging, suggesting a communication between gut microbiota and the brain through neural, humoral and immune pathways (Bercik et al., 2012; Collins et al., 2012; Cryan and Dinan, 2012; McLean et al., 2012; Saulnier et al., 2013). This fascinating area of research will likely expand in coming years. Direct evidence for an effect of gut microbiota on the brain comes mainly from animal studies, with mice administered certain gut bacteria displaying altered behaviours.

The interplay between gut microbiota, gut inflammation and the gut-brain axis in infant colic is an exciting and credible concept that is currently supported by limited evidence. Further research in this area is required to clarify whether this theory can be justified. Nevertheless, it forms the basis for examining whether altering gut microbiota, by the use of probiotics, for example, may have a role in infant colic.

### 12.3 Role of probiotics in infant colic

The last decade has seen emerging evidence of the role of probiotics in infant colic. Most studies have examined the role of one specific probiotic, *L. reuteri*, in treating colic. A handful of studies have studied other strains in treating and preventing colic. The section below discusses the evidence for probiotic use, particularly *L. reuteri*, in infant colic, and the possible underlying mechanisms.
Probiotics in treating infant colic

*L. reuteri* is the most studied strain of probiotic in treating infant colic, and has shown the most promise. In the last decade, six randomised controlled trials (RCTs) have examined its effectiveness in treating colic. Table 12.2 summarises the studies' baseline characteristics.

All six RCTs included infants with colic on the basis of modified Wessel’s criteria (crying ≥3 hours/day for ≥3 days/week) and reported daily crying duration (Savino *et al.*, 2007, 2010; Szajewska *et al.*, 2013; Sung *et al.*, 2014; Chau *et al.*, 2012; Mi *et al.*, 2015). The Sung *et al.* (2014) and Chau *et al.* (2012) studies also included ‘fussing’ in the definition of colic and reported ‘fussing’ in the outcome measures. All RCTs except the Savino (2007) study were placebo-controlled, blinded trials that examined the same dose of *L. reuteri* DSM 17938 at 1×10⁸ cfu per day, compared to placebo. The Savino *et al.* (2007) study was an open-label study comparing *L. reuteri* ATCC55730 (10⁸ cfu/day) to simethicone (60 mg/day).

Table 12.2. Baseline characteristics of *Lactobacillus reuteri* randomised control trials in treating infant colic.

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Feeding method</th>
<th>Country</th>
<th>Median infant age (weeks)</th>
<th>Family history of atopy (%)</th>
<th>Caesarean delivery (%)</th>
<th>Male (%)</th>
<th>Measure of cry/ fuss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Savino <em>et al.</em>, 2007a</td>
<td>83</td>
<td>breast and maternal dairy-elimination</td>
<td>Italy</td>
<td>4.5</td>
<td>47</td>
<td>35</td>
<td>53</td>
<td>Parental recall at end of each day</td>
</tr>
<tr>
<td>Savino <em>et al.</em>, 2010</td>
<td>46</td>
<td>breast and maternal dairy-elimination</td>
<td>Italy</td>
<td>4.4</td>
<td>42</td>
<td>38</td>
<td>58</td>
<td>Parental recall at end of each day</td>
</tr>
<tr>
<td>Szajewska <em>et al.</em>, 2013</td>
<td>80</td>
<td>breast</td>
<td>Poland</td>
<td>4.9</td>
<td>35</td>
<td>15</td>
<td>60</td>
<td>Parental recall at end of each day</td>
</tr>
<tr>
<td>Sung <em>et al.</em>, 2014</td>
<td>167</td>
<td>breast and formula</td>
<td>Australia</td>
<td>7.4</td>
<td>60</td>
<td>40</td>
<td>51</td>
<td>Baby day diary</td>
</tr>
<tr>
<td>Chau <em>et al.</em>, 2012</td>
<td>52</td>
<td>breast</td>
<td>Canada</td>
<td>5.9</td>
<td>39</td>
<td>21</td>
<td>48</td>
<td>Baby day diary</td>
</tr>
<tr>
<td>Mi <em>et al.</em>, 2015</td>
<td>42</td>
<td>breast</td>
<td>China</td>
<td>4.2b</td>
<td>54</td>
<td>26</td>
<td>56</td>
<td>Modified diary</td>
</tr>
</tbody>
</table>

a All RCTs were placebo-controlled and blinded except for the Savino *et al.* (2007) open-label study which used *Lactobacillus reuteri* ATCC55730 drops (10⁸ cfu/day) compared to simethicone drops (60 mg/day).

b The Mi *et al.* (2015) study reported mean age only.
Table 12.3 summarises the median cry/fuss durations recorded at the various study time points. All RCTs, except Sung et al. (year) trial, reported *L. reuteri* to be effective in treating infants with colic.

It is interesting that the largest trial was the only one so far that showed the probiotic to be ineffective in colic (Sung et al., 2014). There are a few possible reasons for Sung et al. (2014) controversial results. It was a pragmatic trial designed to reflect what happens in real life, and therefore had less stringent inclusion and exclusion criteria. It included infants who were formula fed, on anti-reflux medications or hypoallergenic and probiotic-containing formulae. These factors may have influenced gut microbiota and the effectiveness of the probiotic in altering gut microbiota. However, subgroup analysis indicated the probiotic to be ineffective in the breastfed infants. In addition, there is good evidence that anti-reflux medications are ineffective for infant crying or reflux symptoms (Gieruszczak-Białek et al. 2015; Jordan et al., 2006; Loots et al., 2014; Moore et al., 2003; Orenstein et al., 2009), so including infants on these medications was unlikely to have impacted crying duration. Other possible reasons are the larger sample size involved, the older age of infants recruited and geographical or environmental differences. It is possible that the probiotic was ineffective in infants from Australia because of undetermined differences in infant gut microbiota compared with European, Canadian and Chinese infants.

These six RCTs have been variably included in four different systematic reviews. The first two published in 2013, which included the first three trials, concluded *L. reuteri* DSM 17938 to be possibly effective in treating exclusively breastfed infants with colic, however there was insufficient evidence to support its use in formula-fed infants with colic (Anabrees et al., 2013; Table 12.3. Daily infant cry/fuss durations in *Lactobacillus reuteri* randomised control trials in treating infant colic.

<table>
<thead>
<tr>
<th>Study</th>
<th>Median daily infant cry/fuss time (minutes/day): probiotic/placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>Savino et al., 2007</td>
<td>197/197</td>
</tr>
<tr>
<td>Savino et al., 2010</td>
<td>370/300</td>
</tr>
<tr>
<td>Szajewska et al., 2013</td>
<td>240/240</td>
</tr>
<tr>
<td>Sung et al., 20144 (Cry time only)</td>
<td>176/160</td>
</tr>
<tr>
<td>Sung et al., 20144 (Cry + fuss time)</td>
<td>322/350</td>
</tr>
<tr>
<td>Chau et al., 20124 (Cry + fuss time)</td>
<td>131/122</td>
</tr>
<tr>
<td>Mi et al., 20155</td>
<td>201/201</td>
</tr>
</tbody>
</table>

a Sung et al. (2014) and Chau et al. (2012) RCTs reported crying and fussing times; all other studies reported crying times only.

b The Mi et al. (2015) study reported means only.
Sung, 2013). Xu et al. (2015) systematic review included six RCTs, two of which were in fact the same trial. Schreck Bird et al. (in press) systematic review included five of the RCTs. Both reviews reached the same conclusion as the previous two reviews, noting the heterogeneity of the included trials. Authors of the Italian, Polish, Australian and Canadian RCTs have been collaborating to conduct an individual participant data meta-analysis (IPDMA) which pools raw data from individual trials to create sufficient power for sub-group analysis (Sung et al., 2014). Preliminary data from this IPDMA suggest L. reuteri DSM 17938 to be effective in treating breastfed infants with colic, but not formula fed infants (Sung, unpublished data).

Apart from the six trials involving L. reuteri, four other trials have investigated the effectiveness of LGG in treating infant colic. Dupont et al. (2010)’s blinded RCT randomised 53 formula-fed infants with colic to a combination of LGG, Bifidobacteria infantis M63 and alpha-lactalbumin versus placebo. Mentula et al. (2008) study randomised nine breastfed infants with colic to receive the combination of LGG, L. rhamnosus LC705, Bifidobacterium breve Bbi99, and Propionibacterium freudenreichii ssp. shermanii JS versus placebo. Infant crying were not primary outcomes in either trials and how crying was measured was unclear in both studies. Both studies indicated the probiotic combinations to be ineffective in treating colic. Pärtty et al. (2015b) trial randomised 30 breast and formula-fed infants with colic to receive LGG ATCC 53103 versus placebo. The trial suggested LGG to be effective by parental report, but not by the validated prospectively recorded Baby Day Diary. Fatheree et al. (2016) trial randomised 20 infants with colic to receive a hypoallergenic formula with LGG versus hypoallergenic formula without LGG. The study suggested a trend in reduced fussing time in the probiotic group, however the results were not statistically significant.

**Probiotics in preventing infant colic**

Only one study has examined the role of probiotics in preventing infant colic as a primary outcome. Indrio et al. (2014) study randomised 589 term new-born infants to receive L. reuteri DSM 17938 or placebo for 90 days, to investigate the probiotic’s effectiveness in preventing the onset of colic, gastroesophageal reflux and constipation in term new-borns (Indrio et al., 2014). At one month, the probiotic group had significantly shorter mean inconsolable crying duration than the placebo group, and the effect was sustained at three months. The study authors concluded that the probiotic was effective in reducing crying time in term new-born infants. However, there may be some study limitations. First, it is unclear whether the study had adequate power to detect clinically meaningful outcomes. Second, the outcome of infant colic was not defined. Third, unexplained protocol violations seemed to have contributed to the loss of follow-up in the probiotic group. The study’s conclusion therefore needs to be considered with caution.

Apart from Indrio et al. (2008) study, seven other randomised trials have examined the effectiveness of probiotics in preventing infant crying, however none measured infant crying as a primary outcome (Indrio et al., 2008; Kukkonen et al., 2008; Pärtty et al., 2013b; Rinne et al., 2006; Roze et al., 2012; Vendt et al., 2006; Vlieger et al., 2009; Weizman and Alsheikh, 2006).
The studies differed widely in the strain types, concentrations and dosing of probiotics used, in their populations of infants and outcome measures. There is still insufficient evidence to support the use of probiotics in preventing infant colic.

**Proposed mechanisms of probiotics in infant colic**

How probiotics may work in infant colic relies on the assumption that infant crying may be caused by gastrointestinal factors. Probiotics can decrease gut inflammation through the reduction of tumour necrosis factor (TNF) and modulation of toll-like receptors and cytokine levels (Ganguli et al., 2013; Rodes et al., 2013; Liu et al., 2012; Livingston et al., 2010). In *in vivo* human studies, probiotics can reduce faecal calprotectin levels in preterm infants (Mohan et al., 2008) and children with cystic fibrosis (Yousefi et al., 2013). However, this has not been replicated in clinical studies of *L. reuteri*. Faecal calprotectin levels did not differ between infants with colic randomised to *L. reuteri* versus placebo in a 2014 Australian study (Sung et al., 2014), and were higher (though still normal) in adults randomised to take *L. reuteri* than those on placebo (Mangalat et al., 2012).

*L. reuteri* may have many unique mechanisms that modulate infant crying. In animal studies, *L. reuteri* can change gut-mediated pain perception, modulate neuroregulatory factors in the central nervous system, change gut motility through calcium channel blockade in enteric neurons and suppress gut inflammation by reducing bacterial translocation through its protective effects on the mucus layers of the gut. *L. reuteri* can kill bacteria, via reduction of gut glycerol to produce reuterin, a substance with direct bactericidal effects (Cleusix et al., 2007; De Weirdt et al., 2012; Schaefer et al., 2010; Spinler et al., 2008). Its other bactericidal effects may be carried out through its production of reutericyclin and lactic acid. *L. reuteri* can also ferment dietary carbohydrates to produce short chain fatty acids which are known to have protective effects in the gut (Wong et al., 2006). In human studies, *L. reuteri* has been shown to reduce gastric distension and promote gastric emptying in infants with regurgitation (Indrio et al., 2011). A study of children given oral *L. reuteri* indicated that the probiotic can modulate cytokine production *in vivo* (Miniello et al., 2010).

It is worthwhile noting that *L. reuteri* has the potential to inhibit two potentially important bacteria, *E. coli* and *H. pylori*. *E. coli* may play a role in infant colic, with several studies indicating it is found in higher concentrations in infants with colic than those without (Mentula et al., 2008; Savino et al., 2009, 2011). *Lactobacillus* strains can inhibit the growth of *E. coli* strains *in vitro* (Bujnakova and Kmet, 2012; Cleusix et al., 2008; Mogna et al., 2012). Meanwhile, two studies have recently implicated a role of *H. pylori* in infant colic (Ali, 2012; Ali and Borei, 2013). *In vitro* studies show *Lactobacillus* strains can induce inhibitory and antibacterial activities against *H. pylori* (Figueroa et al., 2010; Mukai et al., 2002). *In vitro* studies also show *Lactobacillus* concentrations to be inversely proportional to *H. pylori* concentrations in gastric biopsies of adults with gastrointestinal symptoms (Garcia et al., 2012). In fact, numerous adult studies and a recent systematic review and meta-analysis have concluded that *L. reuteri* is efficacious in...
eradicating *H. pylori* in adults (Emara *et al.*, 2014; Francavilla *et al.*, 2014; Zheng *et al.*, 2013). There is, however, no clear evidence for this in children (Lionetti *et al.*, 2012), although one study suggested it reduced antibiotic-associated side effects during eradication therapy for *H. pylori* in children (Lionetti *et al.*, 2006).

It is interesting to note that *L. reuteri* has been shown to be effective only in breastfed infants with colic. *Bifidobacteria* have been reported to dominate the gut microbiota of breastfed infants, while the microbiota in formula-fed infants is more diverse (Harmsen *et al.*, 2000, Rautava *et al.*, 2012). These differences might be due to the unique composition of maternal milk or possible direct effects of microbes in breast-milk (De Weerth *et al.*, 2013a; Rautava *et al.*, 2012). However, breast-feeding *per se* has not been shown to provide a protective effect on the development of infant colic (Clifford *et al.*, 2002a; Lucassen *et al.*, 2001). The effectiveness of *L. reuteri* in infant colic seems to be linked to breastfeeding, but the exact mechanism remains unknown.

**Possible role of probiotics in influencing infant behaviour and mental health**

Many mental health conditions have early life antecedents and therefore determining the behaviour-gut associations in early life may be important. A recent American study of 77 children aged 18 to 27 months indicated infant temperament to be associated with gut microbiota (Christian *et al.*, 2015). Specifically, the study found alpha and beta diversity as well as the structure and specific bacterial taxa of the gut microbiome to be associated with temperament in toddlers. The gut microbiota may be more malleable during the first years of life than later and therefore early modification of gut microbiota may play a crucial role in influencing behaviour (Clarke *et al.*, 2013). There is accumulating evidence from preclinical trials that probiotics have effects on emotional behaviour (Saulnier *et al.*, 2013). However, there is very limited evidence for the efficacy of probiotic interventions on symptoms of psychiatric disorders in clinical trials (Romijn and Rucklidge, 2015).

Two studies have examined the effect of probiotics on infant behaviour other than crying. A 2014 Australian study randomised 167 infants with colic to receive *L. reuteri* or placebo for four weeks, and reported infant sleep duration and infant physical, emotional, social and cognitive functioning at one and six months. The probiotic did not affect infant functioning scores at one and six months. Interestingly, the probiotic group slept 47 minutes less per day than the placebo group at one month, but the difference disappeared by six months (Sung *et al.*, 2104).

A recent Finnish study suggested an association between the probiotic LGG and later childhood behaviour (Pärty *et al.*, 2015a). The study followed up 75 infants who received LGG or placebo in the first six months of life, and found that 13 years later the group of children who were previously assigned the placebo had higher rates of attention deficit hyperactivity disorder and/or autism diagnoses (17% of placebo group versus 0% of probiotic group). The diagnosed children had lower concentrations of *Bifidobacterium* species in their faeces in the first six months of life.
12. Intestinal microbiota and infant crying

compared to the controls, although no single consistent microbiota composition or change was detected (Pärtty et al., 2015a).

12.4 Recommendations for treating infant colic

Clinical guidelines around the world remain simple and minimal in their intervention advice. Typically, they recommend exclusion of medical causes, providing empathy and support to families, and reassuring caregivers that it is a self-resolving condition (Crotteau et al., 2006; Gupta, 2007; Hiscock, 2006; Hiscock and Jordan, 2004; NICE guidelines, 2014; Roberts et al., 2004; Royal Children’s Hospital, 2014). A limited trial of hypoallergenic formula, or maternal cow’s milk protein elimination diet in exclusively breastfed infants, is often recommended for infants with colic who also have other indicators of cow’s milk protein allergy, such as bloody or mucousy diarrhoea, failure to thrive, poor feeding, significant vomiting, eczema and family history of atopy (NICE guidelines, 2014; Royal Children’s Hospital, 2014).

With the emerging evidence of probiotic effectiveness in treating infant colic, a trial of five drops per day of *L. reuteri* DSM 17938 at 0.2x10⁸ cfu per drop may be considered for exclusively breastfed infants with colic, except perhaps for those who live in Melbourne, Australia. There is currently no evidence of the probiotic’s benefit in formula fed infants with colic, nor is there sufficient evidence for its routine use in preventing infant colic.

12.5 Suggestions for future clinical research

The use of *L. reuteri* DSM 17938 in treating infant colic has been promising. However, there is as yet a lack of evidence of gut microbiota and inflammatory marker changes to accompany the clinical effectiveness demonstrated in breastfed infants. Further energy should be spent on examining gut microbiota and inflammatory changes with *L. reuteri* DSM 17938 supplementation in breastfed infants with colic using uniform, reliable and efficient microbial analysis techniques. In addition, further large and rigorously designed trials need to examine the use of *L. reuteri* DSM 17938 in formula fed infants with colic. These trials should be pragmatically designed to reflect the real-world setting where infants with colic are often on non-evidence based anti-reflux medications and probiotic/prebiotic formulae.

The role of *L. reuteri* DSM 17938 in preventing infant colic needs further exploration by large, rigorously designed multi-centred trials using well-defined outcomes and using validated outcome measures. In particular, research to confirm the absence of long-term adverse effects of *L. reuteri* DSM 17938 supplementation in early life is required before its routine use can be considered. The recent formation of the international IPDMA collaboration is a vehicle to pool data from trials from all around the world to create sufficient power to answer these important questions.
Conflict of interest

The authors have no conflict of interest to declare.

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12. Intestinal microbiota and infant crying


Part VI.

Consequences of dysbiosis outside of the gut
Chapter 13
The role of the local microbiomes in inflammatory and infectious diseases of the respiratory tract and oral cavity

J. Gerritsen* and J.A. Younes
Winclow Probiotics, Hulstweg 11, 1032 LB Amsterdam, the Netherlands; c.gerritsen@winclow.nl

Abstract

Recent microbiological insights point towards a large role for the local respiratory and oral microbiomes in the aetiology of inflammatory and infectious diseases that affect the respiratory tract and oral cavity during childhood. Complex interactions exist in the respiratory tract and oral cavity between viral, bacterial and fungal pathogens, and non-pathogenic members of the respiratory and oral microbiomes. The recent application of culture-independent molecular methods have highlighted the substantial differences in microbiota composition and activity between the niches within these regions. It has been demonstrated that many bacterial pathogens involved in respiratory or oral disease are in fact common local residents of these niches. In addition, the presence of certain bacterial members of each local microbiome have been associated with respiratory and oral health or disease. Altogether it is becoming more evident that disease might result from dysbiosis of the normal functioning microbiome and associated biofilms supporting a polymicrobial involvement in inflammatory and infectious diseases of the respiratory tract and oral cavity. These emerging microbiological insights can be used to develop ecological approaches towards diagnostics and therapeutic strategies for the management of respiratory and oral inflammatory and infectious diseases, with a focus on the long-term health consequences of the therapeutic strategies used. This chapter will address common respiratory or oral diseases that affect children and those that highlight different mechanisms of microbial involvement. It will especially emphasise the role of microbial dysbiosis in health and disease in acute respiratory tract infections, dental caries, periodontal disease, and oral candidiasis.

Keywords: respiratory tract, oral cavity, dysbiosis, microbiome, biofilm, respiratory tract infections, caries, periodontal disease, candidiasis

13.1 Introduction

Inflammatory and infectious respiratory diseases are among the leading causes of mortality and morbidity in infants and young children (Mizgerd, 2012). Common respiratory inflammatory and infectious diseases include diseases such as the common cold, rhinosinusitis, pharyngitis, otitis media, bronchitis and pneumonia, which are some of the most common reasons for a medical visit among children. Traditionally, the respiratory system is divided into the upper respiratory tract (the nasal, sinus and oral cavities, the pharynx, and the portion of the larynx above the vocal cords) and the lower respiratory tract (the portion of the larynx below the
vocal cords, the trachea, bronchi, bronchioles, and alveoli) (Figure 13.1). Especially in the oral cavity and the pharynx, the respiratory system is closely associated with the digestive system. Both systems share common spaces and while structurally similar, their functions differ. The respiratory system is lined by mucosal epithelial surfaces and it is at this interface with the external environment that distinct microbial communities have co-evolved and colonised (Armstrong, 2015; De Steenhuijisen Piters et al., 2015).

Only recently have the direct and indirect contributions of resident microbial communities to human health begun to be understood and appreciated. Oral and respiratory microbial communities work in the direct line against pathogens through activities such as competition for attachment sites, local secretion of antimicrobial substances, and competition for nutritional resources, thus assisting the host to withstand unwanted microbial invasion (Bosch et al., 2013; De Steenhuijisen Piters et al., 2015).

Figure 13.1. A schematic overview of distinct microbiological niches within the respiratory system, including the oral cavity. Indicated are characteristic environmental determinants for the composition and activity of the microbiomes within these niches.
Kuramitsu et al., 2007). Indirectly, these microbial communities are involved in human health via the regulation of mucosal cell maturation, barrier integrity, and immune modulation (Bosch et al., 2013; De Steenhuijsen Piters et al., 2015; Vissers et al., 2014).

Microbial colonisation begins before, during, and directly after birth and during childhood distinct microbial communities develop within different oral and respiratory niches. These local microbial ecosystems, also called microbiomes, are influenced by multiple factors such as mode of delivery, feeding regime, host genetics, and age. The external environment, for example social status, antibiotic use, vaccination status, season, parental smoking, and specific pattern of social contacts (e.g. day-care attendance, number of siblings, etc.) (Armstrong, 2015; de Steenhuijsen Piters et al., 2015; Teo et al., 2015) also has a strong influence on local microbiomes. As indicated in Figure 13.1, local niche conditions such as temperature, pH, redox potential, atmospheric conditions, salinity, and water activity from saliva and mucus, epithelial cell types also influence composition and activity of each local microbiome (Avila et al., 2009; De Steenhuijsen Piters et al., 2015; Nagalingam et al., 2013). In addition, new microbes are continually introduced to the respiratory tract and oral cavity by virtue of exposure to air, fluids, and food. In the past, studies using culture-based methods have indicated that there are substantial differences in the composition and function of the local microbiomes that inhabit these different microenvironments. Especially the oral cavity contains an impressive variety of microenvironments, including shedding and non-shedding surfaces (Segata et al., 2012). Some areas are more aerobic, such as the tongue and buccal surfaces, while others offer anaerobic conditions to the microbiome, such as the subgingival plaques. Furthermore, salivary flow exposure is another distinguishing factor for microbial communities, as well as the choice of hard dentin or soft mucosal tissue for microbial colonisation.

The recent application of culture-independent molecular methods for the identification of microbial composition, such as next-generation sequencing, have undeniably demonstrated the substantial differences in microbiota composition between the multiple niches within the respiratory tract and oral cavity (Huse et al., 2012; Lemon et al., 2010; Segata et al., 2012; Simon-Soro et al., 2013; Wang et al., 2013; Zaura et al., 2009). The clinical significance of the application of these culture-independent methods is reflected in, for example, the findings that there is a functioning lung microbiome in hard contrast with the classical view of a sterile healthy lower respiratory tract (Charlson et al., 2011; Dickson et al., 2013). To date, such in-depth microbiome surveys of the communities present in each respiratory tract and oral niche have still largely only focused on the bacterial microbiome, while it is known that viruses and fungi are also abundantly present and thus it follows, also play a role in health.

It is important to note that in the human body microbes are found as functioning and distinct microbial communities (biofilms) attached to a surface (mucosal or other) and protected by a polymeric matrix of polysaccharides, proteins and nucleic acids (Høiby et al., 2011). In other words, microbes almost never exist in the body as planktonic organisms but as biofilms. The direct influence of the biofilm structure on pathogenesis, symptoms, and treatment of infections
should not be underestimated. Some unique biofilm features that especially contribute to infection treatment failure and recurrence include a different mode of growth than planktonic bacteria (Donlan and Costerton, 2002), the biofilm matrix which limits the penetration and killing effect of antimicrobials to the only the outermost biofilm layers (Cerca et al., 2005; Donlan and Costerton, 2002), and the metabolic and genetic interdependence and resilience of each biofilm resident. In addition, the formation of intracellular bacterial communities (IBCs), a process facilitated by internalisation of bacteria into host cells due to changes in extracellular conditions, is a mechanism that enables rapid bacterial expansion within the host and contributes to bacterial persistence (Duell et al., 2016; Kostakioti et al., 2013).

The classical view on infectious diseases is that infections are caused by a single pathogenic agent, such as a bacterium, virus, or fungus, and inflammation is one of the resulting clinical manifestations. However, this ‘single causative agent’ concept does not apply any longer to many of the inflammatory and infectious diseases of the respiratory tract and oral cavity as the evidence against this idea is becoming stronger; clinical and pathologic manifestations are associated by the presence of multiple microorganisms (Brogden and Guthmiller, 2002; De Steenhuijsen Piters et al., 2015). In addition, a growing number of studies support the idea that many of the microorganisms historically classified as bacterial pathogens are in fact normal colonisers of the respiratory and oral niches – henceforth commonly referred to as pathobionts – that only become virulent when local conditions are disturbed (De Steenhuijsen Piters et al., 2015; Teo et al., 2015). While colonisation is a prerequisite for most infections, by itself it is not sufficient and colonisation by pathobionts is usually asymptomatic.

If pathobionts are in fact local residents, it follows that when a microbiome is disturbed (becomes dysbiotic through a metabolic, genetic, physical, or ecological insult), the change in composition or function may favour the overgrowth and dominance of said pathobiont. Depending on the state of the host, (polymicrobial) infection might become inevitable (Figure 13.2). The exact mechanisms behind these ecological changes are still largely unknown and the involvement of the local microbiome in onset of respiratory diseases is still largely neglected in current research. When compared to the respiratory tract, it is much more recognised in the oral cavity that disease can be caused by consortia of microorganisms rather than a single pathogen (Jenkinson and Lamont, 2005). Though relatively stable, it is well accepted that shifts in the oral microbiome composition are an important step in the progression of oral disease (Chen and Jiang, 2014; Crielaard et al., 2011). It is becoming increasingly apparent that disease might in fact result from dysbiosis of the normal functioning microbiome and associated biofilms supporting a polymicrobial involvement in inflammatory and infectious diseases of the respiratory tract and oral cavity. This chapter will explore common inflammatory and infectious childhood diseases in the respiratory tract and oral cavity. It will especially highlight different mechanisms of microbial involvement and emphasise the role of microbial dysbiosis in health and disease for acute respiratory tract infections, dental caries, periodontal disease and oral candidiasis (OC).
13.2 Acute respiratory tract infections

A large part of the inflammatory conditions affecting specific niches in children within the respiratory tract (rhinitis, sinusitis, tonsillitis, pharyngitis, pharyngotonsillitis, laryngitis, otitis media, pneumoniae, etc.) are caused by an acute infection. The average child may get six to eight episodes of acute upper respiratory tract infections (URTIs) per year (Chonmaitree et al., 2008; Nokso-Koivisto et al., 2002). Lower respiratory tract infections (LRTIs), like pneumoniae, occur less frequent than URTIs, but they are generally more serious than URTIs. Though more benign, acute URTIs represent an enormous economic burden for society in terms of visits to doctors and other health-care providers, treatments, absences from work, school or day-care (Lenoir-Wijnkoop et al., 2015). Most respiratory tract infections (RTIs) are highly communicable and when highly virulent strains appear outbreaks may occur in paediatric populations, especially on day-care centres or schools.

Acute RTIs are usually either viral or bacterial in origin. In children, a large part of the acute URTIs are of viral origin. Over 200 viruses are implicated in the cause of RTIs, including adenovirus, bocavirus, coronavirus, influenza virus, metapneumovirus, parainfluenza virus, respiratory syncytial virus and rhinovirus (~115 distinct serotypes) (Canducci et al., 2008; Kusel et al., 2006). In addition, a number of bacterial agents have been associated with RTIs (e.g. Streptococcus pneumoniae, Streptococcus pyogenes, Moraxella catarrhalis, Haemophilus influenzae, Neisseria meningitidis, Staphylococcus aureus).
Microbiome contributions to aetiology

The local respiratory microbiome

The commensal bacterial communities found within the different respiratory niches act as the first-line of defence against acquisition, overgrowth, and invasion of respiratory pathogens. Consequently, they have the potential to influence susceptibility to RTIs. It has been found that the predominant commensal bacterial species found within the respiratory niches of children belong to the genera *Corynebacterium*, *Dolosigranulum*, *Flavobacterium*, *Fusobacterium*, *Haemophilus*, *Moraxella*, *Neisseria*, *Propionibacterium*, *Prevotella*, *Staphylococcus*, *Streptococcus*, *Veillonella* (Bogaert *et al.*, 2011; Stearns *et al.*, 2015; Teo *et al.*, 2015). In addition to the variation in composition between different respiratory niches, there is large inter-individual variation and seasonal shifts within microbiota composition (Bogaert *et al.*, 2011). The presence of certain bacterial members of the local microbiome have been associated with respiratory health and disease (Hilty *et al.*, 2012; Laufer *et al.*, 2011; Teo *et al.*, 2015). For example, it was found that the protective effect of breastfeeding against URTIs may be contributed to an increased nasopharyngeal presence and abundance of the lactic acid bacteria *Dolosigranulum* and *Corynebacterium* (Biesbroek *et al.*, 2014a). In addition, it has been observed that the composition of the respiratory microbiota early in life determines bacterial succession patterns and impacts respiratory health also later in life (Biesbroek *et al.*, 2014b; Teo *et al.*, 2015). However, there is only limited knowledge on how the total bacterial load or species diversity changes preceding, during or following RTIs (Allen *et al.*, 2013).

Source of pathogenic organisms

The detailed pathogenic mechanisms of the various bacterial pathogens and respiratory viruses can be very different, as indicated by the primary site of colonisation/replication and the associated mechanism of immune response. Although some pathogenic agents limit themselves to specific niches within the respiratory tract most respiratory pathogens are generally implicated in acute infections throughout the entire respiratory tract. In addition to direct pathogen-host interactions, recent insights point towards a large role for the local microbiome in the aetiology of acute RTIs (De Steenhuijsen Piters *et al.*, 2015; Vissers *et al.*, 2014). Additionally, data are being obtained on how bacterial microbiota in the nose and nasopharynx might influence the course of viral illnesses. For example, bacterial members of the local microbiome might influence the host response to a specific virus (Vissers *et al.*, 2014). Furthermore, as aforementioned, it has been demonstrated that many bacterial pathogens causing RTIs in children are in fact common residents of the respiratory tract (Adegbola *et al.*, 2014; Garcia-Rodriguez and Fresnadillo Martinez, 2002). For example *S. pneumoniae*, considered to be the most common cause of bacterial pneumonia in children, has frequently been found in nasopharyngeal swabs obtained from healthy individuals (Bogaert *et al.*, 2004a; Chao *et al.*, 2014). The human nasopharynx is considered to be a reservoir for many other bacterial pathogens associated with acute RTIs as well (Bogaert *et al.*, 2011; Garcia-Rodriguez and Fresnadillo Martinez, 2002; Teo *et al.*, 2015).
Pathogenic shifts in the respiratory microbiome

Microbiome-related mechanisms such as shifts in local bacterial community composition, viral infection, or changes in host immune factors are likely to affect the possible transition from asymptomatic (nasopharyngeal) colonisation to respiratory disease. For instance, it is not uncommon that bacterial infections are preceded or co-occur with viral infections (Vissers et al., 2014). In addition, during infancy bacterial pathobionts that are present in the nasopharynx microbiome at the time of viral URTIs are a significant risk determinant for the spread of infection to the lower respiratory tract (Bosch et al., 2013; Vissers et al., 2014). In a healthy situation, the microorganisms found in the lower respiratory tract are most likely continuously inoculated by the constant flow of liquid (e.g. saliva) from the mouth to the throat. This can be concluded from the observations that the bacterial communities present in the healthy lung overlap more with those found in the oral cavity and less with nasal or nasopharyngeal communities (Bassis et al., 2015; Charlson et al., 2011). However, it is hypothesised that during periods of ill health, such as viral URTIs, allergies or sinus infections, the liquid flow from the nasal cavity increases, delivering more microorganisms (including nasopharyngeal pathobionts), from the nasopharynx to the throat. This puts the patient at higher risk for development of a LRTI. Complex interactions, both synergistic and competitive, exist in the respiratory tract between respiratory viruses, bacterial pathobionts, and non-pathogenic members of the respiratory microbiome supporting a polymicrobial view on aetiology of RTIs (Bosch et al., 2013; Van den Bergh et al., 2012; Vissers et al., 2014). Furthermore, biofilms containing pathobionts have been detected on mucosal surfaces during diseases such as pneumonia and otitis media, suggesting a role for biofilm formation in the disease process (Chao et al., 2014; Marks et al., 2013). Altogether, the main message is that pathogenicity of pathobionts can be enhanced or impaired by shifts in bacterial communities.

Role of the host and the immune system

For some inflammatory respiratory diseases (such as sinusitis and otitis media) it is often questionable whether the condition is caused by an infection or that it is in fact a sterile inflammation. For a long time, niches such as the sinuses and the middle ears, were thought to be sterile (Lee et al., 2016). In case of ineffective clearing of mucous secretions from these enclosed niches, fluid might accumulate and cause the complaints that are associated with these conditions such as facial pain or loss of hearing. Ineffective clearing might be the result of a physical blockage of the entry way (e.g. by polyps or large adenoids) or due to mucosal swelling. Such swelling, is an inflammatory tissue reaction of the mucous membranes within in these niches in response to an acute infections elsewhere or as results of other conditions such as allergic rhinitis (Hong et al., 2008). In the past, clinicians have often been puzzled by the fact that normal respiratory commensals have been found in these mucous secretions within healthy individuals. However, given that mucus is highly nutritious, these niches are very attractive environments to certain microorganisms. In healthy individuals, physical entry of microorganisms is prevented by the action of the ciliated epithelium. However, whenever the mucociliary system is impaired (e.g.
by a respiratory virus infection), this primary mechanical defence against bacterial invasion is disrupted. It is often that microorganisms entrapped in an obstructed niche have the possibility to outgrow and cause a superimposed (polymicrobial) infection (Brook, 2016; Marom et al., 2014; Revai et al., 2007). *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. pyogenes*, *S. aureus*, or more anaerobic species such as *Prevotella*, *Porphyromonas*, *Fusobacterium*, and *Peptostreptococcus* species are among the most common bacterial isolates from these niches (Brook, 2016). It has been hypothesised that especially recurrent infections are caused by the constant shedding of pathobionts from biofilms within the nasal and nasopharyngeal cavity that are able to reach the more inaccessible niches such as the sinuses and middle ear and cause infection (Chao et al., 2014; Duell et al., 2016; Marks et al., 2013).

**Other considerations unique to the paediatric situation**

Children in general are a population at higher risk for the development of acute RTIs. It has been suggested that their immature immune system makes infant and small children especially vulnerable to RTIs (De Martino and Ballotti, 2007; Malloy et al., 2013). For example, particular severe forms of ‘strep throat’ are seen in infants and children as they have not yet developed any protective immunity against *S. pyogenes*, a pathogen also known as Group A *Streptococcus* (GAS). Due to development of an adaptive immune response (including cumulative build-up of antibodies) to many different *Streptococcus* serotypes over the years, infections become less frequent with age (Mortensen et al., 2015). However, it must be noted that *S. pyogenes* is widely distributed among humans (within biofilm structures as well) and people, including children, are usually asymptomatic carriers (Marshall et al., 2015; Roberts et al., 2012; Shaikh et al., 2010). That being said, it has been observed that compared to adults, children have higher colonisation rates of potential pathogens (pathobionts) in the upper respiratory tract which puts them at higher risk for the development of acute RTIs as well (De Steenhuijsen Piters et al., 2015). For instance, nasopharyngeal carriage rates of *S. pneumoniae* are greater in children (20-50%) compared to adults (5-20%) (Chao et al., 2014; Marks et al., 2013). Furthermore, their anatomically immaturity makes children more prone for acute RTIS as well. For example, the infants and children, the Eustachian tube is shorter in length and joins the nasopharynx at a more acute angle compared to adults and this makes it easier for microorganisms to move into the middle ear and cause infection (Revai et al., 2007).

**13.3 Dental caries**

In contrast to acute RTIs, dental caries (DC) is much better understood as a polymicrobial disease: the interaction of multiple species residing in dental plaque biofilms results in the demineralisation and breakdown of hard dentin tissue. According to the 2004 National Health and Nutrition Examination Survey, 42% of children in the age 2-11 have had DC in their primary teeth and have an average of 1.6 decayed primary teeth and 3.6 decayed primary surfaces. Prevalence of DC for children over 2 years of age vary by country with developed countries (below 1-32% in some European countries like Sweden, UK and Finland) having lower rates
than developing countries (ranging from 22-61% in some Middle Eastern countries), excluding high risk populations (Çolak et al., 2013). DC presents in children as decayed, missing or filled smooth surfaces in primary maxillary anterior teeth (Çolak et al., 2013). It should be noted that there are multiple names for DC, often relating to the age of the child: early childhood tooth decay, early childhood caries, baby bottle-fed tooth decay, early childhood dental decay, comforter caries, nursing caries, maxillary anterior caries, and rampant caries. The term 'early childhood caries' is also becoming increasingly popular with dentists and researchers alike for use in children under 72 months.

**Microbiome contributions to aetiology**

The aetiology of DC is well-established to be multifactorial and points in one direction: dysbiosis of the normal functioning microbiome and associated biofilms (Hajishengallis et al., 2015). These biofilms have been defined as the microbial community residing on the tooth surface embedded in a matrix of polymers of bacterial and salivary origin (Marsh et al., 2011). It is not yet clear if children with DC have an entirely distinctive oral microbiome composition as opposed to an under-abundance of health-associated species (Corby et al., 2005), as a consensus on the composition of a healthy or asymptomatic core oral microbiome has not yet been reached. A recent study reported that a higher level of microbial diversity was found in children with a healthy oral cavity in comparison with children suffering from severe tooth decay (Kanasi et al., 2010).

Poor diet and oral hygiene are frequently associated with DC. Cariogenic microorganisms initially colonise in low numbers early in life (Corby et al., 2005) and can subsequently emerge and increase in abundance to cause DC under favourable conditions (Scheie and Petersen, 2004). For instance, there is a very strong link between increased (fermentable) carbohydrate intake and DC (Grigalauskiene et al., 2015). Acids are produced during the fermentation process which demineralise and destroy the enamel and dentin, ultimately leading to DC (Grigalauskiene et al., 2015). Other events such as changes in pH, salivary composition and flow rate, intake of other selective nutrients contribute selective niche pressure skewing the microbiome towards a more cariogenic-dominated composition (Grigalauskiene et al., 2015; Marsh, 2006; Ruby et al., 2010).

The bacterial genera which are uniquely associated with the absence of childhood DC include *Neisseria, Cardiobacterium, Rothia, Kingella, Aggregatibacter* or *Mannheimia* (Alcaraz et al., 2012). Even on the species level, caries-free subjects exhibit an overabundance of certain known beneficial species, i.e. *Streptococcus parasanguinis, Abiotrophia detectiva, Gemella haemolysans, Streptococcus mutans, Streptococcus mitis/oralis, Streptococcus cristatus and Streptococcus sanguinis* (Tanzer et al., 2001). Conversely, bacterial species of the genera *Veillonella, Cryptobacterium, Lactobacillus, Bifidobacterium, Megasphaera, Olsenella, Flavobacterium, Neisseria, Bergeyella, and Propionibacterium*, low-pH non-*S. mutans* streptococci, *Actinomyces* species, and *Atopobium* species, are found in children with DC and likely play important roles in caries progression (Aas et al., 2008; Crielaard et al., 2011). Contrary to popular opinion, *S. mutans* is not the only
species that has been implicated in DC prediction. Recently, microbiome analysis data have also shown that high levels of *Veillonella* species were a good predictor of caries in children (Gross *et al*., 2012), highlighting the metabolic dependency between acid-producers (e.g. *S. mutans*) and acid-consumers (*Veillonella* species).

### 13.4 Periodontal diseases

Gingivitis and periodontitis are regarded as common periodontal diseases (PD) in children (Al-Ghutaimel *et al*., 2014; Highfield, 2009). Gingivitis literally means ‘inflammation of the gingiva’ and is in essence an inflammatory reaction of the marginal gingiva. Prevalence rate data is limited to older studies. Gingivitis prevalence rates in Zurich schoolchildren (aged 8-17 years) in the 1970s were reported to exceed 90% at all ages (Curilovic *et al*., 1977), while in Sweden in the early 1980s gingivitis was present in 36% of 3-year-olds, 64% of 5-year-olds, 97% of 10-year-olds, 74% of 15-year-olds and 97% of 20-year-olds (Hugoson *et al*., 1981). The term ‘periodontitis’ includes infections or disorders of the tissues surrounding or supporting the teeth (gingiva, cementum, periodontal ligament, and alveolar bone) (Pihlstrom *et al*., 2005). Periodontitis is destructive and (usually) non-reversible, resulting in potential loss of tooth connective-tissue attachment to bone and loss of any involved teeth. Different assessment methods and few studies limit periodontitis epidemiology information and make the data difficult to interpret and potentially unreliable. However, some estimates place the prevalence of periodontitis in European children at 5% (Matsson *et al*., 1995). Higher subject and site prevalence rates in other ethnicities may be related to racial predisposition or poor oral hygiene. While there are high prevalence rates of PD among children around the world (Gjermo *et al*., 2002; Ketabi *et al*., 2006), experts have noted that the initial damage caused in children is reversible with good clinical management (Pinkham *et al*., 2005).

**Microbiome contributions to aetiology**

The primary aetiology of PD is the formation and development of microbial plaque on periodontal structures (Oh *et al*., 2002). Dental plaque is a biofilm community on the surface of soft and hard tissue and it has been known since the mid-1970s through the work of Page & Schroeder, that microbial biofilms elicit most periodontal tissue injury indirectly through mechanisms that initiate and propagate inflammatory host tissue reactions. The inflammatory infiltrate is characterised by polymorphonuclear leukocytes, macrophages, lymphocytes, plasma cells, and it is associated with both acute and chronic cellular reactions, including the substantial loss of collagen (Tatakis and Kumar, 2005). The significant microbial involvement in the aetiology of PD is strongly supported by the fact that prevention or treatment of PD can be achieved with mechanical and/or chemical antibacterial treatment.

During childhood, there are anatomical differences such as large interdental gaps in the gingiva which favour microbial plaque deposit and biofilm development even from an early age. Some studies suggest that PD develop when the numbers of Gram-negative bacteria and anaerobes
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in subgingival plaque increase (Ramberg et al., 2003; Ximénez-Fyvie et al., 2000). In healthy children (3-12 years old), Streptococcus species are primary colonisers and it has been found that they dominate soft tissue microbial communities in which they are present in higher abundance than in tooth-associated communities (Lucas et al., 2000; Papaioannou et al., 2009). Primary colonisers are especially unique in their abilities to co-aggregate with other bacteria, acting as bridging species between early and late colonisers to allow biofilm formation and maturation (Kolenbrander et al., 1989). Some primary colonisers such as Fusobacterium nucleatum subspecies have an ability to adhere to mammalian cells, such as fibroblasts and epithelial cells (Han et al., 2000).

While the risks and predisposing factors in children for the development of PD are varied and not as well defined, one of the most important local factors in children remains poor oral hygiene habits (Oh et al., 2002). When plaque and food debris accumulate in poor oral hygiene, microorganisms also accumulate initiating the inflammatory process. Much evidence indicates that gingivitis precedes the onset of periodontitis; however, not all gingivitis cases develop into periodontitis (Clerehugh, 2008). The reason for this is that accumulation of gingival or dental plaque is not solely sufficient for the development of periodontitis; host susceptibility being one of the missing factors. Also a small number of bacterial species associated with periodontitis, but not gingivitis have been recently identified, including members of the genera Prevotella, Treponema and Selemonas (Shaw et al., 2016).

13.5 Oral candidiasis (thrush)

Bacterial species are not alone in the respiratory tract and oral cavity, and fungal species are also important but neglected members of the microbiomes in these niches. In the oral cavity, they participate, for example in oral biofilm structure and function. Candida is an opportunistic fungal organism that is highly present in biofilm communities in most healthy mouths (Bahrani-Mougeot et al., 2008). In a 2007 study, 66% of North American children had oral fungal growth, and Candida albicans isolates were recovered in 56% of children (Jabra-Rizk et al., 2007). OC or thrush is a fungal infection of the mouth. In Italian children, aged 0-12 years old, the presentation frequency of oral mucosal lesions characteristic of OC is 28.4% (Majorana et al., 2010). Asymptomatic OC in children is a relatively common finding: a Polish group found that the overall incidence of OC in healthy preschool and school children is approximately 40% (Rozkiewicz et al., 2006). In OC, C. albicans generally accounts for around 50% of cases (Thompson et al., 2010), but other Candida species have been increasingly reported (Krcmery and Barnes, 2002); undoubtedly the increased reports are due to improved diagnostic methods and changes in medical practices and immunosuppressive therapy (Falagas et al., 2010).

**Microbiome contributions to aetiology**

The interactions and function of the ‘mycobiome’ (fungal community) in connection with the microbiome and host in the child’s oral cavity remains thus far relatively poorly explored, so
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it is difficult to draw conclusions as to the role of these fungal organisms. However, a change from the harmless commensal existence of *Candida* to a pathogenic state can occur following alteration of the oral cavity environment to one that favours the growth and virulence of *Candida*. Such predisposing factors most often relate to the weakening of the host immune defences, for example through medical or dental devices, reduced salivary flow, high intake of fermentable carbohydrates, endocrine disorders, immunosuppression, antibiotics, or nutritional deficiencies (Campisi *et al.*, 2008). Other factors can depend on e.g. age, immune maturation, diet, and environmental changes (Kadir *et al.*, 2005; Van Wyk and Steenkamp, 2011).

Overgrowth and domination of *Candida* in the oral cavity leads to desquamation of epithelial cells and accumulation of additional microorganisms, keratin and other proteins, and sometimes necrotic tissue. No single primary virulence factor has been identified with *Candida* species, however, a number of putative virulence factors contributing to OC aetiology have been proposed. Virulence of *Candida* is related to multiple mechanisms including adhesion to oral surfaces through various cell wall glycoproteins, biofilm formation and colonisation on and inside host tissue, host-damaging enzymes that allow tissue invasion damage, immune cell lysis, production of toxins or enzymes, and persistence during active infection (Thompson *et al.*, 2011). *Candida* is an organism that has evolved to flourish under different circumstances and these virulence factors are strongly related to a unique ability of *Candida*: polymorphogenic switching from yeast to hyphae. Polymorphogenic switching especially has pathogenic significance as the yeast form allows for rapid transport across mucosal surfaces via bodily fluids (e.g. saliva, vaginal secretions, and intestinal fluid); in contrast, the hyphal form allows for rapid attachment, biofilm formation, and nutrient scavenging in specific regions of the mucosa and is responsible for induction of pro-inflammatory responses at mucosal surfaces (Thompson *et al.*, 2011).

Similarly to the respiratory pathobionts abundantly found as commensal microbial community members in the nasopharynx, *Candida* can be found in all its polymorphic forms even in healthy individuals (Sudbery *et al.*, 2004). Recent emerging evidence even suggests a role for *Candida* in the oral cavity in health, co-existing with bacteria (Krom *et al.*, 2014). These inter-kingdom relationships occur through physical, chemical and metabolic interactions. *Candida* species are known to physically co-aggregate with oral commensal bacteria, acting as bridging or scaffold organism upon which an oral biofilm can be formed. This provides a protective environment for bacterial biofilms by helping them resist from being removed by saliva or swallowing and also makes them less susceptible to antibiotic treatment (Harriott and Noverr, 2009). In addition, because *C. albicans* can grow under both aerobic and anaerobic conditions, when it utilises limited oxygen in oral biofilms, it ensures favourable conditions for anaerobic bacterial species. *Candida* and bacteria have co-evolved together and have developed a chemical communication system (known as quorum sensing) that allows inter-kingdom signalling in order to influence multiple processes including growth, virulence, and adhesion among others. *C. albicans* itself has evolved to allow it to utilise various carbon sources (e.g. sucrose and glucose as well as the non-fermentable lactate) including those derived from inter-kingdom cross-feeding relationships.
Some research posits that the evidence suggests that *C. albicans* growth is stimulated by oral species that produce lactate such as *S. mutans* (Krom et al., 2014).

### 13.6 Existing diagnostics related to the microbiome

As outlined earlier, there is a role for the microbiome in respiratory tract and oral cavity diseases, yet current diagnostic practices do not always take this into account. The exception is for OC where diagnosis relies on direct microscopic identification of *Candida*. The clinical presentation of OC is creamy white lesions on the mucosal surface that can be easily removed through gentle scraping, leaving behind an underlying erythematous mucosal surface (Reichart et al., 2000). The recovered pseudomembranes are then visually examined using microscopy or histology, and typically reveal both yeast and filamentous forms of *Candida* in addition to desquamated epithelial cells. However, for other respiratory and oral diseases, such as RTIs, DC, and PD, the diagnosis is primarily based on observations of clinical signs and symptoms. Yet, based on clinical symptoms, it is often difficult to determine the extent of microbial involvement in these conditions. First of all, the symptoms and signs of infectious diseases often overlap with those of other conditions (e.g. allergic rhinitis). Secondly, for most causative agents there is a lack of specific correlation between the agent and the clinical manifestations as a diverse array of overlapping symptoms can be expected dependent on the causative agent.

In the case of suspected viral involvement, there are no universal standard procedures for direct examination of clinical specimens or for serological diagnosis, as standards differ between clinics/hospitals, regions, and countries. Clinical practice commonly avoids isolation of the suspected viral agent as the slow identification process is prohibitive. Improvements in viral detection techniques, such as viral antigen detection and PCR-based assays, have made it possible to identify viral presence in clinical specimens, but this is still rarely done in clinical settings since such tests require knowledge of expected targets beforehand. However, as these new diagnostic techniques became more readily available in clinical settings, studies have been showing, for example, a much higher prevalence of respiratory coinfection in paediatric patients than previously found (Lim et al., 2016). The diagnosis of a bacterial infection has traditionally been performed by culture-dependent methods and rests on the demonstration of a bacterial pathogen in tissue, exudates, blood, or other bodily fluids. Rapid strep tests based on antigen detection, have proven useful for the diagnosis of streptococcal pharyngitis, though the specificity and sensitivity vary widely. As many pathogens are members of the normal microbiota, culturing of nose or throat specimens is not indicative for the identification of the causative agent.

Due to their polymicrobial nature, RTIs could benefit from microbiome-centred diagnostics to identify possible causative microbiome communities. There are no current accepted procedures for direct examination and analysis of microbiome specimens for diagnostic purposes. Sampling for microbiome analysis can be challenging within the human respiratory tract, due to inaccessibility of certain niches, the relative low numbers of microorganisms present in some...
niches, and the possibility of cross-contamination during sampling. To date, analysis of causative microbiome communities in clinical practice is non-existent because of the lack of an accepted healthy core microbiome for each niche.

13.7 Microbiome-supportive therapeutic strategies

With respect to oral health, good oral hygiene is key. The critical window for children to develop good oral hygiene practices in children is from 3 months to 3 years of age (Douglass, 2000; Kranz et al., 2006; Lif Holgerson et al., 2015). This is because the oral microbiome profiles change significantly during the first years of life (Lif Holgerson et al., 2015), as the result of physiological changes, such as immune development and tooth eruption (Colombo et al., 2009), and environmental changes, such as dietary practices and siblings (Mattos-Graner et al., 2001; Stahringer et al., 2012). Most hygiene centres around physical (Hope and Wilson, 2003) and chemical (Filoche et al., 2005; Fine, 1988) reduction of microbial load in the oral cavity. Another priority in the treatment of oral diseases is the alleviation of any identifiable predisposing factors. Simple direct microbial management strategies such as avoidance of excess fermentable carbohydrates and proper biofilm (plaque) removal during oral hygiene practices have been shown to enhance oral health and reduce the incidence of DC in children (Grigalauskiene et al., 2015). Effective control of the plaque biofilm and preventive measures are also fundamental to therapeutic success in PD. Cautious use of antimicrobial toothpaste and mouth rinses should be employed. Even though some products, for instance those containing triclosan or fluoride, have not shown any effect on the oral microbiome in the short-term (Koopman et al., 2015; Poole et al., 2016), their long-term effects on the paediatric microbiomes and oral and respiratory health have not yet been studied.

Most current therapeutic therapies used in paediatric patient populations to support respiratory health, such as antibiotics and vaccines, aim to eliminate (potential) pathogens. Some of these therapeutic strategies rely more on preventative control measures regarding pathogen transmission than on actual treatment. For example, vaccines have been successful in eliminating specific pneumococcal serotypes from immunised populations. However, this strategy is not sustainable since serotypes that are not targeted by the vaccines have been shown to fill in vacant nasopharyngeal niches (Biesbroek et al., 2014c; Bogaert et al., 2004b; Hanage et al., 2010; Spijkerman et al., 2011). As a result, overall pneumococcal carriage rates have not, or only temporarily, decreased. Furthermore, by reducing nasopharyngeal carriage of specific pathobionts, the use of specific elimination strategies may also create ecological niches for colonisation by alternative respiratory pathogens (Biesbroek et al., 2014c; Bogaert et al., 2004b; Revai et al., 2006).

As pathogens are highly adaptive, complete elimination is nearly impossible and severe impairment of the respiratory tract and oral microbiomes is an undesirable but prominent side effect. For example, (broad-spectrum) antibiotics are prescribed to eliminate specific pathogens, however, also the resident bacterial microbiome is affected by these antibiotics (Jakobsson et
Antibiotic usage in paediatric populations is still varied; despite the fact that many RTIs are viral in origin, antibiotics are often prescribed with the belief that they may prevent secondary bacterial infections (Kenealy and Arroll, 2013). Cautious use of antibiotics is indeed warranted when coupled with the possibility of misidentification of the causative agent(s) as seemingly innocent URTIs can sometimes develop into severe diseases such as meningitis, sepsis, or rheumatic fever. For example, treatment of ‘strep throat’ by antibiotic treatment is primarily aimed at preventing non-suppurative (in particular, rheumatic fever) and suppurative complications in paediatric patients and to prevent bacterial transmission to other individuals. However, the emergence of antibiotic-resistant bacteria due to overuse of antibiotics is a serious global health concern. Therefore, nowadays many clinicians are moving towards a ‘wait-and-see’ non-treatment approach for most acute URTIs, since most infections will recover naturally (Kenealy and Arroll, 2013; Venekamp et al., 2015).

The consequences of treatment failure or non-treatment are significant: lingering complaints, secondary infections, recurrence, and the development of treatment-resistant pathogens. The difficulty of making a reliable diagnosis is one of the primary factors leading to inappropriate treatment. This suggests that a more evidenced-based ecological approach should be adopted to avoid long-term health consequences of the therapeutic strategies used for the management of inflammatory and infectious diseases of the respiratory tract and oral cavity.

Alternative therapies that support the commensal microbiome in the respiratory tract and oral cavity are highly desired. Microbiome-supportive therapies, such as probiotics, have shown their clinical potential in the prevention of (recurrent) inflammatory and infectious diseases of the respiratory tract and oral cavity. For example, the oral use of probiotics has been shown to reduce the abundance of certain cariogenic species when taken prophylactically (Cagetti et al., 2013). Moreover, recent studies have suggested that probiotics may reduce the risk of various symptoms of URTIs (Hao et al., 2015; King et al., 2014). Probiotics (mainly *Lactobacillus*- and *Bifidobacterium*-containing products) have shown their potential clinical efficacy by preventing the recurrence of ear infections, reducing the number of antibiotics prescriptions and cold-related school absences in acute URTIs. However, there is enormous heterogeneity between current probiotic studies with respect probiotic strains used, dosage, duration, population demographics, primary and secondary outcomes, and parameters studied. Nevertheless, the current available data suggest that the use of probiotics, including certain *Lactobacillus* and *Bifidobacterium* strains, is potentially effective in the prevention of acute RTIs. However, there is also tremendous potential for the isolation of new potential probiotic strains from the local microbiome, as commensal non-pathogenic members have evolved to compete with pathogens. For example, oral and pharyngeal-derived *Streptococcus* species have shown to be able to reduce the number of episodes of streptococcal pharyngeal infections (Di Pierro et al., 2014; Roos et al., 1993, 1996). The mechanisms behind the possible beneficial effect of probiotics are not completely clear but may be related to the stimulation of the immune response and colonisation of the URT by probiotic bacteria (Nagalingam et al., 2013; Popova et al., 2012). However, as the beneficial clinical effects are strain specific, as is the safety profile of strain, results should not
be extrapolated and more extensive studies are needed to be able to provide recommendations for the use of specific probiotic strains (Szajewska, 2016).

13.8 Goals for future research

As the microbiome has a role in aetiology of many diseases, this clearly has potential implications for clinical management of paediatric patients with respect to diagnostics, treatment and prevention of disease. Unfortunately with respect to inflammatory and infectious diseases of the respiratory tract and oral cavity, the majority of the research on the microbiome in the different respiratory and oral niches is still focused on adults and this restricts the progression of the paediatric field. Especially since it has been found that early-life microbiome development has health implications also later in life, priority should be given to studies in paediatric populations with the following aims: (1) identification of key species within the different respiratory and oral niches and their role in paediatric respiratory and oral health; (2) understanding the assembly of the respiratory and oral microbiomes and identify paediatric developmental milestones; (3) unravelling the mechanisms behind IBC and biofilm formation, development, and penetration in order to improve treatment and diagnosis; and (4) consequences of antibiotic use in early life regarding respiratory diseases.

Since changes in local microbiomes have been associated with a growing number of inflammatory and infectious diseases of the respiratory tract and oral cavity, there are some pressing research questions regarding microbial dysbiosis that have emerged. Firstly, do disturbances within the microbiome indeed contribute to disease aetiology or is it merely a marker of injury and inflammation? Secondly, how does the diverse and dynamic homeostasis within a local microbiome collapse? Lastly, can the local microbiome be manipulated therapeutically to change exacerbation, frequency, or disease progression? This polymicrobial view on inflammatory and infectious diseases may provide new insights in therapeutic strategies with a more ecological approach to disease management. The regular occurrences of mild respiratory diseases in childhood are natural events; for a large part related to their developing immune system. However, especially those children that suffer from recurrent respiratory diseases may benefit from an early-stage intervention on a microbiome-level. The application of culture-independent molecular methods in future research both to study microbial composition as well as activity, combined with measuring differences in host responses (e.g. epithelial gene expression, immune cell activation) will progress our understanding of healthy microbiome homeostasis. In addition, future research should focus on therapies that have the potential to modulate the local ecosystem in order to reselect for beneficial commensals. To this end, adjunct or stand-alone therapies that support or target restoration of microbiome homeostasis (e.g. probiotics, prebiotics and synbiotics) hold great promise.
Conflict of interest

Jessica Younes works as a Science Liaison at Winclove Probiotics in Amsterdam. Coline Gerritsen works as a research scientist at Winclove Probiotics in Amsterdam.

References


13. Microbiomes of the respiratory tract and oral cavity


13. Microbiomes of the respiratory tract and oral cavity


Chapter 14
The paediatric urinary tract: emerging lessons from the adult urinary microbiome

J.A. Younes* and J. Gerritsen
Winclove Probiotics B.V., Hulstweg 11, 1032 LB Amsterdam, the Netherlands; j.younes@winclove.nl

Abstract

Urinary tract infections (UTIs) are common bacterial infections in children with incidence rates varying depending on age and sex; the risk of acquiring a UTI during the first decade of life is approximately 3% in girls and 1.1% in boys. Dogma dictates that healthy urine is sterile. Central to aetiology and pathogenesis is evidence that uropathogens migrate to the bladder and can be identified in patient’s urine upon infection. Identification of uropathogens from extra-urinary reservoirs and the subsequent re-identification of the same uropathogens in recurrent UTIs lends further support. Within this dogma, the bladder functions as a sterile storage and secretory vessel for systemic waste yet somehow pathogenic bacteria are somehow able to cause an infection and thrive. Recent discovery of live bacterial communities in healthy female adult bladders nullifies this paradigm. This chapter aims primarily to offer the reader information about the newly discovered urinary microbiome and suggest links from adult research to the paediatric context.

Keywords: children, urinary tract, microbiome, aetiology, diagnostics, treatment failure

14.1 Introduction

Urinary tract infections (UTIs) occur in both genders during the first few months of life, but thereafter UTIs predominate in females. Data in children suggests that Gram-negative uropathogenic Escherichia coli (UPEC) is responsible for the majority of UTI cases (>90%), whereas Gram-positive bacteria (particularly enterococci and staphylococci) represent 5-7% of cases (Shapiro, 1992). The urinary tract is thought to be normally sterile except during UTIs or, less commonly, in conditions of asymptomatic bacteriuria. Since the early 1990s, there has been an increasing trend towards the isolation of other suspected causative pathogens (Abrahamsson et al., 1993). Most notably, the detection of bacteria in urine has puzzled some clinicians (Maskell, 2010). With varying sensitivity and specificity ranges, the vast majority of bacteria found in urine are not or cannot be cultured by standard clinical culture techniques. Furthermore, in young girls, bacteria isolated from urine can be easily mistaken for and contaminated with vaginal colonies, and the dual anatomical function of the urethra prevents direct uncontaminated urine sampling in young boys. With little ability to detect or rule out new or previously unappreciated uropathogens or other non-pathogenic bacteria within standard culture and even though
additional culture techniques consistently yield non-pathogenic bacteria such as lactobacilli (Maskell, 2010), standard culture results still govern UTI diagnosis, treatment, and research.

The identification of a live functioning urinary microbiome (UM) in healthy female adult bladders fundamentally has nullified the sterile urine hypothesis. With the introduction of new bacterial identification techniques, such as 16S rRNA gene analysis and high-throughput DNA sequencing, a much wider spectrum of uncultured bacteria has been identified in the bladder of both healthy adult female individuals and patients with UTIs (Hilt et al., 2014; Pearce et al., 2014; Thomas-White et al., 2016b). Such emerging knowledge demands that clinicians and scientists reassess their assumptions concerning the aetiologies of urinary tract paediatric disorders as more evidence continues to accumulate (Figure 14.1). This chapter will walk the reader through the current state of knowledge of the UM and highlight some take-home messages in the context of paediatric aetiology, diagnosis and treatment.

![Figure 14.1. How the urinary microbiome will change and catalyse new opportunities in clinical paediatric patient management practices.](image-url)
14.2 Microbiome contributions to aetiology

In adults, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, and occasionally *Klebsiella pneumoniae* and *Proteus mirabilis* have been identified as microorganisms responsible for UTIs, while in children under 5 years old, *E. coli* and *K. pneumoniae* are more commonly isolated and identified (Garout *et al.*, 2015). New 16S sequencing and expanded culture data show that bacterial composition of the healthy adult UM has low species diversity and, like the vagina, tends to be dominated by one or two species (Hilt *et al.*, 2014; Pearce *et al.*, 2014; Thomas-White *et al.*, 2016b). *Lactobacillus* species have been found in almost all urine samples to date and *Gardnerella* species are also very common (Pearce *et al.*, 2014). Urine from adult patients with urinary urgency versus matched controls show similar microorganisms but the ratio of species is different. Lactobacilli are more common in non-urinary urgency-derived urine, while the opposite is true for *Gardnerella* species (Pearce *et al.*, 2014). UPEC and other pathogens can be found in healthy urine, but in very low amounts. Such ecologic dualism not only suggests that uropathogens can be commensal or pathogenic in nature, but also the aetiology of UTIs is not as simplistic as previously thought.

The origin of uropathogens (and commensal microorganisms found in the bladder in general) has long been a point of discussion. Given the close proximity of the anus to the vagina, these two niches represent attractive reservoir candidates in female patients. Indeed, as the urinary tract is a self-contained organ with an impermeable lining, retrograde microbial ascent is thought to be the most common mechanism of infection. Cultures of urine-isolated microorganisms from women have been identified as identical to those cultured from vaginal samples (Fede, 1983; Pierce and Hart, 1992) and faecal microorganisms (Gorbachinsky *et al.*, 2014). Interestingly, breastfeeding in general has been shown to be protective against childhood UTIs (Marild *et al.*, 1989; Pisacane *et al.*, 1992), and constipation has been associated with UTI (Cayan *et al.*, 2001), suggesting that the gastrointestinal microbiome may play an important role in urinary health.

Associations between genital and bladder health also exist: young girls with vulvitis are eight times more likely to have a culture-diagnosed UTI (Gorbachinsky *et al.*, 2014), while conversely girls without vulvitis were more likely to yield non-uropathogens, such as *Lactobacillus* species in their urine than girls with vulvitis (81 vs 21%, respectively, *P*<0.0001) (Gorbachinsky *et al.*, 2014). In young males, it is less clear: studies of uncircumcised infants show a number of microorganisms in the urethral meatus and periurethral areas (Fussell *et al.*, 1988; Wiswell *et al.*, 1988). Indeed, a meta-analysis representing over 400,000 children determined that circumcision is associated with a significantly reduced risk of UTI in young boys (Singh-Grewal *et al.*, 2005). Traditional culture methods have been applied in these studies, so the potential wealth of information about identification of non-pathogenic microorganisms in children is still unknown. The first take-home message is that if uropathogens can ascend from extra-urinary reservoirs and colonise the bladder, it logically follows that the same is possible for non-uropathogens.
A second take-home message is that it is critical to understand that newly discovered (commensal) microorganisms in the bladder are alive (Hilt et al., 2014; Pearce et al., 2014; Thomas-White et al., 2016a) and as such this implies a function for them in the bladder niche. Perhaps of particular clinical interest is the association between the UM phenotype and infection recurrence and also to medication response in certain patient populations. UTI recurrence in adults has been shown to have been caused by the same pathogen for up to three years later (Foxman, 2002; Russo et al., 1995). In urinary urgency, the UM phenotype of responders versus non-responders to anticholinergics (solifenacin) relates to their baseline microbiome composition, with responders having fewer species and lower diversity at baseline (Thomas-White et al., 2016b). It could be that the UM composition and function is influenced by long-term medication. Alarmingly, it has also been shown that antibiotic treatment can alter the UM for up to 12 weeks afterwards (Thomas-White et al., 2016b). This is relevant because of the long term use of low-dose prophylactic antibiotics in paediatric populations to prevent renal damage, for instance with ureteral reflux. Antibiotics are unable to alter the receptivity of urothelial cells to adherence by pathogens (Reid et al., 1990), so there might be additional unknown health ramifications to their singular purpose of eradication of the live functioning UM members.

Prior to the discovery of intracellular bacterial communities (IBCs), it was previously thought that UTI pathogenesis occurred extracellularly. However, certain pathogens are notorious for their ability to initiate internalisation pathways by bladder urothelial cells (Flores-Mireles et al., 2015; Nielubowicz and Mobley, 2010). Pathogens in IBCs are able to escape expulsion by exocytosis and endocytic pathways (Eto et al., 2006) and it has been shown that certain compounds produced by UPECs also trigger epithelial exfoliation (Flores-Mireles et al., 2015). Furthermore, urothelial cells isolated from patients with UTIs have a much higher exfoliation rate, leaving poorly differentiated cells highly susceptible to microbial adhesion and invasion (Flores-Mireles et al., 2015; Nielubowicz and Mobley, 2010). When inoculated into the bladders of mice, UPECs isolated from the urine of adult female UTI patients were able to form IBCs but not the control bacteria (Garofalo et al., 2007). It is unknown whether certain commensals offer protection against IBC formation; it might be that there is a protective UM composition that forms harmless IBCs to outcompete pathogens for internalisation routes. This intrinsically implies that pathogenicity is not equal for all bacterial species and it is known that from other microbial niches that inter-microbial communication can also influence pathogenesis and virulence.

This simple change from an extracellular to intracellular location affords the microbes a very nutrient-rich environment (Flores-Mireles et al., 2015) in addition to protection from antibiotics, urine shear forces, and detection by neutrophils and the immune system (Scott et al., 2015). Furthermore, mice models have shown that pathogens can remain entrenched and quiescent within urothelial cells for months (Schilling et al., 2002), also serving as a subsequent source reservoir for UTI pathogens (Hunstad and Justice, 2010). In fact, the ability to form IBCs has been noted in a majority of bacteria isolated from adult patients with acute/recurrent cystitis, other recurrent urinary infections (Anderson et al., 2003), and children (Robino et al., 2014).
Cumulatively, these data suggest a third take-home message: there exists an interdependency between the UM, UPECs and virulence, and clinical symptoms.

### 14.3 Diagnostic challenges related to the microbiome

The current gold standard of UTI diagnosis is based on the culture and enumeration of colony forming units per millilitre of urine (cfu/ml). Some scientists posit that non-pathogenic bacteria have not been detected regularly in urine primarily because gold standard culture techniques were developed to detect easily culturable aerobic bacteria, such as *E. coli* (Maskell, 2010; Thomas-White *et al.*, 2016a) and not fastidious and anaerobic bacteria, which make up a significant portion of the UM (Hilt *et al.*, 2014; Pearce *et al.*, 2014). It is thought that commensal microbiome members are not detected or reported because culture thresholds are too high (usually $10^3$ cfu/ml) and/or because growth of these bacteria require special culture conditions not used in routine urine culture. If a clinical specimen does not yield uropathogen colonies, it is considered to be sterile, or categorised as asymptomatic bacteriuria. Optimal quantification and identification of microbes within the bladder is further complicated by IBCs. A single exfoliated urothelial cell with an IBC, which is normally difficult to detect and identify, can contain a high load of bacteria (up to $10^5$ cfu/ml) but unless homogenised properly, yields only 1 cfu/ml of bacterial colonies.

There is, at the moment, insufficient diagnostic evidence to confirm any relationships between the UM and urinary tract symptoms in children. Such normative data is lacking and requires large, longitudinal studies across diverse populations. Most research to-date employs the gold standard culture methods to focus on understanding uropathogenesis so as to enhance treatments, however, the emerging knowledge on the existence of a functioning UM will have powerful ramifications for diagnostics. Recently, an expanded quantitative urine culture (EQUC) has been developed that demonstrates the presence of other microbes in urine than just simply uropathogens (Hilt *et al.*, 2014). Paediatric populations with recurrent UTIs (rUTIs) perhaps have the greatest long term health risks by virtue of their age and physiological immaturity. Many clinicians are beginning to practice more prudent use of antibiotics, especially prophylactic use, but this is challenging in such populations. The modern clinician is advised to consider the integration of the EQUC technique into routine patient care so as to provide more tailored diagnostic information for the individual paediatric patient.

### 14.4 Treatment failure due to unaccounted for microbial characteristics

Treatment of UTIs in paediatric populations has four main goals: elimination of symptoms and bacteriuria in the acute episode, prevention of renal and urological complications, prevention of recurrence, and correction of any associated urological lesions. This is accomplished with varying antibiotic regimes, depending on severity, symptoms, and identified pathogens. It has been calculated that children under 5 years old with recurrent UTIs have a 5-fold increased risk of associated urological abnormalities compared with those with single UTI (Garout *et al.*, 2015).
Transient renal failure occurs in 40% of paediatric patients with UTIs and permanent renal damage in 5% of paediatric patients (Williams et al., 2001), so antibiotic treatment failure-associated risks are severe.

In adults, rUTIs have distinctive microbial patterns that can perhaps help to partially explain antibiotic treatment failure. Certain microbes are somehow able to outcompete commensal microbes and substantially increase their biomass in the bladder. This is shortly followed by dysfunctional immune defence responses in the bladder and depending on the pathogen, IBC formation. In the optimal scenario, the causative pathogen is first identified and then, once appropriate antibiotics are used, eliminated. However, this is unfortunately not the norm; IBCs have been found in exfoliated cells in urine from adult female patients with UTIs, suggesting that pathogens can reside in the bladder and remain completely undetected in routine culture (Russo and Johnson, 2003). The increase in nutrients after antibiotic use from the dead and potentially lysed microorganisms, combined with a destabilised and impoverished microbial community (and a consequent lack of competition), favours fast-growing pathogens to colonise and dominate the bladder environment, further exacerbating the infection. Furthermore, the application of topical antibiotics applied to the perineum have not been able to prevent rUTIs in adult females (Warren et al., 2000), suggesting that either pathogens have additional unknown reservoirs or that the antibiotics are ineffective at eradicating their microbial targets.

Given the existing safety efficacy evidence in adults, therapeutic options with a high safety profile and fewer side effects (such as probiotics) should be considered and tested further in children. A meta-analysis reported a statistically significant decrease in rUTIs in adult patients given Lactobacillus strains, denoted by the pooled risk ratio of 0.51 (95%CI: 0.26-0.99; P=0.05) (Grin et al., 2013). The clinician should always weigh the options using the evidence and their own intuition: compared with traditional long-term antibiotic prophylaxis, probiotic treatment may generally have a slightly inferior therapeutic effect (Beerepoot et al., 2012), but will result in minimal negative disturbances of the non-target and systemic microbiomes with no risk of antibiotic resistance and systemic side effects. Furthermore, the EQUC protocol can precisely demonstrate what microbes are present and is easily adopted into clinical practice. To achieve optimal patient care, the willing clinician should consider to be guided by clinical logic and their own experience in the absence of standard guidelines for alternative approaches when evaluating options such as probiotics. Other scientific advances may facilitate the identification of new aetiological agents and therapeutic targets for UTIs in children. A renewed interest in the aetiology and management of UTI has surfaced over the past few years. The need for accurate and updated population surveillance data is apparent, particularly in light of concerns regarding antimicrobial resistance. This information will ultimately direct selection of optimal empiric therapy for UTI in children.
14.5 Conclusions

The exploration of the UM in adults has preceded evaluation of the paediatric population, and accordingly lessons can be learned to help guide the direction of future research and paediatric patient care. Evidence in adults suggests that UTIs in general are not caused by one or two pathogens, but rather might be a polymicrobial condition. This observation raises more questions than answers at the moment and future research in children should be focused on the following goals:

- characterisation of the core microbiome of the urinary tract and how it translates to in terms of clinical outcomes in children especially for UTIs;
- identify UM assembly and composition, contributing factors, and vulnerable moments across childhood;
- determine appropriate length of antibiotic therapy in children and better risk profiling to reduce unnecessary long term prophylactic antibiotics;
- determine if sensitive, time-efficient microbial identification can optimally influence paediatric patient management compared to treatment prescribed without such information;
- determine protective factors and behavioural practices for paediatric populations such as breast-feeding;
- identify hidden at-risk populations such as sexual abuse victims or children with early sexual debuts.

Clinicians should become aware of exciting new evidence that promises to change clinical management strategies for paediatric UTIs (Figure 14.1). Bladder colonisation with specific genera in early child/adulthood (asymptomatic or symptomatic) is thought to influence propensity to bladder pathology later in life as one factor in the multifactorial basis of disease pathogenesis (Lewis et al., 2013; Peters et al., 2009). While we do not yet know that exact fingerprint for the UM in children, its very existence promises new opportunities to advance and improve patient care.

**Conflict of interest**

Jessica Younes works as a Science Liaison at Winclove Probiotics in Amsterdam. Coline Gerritsen works as a research scientist at Winclove Probiotics in Amsterdam.

**References**


14. Paediatric urinary tract knowledge is still in the cradle


Part VII.
Assessment of microbiota
Chapter 15
Methodologies for microbiota assessment in infancy and childhood

D. Radjabzadeh1, S.R. Konstantinov2,3, H.A. Moll4, A.G. Uitterlinden1,5, E.G. Zoetendal6 and R. Kraaij1,5*

1Department of Internal Medicine, Erasmus MC, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands; 2Department of Gastroenterology and Hepatology, Erasmus MC, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands; 3Janssen Vaccines & Prevention, P.O. Box 2048, 2301 CA Leiden, the Netherlands; 4Department of Pediatrics, Erasmus MC, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands; 5Department of Epidemiology, Erasmus MC, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands; 6Laboratory of Microbiology, Wageningen University, P.O. Box 8033, 6700 EH Wageningen, the Netherlands; r.kraaij@erasmusmc.nl

Abstract

To design a study involving gut microbiota, it is important to realise what information is needed. The two main questions concerning microbiota are ‘Which microbes are there?’ and ‘What are they doing?’. These questions can then be addressed by several technical approaches: 16S ribosomal RNA profiling, metagenomics, metatranscriptomics, metaproteomics and metabolomics. In this chapter, we will provide an overview of the different approaches with their pros and cons. Furthermore, we will give an overview on the current status of sample collection and sample handling.

Keywords: 16S rRNA, metagenomics, metatranscriptomics, metaproteomics, metabolomics, faecal sample, collection, storage, study design

15.1 Introduction

The intestinal microbiota is crucial for human life and studying its composition and function in health and disease is currently a hot topic. This is in part due to the fact that traditional cultivation-based approaches to study the microbiota have been replaced by high-throughput cultivation-independent approaches, which have now also been adopted in the fields of epidemiological cohort studies and clinical practice. These studies have tremendously increased our knowledge about the intestinal microbiota, notably those represented by the faeces.

Depending on the research question, DNA, RNA, protein or metabolites or combinations of these need to be extracted from the stool sample for subsequent phylogenetic or functional profiling, and these require different sampling and storage conditions. Typical questions concerning microbiota in relation to health and disease are ‘Which microbes are there?’, ‘What are they doing?’ and ‘How can we influence them?’. These questions can be addressed by several
approaches targeting a wide variety of microbial biomarkers that include 16S ribosomal RNA gene profiling, metagenomics, metatranscriptomics, metaproteomics and metabolomics.

15.2 Sampling and sample handling

Faecal samples are most frequently used to study the intestinal microbiota as they are easily collected. Nevertheless, several studies showed that the bacterial community in stool is different from those derived from the mucosa at different sites along the large intestine (Eckburg et al., 2005; Zoetendal et al., 2002). Moreover, recent studies have demonstrated that distinct microbial ecosystems exist in the small and large intestine (Hayashi et al., 2005; Zoetendal and de Vos, 2014). In numbers, the small intestine has 1000-times less microbes than the large intestine (Berg, 1996; Booijink et al., 2010; Ley et al., 2006), and, in contrast to the faecal microbiota, the microbiota of the small intestine is driven by fast uptake and utilisation of simple carbohydrates (Zoetendal et al., 2012). Hence, a faecal sample is only a good proxy for studying the intestinal microbiota of the distal luminal part of the intestine. Sampling at other locations of the intestine needs more invasive procedures, which complicate studying the dynamics of the microbiota over time. New sampling techniques, including smart devices that are swallowed by the participant and sample luminal content along the gastro-intestinal tract, are being developed and will become available in the near future (reviewed in Amoako-Tuffour et al., 2014).

Ideally, collection of a stool sample should follow these rules: the entire stool is homogenised immediately after defecating followed by immediate sample preparation using part of the homogenate (Gorzelak et al., 2015). However, in most situations this will not be possible when samples are collected at the home of the volunteers. Therefore, deviations from this protocol need to be made and optimised for the research question that is addressed.

The main issue regarding sampling is timing of defecation and what biomarkers are needed to address the research question. Since a wide variety of studies requires recruitment of volunteers, sample production at home is often necessary. Furthermore, in large studies it is often practically and economically impossible to collect these samples from the participants’ homes and samples are sent to the research centre by regular mail. However, this procedure cannot be performed when the activity of the microbiota will be studied. When RNA, protein or metabolites are to be studied, direct storage at -80 °C or direct sample preparation is needed. DNA, on the other hand, is more stable and allows for less stringent approaches, such as collection at ambient temperature for a short period of time. Several studies have addressed the effects on microbiota composition when collection is performed at room temperature (Carroll et al., 2012; Dominianni et al., 2014; Gorzelak et al., 2015; Lauber et al., 2010; Sinha et al., 2016; Tedjo et al., 2015). Longer periods of time at ambient temperature will affect composition and will decrease diversity in the sample. Variation in composition may reach up to 10% upon four days at room temperature. Nonetheless, it is recommended that samples should be stored at -80 °C as soon as possible. Alternatives to collection at ambient temperature are temporary storage at the participant’s home freezer, either at 4 or -20 °C, and subsequent collection of the sample by the researchers. Modern freezers,
however, may defrost during night time which may influence the composition when too many of such cycles have occurred (Gorzelak et al., 2015). Also, the addition of stabilising solutions, such as RNA later or 70% ethanol can be considered as they have proven to stabilise the composition for DNA-based analyses as well as those targeting RNA and metabolites (Sinha et al., 2016). However, in these cases safety procedures when used by non-professionals are compromised.

In addition to the biomarker-associated sampling issues, there are also practical issues concerning collection of samples. For example, collection of entire stool samples from people’s homes is often regarded as impractical and, therefore, the study participants take subsamples. To standardise this step in the sampling procedure as much as possible, participants should be carefully instructed by giving clear sampling instruction with pictograms and the instruction not to mix urine and faeces. Once the samples arrive at the research centre, direct preparation of the samples may not be performed for convenience. Also, when samples are collected within large cohort studies, samples arrive at the research centre over longer periods of time (up to years) and sample preparations spread over such time period will introduce batch effects. Therefore, samples are stored for longer periods of time at -80 °C and extracted once all samples have been collected. When dealing with large cohorts of volunteers, storage of samples for longer periods of time at -80 °C or lower may result in compositional changes (Carroll et al., 2012). These variations are, however, very minor and within the range of sampling and technical variations, and much lower than the inter-individual variation.

Sample preparation very much depends on the type of analysis and falls beyond the scope of this chapter. One general rule should be taken into account that every method introduces specific biases. Therefore, standardisation, through adhering to the chosen method as strict as possible preferably by means of automation, is the general rule in sample preparation.

15.3 Microbiota profiling

Several microbiota profiling approaches are available that overlap and complement each other. The different approaches are described in this section and schematically illustrated in Figure 15.1.

16S rRNA gene profiling

The 16S rRNA gene encodes the 16S ribosomal RNA that is part of the small subunit of ribosomes, which can be found in Bacteria and Archaea (eukaryotes have 18S rRNA). The 16S rRNA consists of nine variable regions (V1-V9) surrounded by ten conserved regions. The conserved regions can be used to design primers that target as many species as possible, while the variable regions can be used for phylogenetic characterisation of microbes (Chakravorty et al., 2007). Random cloning and sequencing of 16S rRNA genes as well as sequence-based electrophoretic approaches, such as temperature- and denaturing-gradient gel electrophoresis, have been used to profile the microbiota composition and diversity (Eckburg et al., 2005; Muyzer et al., 1993; Zoetendal et al., 1998). However, nowadays these approaches have largely been replaced by next
Since the 16S rRNA gene is too large to be sequenced in its entire length in a cost effective manner, several variable regions have to be selected during study design. Based on the read lengths produced from the sequencing platform, for example 2×300 base pairs for Illumina MiSeq sequencing, several variable regions can be selected. Selection of variable regions in combination with the PCR-based approach that depends on the conserved regions to target as many species as possible will introduce biases in the pipeline (Walker et al., 2015). Furthermore, different microbiota prefer different regions to allow proper profiling of important species (Chakravorty et al., 2007). For gut microbiota, inclusion of V4 is recommended. At this point, third generation sequencing techniques, in particular PacBio SMRT sequencing, are generating longer reads with lower error rates at lower costs. As a result, the decision on which variable
region to target will not have to be made in the near future and more optimal profiling using the entire 16S rRNA gene can be performed (Myer et al., 2016).

After sequencing a bioinformatics pipeline is needed to perform taxonomic analysis. Typically, reads have to be demultiplexed and, in case of short read paired-end sequencing, merged. Then, reads are clustered based on sequence identity, often set to be 97% or higher, and designated an operational taxonomic unit (OTU). This de novo approach is up until this point independent of any known taxonomy and allows to analyse species that have not been identified yet. Taxonomy can be assigned to OTUs which have been identified and listed in public databases such as RDP (Cole et al., 2014), SILVA (Quast et al., 2013) or Greengenes (DeSantis et al., 2006). The resulting OTU table will contain known and unknown taxonomies at different taxonomic levels and their relative abundances in the sample, based on the number of reads in the OTU. Several public pipelines are available for 16S rRNA profiling, in particular QIIME (Caporaso et al., 2010) and mothur (Schloss et al., 2009). The 16S rRNA phylogenetic profile and partial taxonomy can be used to estimate the diversity in the sample (α diversity) and calculate distances between samples (β diversity). These metrics are the basis of more sophisticated analysis that are included in several software packages, such as phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2013).

Typical drawbacks of the general 16S rRNA profiling are biases introduced by primer design and variable region selection, as well as overestimation of α diversity, and difficulty to compare and contrast samples with varying numbers of reads. These limitations can be corrected for by means of bioinformatics as new pipelines become available, such as NG-Tax, which demonstrates high robustness against choice of region and other technical biases commonly associated with 16S rRNA profiling (Ramiro-Garcia et al., 2016). Furthermore, it has to be realised that sequencing-based microbiota profiling does not provide accurate numbers due to the PCR of 16S rRNA genes. However, quantitative approaches based on 16S rRNA or its encoding gene have also been developed, which include fluorescent in situ hybridisation (FISH) and quantitative PCR. These can be used for specific and more accurate quantification of specific species or groups of microbes (reviewed in Zoetendal et al., 2008).

**Metagenomics**

Instead of targeting the 16S rRNA gene that allows to estimate phylogenetic profiles in a sample, sequence-based metagenomics is an untargeted or shotgun approach by just directly sequencing the DNA extracted from the sample (Riesenfeld et al., 2004; Schloss and Handelsman, 2003). The first advantage is that this technique targets all organisms in the sample including Bacteria, Archaea, Eukaryotes, such as the human host, Fungi, and viruses, including bacteriophages. The second advantage is that it allows for both taxonomic and functional classification. However, the depth of analysis is generally less compared to 16S rRNA gene profiling approaches.
Starting from DNA isolated from the samples under investigation, a library for sequencing is prepared, typically Illumina sequencing being currently the most cost effective platform. Sequencing is performed as deep as possible to allow for optimal profiling, although this comes at a price. An optimal balance between costs and depth is generally set between three and five gigabases per sample. Then, profiling will follow two directions: taxonomy and functional profiling, and, eventually, the presence of viral, fungal and antibiotic-resistance genes can be assessed.

Taxonomy profiling is performed by mapping the sequencing reads to a microbiome reference genome, such as those generated by MetaHIT consortium (Qin et al., 2010) or The Human Microbiome Project consortium (Gevers et al., 2012; Human Microbiome Project, 2012). The number of reads that map to a certain species determines its relative abundance in the sample. This approach can also be used to quantify gene functions and functional pathways that are present in the sample. By selecting protein-coding reads and subsequent mapping to protein databases as KEGG and COG, functional profiles can be obtained. A more compute-intensive approach that can be performed is to assemble the reads in contigs prior to mapping to a database, which allows reconstruction of (partial) genomes of the microbes present in the sample. Bioinformatics pipelines for metagenomics data analysis are complex, but metagenomics generate less biased and more comprehensive profiles than 16S rRNA profiling (Sharpton, 2014). Several tools have been developed to analyse the data, such as HUMAnN (Abubucker et al., 2012), MEGAN (Huson et al., 2007) and the online MG-RAST server (Meyer et al., 2008). Finally, metagenomics also allows for microbiota characterisation beyond the genus level (Li et al., 2016) as well as SNP analysis (Schloissnig et al., 2013).

**Metatranscriptomics, metaproteomics and metabolomics**

As mentioned above, 16S rRNA and metagenomics profiling can address the questions ‘Which microbes are there?’ and ‘What is their functional capacity’. Better functional profiling in terms of ‘What are they doing?’ can be obtained through metatranscriptomics, metaproteomics and metabolomics. These approaches measure the actual functional activity of the community under investigation.

Expression of genes is measured by sequencing RNA from the sample. Since bacterial RNA does not have an A-tail and since 95-99% of total RNA consists of rRNA it is difficult to enrich mRNA by removing rRNA completely from the sample. Hence, pipelines for metatranscriptome analysis have to deal with removal of the residual rRNA reads. The analysis pipeline for metatranscriptomics is essentially the same as the pipeline for functional annotation of metagenomics data: direct mapping of the reads to reference genomes or constructing larger contigs by means of de novo assembly prior to mapping (Bashiardes et al., 2016). The same analysis tools can be used, such as HUMAnN and MG-RAST, as well as general transcriptomics tools, such as Trinity (Grabherr et al., 2011). Furthermore, dedicated metatranscriptomics pipelines have been developed (Leimena et al., 2013; Martinez et al., 2016). Concerning studies with large numbers of volunteers, a major
challenge remains the logistics to obtain and collect samples in which RNA quality will remain as discussed earlier.

Metaproteomics, which is community protein profiling, is another approach that can be used to address the question ‘What are intestinal microbes doing?’. Basically, this technique is a combination of a separation technique, such as high performance liquid chromatography (HPLC), and mass spectrometry to obtain protein signatures of the sample, followed by a computational workflow to identify proteins (reviewed in Ruiz et al., 2016; Xiong et al., 2015). Since this technique targets proteins, which are more stable than RNA, it does not have the disadvantages that come with sampling procedures that are required to stabilise RNA. It is, therefore, also likely that the collective protein content in faeces better reflects the community activity than that of mRNA. Because of these advantages, metaproteomics offer the potential of targeting higher numbers of volunteers. As opposed to the previously described technologies, metaproteomics does not involve sequencing but is driven by advances in the fields of chromatography and mass spectrometry that include higher throughput and more and better analysis tools. The number of reports on metaproteomics of human intestinal microbiota are currently increasing and already provide novel insights into microbiota activity in healthy as well as obese subjects (Kolmeder et al., 2015, 2016).

Last but not least, the metabolites produced by intestinal microbes can also be targeted and provide insight into microbiota activity. These molecules are targeted by separation techniques as gas and liquid chromatography in combination with detection by mass spectrometry or nuclear magnetic resonance (NMR) spectroscopy (reviewed in Aldridge and Rhee, 2014; Vernocchi et al., 2016). Classical chromatography analyses of organic acids already demonstrated that carbohydrate fermentation in the intestine leads to the production of short chain fatty acids. Furthermore, high throughput and deep analysis of metabolites related to nutrition, called metabonomics, have also successfully been applied and demonstrate the wide diversity of metabolites that are produced by gut microbes. These approaches offer great potential for human metabolic phenotyping (Kinross et al., 2014) as well as demonstrating the impact of diet switches on microbiota activity (O’Keefe et al., 2015). In contrast to the previously described approaches, a major disadvantage of metabonomics is that no phylogenetic information can be obtained from the metabolites, and combinations with other profiling techniques are required.

15.4 Study design recommendations

The design of a study is dependent on many variables, but mostly on the microbiota in relation to health and disease to be investigated. The (normal) biology of the gut microbiome is actively under investigation and will serve as a reference framework for future studies. It is clear that inter-individual variations in microbiota composition and function are large and for human studies many subjects should be included. Today, many existing larger scale cohort studies are including microbiomics (Fu et al., 2015; Goodrich et al., 2016; Zhernakova et al., 2016) or new microbiomics-driven cohorts are initiated (Falony et al., 2016).
These association studies depend on large sample sizes and, therefore, the less expensive 16S rRNA approach is often chosen to determine microbiota composition. This approach will identify states of dysbiosis although further investigations on functional alterations are limited. Therefore, the use of complementing approaches that target microbiota composition and activity simultaneously can be used to allow reconstruction of the intestinal ecosystems via a so-called ecosystems-biology approach to model this complex biological system mathematically (Aguiar-Pulido et al., 2016; Bikel et al., 2015; Jovel et al., 2016; Zoetendal et al., 2012). Furthermore, applying phylogenetic profiling to cluster samples within a study and zooming in on a small number of selected samples from the different clusters using more expensive meta-omics approaches, is considered a cost-effective alternative.

Once associations have been established, causality needs be addressed. Case-control, animal and clinical intervention studies will narrow down the biological significance of the gut microbiome and the remaining question will be ‘How can we influence them?’. Such studies will be more controlled than the cohort studies and optimal sampling and analysis approaches will be needed.

Practical advice:
- Optimal sample collection is to store the faecal sample as soon as possible at -80 °C, but deviations from this protocol are often needed and should be evaluated beforehand.
- Sampling from infants offers the advantage to collect from a diaper. Besides this, we do not see any differences that should be taken into consideration when microbiota in children are investigated as opposed to microbiota in adults.
- When participants are involved in sample collection, clear instructions with clear pictograms should be used.
- Meta-omics techniques to profile microbiota complement each other. The approach or combinations of approaches to be chosen should be selected based on the research question and sample size.

15.5 Recommendations for future research
- Microbiome research has mainly focused on Bacteria in the faeces. Although there is a clear numerical dominance of this taxonomy in this niche, more research should be initiated on Archaea, viruses and Fungi, as well as on other niches, such as other intestinal tract locations, skin and vagina.
- Microbiome research is now mostly determining associations between microbiota and traits of health and disease. We need to move from correlation to causality by initiating dedicated intervention and case-control studies.

Conflict of interest

The authors declare no conflict of interest.
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Part VIII.
Manipulating the gut microbiota
Chapter 16
Probiotic interventions to optimise the infant and child microbiota

Y. Vandenplas* and K. Huysentruyt
Department of Paediatrics, UZ Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium;
yvan.vandenplas@uzbrussel.be

Abstract

The optimal healthy microbiota during early life still needs further evaluation. Pre- and probiotics are commonly used as supplementation in infant formula. Prebiotic oligosaccharides stimulate the growth of bifidobacteria aiming to mimic the gastrointestinal microbiota of breastfed infants. In general, results with prebiotics in therapeutic indications are disappointing. Studies suggest that probiotic supplementation may be beneficial in prevention and management of disease, e.g. reducing the risk of necrotising enterocolitis in preterm infants, prevention and treatment of acute gastroenteritis in infants. Although many studies show promising beneficial effects, the long-term health benefits and eventual risks of probiotic supplementation during early life are not clear. It is likely that ongoing research will result in the use of specific probiotic organisms and/or prebiotic oligosaccharides during the first 1000 days of life, with the goal to develop a healthy microbiota from conception over birth into the first two years of life, while lowering risk of infections and inflammatory events.

Keywords: gastrointestinal microbiota, infant feeding, prebiotic, probiotic, synbiotic

16.1 When is infant microbiota optimal?

The question arises immediately: do we know the optimal healthy (gastro-intestinal (GI)) microbiota for the infant and child? The answer is probably negative. The important differences between the GI microbiota development between infants born through caesarean section versus natural delivery or standard infant formula feeding versus breast-feeding are well known. It is also known that the GI microbiota of the mother is influenced by medications (i.e. antibiotics, anti-acid medications), diet, stress and many other factors (Odamaki et al., 2016). The GI microbiota of the breastfed baby born vaginally is in general considered as the ‘healthy microbiota’, but depends on the GI microbiota of the mother. More data needs to be collected to better define the ‘optimal healthy GI microbiota’. The stepwise microbial gut colonisation process may be initiated already prenatally by a distinct microbiota in the placenta and amniotic fluid (Collado et al., 2016). The clinical meaning of these findings needs to be further evaluated.

A necrotising enterocolitis (NEC)-associated gut microbiota has been identified in meconium samples. *Clostridium perfringens* continues to be associated with NEC from the first meconium
till just before NEC onset (Heida et al., 2016). In contrast, in post-meconium, increased numbers of staphylococci were negatively associated with NEC (Heida et al., 2016). Pre-term birth, caesarean section, formula feeding, antibiotic use and malnutrition have been linked to dysbiosis, which in turn is associated with several pathologies such as NEC, inflammatory bowel diseases, colic, and allergies during childhood.

### 16.2 Probiotics and prebiotics: definitions

Probiotics are living microorganism that, when administered in sufficient amount, have a health benefit for the host (Hill et al., 2014). The classic definition for prebiotics is: ‘prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, which can improve host health’. Recently, a new definition has been proposed. Dietary prebiotics are defined as a selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (Valcheva and Dieleman, 2016). Application of prebiotics may then restore the gut microbiota diversity and activity (Valcheva and Dieleman, 2016). Synbiotics are a mixture of pre- and probiotics.

Based upon the current literature, a case can be made for the use of specific sets of probiotic organisms with the goal of promoting a healthy pregnancy to birth, and a healthy start to life with lowered risk of infections and inflammatory events (Valcheva and Dieleman, 2016). The mechanisms of specific probiotic strains administered during the perinatal period suggest that probiotic interventions in early life can be envisaged for disease prevention in both healthy offspring and offspring at risk of chronic disease. There is evidence that manipulation of the infant microbiota by using pre- or probiotics can restore the ecological balance of the microbiota and may mitigate potential negative effects on the developing immune system, when use of antibiotics cannot be avoided (Rutten et al., 2015).

Unfortunately, the term ‘probiotic’ seems to cause confusion. The taxonomic ranking for classification of probiotics is as follows: there is a domain (bacteria, fungi), a lineage (e.g. Firmicutes), a class (e.g. Lactobacillus), an order (e.g. Lactobacillales), a family, a genus, a species and subspecies. Most probiotics are bacteria, but the yeast Saccharomyces boulardii is one of the best studied, especially for gastroenterological indications (Figure 16.1). Probiotics do not colonise the host. This means that one to two weeks after stopping the administration of a probiotic strain, it disappears from the GI tract. The European Food Safety Authority (EFSA) considers ‘probiotics’ as a medical claim, which has been resulting in the prohibition of the term on products or for communication by companies. More and more patents are given to specific probiotics with specific health claims; including, amongst others, claims for fat metabolism, obesity, oral health, anticancer treatments, cardiovascular disorders, diabetes, immunomodulation, allergy, viral diseases such as AIDS. A lot of research on probiotics is now focusing on other areas such as veterinary medicine and dermatology.
16. Probiotics and microbiota

Fermented food

Traditional cuisines do contain a lot of fermented foods and thus probiotics. Fermented foods are foods produced or preserved by the action of microorganisms. The use of bacteria, such as *Lactobacillus*, results in the making of foods such as yoghurt and sauerkraut. The science of fermentation is known as zymology. Fermentation is most of the time the process that is applied to prepare pickled or soured foods. It is well known that pickled and sour food preserves better and has some health benefits. Infant formula (re-)discovered this process recently, and fermented infant formula is now commercialised claiming health benefits in comparison to standard infant formula (Huet *et al*., 2016). Fermented infant formula was popular more than 30 years ago, and has continued to be on the market in some countries.

Food supplements

The market is overloaded with food supplements containing probiotics. However, there is no guarantee for the quality of such a product. There can be problems at the manufacturing level, but also at the level of survival during shelf life, or poor resistance to gastric acid (Hamilton *et al*., 1996; Huys *et al*., 2013 Vanhee *et al*., 2010;). In other words, there is a world of difference between using probiotics as a healthy eating habit or using probiotics as medication. Probiotics

Figure 16.1. Illustration of the size difference between a yeast probiotic (*Saccharomyces Boulardii*) and bacteria (*Salmonella*).
used for medical purposes are often commercialised as pills or sachets. Some products are also on the market in liquid form and are administered as drops. Slow release tablets to be put in a specially designed infant pacifier, are also available (Taipale et al., 2011). In addition, for respiratory tract indications, probiotics sprays have been developed (Santagati et al., 2015). A novel probiotic delivery system has also been developed in which probiotics are grown as a biofilm on microspheres, allowing enhanced efficacy with only a single treatment (Navarro et al., 2017). A single dose of Lactobacillus biofilm grown on biocompatible microspheres was shown to significantly reduce NEC incidence and severity in rats (Olson et al., 2016). There are no data that compare efficacy of the different ways of administration.

**Probiotics in food**

Probiotics can just be added to food, such as in infant formula. Probiotics are also added to foods such as chocolate and ice cream. Many yoghurts are enriched with probiotics. However, it is obvious that the targeted effect of probiotics in food differs in both situations substantially: while in infant formula there is a clear medical purpose, probiotics added to food such as ice cream and chocolate have a merely marketing goal claiming to improve general wellbeing.

**Strain specificity**

It is beyond doubt that for specific medical purposes the effect of probiotic strains is strain-specific. This is illustrated by the following findings. Breastfeeding women received probiotic milk or placebo from 36 weeks of gestation up to three months postnatal while breast-feeding (Dotterud et al., 2015). The probiotic milk contained Lactobacillus rhamnosus GG (LGG), Lactobacillus acidophilus La-5, and Bifidobacterium animalis lactis subsp. Bb-12. Only the L. rhamnosus GG bacteria colonised the children at 10 days and at 3 months of age (Dotterud et al., 2015). Five out of 50 lactobacilli strains isolated from the faeces of breastfed infant have probiotic properties (Panya et al., 2016). These five isolates showed susceptibility to all antibiotics tested except for vancomycin, ability to survive in the simulated gastrointestinal conditions of both acid and bile salt solution, and ability to inhibit growth of Escherichia coli O157: H7 and Vibrio cholera (Panya et al., 2016). Bacterial species identification revealed that all five isolates were firmly identified as L. rhamnosus species (Panya et al., 2016). However, the relevance of strain specificity depends on the probiotic property that is looked for. Lactobacilli that survive gastric acid and bile will be fermented in the colon.

**Combination products, dosage and timing of administration**

Many probiotic products are a combination of different strains. Data show that combination products can be more effective than the single strains. But single strains can also be more effective than combination products (Chapman et al., 2012; Grandy et al., 2010).
In general, high dosages are more effective than low, although the number of dose-efficacy studies is limited (Shornikova et al., 1997). However, some meta-analyses come exactly to the opposite conclusions: in patients with irritable bowel syndrome, single probiotics at a low dose and with a short treatment duration appear to be more effective in improving overall symptom response and quality of life (Zhang et al., 2016).

Older literature also suggest that the earlier probiotics are started (in the treatment of acute gastroenteritis), the greater the effect (Rosenfeldt et al., 2002). The impact of probiotics on the development of the immune system in formula fed infants was recently illustrated by the findings of the TEDDY study (Uusitalo et al., 2016). Early probiotic supplementation (at the age of 0–27 days) was associated with a decreased risk of islet autoimmunity when compared with probiotic supplementation after 27 days or no probiotic supplementation (Uusitalo et al., 2016). It was concluded that early probiotic supplementation may reduce the risk of islet autoimmunity in children at the highest genetic risk of diabetes (Uusitalo et al., 2016).

Safety

In general, probiotics have the label to be ‘GRASS’ (generally regarded as safe). However, side effects, mostly sepsis, are reported. Nevertheless, probiotics have also been used safely in patients with immune deficiencies, showing a beneficial effect on growth only in HIV-positive infants (Steenhout et al., 2009). The increased use of probiotics in vulnerable immune insufficient preterm infants may, however, lead to an increased risk of severe complications such as sepsis with the probiotic (Zbinden et al., 2015).

Efficacy of probiotics

Although generalisation should be avoided, as efficacy for medical indications is strain specific, it can be said that there is evidence that some strains do prevent atopic dermatitis (especially if probiotics are also given to the mother during pregnancy). To date, expert bodies do not generally recommend probiotics for allergy prevention, although the World Allergy Organization (WAO) in their recently developed guidelines suggests considering using probiotics in pregnant women, during breastfeeding and/or to the infant if at high risk of developing allergic disease (based on heredity) (West, 2016). However, in concordance with other expert bodies, the WAO guideline panel stressed the low level of evidence and the need for adequately powered randomised controlled trials and a more standardised approach before clinical recommendations on specific strains, dosages and timing can be given (West, 2016). Several meta-analyses have reported a moderate benefit of probiotics for eczema prevention, and the most consistent effect has been observed with a combined perinatal intervention in infants at high risk of allergic disease due to familial predisposition (West, 2016). Multi-strain probiotics appeared to be most effective for eczema prevention (West, 2016).
Probiotic strains have also been shown to prevent infectious disease such as community-acquired and nosocomial gastroenteritis, respiratory tract and urinary tract infections. Infants receiving Bifidobacterium BB-12 were reported to have experienced fewer respiratory tract infections (risk ratio (RR): 0.87; 95% confidence interval (CI): 0.76, 1.00; P=0.033) than controls (Taipale et al., 2016). No significant differences between the groups were observed in reported GI symptoms, otitis media, or fever (Taipale et al., 2016).

Probiotics also decrease the incidence and severity of NEC in preterm. Strategies such as antenatal glucocorticoids, postnatal breast milk feeding, and cautious approach to enteral feeding failed to eliminate NEC because these strategies did not address the complexity of the pathogenesis (Chen et al., 2014). Probiotics seem to be the most significant advance in NEC prevention at present because of the significant range of beneficial effects at various levels of gut function and defence mechanism (Chen et al., 2014). However not all studies are positive, as shown by a study with Bifidobacterium breve BBG-001 for prevention of NEC and late-onset sepsis in very preterm infants, which showed absence of benefit (Costeloe et al., 2016). Therefore, the risk/benefit should be considered before starting routine administration of probiotics to preterm infants. Standard care in the Neonatal Intensive Care Units in the western world has resulted in a very low incidence of NEC. However, absence of benefit does not mean that there would be an increased risk for adverse effects. Oral probiotic given to very low birth weight infants are considered to be safe, as it has been shown that they do not affect neuromotor, neurosensory and cognitive outcomes at 18-24 months' corrected age (Akar et al., 2016). Rapid changes of nasogastric tubes may lead to a decreased incidence of NEC without the risk for sepsis (Petersen et al., 2016).

Probiotics also shorten the duration of infectious diseases, such as gastroenteritis. Probiotics added to infant formula have been shown in many older studies to possibly protect for infectious gastroenteritis, although some studies did not show a benefit. Additionally, there was no study that suggested an increased risk of gastroenteritis. Probiotics are also effective in the prevention and treatment of antibiotic associated diarrhoea (AAD). Moderate quality evidence suggests a protective effect of probiotics in preventing AAD (RR 0.46; 95% CI 0.35 to 0.61), with a number needed to treat of 10 (Goldenberg et al., 2015).

Probiotics may also have a role in infantile colic and in irritable bowel syndrome, especially in older children and adults. The management of infantile colic in formula fed infants is still a challenge. Lactobacillus reuteri DSM 17938 was ineffective in formula fed infants (Xu et al., 2015). LGG in infants treated in tandem with behavioural support and a cow’s milk elimination diet did not provide additional treatment effect for diary-verified colic crying although parental report of crying suggested the probiotic intervention effective (Pärty et al., 2015). A synbiotic (a mixture of seven probiotic strains plus FOS) significantly improved colic symptoms in comparison with placebo (Kianifer et al., 2014). Several studies were performed with L. reuteri DSM 17938 in breastfed infants presenting with infantile colic (Harb et al., 2016).
Probiotics may also decrease regurgitation. Treated infants demonstrated a reduction in daily regurgitations at the end of treatment, three neonates in the placebo group only needed simethicone for GI pain, sIgA level was similar in both groups (Garofoli et al., 2014).

Probiotics result in a 10% eradication rate of Helicobacter pylori infection. It is not known whether this is due to a true better eradication, or better compliance to the eradication treatment because of a decrease of adverse effects (AAD)). Probiotics have been shown to be effective in the treatment of constipation in adults, but not in children. Literature on the efficacy of probiotics in inflammatory bowel disease is in general disappointing, certainly in paediatrics. The above list is not exhaustive, but indicates the broad spectrum of possible indications, going from general wellbeing over immune mediated diseases to infections. But negative results have also (seldom) been reported. L. acidophilus (LAVRI-A1) was reported to increase the risk of atopic dermatitis compared to placebo (Taylor et al., 2007).

**Probiotics during pregnancy**

Probiotics administered during pregnancy (and breastfeeding) to the mother can be found in the GI microbiota of the woman and have been shown for some strains to possibly have a preventive effect of the frequency and severity of atopic dermatitis in the infant. However, the literature on the effect of probiotics on the infant when given to the mother during pregnancy is quit contradictory. According to some authors, currently evidence does not indicate that probiotic supplementation reduces the risk of developing allergy in children.

Current analysis of the role of probiotics in the prevention of atopic dermatitis reveals that a positive effect may be related to the type of probiotic strain used, the method of administration, onset time, as well as the dose size and duration of treatment (Rather et al., 2016). Panduru et al. (2015) concluded in a meta-analysis that probiotics have a protective role in atopic dermatitis prevention if they are administered during the pre- and postnatal period, in both the general and at allergic risk population. Maternal probiotic ingestion alone may be sufficient for long term reduction in the cumulative incidence of atopic dermatitis, but not other allergy related diseases (Simpson et al., 2015).

The guideline of the World Allergy Organisation (WAO) panel determined that there is a likely net benefit from using probiotics resulting primarily from prevention of eczema. The WAO guideline panel suggests: (1) using probiotics in pregnant women at high risk for having an allergic child; (2) using probiotics in women who breastfeed infants at high risk of developing allergy; and (3) using probiotics in infants at high risk of developing allergy. All recommendations are conditional and supported by very low quality evidence (Fiocchi et al., 2015).
16.3 Prebiotics

Many of the production and conservation difficulties, such as survival during production and shelf-life with probiotics, are not valid for prebiotics. Prebiotics stimulate the GI microbiota of the host, whereas probiotics just add one or couple of strains. According to some data, they may have prolonged effects. Some data suggest that if infants are supplemented from birth up to six months, that the bifidogenic effect can still be observed at the age of 12 months (Salvini et al., 2011). Also prebiotics are generic, and findings of one prebiotic cannot be extrapolated to another one (Veereman et al., 2011). The number of comparative and dose-efficacy studies is extremely limited.

The advantages of prebiotics in infants and children is mostly limited to prevention studies with infant formula supplemented with prebiotics oligosaccharides. While some studies show a benefit, others fail to do so, but the outcome in the prebiotic group is never inferior to the comparator group. Most studies on the efficacy of prebiotics in therapeutic indications are disappointing. The effect of some prebiotic oligosaccharides on the gastro-intestinal microbiota is clear, as the effect on stool frequency and composition in non-constipated infants, bringing defecation pattern in formula fed infants closer to the pattern in breastfed infants. Literature is not conclusive on other possible effects such as decrease of infection, decrease of atopic dermatitis.

Compared to probiotics, for which side effects are scarcely reported, adverse effects of prebiotics in infants are not reported.

16.4 Synbiotics

Data on the combination of pre- and probiotics in infants and children are quit limited. Most studies regard short term therapeutic interventions. Mean±standard deviation infection rates in infants followed up to 12 months were 4.9±3.2 per infant per year in the B. lactis+GOS/FOS group and 4.5±3.0 per infant per year in the B. lactis group (p=0.18). Mean daily weight gain was slightly lower in the B. lactis+GOS/FOS than the B. lactis group (16.1±2.9 vs 16.6±2.6 g/day, P=0.046), but was not clinically significant (Bocquet et al., 2013). Other outcomes were not significantly different between groups. Formulas containing B. lactis+GOS/FOS did not reduce infection rates beyond those containing only B. lactis (Bocquet et al., 2013). Chang et al. (2016) conclude in their meta-analysis that evidence supports the use of synbiotics for the treatment of atopic dermatitis, particularly synbiotics with mixed strains of bacteria and for children aged 1 year or older (Chang et al., 2016). Nevertheless, infant formula companies promote the combination of pre- and probiotics in infant formula. Although this evolution does not seem to induce any increased risk for adverse effects, the benefit has not been shown.
16.5 Conclusions

The knowledge on the importance of the GI microbiota to the development of wellbeing and general health is increasing, and has been a focus of research during the past 10 years. The development of a healthy GI microbiota from conception throughout the first years of life will have lifetime long consequences. The use of prebiotics, probiotics and synbiotics in the prevention and treatment of different health conditions is increasing. The discovery that a subset of commensal bacteria can provide colonisation resistance against many of the most threatening antibiotic-resistant pathogens causing disease in patients is exciting, and their development as preventive or therapeutic agents is important (Palmer, 2016). For commercialised probiotic and prebiotic products that are intended to be used to treat a particular medical condition or yearning a health claim, at least two independent clinical studies need to be conducted with these products. If products are commercialised with the intention to contribute to healthy eating habits, such high quality research is not needed. In that case, it is not justified to have any claim. In order to reduce confusion, different terminology for ‘healthy foods with pre- and probiotics’ and ‘specific products claiming a targeted health benefit’ would be useful. The earlier proposed term ‘biotherapeutic agent’ does not fulfil the requirements, as the concerned effects regard mostly prevention, more than therapy. The more we learn about the microbiota, the more questions arise and the more we realise that we are entering a novel world of complex interactions. In fact, nothing is known yet.

Gaps in our knowledge:
- What is the composition of a ‘healthy well balanced gastro-intestinal microbiota in the newborn’?
- What is the role of (epi-)genetics in the composition of the microbiota? Can we really manipulate the composition of the microbiota?
- If there is really a ‘window of opportunity’ to skew the development of the immune system, long-term follow-up studies are needed to secure safety.

Conflict of interest

Y. Vandenplas has participated as a clinical investigator, and/or advisory board member, and/or consultant, and/or speaker for Abbott Nutrition, Aspen, Biogaia, Biocodex, Danone, Hero, Nestle Nutrition Institute, Nutricia, Mead Johnson Nutrition, Merck, Orafti, Phacobel, Rontis, Sari Husada, United Pharmaceuticals, Wyeth and Yakult. K. Huysentruyt has no conflict of interest to declare.
References


16. Probiotics and microbiota


16. Probiotics and microbiota


Chapter 17
Safety of probiotics in infants and children

M. Van den Nieuwboer1*, P.D. Browne2,3,4 and E. Claassen1
1New-Med Solutions, Riouwstraat 61A, 1094 XH Amsterdam, the Netherlands; 2Clinical Research Rotterdam, Marconistraat 16, 3029 AK Rotterdam, the Netherlands; 3Faculty of Earth & Life Sciences, Athena Institute, VU University, De Boelelaan 1085, 1081 HV Amsterdam, the Netherlands; 4Department of Developmental Psychology, University of Nijmegen, P.O. Box 9104, 6500 HE Nijmegen, the Netherlands; m.vdnieuwboer@gmail.com

Abstract

Since the PROPATRIA-trial in 2008, probiotics have been placed under scrutiny and concerns have been raised regarding the safety of administration of live microorganisms. When probiotics are administered to immune-incompetent or immune-deficient individuals, probiotic strains can potentially cause systemic infections. As probiotic intake and gut modulation might be most effective early in life, no uncertainties should remain regarding safety administration of probiotics during the perinatal period and childhood. This chapter provides a comprehensive overview of the safety of probiotics based on data from clinical trials up to 2016. These studies indicate that probiotics are safe for infants and children, even in frail preterm infants.

Keywords: probiotics, safety, adverse events, preterm neonates, infants, children

17.1 Introduction

Probiotics have been widely used in many countries by consumers in clinical practice and research. Nevertheless, since the last decade concerns regarding the safety of probiotics have increased, particularly for neonates born prematurely and children. The most important concern with the safety of probiotics for infants and children is the risk of sepsis. Over the last decade, some case reports of sepsis related to probiotic use have been published (De Groote et al., 2005; Kunz et al., 2004; Land et al., 2005). Other reasons for vigilance were the result of the PROPATRIA-trial (2008). This multicentre, double-blind, placebo-controlled clinical trial evaluated the effect of administration of a multispecies probiotic supplement in patients suffering from severe pancreatitis. In this study there was an unprecedented significantly higher mortality rate of 16% in the group receiving probiotics compared to 6% in the placebo group (Besselink et al., 2008). Despite the fact that no causal relationship between the administered probiotics and higher mortality rate were found in later analyses, and although the study only included adult patients, concerns surrounding the safety of probiotics for infants and children increased (Morrow et al., 2012; Van Baal, 2014).
Since microbial modulation might be most effective early in life, a good safety profile of probiotics is paramount for infants, children and their parents (Martinez, 2014). In addition, when aiming at long-term intake of high dosages no ambiguities should remain regarding the safety aspects and potential hazards of these food supplements (Hempel et al., 2011; Van den Nieuwboer et al., 2014a,b; 2015). In this chapter, we review the most recent data from human clinical trials (up to 2016) on safety of probiotic treatment in infants and children (0-18 years). We start with discussing the regulatory approaches of probiotic consumption in the United States and Europe. We then highlight several concerns regarding probiotic consumption and review cases of probiotic infection in infants and adults. Finally, this chapter provides conclusions on safety of probiotic consumption in infants and children based on the most recent data from clinical trials.

17.2 Regulation of probiotics

There are several regulatory approaches to probiotic safety in the European Union (EU) and United States (USA). In the USA, a microorganism or probiotic strain used in food as an additive needs to be approved according to the Food Drug and Cosmetic Act (FDCA) on the basis of safety and efficacy data. For the EU, probiotic strains that have not been used before 1997 are considered novel and to obtain market approval for these strains, comprehensive safety data needs to be provided to regulatory authorities. In addition, when a microorganism or probiotic strain is ‘generally recognised as safe’ it receives a ‘GRAS’ status whereby it can bypass the standard approval steps (Von Wright, 2005). The European counterpart of the GRAS is the ‘qualified presumption of safety’ (QPS) status. Probiotics may be introduced to the market as long as they are considered GRAS or QPS.

17.3 Concerns related to probiotic administration

Whereas probiotic administration in healthy adults is scarcely associated with serious adverse events, administration particularly in infants, young children and immune compromised individuals may pose several safety concerns. Here, we review three concerns directly related to ingestion of probiotics by infants and children and three concerns related to public health or regulatory affairs.

One theoretical concern is the adherence of bacteria to the intestinal mucosa possibly leading to increased bacterial translocation (Boyle et al., 2006). Bacterial translocation is the passage of viable bacteria from the gastrointestinal tract to other sites in the body, for example to the bloodstream and lymph nodes (Liong, 2008). During the neonatal period, the infant gastrointestinal tract is immature and still developing resulting in an increased intestinal permeability of the infant gut. Increased gut permeability can consequently lead to translocation of pathogens, which susceptibility to inflammation and infection (Van Elburg et al., 2003). Another concern is that infants are more susceptible to infection compared to adults due to their underdeveloped gastro-intestinal tract, gut-immune system and immature intestinal epithelium. For example, it is suggested that in particular the preterm infant gut contains fewer Paneth cells, a lower
expression of α-defensin, a reduced expression of mucin (Battersby and Gibbons, 2013). As a result, probiotics could trigger a suboptimal host immune response. Moreover, they could potentially have an unfavourable immunologic effect on the infant. For instance, probiotics could theoretically induce local inflammation or skew the host immunity to a more T\textsubscript{2} response, which is associated with the development of allergies (Hibberd and Davidson, 2008). A third concern could be the metabolic activity of probiotic species and their microbial products. For example, probiotic species that produce D-lactate may induce D-lactic acidosis in individuals with short bowel syndrome (SBS), a condition characterised by a reduced functional gut mass, associated with increased intestinal permeability and inflammation (Cole \textit{et al.}, 2010; Miquel \textit{et al.}, 2015; Ku \textit{et al.}, 2006; Munakata \textit{et al.}, 2010).

Regarding public health safety, probiotics could potentially contribute to the spread of antibiotic resistance by carrying antibiotic resistant genes. These genes are located on easy transmissible plasmids and transposons and may lead to unwanted multi-resistant bacterial strains, which may challenge the treatment of infections in the future. Secondly, given the long history of safe use, investigators and consumers often assume that probiotics in general are safe. For example, lactobacilli and yeasts have been used for the preparation and preservation of a variety of traditional fermented foods for thousands of years. However, the properties of probiotics are strain specific and safety data of one strain cannot be simply extrapolated to other strains (Hempel \textit{et al.}, 2011). Thus, researchers and consumers should remain alert when using probiotics. Thirdly, some probiotic strains that have the GRAS of QPS status are combined into multispecies probiotic formulations. When combined, this status might not be applicable, especially when supplementing probiotic species in high dosages to non-healthy individuals.

### 17.4 Example cases of probiotic infections

The amount of cases of bacteraemia in adults and children are low in proportion of people consuming probiotics. In Finland, where probiotic intake is part of the standard diet, there has been no increase in frequency of \textit{Lactobacillus} bacteraemia cases since the 1980’s, despite a significant increase in probiotic consumption (Salminen \textit{et al.}, 2002). In addition, over a period of 10 years, covering a population of 1.3 million individuals, 89 \textit{Lactobacillus} bacteraemia cases were identified in individuals with severe underlying diseases. The cases were not directly related to probiotic intake, and antimicrobial treatment was effective (Salminen \textit{et al.}, 2004). Based on the general and widespread safe use of probiotics, one may argue that probiotic consumption can be regarded as safe in healthy individuals.

Nonetheless, oral ingestion of probiotics can cause infections in infants and adults. There are case reports reporting that probiotic species, such as \textit{Lactobacillus} ssp. can translocate through the gastrointestinal barrier causing bacteraemia. For example, bacteraemia was reported in three infants with SBS after ingestion of \textit{Lactobacillus rhamnosus} GG (De Groote \textit{et al.}, 2005; Kunz \textit{et al.}, 2004). An analysis by Boyle \textit{et al.} (2006) reported that bacteraemia was associated with the use...
of Lactobacillus acidophilus (n=1), L. rhamnosus (n=12) and Bacillus subtilis (n=5). Fungaemia after concurrent use of Saccharomyces boulardii was reported in 27 cases (Boyle et al., 2006).

In adult studies, infection after probiotic intake is incidentally reported. For example, over a period of 53 years, Cannon et al. (2005) conducted a comprehensive study on Lactobacillus infections. Cases of bacteraemia (n=129), endocarditis (n=73) and localised infections (n=39) were found (Cannon et al., 2005). In most of the reported cases pre-existing underlying medical conditions were present in patients (Avlami et al., 2001; MacGregor et al., 2002; Land et al., 2005).

Other risk factors of infection, independent of age or medical condition, include the presence of long-term venous access (e.g. a ‘central line’) that can potentially be contaminated (Boyle, 2006; Land, 2005). Given these reported cases of bacteraemia and infections in children and adults probiotic intake individuals with underlying diseases should be closely monitored.

17.5 Measuring safety in the clinical trial setting

Conclusions on safety of probiotics should eventually be based on events that occur in a controlled and well-documented setting, such as in randomised controlled clinical trials (RCTs). In RCTs confounding factors can be better controlled for that in case control studies. Moreover, it is easier to define causality with larger subject population than in a single case. In the clinical research setting, probiotic safety is measured by documenting the occurrence of adverse events (AEs). AEs are defined as complications, illnesses or the worsening of a clinical condition throughout the study duration. When life threatening or leading to death, AEs are classified as serious adverse events (SAEs) (ICH, 1997). AEs, whether a consequence of the study product or not, are categorised according to the Common Terminology Clinical Adverse Events (CTCAE; v4.0) classification system. It includes 26 CTCAE categories reporting on severity and one category listing ‘unspecified AEs’ as illustrated in Table 17.1. Several safety studies using this classification system in their safety analysis concluded that probiotic consumption is safe. For example, Hempel et al. (2011) analysed safety data of probiotics in controlled studies between 1966 and 2010. The pooled relative risk (RR) for experiencing an AE for probiotics groups relative to control groups was 1.00 (95% CI: 0.93, 1.07, P=0.999; Hempel et al., 2011). An update by Van den Nieuwboer et al. (2014a,b; 2015) concluded that there was no indication that probiotic administration was unsafe for infants under two years of age, children under 18 years of age and immune compromised adults in a controlled setting of clinical trials.

17.6 Probiotic safety in infants

To provide the most up to date information on the safety of probiotic administration in infants (0-2 years of age), we analysed 93 controlled clinical studies including a total of 20,732 infants. 44 different strains were used in these studies, with a daily intake ranging from 7×10⁶ colony forming units (cfu) to 2×10¹² cfu. Of the total group of 20,732 infants, 10,811 infants received a probiotic product, probiotic mixture or synbiotic product during the study period. The majority
of these participants were preterm infants (<37 weeks gestational age) with a low birth weight (<2,500 g) to a very low birth weight (<1,500 g). The infants in the control groups were healthy infants, infants with an increased risk of allergy and critical ill infants.

Although more preterm infants received probiotics compared to the control (n=5,819 vs n=4,964 respectively), there were no significant differences in reported adverse effects (Figure 17.1A).

Table 17.1. The Common Terminology Clinical Adverse Events (CTCAE) version 4.0. This classification system allows a standard terminology and categorisation for the reporting of adverse events according to their severity in 26 categories. A 27th category is added for unspecified AEs.

<table>
<thead>
<tr>
<th>Category</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood and lymphatic system disorders</td>
<td>I</td>
</tr>
<tr>
<td>Cardiac disorders</td>
<td>II</td>
</tr>
<tr>
<td>Congenital, familial and genetic disorders</td>
<td>III</td>
</tr>
<tr>
<td>Ear and labyrinth disorders</td>
<td>IV</td>
</tr>
<tr>
<td>Endocrine disorders</td>
<td>V</td>
</tr>
<tr>
<td>Eye disorders</td>
<td>VI</td>
</tr>
<tr>
<td>Gastrointestinal disorders</td>
<td>VII</td>
</tr>
<tr>
<td>General disorders and administration site conditions</td>
<td>VIII</td>
</tr>
<tr>
<td>Hepatobiliary disorders</td>
<td>IX</td>
</tr>
<tr>
<td>Immune system disorders</td>
<td>X</td>
</tr>
<tr>
<td>Infections and infestations</td>
<td>XI</td>
</tr>
<tr>
<td>Injury, poisoning and procedural complications</td>
<td>XII</td>
</tr>
<tr>
<td>Investigations</td>
<td>XIII</td>
</tr>
<tr>
<td>Metabolism and nutrition disorders</td>
<td>XIV</td>
</tr>
<tr>
<td>Musculoskeletal and connective tissue disorders</td>
<td>XV</td>
</tr>
<tr>
<td>Neoplasms benign, malignant and unspecified (incl. cysts and polyps)</td>
<td>XVI</td>
</tr>
<tr>
<td>Nervous system disorders</td>
<td>XVII</td>
</tr>
<tr>
<td>Pregnancy, puerperium and perinatal conditions</td>
<td>XVIII</td>
</tr>
<tr>
<td>Psychiatric disorders</td>
<td>XIX</td>
</tr>
<tr>
<td>Renal and urinary disorders</td>
<td>XX</td>
</tr>
<tr>
<td>Reproductive system and breast disorders</td>
<td>XI</td>
</tr>
<tr>
<td>Respiratory, thoracic and mediastinal disorders</td>
<td>XXII</td>
</tr>
<tr>
<td>Skin and subcutaneous tissue disorders</td>
<td>XXIII</td>
</tr>
<tr>
<td>Social circumstances</td>
<td>XXIV</td>
</tr>
<tr>
<td>Surgical and medical procedures</td>
<td>XXV</td>
</tr>
<tr>
<td>Vascular disorders</td>
<td>XXVI</td>
</tr>
<tr>
<td>Unspecified</td>
<td>XXVII</td>
</tr>
</tbody>
</table>
Figure 17.1. Adverse events (AEs) in (A) preterm infants (gestational age <37 weeks; (B) infants (age 0-2 years) and (C) children ageing 0-18 years in the probiotic group (dark) or control group (light). AEs are categorised according to the Common Terminology Clinical Adverse Events (CTCAE) version 4. XI: infections and infestations, VII: intestinal disorders, XXII: respiratory, thoracic and mediastinal disorders.
The 8,537 AEs reported in total (4,206 vs 4,331 in the probiotic and control group respectively) were infections and infestations (e.g. sepsis and respiratory tract infections), gastrointestinal disorders (e.g. diarrhoea and vomiting) and respiratory, thoracic and mediastinal disorders (e.g. wheezing and asthma) (Figure 17.1B).

Furthermore, none of the 93 studies reported on serious adverse events related to the placebo of probiotic intervention. In the cases of sepsis the administered study products were not grown on positive blood cultures. In general, probiotic study interventions are well tolerated by infants in the research setting (see Appendix 17.1).

Probiotic consumption during early life by healthy infants does not seem to cause any negative long-term consequences for health. In long-term follow-up studies evaluating adverse effects from 1 to 10 years post probiotic administration, administered probiotics did not seem to influence body composition, growth or incidences of infection and allergic diseases during childhood (Abrahamsson et al., 2013; Maldonado-Lobón et al., 2015; Videhult et al., 2015).

17.7 Probiotic safety in children

A clear safety profile is also important for safe administration of probiotics in older children. Therefore, we also analysed 94 controlled probiotic clinical intervention studies including children between 0 and 18 years. The probiotic treatment group consisted of 9,712 children in total, ranging from healthy to immune compromised children. In these studies, 64 specific strains were used with a daily intake ranging from $3.0 \times 10^6$ to $1.5 \times 10^{11}$ cfu. The control group consisted of 8,621 children.

There was no significant difference in AEs in the treatment group compared to the control group. 3,541 AEs occurred in the treatment group versus 4,012 AEs in the control group. None of the SAEs were related to the study products. Three studies reported on more flatulence, higher occurrence of nasal adverse events and increased change in bowel habits in the probiotic treatment group compared to placebo group (Agustina et al., 2012; Marchisio et al., 2015; Mayes et al., 2015). The majority of these AEs were categorised as gastrointestinal disorders (e.g. diarrhoea and vomiting), infections and infestations (e.g. upper respiratory tract disorders) and respiratory, thoracic and mediastinal disorders (e.g. asthma and wheeze). The probiotic products were well tolerated by children (Figure 17.1C).

17.8 Safety of probiotics during pregnancy

The effect of probiotic administration during pregnancy has been evaluated in multiple controlled clinical trials. In none of the trials consumption of probiotics were related to SAEs or complications (Rautava et al., 2012; Nikniaz, et al., 2013; Maldonado-lobón et al., 2015; Aaltonen et al., 2011). In addition, there were no statistical significant differences in adverse pregnancy outcomes (e.g. spontaneous abortions, preterm births and malformations) in women consuming
probiotics compared to women taking a placebo during pregnancy (Lee et al., 2012; Lindsay et al., 2015). These findings are supported by a meta-analysis including >1,500 women showing that probiotic intake during pregnancy has no effect on the incidence of caesarean section, birth weight or gestational age (Dugoua et al., 2009). In addition, there are no indications that perinatal maternal probiotic consumption has a negative effect on child health, on both short and long-term (Rutten et al. 2015).

17.9 Conclusions

Based on the current data available, we conclude that probiotic administration in preterm infants, infants and children can be regarded as safe in the setting of controlled clinical trials. Interestingly, there was a tendency of less AE’s in the probiotic groups (4,206 vs 4,331 in the probiotic and control group respectively). For example, there was a higher frequency of diarrhoea, frequent itchy and red eyes, vomiting, green loose stools and feed refusal noted in the control groups compared to probiotic treatment group (Firmansyah et al., 2011; Gore et al., 2012). We therefore encourage future research to further explore the effectiveness of probiotic strains in preventing or treating infant and childhood medical conditions.

In our study, we focused on the safety of probiotic strains by analysing the reported SAEs in clinical trials. However, there are drawbacks in the current data from clinical trials that prevent providing conclusive probiotic strain specific safety profiles (Van den Nieuwboer 2014a,b; 2015). First of all, there is a large variety in different strains and large variety in dosages used in studies, sometimes differing a factor 1000. Secondly, many studies do not provide the specific strain designation, do not sufficiently address the safety aspects or do not adequately report on all AEs or SAEs in their results. Thirdly, treatment regiments differ greatly between studies making data less comparable. For example, some studies used a single strain probiotic product, while others administered a multispecies product or synbiotic. Moreover, the duration of the probiotic intervention also significantly differs between studies. These drawbacks may lead to an underrepresentation of the potential risks of probiotic ingestion by infant and children in clinical trial setting. Clinical researchers are encouraged to consequently report on AEs and SAEs using the CTCAE in future clinical trials. This should also be stimulated and monitored by IRBs.

In sum, based on the current knowledge, investigators can safely supplement available probiotics with GRAS and QPS status to mothers during pregnancy, infants and children. Nonetheless, when administering to infants and children with severe immune compromised conditions, in high dosages or long term, investigators should remain aware of potential complications.

Conflict of interest

M. Van den Nieuwboer declares no conflict of interest. P. Browne has participated as a speaker for Winclow Probiotics B.V. E. Claassen is CEO of Vironovative.
17. Safety of probiotics in infants and children

References


17. Safety of probiotics in infants and children


Appendix 17.1
Clinical trials with infants and children

Infant studies


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17. Safety of probiotics in infants and children


Children studies


17. Safety of probiotics in infants and children


17. Safety of probiotics in infants and children


17. Safety of probiotics in infants and children


**Other**


17. Safety of probiotics in infants and children


