Antibiotics and microbiota colonization in infancy

What lessons can we learn?

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Publication date
2023

Document Version
Final published version

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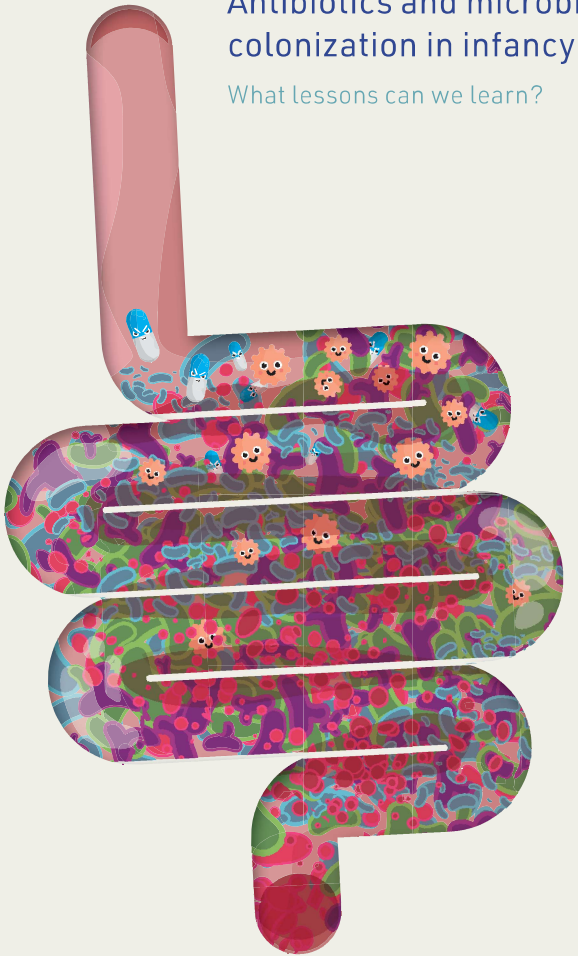

Thomas Dierikx

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. P.P.C.C. Verbeek
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op vrijdag 23 juni 2023, te 16.00 uur

door Thomas Harry Dierikx
geboren te AMSTERDAM

Colofon

Cover design: Proefschrift-AIO
Lay-out: Proefschrift-AIO
Print: Proefschrift-AIO
ISBN: 978-94-93315-68-6

Financial support for printing of this thesis was kindly provided and supported by the Amsterdam Gastroenterology Endocrinology Metabolism (AGEM) research institute, the Amsterdam Reproduction & Development (AR&D) research institute and Winclove Probiotics B.V.

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CHAPTER 1

General introduction and outline of the thesis

The human gut is inhabited by trillions of micro-organisms, mostly bacteria, which are called the gut microbiota. All micro-organisms from the intestinal microbiota and their genes shape the microbiome, although the term microbiota and microbiome are often used interchangeably.[1] Bacterial colonization and development of a healthy gut microbiota early in life is essential for human health, since host-microbe interactions play a key role in multiple physiological processes.[2,3] The intestinal bacteria are e.g. involved in different metabolic pathways and are important for the synthesis of essential vitamins. Microbial colonization of the infant gut also plays a crucial role in the development and maturation of both the native and adaptive immune system. It is consequently believed that the risk of developing numerous non communicable diseases later in life is programmed during infancy when the intestinal microbiota develops.[2,3] Bifidobacteria for example produce acetate and lactate which act as a barrier against enteropathogenic infections. Delayed colonization with bifidobacteria has been associated with a decreased number of memory B-cells later in infancy and with immune dysregulations.[4-6] Furthermore, chronic conditions such as inflammatory bowel disease (IBD), obesity, asthma, allergy type 1 diabetes and many more have been associated with microbiota perturbations early in life.[7-9] It is therefore pivotal to understand the underlying mechanisms of bacterial colonization and development of a healthy microbiota. Subsequently, knowledge on which factors may lead to perturbations in the development of the early microbiota and how to keep these perturbations to a minimum is needed.

During birth millions of micro-organisms are transferred from the mother to the infant gut. In the hours, days and months following birth, even more micro-organisms from the outer environment colonize the infant gut.[10] Particularly in the early phase, the microbiota is highly dynamic and develops rapidly. Directly after birth, the infant gut is mostly inhabited by facultative anaerobes such as *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Escherichia* and *Enterococcus*. In the first days of life these bacteria use up the oxygen and the infant gut turns anaerobic. This allows strict anaerobic genera such as *Bifidobacterium* and *Bacteroides* to colonize the infant gut.[11,12] Both genera play a crucial role in the immune development, and decreased abundance of these genera has been associated with a broad range of diseases, such as asthma, eczema and obesity.[13,14] Vaginally born infants are exposed to the maternal vaginal (and fecal) microbiota and their gut is predominated by *Lactobacillus* early in life. *Lactobacillus* can also modulate the host immune response and suppress inflammation by inducing T-cells.[15] In contrast to vaginally born infants, infant born via caesarean section (CS) circumvent the birth canal and exposure to maternal vaginal microbiota. Consequently their gut microbiota resembles a more skin-like microbiota. During infancy, the microbiota is further shaped by environmental

factors such as feeding habits, medication and geographical location (Figure 1). At the age of three years a more stable, adult-like microbiota has been formed.[11,12]

It is known that early life microbiome acquisition and development can be compromised by multiple external factors such as delivery via CS, formula feeding and exposure to antibiotics.[16] This thesis focuses on (1) the effects of antibiotic exposure during childhood and infancy on the microbiota colonization, (2) on strategies aiming at the reduction of unnecessary antibiotic exposure in newborns and (3) on interventions to reduce adverse effects of antibiotic exposure in childhood.

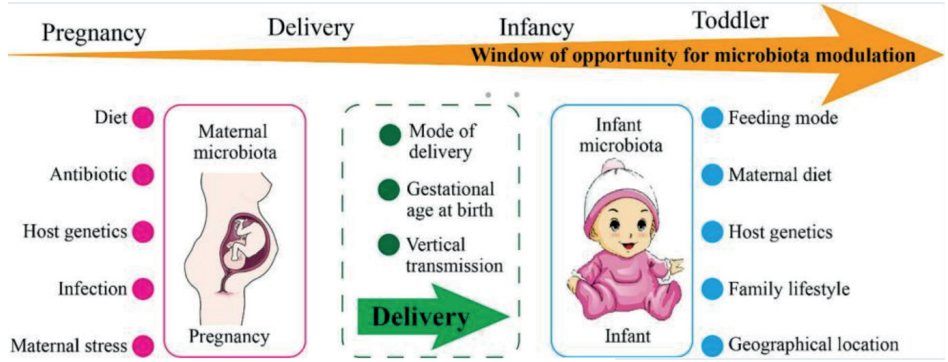

Figure 1. Overview of the most important pre-, peri- and postnatal factors influencing infant microbiota colonization and development (Yao et al. (2021), Front Immunol)

Acute effects of antibiotics, possibly by modulation of the microbiota, include antibiotics-associated diarrhea (ADD) and an increased risk for life-threatening conditions in preterm neonates, such as necrotizing enterocolitis (NEC).[17,18] In general, antibiotic exposure leads to a decreased diversity, decreased abundance of commensal bacteria such as *Bacteroides* and *Bifidobacterium* and an increased abundance of pathogenic bacteria including Enterobacteriaceae spp.[19] Previously, it has been demonstrated that antibiotics administered to pregnant women are transferred over the placenta and consequently reach the fetus bloodstream.[20] As liver and renal functions of infants are relatively compromised at birth, drug metabolism and extraction is delayed compared to adults and half-life of antibiotics is increased in infants.[21] Despite this knowledge, prescription of maternal intrapartum antibiotic prophylaxis (IAP) has increased dramatically over the last decades, resulting from implementation of adjusted obstetric guidelines aiming to reduce maternal and neonatal infection.[22,23] As implementation of these adjusted guidelines have resulted in an increased use of antibiotics antenatally,[22,23] concerns on early-life exposure to broad-spectrum antibiotics and associated pervasive effects on the gut microbiome development and various disorders later in life are

growing.[24] Besides, early-life antibiotic exposure may increase the risk of multi-resistant bacterial (MRB) infections in neonatal patients.[25] Recent epidemiological and mechanistic data on the association between early antibiotic use, dysbiosis and disease support these concerns.[26] In **Chapter 2** we therefore systematically summarized all data available on the influence of maternal IAP on the infant microbiota colonization and on health effects.

One of the revised international obstetric guidelines leading to an increased exposure to antibiotics, is the National Institute for Health and Care Excellence (NICE) (2011) guideline for CS.[22] Yearly, around 30 million infants are born by CS worldwide. In the revised guideline, it is advised to administer maternal prophylactic antibiotics prior to skin incision, instead of after clamping of the umbilical cord. This policy has been shown to reduce the maternal risk on infectious morbidities, particularly of endometritis and wound infections, from 7 to 4%.[27] Consequently, all infants born by CS are currently exposed to broad-spectrum antibiotics via the umbilical cord when adhering to this revised guideline. Although no increase in incidence of neonatal sepsis was observed,[27] effects on the gut microbiota colonization and long-term health consequences following this guideline adjustment remain largely unknown. In **Chapter 3**, we studied this effect by comparing the microbiome composition of CS born infants in a randomized controlled trial (RCT), by comparing colonization in infants with and without intrauterine antibiotic exposure according to the revised and previous protocol, respectively.

Despite implementation of these adjusted guidelines aiming at reduction of maternal and neonatal infections, neonatal sepsis remains one of the leading causes of morbidity and mortality at the neonatal intensive care unit (NICU) and antibiotics still are one of the most prescribed drugs in this population.[28] Neonatal sepsis is divided into early-onset sepsis (EOS) and late-onset sepsis (LOS), based on the timing of clinical onset. EOS reflects vertical transmission of pathogens from the mother shortly before or during delivery and has onset within 72 hours of life, whereas LOS occurs after 72 hours.[29,30] To date, accurate and quick diagnosis of EOS is challenging, mainly due to the non-specific signs and symptoms in combination with the suboptimal gold standard, a peripheral blood culture (PBC).[31]

There are certain disadvantages of a PBC.[32] First, PBC is a painful procedure and a relative large volume is needed increasing the risk for iatrogenic anemia, especially in very low birth weight (VLBW; <1500 g) infants [33,34]. Second, it can be a challenging technique for the physician to obtain an adequate blood volume from a peripheral vein.[32] Third, the sensitivity of a PBC for EOS is low, especially when an inadequate sample volume is collected or when mothers received IAP.[33,34] A PBC often provides false negative results. The exact sensitivity of PBC for neonatal EOS is unknown, but is estimated to be around 25%.[35,36] The use of umbilical cord blood culture (UCBC) has been suggested as an alternative diagnostic test if EOS is suspected at the time of birth. Collection of umbilical cord blood is not painful, it is technically easy to perform and without risk for anemia if a sufficient sample volume can be obtained, potentially increasing the sensitivity.[37] However, studies on the diagnostic accuracy of UCBC compared to PBC included low sample sizes and the results are conflicting. To date, neither a systematic review nor meta-analyses has been performed. Therefore, we systematically identified, appraised and evaluated the diagnostic test accuracy (DTA) of UCBC for the diagnosis of EOS compared to PBC in **Chapter 5**.

Besides aforementioned disadvantages of a PBC, time-to-positivity can be up to 72 hours, leading to delay in diagnosis. Delay in initiation of antibiotic treatment may lead to progressive deterioration in EOS cases. Consequently, a PBC has no use to exclude EOS at the time of initial suspicion, even if the sensitivity in cord blood appears to be high. Therefore, a lot of neonates are unnecessarily treated with empiric antibiotics for 36-72h, awaiting culture results. The incidence of EOS is estimated to be 0.1% in all neonates, and up to 1% in very low birthweight (VLBW; <1500g) and preterm infants.[38] Despite the relative low incidence, about 5% of infants and over 75% of very preterm born infants (gestational age < 30 weeks) are exposed to empirical antibiotics shortly after birth under suspicion of EOS.[39,40] Seen the high risk for false negative results of a PBC, the decision to prolong antibiotics is often based on the clinical condition of the infants, disregarding the outcome of the PBC. In very preterm infants, empirical antibiotics for EOS are continued for at least 7 days in roughly 30% despite negative PBC results. This enormous number of infants unnecessarily exposed to antibiotics increases the risk of antibiotic resistance, microbial aberrations and associated impact on short- and long-term outcomes, as aforementioned.[41]

Antibiotic exposure causes dysregulation of microbial gut colonization by decreasing the diversity and promoting overgrowth of potential pathogens [42]. It has been demonstrated in VLBW infants that every additional day of antibiotic exposure is associated with worse composite outcome of multiple adverse events, including NEC and LOS [43]. However, these findings have recently been questioned by observational and animal model studies, suggesting a mitigating effect of antibiotics on NEC [44,45]. In murine models, antibiotics decrease bloodstream infections, potentially by delaying colonization and thus protecting the immature gut [46]. This hypothesis is supported by a recent cohort study in premature infants.[44] Previous studies, however, did not focus specifically on empirical antibiotic exposure for EOS suspicion. They also did not focus on specific groups divided by duration of empirical antibiotic exposure as

described previously: infants not exposed to antibiotics, exposed for 36-72 hours until confirmation of negative PBC or more than 72 hours despite negative PBC. In **Chapter 4** we therefore aimed to explore the association between the duration of early empirical antibiotic exposure with NEC and LOS in a large multicenter cohort.

In order to decrease unnecessary antibiotic exposure and antibiotic related complications in uninfected infants, a rapid diagnostic tool with high accuracy at initial EOS suspicion is urgently needed. This would guide clinicians when not to start antibiotics in uninfected neonates, preventing unnecessary harm to the developing microbiota. The diagnostic value of a large set of biomarkers such as C-reactive protein (CRP), Procalcitonin (PCT) and different interleukins have been studied for this purpose, but these had unreliable accuracy when performed directly after birth.[38,47] Presepsin, however, might be promising as an early and accurate biomarker. Presepsin is expressed on the cell surface of monocytes and macrophages as CD14, a member of the Toll-like receptors (TLR), and is immediately released after binding of CD14 to bacterial ligands such as lipopolysaccharides (LPS).[48,49] Concentrations increase very early and rapidly in infected patients and presepsin might consequently be an accurate biomarker immediately at onset of EOS suspicion. The sensitivity and specificity of presepsin seems to be higher than that of CRP and PCT.[50] Besides, only a small amount of blood is needed to determine the concentration of presepsin. Previous diagnostic studies on the accuracy of presepsin for EOS in newborns, however, have methodological flaws and a clear cut-off value with a high negative predicting value is still lacking.[50,51] Therefore, we studied the diagnostic accuracy of presepsin for EOS at first presentation in **Chapter 6** where we consecutively included all infants suspected for EOS.

Besides more accurate biomarkers, advanced rapid culturing techniques might also facilitate rapid diagnosis of EOS. The past years state-of-the-art molecular methods have become available at identifying bacteria.[52,53] One of these advanced molecular culture techniques is called Molecular Culture via IS-pro (MC).[54,55] MC is a rapid unrestricted PCR based technique that detects and identifies bacterial DNA via the 16S-23S rRNA gene interspace regions, of which the length is specific for microbial species allowing for profiling of bacteria at species level.[54,55] A previous report compared results of conventional cultures with MC results in samples sent for conventional culturing from infected adult patients of normally sterile bodily sites. In 100% of conventional culture positive samples, MC was also positive. Besides, in 50% of conventional culture negative samples MC detected clinically relevant pathogens, demonstrating the potential of the MC as diagnostic tool in septic patient.[55] MC generates results within 4 hours, compared to 36-72 hours of the conventional PBC. This may guide clinicians to stop or continue empirically administered antibiotics at a much earlier stage, potentially reducing antibiotic overuse in newborns with all associated beneficial effects. As data on the potential of MC for EOS diagnosis in blood samples are lacking so far, we aimed to evaluate this in a cohort of infants suspected for EOS in **Chapter 7**.

From aforementioned it becomes clear that antibiotics are often prescribed for (presumed) infections during the time-window that the gut microbiota is still developing.[56] In case of bacterial infections, antibiotics are mostly the only proven effective treatment and prescription cannot be averted, despite the known side effects.[57] For that reason, it is important to also study interventions aiming at preventing or reducing the unwanted side effects of antibiotics. A common complication of antibiotic treatment is AAD, estimated to occur in 20% of children exposed to antibiotics.[58,59] AAD is considered to be the result of gut dysbiosis, which provokes overgrowth of specific pathogens, most prominently *Clostridioides difficile*, and also leads to altered function of the microbiota.[60,61] The most thoroughly studied preventive intervention for AAD is the administration of probiotics, defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'.[62] According to a 2019 Cochrane review,[59] probiotics as a group have a moderate protective effect on the prevention of pediatric AAD. Among the 33 included studies, only six RCTs investigated combinations of more than three probiotic strains, with varying results. This variance may be explained by the different strains and limited sample sizes in these RCTs.[63-68] Thus, the question whether multispecies probiotic supplementation reduces the AAD incidence in children remains to be answered. In adult patients, one of the multispecies probiotics which was shown to be effective in reducing the risk of AAD consisted of nine bacterial species,[69,70] which were selected based on their ability to survive in the gastrointestinal tract and *in vitro* inhibition of pathogen growth, including *C. difficile*.[71] In **Chapter 8** we aimed to assess the efficacy of a comparable multispecies probiotic mixture in the prevention of AAD in children in an international, multi-center randomized controlled trial. The presumed underlying mechanism of probiotics in the prevention of AAD, is mitigation of antibiotic induced microbial aberrations. Preventing or decreasing aberrations due to antibiotic exposure during the critical time-window early in life, may also decrease risk for other previously mentioned microbiota related short- and long-term adverse effects. In **Chapter 9** we therefore studied the fecal microbiota from children in included in this RCT, in order to investigate the possible protective effects of probiotics on antibiotic induced microbial aberrations.

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Chapter 2

The influence of prenatal and intrapartum antibiotics on intestinal microbiota colonisation in infants: a systematic review

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Journal of infection 2020 Aug; 81(2):190-204

Abstract

Background: The intestinal microbiota develops in early infancy and is essential for health status early and later in life. In this review we focus on the effect of prenatal and intrapartum maternally administered antibiotics on the infant intestinal microbiota.

Methods: A systematic literature search was conducted in PubMed and EMBASE. All studies reporting effect on diversity or microbiota profiles were included.

Results: A total of 4.030 records were encountered. A total of 24 articles were included in the final analysis. Infants from mothers exposed to antibiotics during delivery showed a decreased microbial diversity compared to non-exposed infants. The microbiota of infants exposed to antibiotics was characterised by a decreased abundance of *Bacteriodetes* and *Bifidobacteria*, with a concurrent increase of *Proteobacteria*. These effects were most pronounced in term vaginally born infants.

Conclusion: Maternal administration of antibiotics seems to have profound effects on the infant gut microbiota colonisation. Interpretation of microbiota aberrations in specific populations, such as preterm and caesarean born infants, is complicated by multiple confounding factors and by lack of high quality studies and high heterogeneity in study design. Further research is needed to investigate the potential short- and long-term clinical consequences of these microbial alterations.

Introduction

The intestinal microbiota plays an essential role in a variety of physiological processes including metabolic and immunologic functions[1] and digestion of nutrients[2]. Evidence for the importance of the infant gut microbiota colonisation on health and disease later in life is rapidly increasing[3]. A blueprint for the final shape of microbiota composition is created in early infancy. During this critical window in early life, commensal micro-organisms interact with the mucosal surface and are responsible for programming of the immune system[4]. Antibiotic induced disruption of this colonisation process early in life has been associated with numerous conditions early and later in life such as bronchopulmonary dysplasia[5], obesity[6,7], asthma[8], eczema[9], inflammatory bowel disease (IBD)[10], and increased antibiotic resistance[11]. The most severe early complication associated with intrapartum antibiotics has been the increase in Gram-negative early onset sepsis[12].

Neonatal intestinal colonisation is influenced by multiple perinatal factors, such as mode of delivery, feeding type, gestational age and neonatal medication use (particularly antibiotics)[13]. However, also other factors, like maternally administered antibiotics, have increasingly been considered to influence this neonatal colonisation process[14]. The majority of prenatally prescribed antibiotics are Beta-Lactams (typically ampicillin or penicillin) administered prophylactically, in accordance with guidelines on the prevention of neonatal Group B *Streptococcus (GBS)* infection and antibiotics to prevent maternal morbidity following caesarean section (CS)[15]. International guidelines on prevention of GBS infection[16] and wound prophylaxis during CS[17] have recently been adjusted, leading to an increase in prophylactic antibiotic administration during delivery and consequently increased antibiotic exposure to the infant. Currently, 20-25% of pregnant women are being prescribed antibiotics[18,19] and nearly 80% of all medications prescribed to pregnant women are antibiotics[20]. These antibiotics are prescribed during delivery (hereafter referred to as intrapartum antibiotics) which are mainly given prophylactically according to guidelines or are given prenatally during pregnancy before onset of delivery (hereafter referred to as prenatal antibiotics), mostly given non-prophylactically. These antibiotics may impact early microbial colonisation via two routes. First, maternally administered antibiotics reach the neonatal bloodstream via the umbilical cord and remain present up to at least ten hours after administration, and are likely to influence early colonisation[21,22]. Secondly, maternally administered antibiotics alter the maternal vaginal and intestinal microbiome and consequently could influence the vertical microbial transmission process[23] and postnatal infant immunity[24]. However, the effects of antibiotics during pregnancy and delivery on neonatal gut colonisation and health related outcomes remain largely unknown.

This review aims to evaluate the effect of prenatal and intrapartum maternal antibiotic use on the development of infantile microbiota and to address health related consequences linked to the intestinal colonisation in infants after maternal antibiotic use.

Methods

Study objectives

The primary aim of this review was to evaluate the effect of prenatal and intrapartum maternal antibiotic use on the infantile microbiota. Our goal was to investigate the effect of maternally administered antibiotics on neonatal microbial diversity and on taxonomic composition. The secondary aim of this review was to identify health related consequences of microbiota alterations associated with maternal antibiotic use before birth.

Study eligibility criteria

We conducted a search with support of a clinical librarian. Studies investigating the intestinal microbiota of children and addressing potential influences of maternal antibiotic use during pregnancy (any prenatal antibiotic exposure) or delivery (intrapartum antibiotics) were evaluated. No age limit for the offspring was used in the inclusion criteria. Studies using conventional culture methods were excluded since these do not cover the entire microbiota composition[25]. Studies analysing the microbiota without reporting data on maternal antibiotic use or when no full-text was available were excluded. Searches were restricted to articles published in English, Dutch, French, German or Spanish.

Information sources and search strategy

A review protocol was developed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)-statement. A comprehensive search was conducted in PubMed and EMBASE in collaboration with a medical information specialist. Databases were searched from inception up to 5 September 2019. The following terms were used (including synonyms and closely related words) as index terms or free-text words: "Anti-Bacterial Agents", "Pregnancy", "Delivery", "Microbiota" and "Infant". The search was performed without date or publication status restriction. Duplicate articles were excluded. References from included studies matching the inclusion criteria, but not found with the used search strategy, were also included. The full search strategies for all databases can be found in the online supplementary 1.

Study selection and data extraction

Search results were independently screened by two reviewers who each assessed potentially eligible full-text papers. In case of disagreement, a third researcher decided whether an article could be included or not. Two authors extracted relevant data from papers as well as any available supplements. Other authors verified data-extraction for completeness and accuracy. Data on alpha and beta diversity and microbiota composition at different taxonomic levels (phylum, family, genus and species level) was extracted. The following data was extracted: year of study, country, study design including study setting, characteristics of study population, number of participants, delivery mode, feeding strategies, timing of antibiotic administration during pregnancy or delivery, antibiotic regimen (substance, dose, administration route, duration), indication for antibiotics, infant and maternal antibiotic use postpartum, infant and maternal probiotic use, time-points of collection of stool samples and methods of microbiota analysis. The first requisite for articles to be included was *in utero* antibiotic exposure and data on the microbiota composition. Secondary, after meeting these criteria, data on health related outcomes was extracted from included articles.

Presenting extracted data

Multiple perinatal factors such as route of delivery, postnatal antibiotic administration and gestational age have a profound impact on neonatal microbiota[13]. Heterogeneity in patient characteristics concerning these variables limits reliable comparison between studies. To provide a more reliable overview of the impact of maternal administration of antibiotics on neonatal microbiota composition, circumventing bias by heterogeneity in study design, all eligible articles were divided in subgroups. These subgroups were created based on characteristics of included subjects, route of delivery and gestational age. Results will be presented for antibiotics given intrapartum and prenatal exposure during pregnancy before onset of delivery for each of the following subgroups separately:

A. Vaginally born infants only
   - A1. Articles reporting effect of antibiotic exposure for term born infants only
   - A2. Articles reporting effect of antibiotic exposure for preterm born infants (gestational age < 37 weeks) only
   - A3. Articles reporting a combined effect of antibiotic exposure for term and preterm born infants together

B. Caesarean born infants only
   B1. Articles reporting effect of antibiotic exposure for term born infants only
   B2. Articles reporting effect of antibiotic exposure for preterm born infants (gestational age < 37 weeks) only
   B3. Articles reporting a combined effect of antibiotic exposure for term and preterm born infants together

C. Articles reporting a combined effect of antibiotic exposure for vaginally and caesarean born infants together.
   C1. Articles reporting effect of antibiotic exposure for term born infants only
   C2. Articles reporting effect of antibiotic exposure for preterm born infants (gestational age < 37 weeks) only
   C3. Articles reporting a combined effect of antibiotic exposure for term and preterm born infants together

The use of different microbiota detection techniques, such as quantitative polymerase chain reaction (qPCR), metagenomic sequencing and 16S rRNA gene sequencing to determine the microbial composition, and heterogeneity in reported outcomes hampers reliable comparison of results. Therefore, outcomes will be discussed separately for alpha diversity and at different taxonomic levels where possible up to species level. Since the human gut harbours over 1.000 different species[26], it is not feasible to describe outcomes of all species present. Here we present differences in the most prevalent and reported species.

Risk of bias and quality assessment

After selection of studies, evaluation of risk-of-bias was conducted using the "Risk of Bias in Non-randomised Studies of Interventions" (ROBINS-I) tool[27] for nonrandomised studies. The revised Cochrane risk-of-bias tool[28] was used for randomised trials. The Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) group criteria[29] were used to assess the quality of evidence. The quality of evidence was classified as very low, low, moderate or high.

Results

Included studies

We identified a total of 4.030 studies (PubMed 1.928, EMBASE 2.102). A total of 2.558 articles remained after removal of duplicates. Titles and abstracts of these articles were screened and 2.449 records were consequently excluded. Full text of the remaining 109 articles were checked for eligibility; 85 articles were excluded based on exclusion criteria, leaving 24 articles meeting the inclusion criteria for this systematic review (figure 1). These studies included 3.583 infants of which 1.178 mothers were exposed to antibiotics during pregnancy or delivery (intervention group). Mothers of the other 2.377 infants were not exposed to antibiotics, these infants were included as a control group. Data on antibiotic use from the remaining 28 mothers was missing. Included infants provided a total of 6.429 unique stool samples that were analysed from the first day up to twelve months postpartum. Characteristics of included studies are described in the online supplementary 2. Reasons for exclusion were mainly missing data on the influence of maternally administered antibiotics.

Risk of bias and quality of evidence

Results of the assessment of risk of bias are included in the online supplementary information (online supplementary 3). Studies in subgroup A1 were judged as low risk of bias. However, four studies were done by the same research group and there might have been an overlap in participants[30-33]. Two more studies were performed by the same research group[34,35], however participants were recruited in a different time frame, so there was no overlap in participants. In subgroup C two studies were performed by the same research group, including the same participants which may cause selection bias[36,37]. Two studies from subgroup B and all studies from subgroup C were characterised by high risk of bias due to confounding. These studies included infants born by both CS and the vaginal route, without reporting data for both groups separately. Since international guidelines advise to administer antibiotics prophylactically in women delivering via CS, almost all caesarean born infants will fall in the exposed intervention group. A high rate of the premature infants received postnatal antibiotics directly postpartum. None of the studies included in the subgroups performed a sample size calculation to detect effects of maternal antepartum antibiotic use on neonatal microbiota. The overall quality of evidence was classified as low using the GRADE group criteria (table 1).

Table 1. Results of quality assessment using GRADE group criteria

Number of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	№. Of patients		Quality
							Antibiotics	No antibiotics	
24	Randomised trial; observational studies	Serious	Not serious	Not serious	-	None	1.178	2.377	Low ⊕⊕◯◯

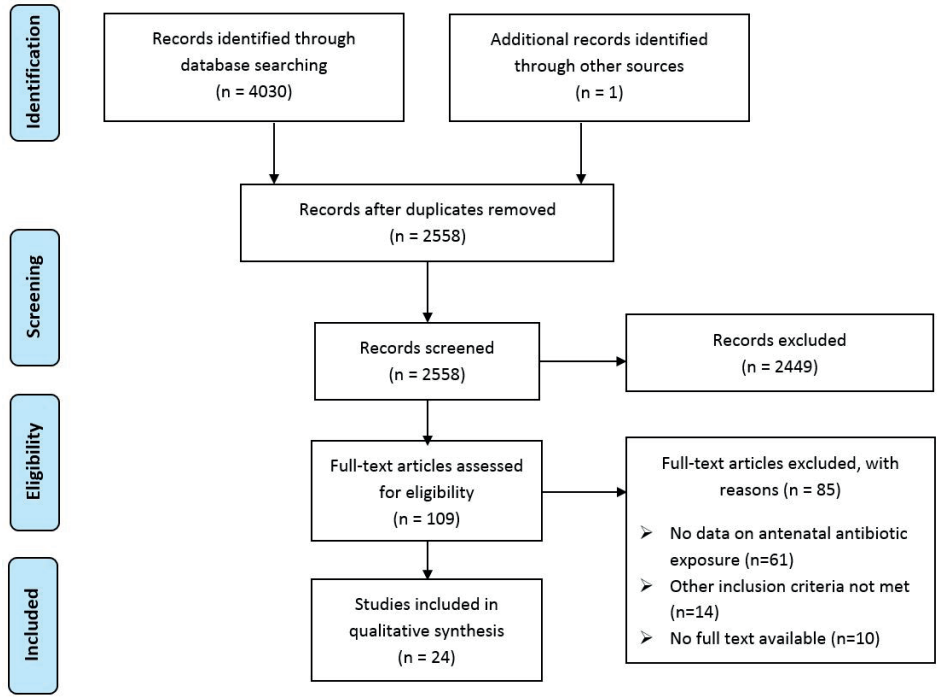

Figure 1. Flow diagram of study selection

Intrapartum antibiotics

Subgroup A: Effect of intrapartum antibiotics in vaginally born infants

All ten studies including only vaginally born infants or presenting data for vaginally born infants separated from results of caesarean born infants, included only infants born at term (subgroup A1)[7,30-33,35,38-41]. No studies were found investigating the effect of *in* utero antibiotic exposure in solely vaginally born infants that were born preterm (subgroup A2) nor studies including both term and preterm vaginally born infants (subgroup A3).

Subgroup A1: Effect of intrapartum antibiotics in term vaginally born infants only

An overview of the study characteristics and main findings from these ten studies is presented in table 2 and displayed in figure 2. Six of these studies included merely women receiving intrapartum prophylactically administered ampicillin[30-33] or penicillin[39,40] for GBS prophylaxis. One study included mainly women receiving antibiotic prophylaxis for GBS prophylaxis or prophylaxis in case of prolonged rupture of membranes (PROM)[35]. It was not specified in this study which type of antibiotic was administered. In the other three studies, the indication for intrapartum antibiotic administration was not mentioned[3,38,41]. Studies included 1.098 vaginal born infants. Mothers from a total of 313 infants received intrapartum antibiotic prophylaxis

(IAP) where the remaining 785 were not exposed to antibiotics and were included as control group. Seven studies used 16S rRNA gene sequencing to analyse the neonatal microbiota[31,33,35,38-41]. Two other studies used qPCR, detecting *Bacteroides fragilis, Escherichia coli* and *Clostridium difficile,* as analysing technique[30,32]. The remaining study analysed stool samples by whole-genome shotgun sequencing[3].

All included infants had a birth weight adequate for their gestational age. Two studies included only breastfed infants[30,31], where other studies included both breastfed and formula fed infants[3,32,33,35,38-41]. However, no differences in baseline characteristics were found between infants from intrapartum antibiotic exposed mothers compared to non-exposed mothers. Three studies included infants who received postnatal antibiotics in their analysis[3,35,38] ranging from 1.5%[38] and 4%[35] directly postpartum, to 36.5% by twelve months postpartum[35]. Faecal samples were collected from the first day after birth up to one year.

Diversity

Diversity was determined in the eight studies analysing sample with next generation sequencing methods. A lower bacterial diversity in faecal samples of neonates from mothers who were exposed to antibiotics was consistently reported in seven studies. Reduced diversity was presented as significant lower score of Chao1 [31,33,35], Shannon diversity indices Shannon indices[31,33,38,40] and overall alpha diversity[39]. A decreased Shannon diversity index was found up to one year after birth[38]. In contrast, no difference in the daily change in microbial diversity was found in one study the first week of life nor at twelve months after birth[41]. However, the beta profiles of infants from antibiotic exposed mothers differed from non-exposed infants already at day one postnatally[41]. Beta diversity profiles of unexposed infants grouped together, whereas microbiota of antibiotic exposed infants, indirect via their mother, did not[41].

Phylum level

The most abundant phyla characterising neonatal microbiota included *Proteobacteria, Actinobacteria, Bacteriodetes and Firmicutes*[31,33,39,40]. In infants from antibiotic exposed mothers, an increase in *Proteobacteria*[31,33,35,39-41] and a concurrent decrease in *Actinobacteria*[31,33,39,40] and *Bacteriodetes*[31,33,35,39,41] during the first ten days of life was observed. These differences seemed to be diminished at 30[32,33] and 90 days[39,40]. However, in one study the abundance of *Bacteriodetes* was still decreased after three months, but not at twelve months[35]. Data on the abundance of *Firmicutes* was contradictory, with one study reporting a delay in colonisation[40], two others a higher abundance[39,41] and other studies no difference.

Family/genus level

At family level, *Enterobacteriaceae* (phylum: *Proteobacteria*) were significantly increased in neonates from antibiotic exposed mothers one week [31,33] and three months after birth[35]. Reported data on the genus *Bifidobacterium* (family: *Bifidobacteriaceae* and phylum: *Actinobacteria*) consistently showed a decreased presence in samples collected during the first month of life [30-33,38-40]. This decrease persisted up to twelve weeks postpartum in one study [40], but was no longer present in another [35]. Furthermore, results on *Bacteroides* showed a decreased taxonomic abundance of this genus in four studies[3,35,38,41]. Most studies did not show data on the abundance of *Lactobacillus* (Family: *Lactobacillaceae* and phylum: *Firmicutes*). However, two studies were unable to show a difference between neonates from antibiotic exposed and non-exposed mothers[30,32].

Species level

Three studies reported data on species level[30,32,38]. One study using qPCR did not demonstrate a difference in *E. coli* and *C. difficile* between infants from antibiotic exposed compared to non-exposed infants one week after birth[30]. No differences were found at seven nor at 30 days in the abundance of *B. fragilis*[30,32]. In another study the abundance of this species was decreased one year postpartum after penicillin use by the mother, whereas *B. fragilis* was increased after maternal cephalosporin use[38].

Table 2. Overview of findings of studies investigating the effect of intrapartum antibiotic prophylaxis on the microbiota of term vaginally born infants (subgroup A1)

Author	Study population	Intervention and indication	Analysis technique	Age of sampling	Outcomes in microbiota samples of neonates from mothers exposed to IAP				
					Alfa diversity	Phylum level	Family level	Genus level	Species level
Aloisio 2014[30]	n total = 52 n control = 26 n IAP = 26	2 g of ampicillin followed by 1 g every 4 h until delivery for GBS prophylaxis	Real time qPCR (*Lactobacilli, Bifidobacteria, B. fragilis, C. difficile, and E. coli*)	6th or 7th day	-	-	-	Lower abundance: *Bifidobacteria* No difference: *Lactobacilli*	No difference: *B. fragilis* *C. difficile* *E. coli*
Aloisio 2016[31]	n total = 20 n control = 10 n IAP = 10	2 g of ampicillin followed by 1 g every 4 h until delivery for GBS prophylaxis	16S rRNA gene sequencing of 16S V2, V3, V4, V6 + V7, V8 and V9 regions and V4 region separately	6th or 7th day	Decreased	Lower abundance: *Actinobacteria* *Bacteriodetes* Higher abundance: *Proteobacteria*	Lower abundance: *Bacteriodaceae* *Lactobacillaceae* Higher abundance: *Enterobacteriaceae* *Streptococcaceae*	Lower abundance: *Bifidobacteria*	-
Azad, 2015[35]	n total = 198 n control = 113 (113 vag) n IAP = 85 (42 vag)†	Intervention not specified. Indication: typically GBS prophylaxis and pre-labour rupture of membranes	16S rRNA gene sequencing of V4 region	3 months, 12 months	Decreased (m3)	Lower abundance: *Bacteriodetes* (m3) Higher abundance: *Proteobacteria* (m3, p<0.10)	Lower abundance: *Bacteriodaceae* (m3) Higher abundance *Clostridiaceae* (m3, m12) *Enterobacteriaceae* (m3)	Lower abundance§: *Bacteroides* (m3) *Acinetobacter* (m12) Higher abundance§: *Clostridium* (m3, m12) *Enterococcus* (m3) *Veillonella* (m12)	-
Coker, 2019[38]	n total = 266 n control = 179 n IAP = 87	Penicillin (n=55), cephalosporin (n=14), multi-drug classes (n=12) other (n=6) Indication not specified.	16S rRNA gene sequencing of V4-V5 region	6 weeks, 12 months	Decreased (6w, 12m)	-	-	Lower abundance (w6 and m12 combined): *Bacteroides, Bifidobacteria, Blautia, Roseburia, Rumicococcus* Higher abundance (w6 and m12 combined): *Oscillospora, Pseudobacter, Veillonella dispar*	Lower abundance after penicillin: *B. fragilis(12m)* Higher abundance after cephalosporin: *B. fragilis(12m)*

Table 2. Continued

Author	Study population	Intervention and indication	Analysis technique	Age of sampling	Outcomes in microbiota samples of neonates from mothers exposed to IAP				
					Alfa diversity	Phylum level	Family level	Genus level	Species level
Corvaglia 2016[32]	n total = 84 n control = 49 n IAP = 35	2 g of ampicillin followed by 1 g every 4 h until delivery for GBS prophylaxis	qPCR (*lactobacilli, Bifidobacteria and B. fragilis*)	7 days, 30 days	-	-	-	Lower abundance: *Bifidobacteria* (d7) No difference: *Lactobacillus* (d7, d30)	No difference: *B. fragilis* (d7, d30)
Mazzola, 2016[33]	n total = 26 n control = 13 n IAP = 13	2 g of ampicillin followed by 1 g every 4 h until delivery for GBS prophylaxis	16S rRNA gene sequencing of V3-V4 region	7 days, 30 days	Decreased (d7)	Lower abundance: *Actinobacteria* (d7) *Bacteriodetes* (d7, p=0.078) Higher abundance: *Proteobacteria* (d7)	Higher abundance: *Enterobacteriaceae* (d7) *Veillonellaceae* (d30)	Lower abundance: *Bifidobacteria* (d7) *Streptococcus* (d30) Higher abundance: *Escherichia* (d7)	-
Nogacka, 2017[39]	n total = 40 n control = 22 n IAP = 18	5 million units of penicillin followed by 2.5 million units every 4 h until delivery for GBS prophylaxis	16S rRNA gene sequencing of V3-V4 region	2, 10, 30 and 90 days	Decreased (all days combined)	Lower abundance: *Actinobacteria* (d10) *Bacteriodetes* (NP) Higher abundance: *Firmicutes* (d10, d90) *Proteobacteria* (NP)	Lower abundance: *Bifidobacteriaceae* (d10) Higher abundance: *Muribaculaceae* (d2, d10, d30, d90) *Prevotellaceae* (d2, d90) *Rikenellaceae* (d2) *Clostridiaceae* (d10) *Campylobacteraceae* (d90) *Helicobacteraceae* (d90)	-	-

Table 2. Continued

Author	Study population	Intervention and indication	Analysis technique	Age of sampling	Outcomes in microbiota samples of neonates from mothers exposed to IAP				
					Alfa diversity	Phylum level	Family level	Genus level	Species level
Shao, 2019[3]	n total = 596 n control = 291 (291 vag) n IAP = 305 (23 vag)†	Intervention and indication not specified.	Shotgun metagenomic sequencing	Day 4, 7 and 21 and between 4-12 months	Decreased	-	-	Lower abundance: *Bacteriodes*	-
Stearns, 2017[40]	n total = 74 n control = 53 (53 vag) n IAP = 21 (14 vag) †	Penicillin G for GBS prophylaxis (dose regimen not reported)	16S rRNA gene sequencing of V3 region	3 days, 10 days, 6 weeks, 12 weeks	Decreased (d10, w6)	Delay in colonisation: *Actinobacteria* *Firmicutes* Prolonged persistence: *Proteobacteria*	-	Lower abundance: *Bifidobacteria* (w12) Higher abundance: *Escherichia* (w12)	-
Tapiainen, 2019[41]	n total = 73 n control = 29 n IAP = 44	Penicillin G (n=38), cefuroxime (n=4) and clindamycin (n=2). Indication not specified.	16S rRNA gene sequencing of V4-V5 region	Daily when hospitalized from day 1 - 7 and at 6 months	No difference	Lower abundance: *Bacteriodetes* (d3, m6) Higher abundance: *Firmicutes* (d3) *Proteobacteria* (d4)	-	Lower abundance: *Bacteriodes* (d2) Higher abundance *Clostridia* (m6)	-

CS = Caesarean born infants, g = gram, GBS = ***Group B streptococcus,*** h = hours, IAP = Intrapartum antibiotic prophylaxis, NP = no p-value shown, qPCR = quantitative polymerase chain reaction, rRNA = ribosomal ribonucleic acid, vag = vaginally born infants

† Results for comparison of vaginally born exposed infants to non-exposed vaginally born infants excluding the caesarean born infants in the analysis

§ Subset shown of > 10 genera reaching statistic significant difference

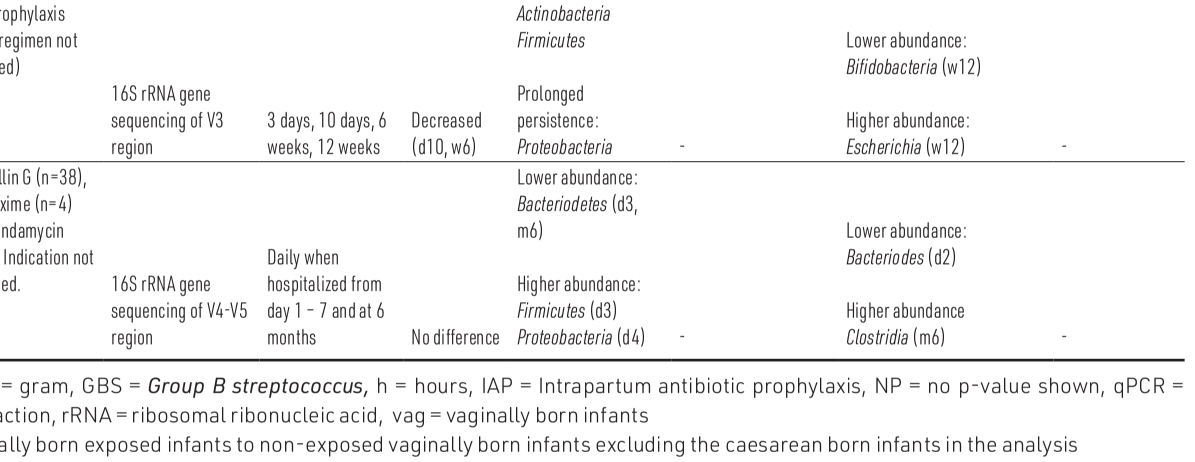

Figure 2. Overview of main pre-, peri- and postnatal factors influencing neonatal microbiota. In vaginal born infants at term, prophylactic intrapartum administration of antibiotics to the mother resulted in a decreased diversity, a decreased abundance of *Bacteroidetes* and *Actinobacteria* and an increased abundance of *Proteobacteria* in the microbiota of the infant.

Subgroup B: Effect of intrapartum antibiotics in caesarean born infants

Three studies reported an effect of antibiotic exposure on the microbiota of solely caesarean born infants[35,40,42]. These studies all included infants born at term (subgroup B1). No studies included caesarean born preterm infants (subgroup B2) or both preterm and term infants (subgroup B3).

Subgroup B1: Effect of intrapartum antibiotics in caesarean born term infants only

An overview of the study characteristics and main findings from these studies is presented in table 3. In all three studies all mothers of the caesarean born infants received antibiotics to lower maternal morbidity. Studies included 258 infants of whom 72 mothers were exposed to IAP before childbirth. Two studies investigated the effect of IAP in caesarean born infants and compared this to vaginally born infants without IAP exposure[35,40]. One randomized controlled trial (RCT) compared microbiota of infants where antibiotics were administered prior to the CS compared

to after childbirth and after clamping of the umbilical cord[42]. All three studies used 16S rRNA gene sequencing to analyse the collected stool samples.

The two studies comparing caesarean born infants from mothers exposed to IAP to non-exposed vaginal born infants showed a decreased abundancy in *Bacteriodetes*[35], *Bacteriodaceae*[35] and *Bacteroides*[35,40] up to twelve months. The abundancy of *Firmicutes* at three months was increased in one study[40] and decreased in the other[35]. Furthermore, both studies showed an increase in *Proteobacteria*[35,40].

In the RCT by Kamal et al., faecal samples were collected at day 10 and after 9 months. After 10 days the microbiota of both groups was dominated by the family *Enterobacteriaceae* (phylum: *Proteobacteria*). No statistical differences were found at phylum, family nor genus level between the antibiotic exposed and non-exposed group. At nine months of age the number of observed species was lower in infants from antibiotic exposed mothers (361 versus 496, p=.012) however, Shannon diversity index did not reach a statistically significant difference (p=.062).

Table 3. Overview of findings of studies investigating the effect of intrapartum antibiotic prophylaxis on the microbiota of term caesarean born infants (subgroup B1)

Author	Study population	Intervention	Analysis technique	Age of sampling	Outcomes in microbiota samples of neonates from antibiotic exposed mothers			
					Alfa diversity	Phylum level	Family level	Genus level
Azad, 2015[35]	n total = 198 n control = 113 (113 vag) n IAP = 85 (43 CS)†	Cefazolin	16S rRNA gene sequencing of V4 region	3 months, 12 months	Decreased (m3, m12)	Lower abundance: *Bacteriodetes* (m3, m12) *Firmicutes* (m3, m12) Higher abundance: *Proteobacteria* (m3, m12)	Lower abundance: *Bacteriodaceae* (m3, m12) Higher abundance *Clostridiaceae* (m3, m12) *Enterobacteriaceae* (m3)	Lower abundance §: *Bacteroides* (m3, m12) Higher abundance§: *Clostridium* (m3, m12) *Enterococcus* (m3) *Akkermansia* (m12)
Stearns, 2017[40]	n total = 74 n control = 53 (53 vag) n IAP = 21 (7 CS)†	Cefazolin (n=5) Ampicillin (n=1) Cephalexin (n=1)	16S rRNA gene sequencing of V3 region	3 days, 10 days, 6 weeks, 12 weeks	-	Delay in colonisation: *Actinobacteria* *Bacteroidetes* Prolonged persistence: *Proteobacteria* *Firmicutes*	-	Lower abundance: *Bifidobacteria* (w12), *Bacteroides* (w12) *Escherichia* (w12) Higher abundance: Uncl. *Enterobacteriaceae* (12w)
Kamal, 2019[42]	n total = 42 (42 CS) n control = 20 n IAP = 22	Cefuroxime	16S rRNA gene sequencing of V3-V4 region	10 days, 9 months	Decreased (9m)	No differences	No differences	No differences

CS = Caesarean born infants, IAP = intrapartum antibiotic prophylaxis, rRNA = ribosomal ribonucleic acid, uncl = unclassified, vag = vaginally born infants
† Results for comparison of caesarean born exposed infants to non-exposed vaginally born infants excluding vaginal born infants in the analysis
§ Subset shown of > 10 genera reaching statistic significant difference

Subgroup C: Effect of intrapartum antibiotics in vaginal and caesarean born infants together

Nine studies evaluated the effect of intrapartum antibiotics on the microbiota of the offspring, without reporting data for caesarean born infants separately of vaginal born infants. Two included only term born infants (subgroup C1.) [34,43], four included merely preterm born infants (subgroup C2)[36,37,44,45], and three included both preterm and term born infants in their analysis (subgroup C3)[46-48].

Subgroup C1: Term vaginally born and caesarean born infants

One study compared the microbiota of eleven caesarean and vaginally born intrapartum antibiotic exposed infants to that of thirteen non-exposed infants. No significant differences were found in any of the 16S rRNA gene sequencing analysis at four months. However, the genus *Blautia* tended to be elevated in exposed infants[34]. Another study with the same design investigated differences between fourteen non-exposed and nineteen exposed infants. At one month postpartum the diversity was decreased in the exposed group. Furthermore, the abundancy of *Bifidobacteria* was significantly decreased. No differences were found in the abundancy of *Bacteriodes*, *Escherichia* or *Clostridium*[43].

Subgroup C2: Preterm vaginally born and caesarean born infants

Four studies were found evaluating the effects of intrapartum administered antibiotics on the microbiota of infants born before 37 weeks of gestation[36,37,44,45]. Gestational age of these preterm infants ranged from 23 weeks[45] up to 36 weeks[44]. A total of 94 infants were included in these studies of whom 42 mothers were exposed to antibiotics during delivery. All studies included both vaginal and caesarean born infants and reported their outcomes for vaginally and caesarean born infants together; between 33%[49] and 74%[36,37] of infants were born by CS. The majority of the infants received antibiotics postpartum. From 63% [36,37] up to 82%[45] of included infants were exposed to antibiotics directly postpartum. None of the studies had any documentation on the indication for the intrapartum antibiotic administration. Twelve mothers were exposed to a combination of ampicillin and erythromycin and two exclusively to ampicillin and penicillin. From all other mothers, data on the type of antibiotic was missing. Stool samples were collected from the first day up to three months postpartum and were analysed by 16S pyrosequencing[45] and 16S rRNA gene sequencing[36,37,44]. A summary of main findings of these studies is given in table 4.

Microbial diversity

Only one study investigated differences in the microbial diversity of preterm neonates from antibiotic-exposed mothers compared to those from non-exposed

mothers[45]. In this study a trend towards lower diversity (p=.06) in the first stool sample was found but not after seven days (p=.75)[45]. The three other studies did not show data on diversity[36,37,44].

Taxonomic composition

Not all studies analysed the microbiota at phylum level. One studies in which data was shown demonstrated a significant increase in the abundance of *Proteobacteria* during the first month of life[37]. Results on the abundance of *Bacteriodetes* showed no difference in the first month postpartum[37]. In two studies, *Bacteriodetes* were almost completely depleted in all preterm infants irrespective of maternal antibiotic exposure up to 90 days[36,37]. *Actinobacteria* and *Firmicutes* were both decreased in one study after seven and 30 days in infants from antibiotic exposed mothers [37]. After 90 days abundance levels of these phyla had normalized and differences had disappeared[37]. The two other studies did not report data on phylum level [44,45].

At family level, *Enterobacteriaceae* were overrepresented at the age of one month[36]. *Bifidobacteria* showed decreased abundance[36] at fourteen and 90 days postpartum. In contrast, this difference was not found in another study[44]. The first month of life, no differences were found in the abundance of *Lactobacilli*[44]. Two studies did not show data on family nor genus level [37,45]. Furthermore, none of the studies reported data on species level.

Subgroup C3: Term and preterm vaginally born and caesarean born infants

Three prospective cohort studies reported on the influence of intrapartum antibiotics, as secondary outcome, on the infant microbiota for preterm and term born infants and caesarean and vaginally born infants together[46-48]. These studies included 390 infants of whom 131 mothers were exposed to antibiotics during delivery. Mothers were exposed to cefazolin (n=24), penicillin (n=12), ampicillin-sulbactam (n=8), ampicillin (n=6), clindamycin (n=5), cephalosporin (n=4), vancomycin and unspecified antibiotics (n=85). Indication for antibiotic administration was not mentioned in any of the three studies. Gestational age ranged from 34 weeks to 42 weeks. A total of 87 infants were born by CS, ranging from 19%[48] up to 56.7%[46] in the included studies. Stool samples were collected directly after birth[48] up to the eight months[46]. Stool samples were analysed by qPCR[47], 16S rRNA gene sequencing[48] or metagenomic sequencing[46]. In table 4 an overview of main findings from these studies is shown.

Microbiota outcomes

One study reported decreased diversity following maternal ampicillin use in samples collected monthly up to eight months[46]. Other studies did not report any

effect on diversity. Studies investigating the taxonomic composition reported several differences between infants from antibiotic exposed mothers compared to those of non-exposed. However, studies did not show data on abundance at phylum level. At family level, samples collected from maternal antibiotic exposed infants during the first eight months contained a higher abundance of *Lachnospiraceae* and *Enterobacteriaceae* in mothers exposed to clindamycin[46]. Infants from antibiotic exposed mothers depicted a decreased abundance of *Bifidobacterium* species[47,48] and especially of *Bifidobacterium breve* and *Bifidobacterium longum*[47]. Species belonging to the genera *Staphylococcus* and *Lactobacillus* were depleted in meconium samples[47].

Table 4. Overview of findings of studies investigating the effect of intrapartum antibiotic prophylaxis on the microbiota of cohorts including both vaginally and caesarean born infants (subgroup C);

Table 4A. term born infants (subgroup C1)

Author	Study population	Intervention and indication	Analysis technique	Age of sampling	Outcomes in microbiota samples of neonates from antibiotic exposed mothers				
					Alfa diversity	Phylum level	Family level	Genus level	Species level
Azad, 2013[34]	n total = 24 n control = 13 (13 vag) n IAP = 11 (6 CS, 5 vag)	Not specified	16S rRNA gene sequencing of V3, V4 and V5 region	Between 3 - 4 months	No significant differences were detected according to intrapartum exposure to antibiotics, although the genus Blautia tended to be overrepresented among exposed infants. Data were not shown.				
Imoto, 2018[43]	n total = 33 n control = 14 (14 vag) n IAP = 19 (9 CS, 10 vag)	GBS prophylaxis (n=4 received ampicillin), PROM (n=6 received ampicillin) and CS (n=9 received cefazolin)	16S rRNA gene sequencing of V4 region	1 month	Decreased	-	-	Lower abundance: *Bifidobacteria* No difference: *Bacteroides* *Escherichia* *Clostridium*	-

Table 4B. Preterm born infants (subgroup C2)

Author	Study population	Intervention and indication	Analysis technique	Age of sampling	Outcomes in microbiota samples of neonates from antibiotic exposed mothers			
					Alfa diversity	Phylum level	Family level	Genus level
Arboleya, 2015[36]	n total = 27 (mean GA 29.6, range 24-32, 7 vag, 20 CS) n control = 5* n IAP = 5*	Penicillin (n=1), ampicillin (n=1), ampicillin + erythromycin (n=12). Indication not specified.	16S rRNA gene sequencing of V3-V4 region	Day 2, 10, 30, 90	-	-	Lower abundance: *Leuconostaceae* (d2) *Micrococcaceae* (d10) *Propionibacteriaceae* (d10) *Comamonadaceae* (d30) *Staphylococcaceae* (d30) *Bifidobacteriaceae* (d30) Uncl. *Actinobacteia* (d30) Uncl. *Lactobacillales* (d30) *Ruminococcaceae* (d90) Higher abundance *Enterobacteriaceae* (d30)	Lower abundance: *Bifidobacteria* (d90)
Arboleya, 2016[37]	n total = 27 (mean GA 29.6, range 24-32, 7 vag, 20 CS) n control = 5† n IAP = 5†	Penicillin (n=1), ampicillin (n=1), ampicillin + erythromycin (n=12). Indication not specified.	16S rRNA gene sequencing of V3 region	Day 2, 10, 30, 90	-	Lower abundance: *Actinobacteria* (d30) *Firmicutes* (d30) Higher abundance: *Proteobacteria* (d30)	-	-
Jia, 2019[44]	n total = 51 (mean GA 31.8, range 26-36, 16 vag, 35 CS) n control = 20 n IAP = 25 n missing = 6	Intervention and indication not specified.	16S rRNA gene sequencing of V3-V4 region	Day 1, 7, 14, 21, 42, 70 and 90	'In a multivariate regression model maternal IAP use was correlated with lower abundance of *Peptoclostridium*. No correlation was found for *E. coli, Klebsiella, Bifidobacterium, Bacteriodes, Enterococcus, Streptococcus, Veillonella, Acinetobacter, Lactobacillus, Clostridium sensus stricto, Staphylococcus, Parabacteroides* and unclassified *Enterobacteriaceae*.'			

Table 4B. Continued

Author	Study population	Intervention and indication	Analysis technique	Age of sampling	Outcomes in microbiota samples of neonates from antibiotic exposed mothers			
					Alfa diversity	Phylum level	Family level	Genus level
Mshvildadze, 2010[45]	n total = 23 (mean GA 29.9, range 23-32 weeks, 10 vag, 13 CS) n control = 16 n IAP = 7	Intervention and indication not specified.	Denaturing gradient gel electrophoresis and 454 based 16S rRNA pyrosequencing	Day 1, weekly	Decreased (d1, p = 0.06)	-	-	-

Table 4C. cohorts including both term and preterm infants (subgroup C3)

Author	N	Intervention and indication	Analysis technique	Age of sampling	Outcomes in microbiota samples of neonates from antibiotic exposed mothers
Baumann - Dudenhoeffer, 2018[46]	n total = 60 (mean GA 37 weeks; IQR 36-38 weeks. 26 vag, 34 CS) n control = 14 n IAP = 46	Cefazolin (n=24), ampicillin (n=6), penicillin G (n=6), vancomycin (n=2), clindamycin (n=4) ampicillin-sulbactam (n=8). Indication not specified.	Metagenomic shotgun sequencing	Monthly from 0 - 8 months	Decreased alpha diversity. Higher abundance of *Lachnospiraceae* and *Enterobacteriaceae*
Forsgren, 2017[47]	n total = 118 (mean GA 39 weeks; range 33-42, 76 vag, 24 CS) n control = 94 n IAP = 24	Intervention and indication not specified.	qPCR (several *Bifidobacterium and clostridium spp., s. aureus* and *akkermansia munciniphila*)	Day 1, 2-4 weeks and 6 months	Lower abundance in the following species: *B. breve* (p=0.06) and *B. longum*.
Tapiainen, 2018[48]	n total = 212 (mean GA 40 weeks, range 35-42, CS 40, 172 vag). n control = 151 (137 vag, 14 CS) n IAP = 61 (35 vag, 26 CS)	Intervention and indication not specified.	16S rRNA gene sequencing of V4 region	Day 1	Lower abundance of: *Staphylococcus spp.* (0.06), *Bifidobacterium spp., Lactobacillus spp.* (p=0.065)

CS = Caesarean born infants, GA = gestational age, GBS = ***Group B streptococcus,*** IAP = Intrapartum antibiotic prophylaxis, qPCR = quantitative polymerase chain reaction, rRNA = ribosomal ribonucleic acid, vag = vaginally born infants

† Infants receiving antibiotics directly postpartum were excluded in the analysis

Prenatal antibiotic exposure

Four studies investigated the effect of antibiotics, not administered prophylactically during the delivery, but earlier in pregnancy before onset of labour. Studies on these prenatally administered antibiotics included both vaginally and caesarean born infants and did not report outcomes for these two groups separately. Two studies included only preterm born infants[49,50] (subgroup C2) and two both at-term and preterm infants[13,51] (subgroup C3). An overview of main patient characteristics and outcomes is presented in table 5.

Subgroup C2: Preterm born vaginal and caesarean born infants

Two studies investigated the effect of antibiotic exposure during pregnancy on the microbiota of the offspring[49,50]. One of these studies included 66 extremely and very premature infants (gestational age 25-31 weeks)[50]. All 31 extremely premature infants received a probiotic supplementation to prevent necrotizing enterocolitis (NEC). Half of the included infants were born via caesarean section. Mothers of twenty infants (30%) were exposed to prenatal antibiotics. The exact timing of administration, the type of antibiotic used nor the indication was mentioned. Stool samples collect at day seven and analyzed by whole-genome shotgun sequencing showed no differences in microbial composition. However, 56 of 66 infants received broad spectrum antibiotics postpartum before collection of the sample.

The second study included twelve preterm infants whose mothers were exposed to cefazolin during pregnancy[49]. Also in this study, indication and timing of administration were not reported. These twelve infants were matched with twelve infants whose mothers were free of antibiotic exposure. Infants were matched based on route of delivery, gestational age and feeding method. Both groups consisted of three caesarean born infants. Samples were collected at day seven and fourteen postpartum and analyzed by 16S rRNA gene sequencing. Prenatal antibiotic exposure resulted in a decreased abundancy of *Proteobacteria* with a concurrent decrease of *Firmicutes* and *Bacteriodetes*. No differences were found in the Shannon diversity between the two groups. In all infants, in both groups, antibiotics were started postpartum.

Subgroup C3: Term and preterm born vaginal and caesarean born infants

Two studies investigated the effect of maternal antibiotic exposure during pregnancy on the microbiota[13,51]. In a large cohort study including 1.032 children, faecal samples were collected one month postpartum to identify factors influencing the early gut microbiota[13]. A total of 108 of the infants were born via CS, and 28 received antibiotics before collection of the sample. Mothers from 38 (3.7%) children were exposed to antibiotics during the last months of pregnancy. The indication or type of antibiotic was not reported. Stool samples were analysed by qPCR, evaluating the abundance of *Bifidobacteria*, *E. coli*, *C. difficile*, *B. fragilis*, *Lactobacillus* and total bacterial counts. These analysis failed to show any difference between the microbiota composition of infants from mothers exposed to antibiotics compared to infants from non-exposed mothers.

The second study aimed to investigate the effect of antibiotic use during pregnancy on the weight-for-length score (WFL-score)[51]. They included 454 infants, of whom 237 were exposed to antibiotics. Timing of antibiotics was divided by trimester. Type of antibiotics or indications were not presented. Infants from women exposed to antibiotics during the second trimester had a significant higher WFL-score after adjusting for potential confounders at twelve months postpartum. Based on this, stool samples from this group were analysed and compared to infants from unexposed mothers to detect a relation with the microbiota. Amplicon sequence variants (ASVs) were determined in stool samples collected at three and twelve months of age. Antibiotic exposed neonates had significantly different abundance of 13 and 17 ASVs at three and twelve months of age respectively. Mainly genera from the phylum *Firmicutes* were decreased at both time points. *Bacteriodetes* were decreased in the microbiota from antibiotic exposed infants. The family *Enterobacteriaceae* were decreased after antibiotic exposure during the second trimester[51].

Table 5. Overview of findings of studies investigating the effect of maternally administered antibiotics on the microbiota of mixed cohorts including preterm and term born infants (subgroup C3)

Author	n	Intervention and indication	Analysis technique	Age of sampling	Outcomes in microbiota samples of neonates from antibiotic exposed mothers			
					Alfa diversity	Phylum level	Family level	Genus level
Esaiassen, 2018[50]	n total = 66 (25 vag, 41 CS) n control = 46 (23 GA <28 weeks, 23 GA 28-31 weeks) n antenatal exposed = 20 (8 GA <28 weeks, 12 GA 28-31 weeks)	Intervention and indication not specified.	Shotgun metagenomic sequencing	Day 7	'We found no significant influence of antenatal antibiotic exposure on the gut microbiota composition on day 7. However, 57/66 (86%) preterm infants also received antibiotic therapy (ampicillin or penicillin + gentamicin) during the first week of life, limiting the possibility to detect isolated effects of antenatal exposure'.			
Penders, 2006[13]	n total = 1.032 (range GA 34 – 42 weeks, 108 CS, 902 vag) n control = 972 n exposed during last month of pregnancy = 38 n missing = 22	Intervention and indication not specified.	Real time qPCR (*Bifidobacteria, E. coli, C. difficile, B. fragilis, lactobacilli* and total bacterial counts)	1 month	'Antibiotic use by the mother during pregnancy had no influence on the infant's gut microbiotic composition'.			
Zou, 2018[49]	n total = 24 n control = 12 (mean GA 32.5, range 30-34, 3 vag) n prenatal exposed = 12 (mean GA 32.7, range 31-35, 3 vag)	Intervention and indication not specified.	16S rRNA gene sequencing of V3 and V4 region	Day 7, 14	No difference	Lower abundance: *Firmicutes* (d7) *Bacteriodetes* (d7, 14) Higher abundance: *Proteobacteria* (d7, 14)	-	Lower abundance: *Bifidobacteria* (d14) *Bacteriodetes* (d14)
Zhang, 2019[51]	n total = 454 (inclusion criteria: > 28 weeks gestation. Number of CS not reported) Only a subset of 68 infants collected stool samples n control = 237 (mean GA 38.74 weeks, microbiota determined of 56 infants) n exposed during pregnancy = 217 (mean GA 38.46 weeks, microbiota determined of 12 infants)	Intervention and indication not specified.	16S rRNA gene sequencing of V4 region	3 and 12 months	Prenatal antibiotic exposure in the second trimester was associated with differential abundance of 13 unique bacterial amplicon sequence variants at age 3 months and 17 amplicon sequence variants at 12 months.			

CS = Caesarean born infants, GA = gestational age, IAP = intrapartum antibiotic prophylaxis, qPCR = quantitative PCR rRNA = ribosomal ribonucleic acid, vag = vaginally born infants

Discussion

An estimated 40% of women are exposed to antibiotics before childbirth[15,52] and approximately 80% of all medications prescribed to pregnant women are antibiotics[20]. In this systematic review we evaluated the influence of prenatal and intrapartum maternal antibiotic use on neonatal microbial gut composition. Intrapartum administration of antibiotics seems to have a profound impact on infant gut colonisation, leading to a decreased diversity, a decreased proportion of the phyla *Actinobacteria* and *Bacteriodetes* with a concurrent increase in *Proteobacteria*. These effects were most evident in term vaginally born infants and persisted up to twelve months.

Recently a review has been published on the effects of IAP on the infant gut microbiome[53]. This study focused solely on the effect of ampicillin administered to GBS positive mothers during delivery of healthy vaginally born infants at term. The current review systematically evaluated the influence on neonatal microbial gut composition of intrapartum and prenatal maternally administered antibiotics for all indications, not just GBS prophylaxis, and we also included preterm and caesarean born infants. Because of the heterogeneity of the indications for antibiotics and the included population, results were categorised and presented in different subgroups, based on route of delivery, gestational age and timing of treatment. Due to this heterogeneity, different outcome measurements and different microbiota detection techniques, it was not possible to pool data and to perform a meta-analysis.

The described microbiota alterations found in vaginally born term infants following IAP were less evident in preterm and caesarean born infants. However, interpretation of the effect of maternally administered antibiotics in these cohorts should be done carefully since most studies within these subgroups were characterised by the presence of multiple confounding factors such as differences in feeding method (formula feeding or breast milk), route of delivery (CS or vaginal delivery), gestational age and postpartum maternal or neonatal antibiotic use. In assessing the effects of maternal use of antibiotics, one must consider the epidemiology of neonatal sepsis since the practice change of widespread antibiotic prophylaxis. Early reports indicated an increase in Gram negative early onset sepsis[12]. More recently, the incidence of Gram negative (*E. coli*) sepsis seemed to have been stable between 2005 and 2014 whereas GBS incidence decreased. However, in very-low-birth-weight infants the odds of mortality of *E. coli* sepsis remain high[54] and a lot of premature infants receive antibiotics after birth.

In studies limited to preterm infants, over 68% of infants received parenteral antibiotics directly postpartum for suspected sepsis. Postnatally administered

antibiotics in infants result in higher proportions of *Proteobacteria* and a decrease in *Actinobacteria*, *Firmicutes* and *Bacteriodetes*[55,56] and decreased diversity[55,56]. In addition, prematurity seems also to result in a higher abundance of *Proteobacteria* and a lower abundance of *Actinobacteria* and *Bacteriodetes*[37] and decreased diversity[57]. Premature infants were often born via CS: the microbiota of caesarean born infants is characterised by decreased proportions of *Actinobacteria* and *Bacteriodetes*[58] and a decreased diversity in the first two years of life[59]. Most hospital guidelines advocate IAP in women delivering via CS, which makes it impossible to investigate the effect of maternal administrated antibiotics in caesarean born infants, as all of them would fall in the 'exposed' group. These observations illustrate that these three variables may obscure the true effects of maternal antenatal antibiotic use, as reported in healthy vaginally born infants. Consequently, the effects of maternal antibiotic use on microbial composition in studies including antibiotic treated infants, preterm infants and caesarean born infants should be interpreted with caution due to possible confounding. We feel that only the study designs and inclusion criteria from studies in subgroup A1 and the RCT from subgroup B1 are sufficiently robust, minimising potential bias, to draw any conclusions on the effect of maternal antibiotic use. The RCT by Kamal et al. was the only study investigating the effect of IAP, taking the effect of route of delivery into account. In 42 infants, these authors compared the microbiota of infants from mothers receiving antibiotics prior to CS compared to infants from mothers receiving antibiotics after clamping of the umbilical cord. No differences were found in taxonomic composition. However, species richness as measured by alpha-diversity was decreased in the antibiotic exposed group after nine months, but not after 10 days. The authors speculated that this difference was caused by bacterial community reorganisation and chance rather than by a direct immediate effect of antibiotics, since one would expect the differences to be more pronounced in the early sample. However, consistent with observations in studies in older subjects, in vaginally born children some studies also reported effects of IAP in samples collected around twelve months that were not seen in early collected samples. These findings illustrate the need for longitudinal studies to assess the true impact of perinatal antibiotic use.

There were some other limitations with respect to the included studies. Some studies retrospectively retrieved data on maternal antibiotic use by a questionnaire which might have caused recall bias since not all mothers will remember whether they have received antibiotics during pregnancy or delivery. Consequently, cases from antibiotic exposed mothers might not have been recognised and might have been analysed in the control group.

Furthermore, not all studies reported which antibiotic had been used and for which indication. Prophylactically administered antibiotics are mainly prescribed to otherwise healthy women, in contrast with therapeutic use of antibiotics. As the microbiota is influenced by many comorbidities, studies of therapeutic use of antibiotics may have included mothers with pre-existing illnesses and associated microbiota alterations, which in turn influence the vertical-transmission to their infants. Since not all studies reported the indication for antibiotic administration, we were unable to investigate different effects of prophylactic versus therapeutic use of antibiotics.

We were also unable to investigate whether the use of specific classes of antibiotics has different effects on the microbial colonisation, for multiple reasons. The majority of studies did not report the indication for antibiotic administration, nor which class of antibiotic was used. In most studies reporting the class of antibiotics, penicillin or ampicillin was administered exclusively. If different classes of antibiotics were used, then only few studies reported effects on the microbiome separately per antibiotic class. The diversity was decreased when stool samples from all infants from antibiotic exposed mothers were analysed and this persisted in infants from mothers exposed to multiple classes of antibiotics (n=12)[38]. In contrast, no significant differences were found in infants from penicillin (n=55) or cephalosporin (n=14) exposed mothers. At species level there was an increase in the abundance of *B. fragilis* following cephalosporin exposure, whereas the opposite was found following ampicillin exposure[38]. This illustrates that different classes of antibiotics may have different effects at diversity and at taxonomic composition of the infant microbiota. Future studies should take effects of different classes into account and move beyond traditional methods towards longitudinal analyses of community-structure[60].

Interestingly, most studies focused on the effect of intrapartum administered antibiotics. Only few articles reported possible effects of antibiotics earlier during pregnancy. Antibiotics administered just before childbirth will likely still be present in the infant bloodstream after birth, where antibiotics administrated earlier in pregnancy will probably already have been eliminated[22]. This might result in a difference in effect on microbiota acquisition.

Antibiotics administrated earlier in pregnancy, will influence the maternal microbial composition in the short- and longer term, which might influence the vertical transmission process and thus the neonatal microbiota[61]. Results from the four studies on the effect of antibiotics earlier in pregnancy are contradictory. However, they were likely influenced by aforementioned confounders, limiting the possibility to draw any firm conclusions.

Term vaginally born infants from mothers receiving antibiotics during labour seemed to have an increased abundance of *Proteobacteria,* which leads to niche-competition with the other species in the healthy intestinal tract such as *Firmicutes*, *Actinobacteria* and *Bacteriodetes*[62,63]. Importantly, *Proteobacteria* consist of several commensal bacteria as well as human pathogens[63]. An uncontrolled overgrowth of *Proteobacteria* reflects gut dysbiosis and is seen in multiple metabolic and inflammatory diseases: whether the expansion of facultative anaerobes, mainly *Proteobacteria*, occurs after, before or in tandem with intestinal inflammation is the subject of intense debate[64,65]. Subsequently, whether the antibiotic induced expansion of *Proteobacteria* in the infant gastrointestinal tract leads to an increased risk of pathology remains as yet unknown[63]. Species belonging to the genus *Bifidobacteria* (and the phyla *Actinobacteria*), which tended to be decreased in infants from antibiotic exposed mothers, are reported to confer positive health benefits[66,67]. *Bifidobacteria* are one of the first colonisers and most abundant genera in infants. In numerous clinical conditions the abundance of *Bifidobacteria* is decreased. Whether this is a cause or consequence of disease is still an ongoing debate[66,67].

Development of a healthy intestinal microbiota during infancy is essential since it plays a major role in the maturation of our immune system[68,69] and the development of a number of clinical conditions[1,70-72]. In the studies investigating the effect of prenatal or intrapartum antibiotics on the infant microbiota, only one study also investigated health related outcomes. In this study, children from mothers exposed to second trimester antibiotics, had an aberrant microbiota and higher WFL scores. Furthermore antibiotics administered during pregnancy and labour have been associated with an elevated risk on atopy, asthma, allergy and obesity[73] later in life and on colitis in murine models[74]. Besides, these antibiotics have been shown to increase the development of antibiotic resistance[11] and an increase in the incidence of Gram negative early onset sepsis[12]. The effects of intrauterine exposure to antibiotics on longer term health remain largely to be elucidated.

Taken together, these observational studies illustrate the need for better understanding of the dynamics of early host-microbiome interactions to mitigate the risk of maternal morbidity and early onset sepsis as well as late onset microbiome-mediated health problems. We are still at the beginning of studying interventions to manipulate early life colonisation such as faecal transplantation[75], vaginal seeding[76], administration of probiotics[77] and diet[78].

In conclusion, maternally administered intrapartum antibiotics seem to significantly alter the infant microbial colonisation process. However, most evidence is of low quality as derived from studies in term vaginally born infants. Whether these effects can be extrapolated to preterm and caesarean born infants remains to be elucidated. Observed dysbiosis, especially in these populations, may be influenced by many confounding factors, including route of delivery, postnatally prescribed antibiotics and feeding practices. Furthermore, studies on effect of antibiotics administrated earlier in pregnancy are limited. Previous studies suggest an association between prenatal antibiotic exposure and clinical conditions such as asthma and obesity, probably due to early microbiota aberrations. However, none of the included studies combined data on antibiotic-induced microbial alterations beyond the age of one year and clinical outcomes. Future research should also focus on whether the antibiotic induced microbial changes have significant short- or long-term health consequences. To improve quality of evidence, these studies should be aware of potential varying effects of different classes of antibiotics, indication for antibiotics (prophylactic versus therapeutic) and take confounding factors into account.

Online supplementary materials:

Online supplementary 1: Search strategy in PubMed and Embase

Online supplementary 2: Table of included studies

Online supplementary 3: Table of risk assessment

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Chapter 3

The influence of timing of Maternal Antibiotic administration during caesarean section on Microbial colonization in Infants (MAMI): a randomized controlled trial

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Gut 2022 Sep; 71(9):1803-1811

Abstract

Objective: Revised guidelines for caesarean section (CS) advise maternal antibiotic administration prior to skin incision instead of after umbilical cord clamping, unintentionally exposing the infant to antibiotics antenatally. We aimed to investigate if timing of intrapartum antibiotics contributes to the impairment of microbiota colonisation in CS born infants.

Design: In this randomised controlled trial, women delivering via CS received antibiotics prior to skin incision (n=20) or after umbilical cord clamping (n=20). A third control group of vaginally delivering women (n=23) was included. Faecal microbiota was determined from all infants at one, seven and 28 days after birth and at three years by 16S rRNA gene sequencing and whole-metagenome shotgun sequencing.

Results: Compared to vaginally born infants, profound differences were found in microbial diversity and composition in both CS groups in the first month of life. A decreased abundance in species belonging to the genera *Bacteroides* and *Bifidobacterium* was found with a concurrent increase in members belonging to the phylum Proteobacteria. These differences could not be observed at three years of age. No statistically significant differences were observed in taxonomic and functional composition of the microbiome between both CS groups at any of the time points.

Conclusion: We confirmed that microbiome colonisation is strongly affected by CS delivery. Our findings suggest that maternal antibiotic administration prior to CS does not result in a second hit on the compromised microbiome. Future, larger studies should confirm that antenatal antibiotic exposure in CS born infants does not aggravate colonisation impairment and impact long-term health.

Introduction

Early life microbiome acquisition and development can be compromised by external perturbations such as delivery via caesarean section (CS), formula feeding and antibiotics.[1] Acute effects of antibiotics on the microbiota range from self-limiting diarrhoea to increased risk for life-threatening conditions in premature neonates.[2,3] The long-term consequences of such perturbations for the human–microbial symbiosis are more difficult to discern, but chronic conditions such as inflammatory bowel disease (IBD), obesity, asthma, allergy and type 1 diabetes have been associated with childhood antibiotic use and an altered intestinal microbiota.[4-6]

Over the last few years, international obstetric guidelines have been revised in order to reduce the incidence of maternal and neonatal infections.[7,8] Because implementation of these adjusted guidelines have resulted in an increased use of antibiotics antenatally,[7,8] concerns on early-life exposure to broad-spectrum antibiotics and associated pervasive effects on the gut microbiome development and various disorders later in life are growing.[9] Besides, early-life antibiotic exposure may increase the risk of multi-resistant bacterial (MRB) infections in neonatal patients.[10] Recent epidemiological and mechanistic data on the association between early antibiotic use, dysbiosis and disease support these concerns.[11] One of the revised protocols leading to an increased exposure to antibiotics worldwide, is the National Institute for Health and Care Excellence (NICE) (2011) guideline for CS.[7] In this revised guideline, it is advised to administer maternal prophylactic antibiotics prior to skin incision, instead of after clamping of the umbilical cord. This policy has been shown to reduce the maternal risk on infectious morbidities, particularly of endometritis and wound infections.[12] Consequently, however, all infants born by CS are currently exposed to broad-spectrum antibiotics via the umbilical cord, when adhering to this revised guideline. Although no increase in incidence of neonatal sepsis was observed,[12] effects on the gut microbiota colonisation and long-term health consequences remain largely unknown. We hypothesized that exposure to antibiotics in children delivered by CS, related to the revised international guidelines, influences the microbial colonisation process and may impact health outcome. In this randomised controlled trial (RCT), we evaluated this effect by comparing the microbiome composition of CS born infants with and without intrauterine antibiotic exposure, according to the revised and previous protocol respectively, up to three years.

Methods

Study design

This RCT was conducted at the obstetrics and paediatrics department of the Amsterdam UMC, location VUmc, a tertiary referral centre. Participants were recruited between March 2015 and November 2017. The study protocol of this study (NTR6000)[13] was approved by the ethics committee VUmc (2014.468). Written informed consent for participation was obtained from all parents. If parents declined participation, mothers received intrapartum antibiotic prophylaxis (IAP) after clamping of the umbilical cord according to the local hospital guideline. The trial is registered with the Dutch Clinical Trial Registry (Trial registration number: NTR6000, https://www.trialregister.nl/trial/5845. The study protocol was published online (doi: 10.1186/s13063-019-3552-8.).[13]

Patient and Public Involvement

Patients and public were not involved in the research question and the study design. Parents of all participants were contacted to evaluate relevant outcome measures and the burden of participation to improve future trials.

Study population

Pregnant women visiting outpatient clinics of the department of obstetrics and gynaecology during the third trimester of an uncomplicated pregnancy and scheduled for a primary CS were eligible to participate. Uncomplicated pregnancy was defined as a normotensive singleton pregnancy, with a normal-weight fetus, delivering at a gestational age ≥ 37 weeks. An overview of all maternal and neonatal exclusion criteria is listed in Table 1. Included women were randomly allocated to be treated according to the current or the previous NICE guideline on timing of prophylactic antibiotic administration during CS. The women treated according to the current NICE guideline[7] received 1500 mg cefuroxime 30 minutes prior to CS (group A). Those women allocated to be treated in accordance with previous NICE guideline,[14] received 1500 mg cefuroxime after clamping of the umbilical cord (group B). Randomisation was done by means of www.randomizer.org in permuted blocks of 10. A third control group of women visiting the outpatient clinic for vaginal delivery was included simultaneously during the study period, in order to compare CS with vaginally born infants (group C). The same eligibility criteria were retained for this group as for the two CS groups. Over time the inclusion rate of the women delivering vaginally was adapted to the primary CS inclusions to facilitate inclusions in the same seasons.

Table 1. Maternal and neonatal exclusion criteria

Maternal exclusion criteria
Delivery < 37 weeks gestation
Aged ≤ 17 years
Hypertensive pregnancy disorder
Multiple pregnancy
Body mass index (BMI) ≥ 25*
Antibiotic use during pregnancy
Antibiotic use during first month postpartum
Immunosuppressive usage within 3 months prior to delivery
Inflammatory bowel disease
Coeliac disease
Rupture of membranes before caesarean section (group A and B)
Prolonged rupture of membranes for >18 hours (group C)
Diabetes Mellitus type I/II
Gestational diabetes requiring insulin
History of major gastro-intestinal surgery
Alcohol or tobacco use in second and third trimester
Drug use during pregnancy
Neonatal exclusion criteria
Small or large for gestational age
Congenital gastro-intestinal anomalies
Gastro-intestinal surgery during first month of life
Antibiotic or immunosuppressive medication use during first month of life

*Was adjusted to BMI ≥ 30 at November 2015. Abbreviations: BMI = body mass index

Blinding

This study was not placebo controlled, since both groups received antibiotics; only the timing of antibiotics differed between groups A and B. The gynaecologist administering the antibiotics during CS was not blinded. However, the investigators analysing the samples and performing the statistics were blinded.

Sample size calculation

Since there is limited literature available on the influence of antibiotics during CS on infantile microbiota colonisation,[15] a formal power analysis could not be performed for this study. We planned 20 inclusions per arm of investigation to enable detection of differences over time in line with the trial by Nogacka et al.[16]

Sample and data collection

Faecal sample collection

The first stool sample (meconium) was collected in a sterile container (Stuhlgefäß 10 mL, Frickenhausen, Germany) by nurse or midwife, and immediately stored at -20°C. When discharged, parents were asked to collect faecal samples at home from their infants in provided containers at seven and 28 days after birth. These samples were stored at home in a regular freezer and subsequently transported in cooled condition to the hospital on the day of the regular postpartum check-up 6 weeks after the delivery. At arrival in the hospital, the samples were collected by the investigator and stored at -20°C until further handling. At the age of three years, parents collected a fourth faecal sample at home and stored them in a regular freezer. After collection, the faecal samples were transported in frozen condition to the hospital. At arrival in the hospital the samples were stored at -20°C until further handling.

Umbilical cord blood collection

To determine to what extent neonates were exposed to cefuroxime administered to the mother, umbilical cord blood was collected from infants of group A directly after clamping of the umbilical cord and delivery of the placenta. Blood samples were collected in an Ethylene-Diamine-Tetra-Acetic acid (EDTA) tube and directly transported to the laboratory. Samples were centrifuged and plasma was stored at -80 °C until the concentration of cefuroxime was determined.

Data on health status

Parents of all included infants were instructed to complete a questionnaire (Online Supplemental Methods) at the age of three years. The questionnaire was slightly adjusted from a previously used questionnaire[17] and included items on feeding practices, anthropometric measurements, medication and health related problems like allergy, respiratory and gastro-intestinal symptoms.

Sample handling

DNA extraction

DNA from faecal samples of days one, seven and 28 was extracted using the NucliSENS® easyMag® (bioMérieux, Marcy l'Etoile, France). NucliSENS® lysis buffer (1 ml), containing guanidine thiocyanate, was added to a vial containing 150 µg of faeces. The vial was shaken at 1,400 rpm (Thermomixer comfort, Eppendorf, Hamburg, Germany) for 5 min and consequently centrifuged for 4 min at 12,000g. The vials were added to the easyMag container and DNA extraction was performed on the easyMag machine with the Specific A protocol as described by the manufacturer. Elution of DNA was performed using 110 µl of buffer. Extracted DNA was stored at 4 °C until further handling.

Due to merging of the laboratory and change in protocols, the follow-up samples collected at the age of three were analysed in a different laboratory compared to the neonatal samples, because of logistic reasons. The DNA was extracted using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). The DNA was extracted with minor adjustments of the manufacturer's protocol: for disruption of the samples, the TissueLyser II (Qiagen, Hilden, Germany) was used for two minutes at 30 Hz. To increase the DNA concentration, 50 µl of buffer solution was used instead of 100 µl for the elution. Extracted DNA was normalised to 5 ng/µl and stored at 4˚C until use for polymerase chain reaction (PCR) amplification.

16S rRNA gene sequencing

All faecal samples were analysed using 16S rRNA gene sequencing to characterise the taxonomic composition. V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified from the DNA extracted from faecal samples collected during the first month of life using universal primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21.[18] Sequencing was performed on an Illumina MiSeq instrument (Illumina, San Diego, USA) using the2 x 300 bp paired-end sequencing protocol by LifeSequencing S.L. (Valencia, Spain). The read pairs were demultiplexed and trimmed (q>20) before being merged using QIIME.[19] Merged reads with q>25 over a window of 15 bases, no ambiguous bases and a minimal length of 300 were retained. These were dereplicated and counted using mothur[20] and reads with a low abundance (less than 2 reads over all samples) were discarded. Chimeras were removed using VSEARCH,[21] using the RDP gold database[22] as reference. Reads which contained PhiX or adapters as defined in Deblur (part of QIIME2)[23,24] were eliminated. Taxonomic assignment was performed using the RDP classifier[25] against the SILVA_119[26] database, from which results where the sequences were aggregated at genus and at phylum level were further explored. Reads with eukaryotic assignments, as well as reads with a low relative abundance up to 0.0005% in all samples were excluded from further downstream analysis. Samples were rarefied, and α-diversity was calculated using the phyloseq[27] and vegan[28] packages in R.[29] On average 30921.4 sequences were generates (ranging from 14216 to 91901 sequences; Online Supplemental Table 1).

For the follow up faecal samples collected at the age of three, 16S rRNA gene amplification and sequencing was done using the Earth Microbiome Project Protocol.[30,31] The V4 region of the 16S rRNA gene was amplified with a custom made 515F forward primer (Sigma-Aldrich, Saint Louis, USA) and 806R reverse primer (Sigma-Aldrich, Saint Louis, USA) by using a one-step, single-indexed PCR approach. The library was paired-end sequenced (2x250bp) on an Illumina MiSeq platform by the department of Cancer and Genomic Sciences at the University of

Birmingham (Birmingham, United Kingdom). On average 59601.5 sequences were generates (ranging from 22986 to 95091 sequences; Online Supplemental Table 1).

Whole metagenome shotgun sequencing

Extracted DNA from samples of days seven and 28 was used for WMS sequencing to further distinguish possible differences in more detail at these time points. These time-points were chosen since the effect of the perinatal antibiotics was expected to be most pronounced with limited influence of confounding environmental factors in these samples. In contrast to meconium, at day seven the amount of human DNA will be decreased with a concurrent increase in DNA of the limited pioneer bacterial species present in the early microbiome.[32] At day 28, the diversity will be increased due to an increased prevalence of *Veillonella*, *Streptococcus*, *Bifidobacterium* and Enterobacteriaceae.[33] Consequently, associations between perinatal factors and taxonomic composition are likely to be more pronounced after one month compared to early samples from the first week of life.[32] DNA from samples collected at the age of three were not sequenced with WMS, since the microbiome has reached a more stable form[34] and differences due to perinatal influences were expected to have disappeared by then.

Approximately 1-5 ng of extracted DNA was used as input for the Illumina Nextera XT DNA Library Prep kit and barcoded using Nextera XT Indices, as per the manufacturer's instructions (Illumina, San Diego, USA). Isolated DNA was "tagmented" (enzymatically "sheared" and tagged with adaptors), single cycle PCR amplified to add barcodes, purified and normalized using Illumina beads. Final libraries were quantified using the Invitrogen Quant-iT dsDNA (high sensitivity) assay (Thermo Fisher Scientific) using a microplate reader, equal amounts of each library were pooled and then sequenced at the Integrated Microbiome Resource (IMR; Dalhousie University, Halifax NS) using 2x150 bp PE reads on an Illumina NextSeq 550 using the High Output v2.0 chemistry. On average 9274349.4 sequences were generated per sample (ranging from 1076734 to 19473464 sequences; Online Supplemental Table 1). Sequence reads were subjected to the MG-RAST pipeline (version 4.0.3) with default settings.[35,36] Sequence reads were taxonomically classified by a sBLAT similarity search against the M5rna database which integrates the SILVA,[26] Greengenes,[37] and RDP[38] databases. Functional classification of the predicted proteins was performed by a sBLAT similarity search against the M5nr database,[39] which provides nonredundant integration of many databases: GenBank, SEED, IMG, UniProt, KEGG, and eggNOGs.

Antimicrobial resistance genes within the WMS data set were predicted with the deep-learning approach, DeepARG.[40] Translated fasta sequence files (all possible

open-reading frames), were used as input for DeepARG. All potential antimicrobial resistance genes were identified using the Comprehensive Antibiotic Resistance Database (CARD)[41] with DeepARG.

Umbilical cord blood

Cefuroxime plasma concentrations (mg/L) were determined using a validated high performance liquid chromatography – ultraviolet detection analysis at the department of Clinical Pharmacy and Pharmacology, University Medical Centre Groningen, The Netherlands. Validation was carried out according to EMA guidelines. The lower limit of quantitation was 0.4 mg/L and upper limit of quantitation was 100 mg/L. Variation coefficient was less than 4% over the entire working range.

Statistical analysis

Demographic data

Demographic data was given descriptively. For health outcome variables at the age of three, comparisons of continuous variables between the three study groups was done using a one-way ANOVA for normal distributed variables and Kruskal Wallis test for non-normal distributed variables. The χ^2 test was used to compare dichotomous outcome variables. Differences were considered significant if the two-sided p value was <0.05.

Statistical analysis 16S rRNA gene sequencing data

At each time-point, differential abundance analysis of the detected taxa was performed with the Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC) (v.1.2.0)[42] in R (v 4.1.0) with phyloseq (v 1.36). ANCOM-BC uses a linear regression framework in order to estimate the unknown compositional as well as sampling fractions from the sequence count data. Both the differences between all CS and vaginally born infants, as well as the differences between the two CS groups A and B were evaluated. The resulting large sets of p-values were corrected for multiple testing by assessing the positive false discovery rate (pFDR)[43] hence all reported p-values are adjusted p-values. The R-package ggplot2 (3.3.5) was used for visualisation.

Within-sample diversity was calculated using the Shannon diversity index on the genus level data for each group at each time point. Between-sample diversity was calculated based on Bray-Curtis distances on the genus level data, and the dissimilarity matrix was then used for the calculation of principal coordinate analysis (PCoA). The PCoA procedure was performed using Canoco 5 software for multivariate data exploration.[44]

Statistical analysis whole metagenome shotgun sequencing data

Differential abundance analysis on the data sets resulting from the WMS sequencing was performed as described above with ANCOM-BC as well. Furthermore, at each time point the same approach was followed for the functionally annotated data sets.

All potential antimicrobial resistance gene (identitifed by DeepARG) counts were subjected to a Wilcoxon Rank Sum test to calculate the p-value between the two CS groups A and B at day 7 and at day 28.

Results

Patient population

During the inclusion period 572 women delivered via a primary CS. After screening and randomisation, 20 women delivering via CS receiving antibiotic prophylaxis prior to skin incision (group A: antenatally antibiotic exposed infants) and 20 after clamping of the umbilical cord (group B: antenatally antibiotic unexposed infants) were included. A total of 23 women delivering vaginally were found eligible to participate in the vaginal control group (group C). The flow of patient selection and reasons for exclusion is given in Figure 1. Demographic and clinical characteristics of included mothers and infants are shown in Table 2. None of the variables differed significantly between the study groups.

Microbiome analysis

16S rRNA based microbiome composition: vaginally versus CS born infants

Compared to vaginally born infants, both CS groups had a significant lower Shannon diversity at day 28 ($p<0.001$) (Figure 2 A). Figure 2 B and C show that the 16S rRNA gene derived taxonomic composition of samples collected from vaginally born infants cluster to the exclusion of samples collected from CS born infants at day seven and 28. In both CS groups, inter-individual differences were apparent and seemed to prevail over potential antibiotic administration induced differences. In the beta diversity plots, principal coordinates from samples of the vaginal group also clustered together at day 28, while samples of both CS groups did not (Supplemental Figure 1A) . After three years, differences in Shannon diversity and the principal coordinate analysis had disappeared (Supplemental Figure 1B and C).

No differences in the microbiota were found on phylum level between vaginally and CS born infants on day one and seven. Compared to vaginally born infants, the microbiota of CS born infants harboured a decreased abundance of Bacteroides ($P<0.001$) on day 28 with a concurrent increase in Firmicutes ($P=0.001$) (Supplemental Figure 2 and Online Supplemental dataset 1a-c). At genus level, numerous differences were found including a decrease in *Bacteroides* with a concurrent increase in *Enterococcus* (Online Supplemental datasets 1d-f). At three years of age, no differences between vaginally and CS born infants were present at phylum nor genus level.

Table 2. Mother and infant baseline characteristics.

Characteristics	Group A (n=20)	Group B (n=20)	Group C (n=23)	P value
Maternal age at birth, median [IQR], years	36.6 [33.4-39.3]	36.0 ([31.7-39.0]	32.3 [30.8-35.9]	0.550
BMI, median [IQR], kg/m^2	22.8 [19.8-24.3]	23.8 [21.2-25.0]	21.9 [20.8-23.3]	0.594
Gravida, median [IQR]	3 [2-4]	3 [2-4]	2 [1-3]	0.620
Para, median [IQR]	1 [1-1]	1 [0-2]	1 [0-1]	0.779
Maternal diet at birth				
Vegetarian	1 (5)	1 (5)	3 (13)	0.970
Non-vegetarian	18 (90)	19 (95)	20 (87)	
Missing	1 (5.0)	0 (0)	0 (0)	
First or repeat caesarean section				
First	5 (25)	9 (45)	NA	0.185
Repeat	15 (75)	11 (55)	NA	
Gestational age, median [IQR], weeks + days	39+0 [37+6 - 39+6]	39+0 [38+5 - 39+2]	39+6 [38+4 - 40+3]	0.383
Birth weight, gram	3518 (380)	3442 (593)	3385 (484)	0.634
Sex				
Female	12 (60)	7 (35)	14 (61)	0.113
Male	8 (40)	13 (65)	9 (39)	
P-value birthweight				
p <10	0 (0)	3 (15)	0 (0)	0.341
p 10-p50	8 (40)	6 (30)	11 (48)	
p 51-p89	9 (45)	8 (40)	10 (44)	
p >90	3 (15)	3 (15)	2 (9)	
Apgar score, median [IQR]				
1 minute	9 [9-9]	9 [9-9]	9 [8-9]	0.947
5 minutes	10 [10-10]	10 [10-10]	10 [9-10]	0.862
Meconium stained amniotic fluid	0 (0)	1 (5)	3 (13)	0.311
Feeding type				
Breastfed	10 (50)	10 (50)	15 (65)	0.403
Formula fed	6 (30)	3 (15)	4 (17)	
Combination	4 (20)	7 (35)	4 (17)	

Women delivering via caesarean section received antibiotics prior to skin incision (group A) or after clamping of the umbilical cord (group B). Comparison between both caesarean groups was done using the χ^2 test or Fisher's exact test for dichotomous variables and Student's t-test or Mann-Whitney U for normally and non-normally distributed continuous data. Vaginally delivering women (group C) were included as a controls and were not exposed to antibiotics.

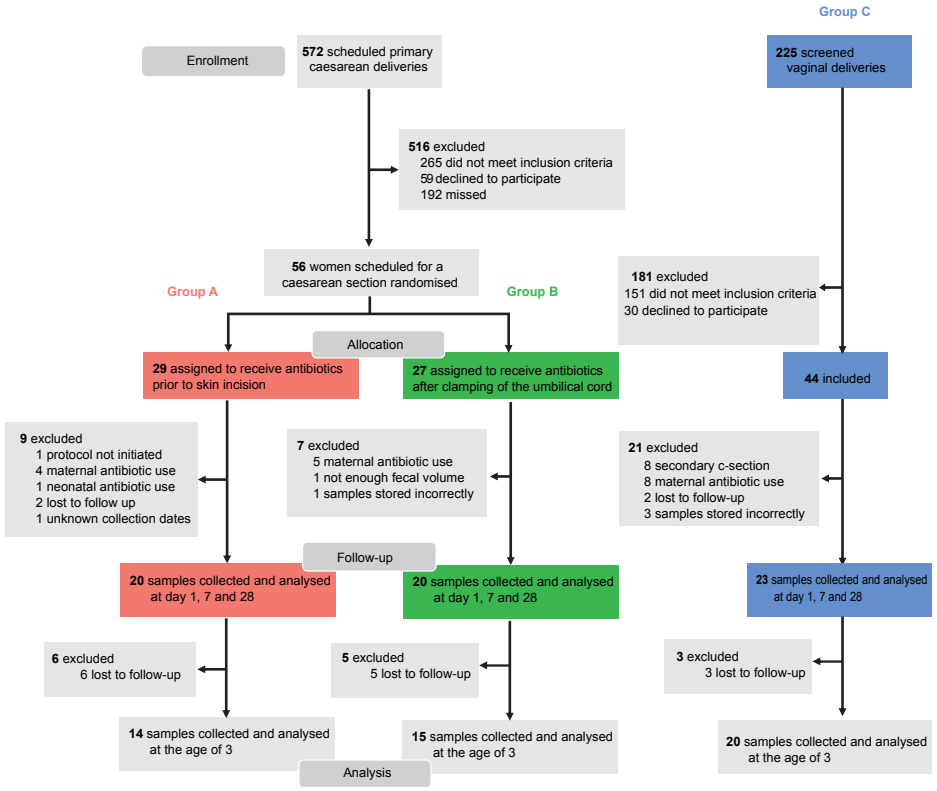


Figure 1. Consort Diagram

Whole metagenome based microbiome composition: vaginally versus CS born infants

At phylum level, a decrease in the abundance of Bacteroidetes and an increase of Lactobacillus was found in CS born infants at day seven. At day 28, also a decreased abundance in Bacteroidetes was present in CS born infants (Supplemental Figure 3 and Online Supplemental dataset 2a and 2b).

At genus level, the microbiota of CS born infants harboured a decreased abundance of *Bacteroides*, *Prevotella* and *Akkermansia* compared to vaginally born infants at day 7. Furthermore, significant differences were found in the abundance of 13 other genera at day 7 (Online Supplemental dataset 2c). Also at day 28, the abundance of the genera *Bacteroides*, *Prevotella* and *Akkermansia* was decreased in CS born infants. The abundance of *Klebsiella*, *Pseudomonas*, *Enterococcus*, *Clostridium* and *Enterobacter* were significantly increased in CS born infants, along with changes in the abundance of 57 other genera at day 28 (Online Supplemental dataset 2d). On species level, there were significant differences in 118 and 188 species at day 7 and 28 respectively. These species did mainly belong to the previously mentioned genera and to members of the genus *Bifidobacterium* (dataset 2e and 2f).

The abundance of numerous function genes did significantly differ between vaginally and CS born infants day 7 (133 genes) and day 27 (663 genes). An overview of these genes are depicted in Online Supplemental dataset 2g an 2h.

16S rRNA based microbiome composition: CS groups

No differences in Shannon diversity indices were found at all four time points between the two CS groups (Figure 2 A). Beta diversity plots also showed no differences (Supplemental Figure 1). A heatmap of samples collected at day seven and 28 showed that the vaginal group clustered together, but did not demonstrate clear difference between the two CS groups (Figure 2 B and C). No differences were found in taxonomic composition between group A and B at all four time points on phylum level (Supplemental Figure 2) nor on genus and species level. An overview of the phyla and genera compared between the two CS groups based on the 16S sequenced data along with adjusted p-values are demonstrated in Online Supplemental Datasets 3a – 3f. Furthermore, no differences in (potential) antimicrobial resistance genes were found during the first month of life (supplemental Figure 4).

Whole metagenome based microbiome composition: CS groups

At phylum level, no differences were found between the two CS groups at day seven nor at day 28 (Supplemental Figure 4 and Online Supplemental dataset 4a and 4b). Also at genus and species level, no significant differences were found between antenatally antibiotic exposed and unexposed CS born infants (Online Supplemental datasets 4c-4f).

Analyses of subsystems (sets of functional roles that together implement a specific biological process or structural complex)[45] did not reveal any differences between both CS protocols. At day seven (Online Supplemental dataset 4g) and 28 (Online Supplemental dataset 4h) the abundance of none of the analysed functions did significantly differ between both CS groups

Functionality was further investigated specifically for the (potential) antimicrobial resistance genes but these were not significantly different between the CS groups at day 7 ($p = 0.88$), nor at day 28 ($p = 0.20$) (Supplemental Figure 4).

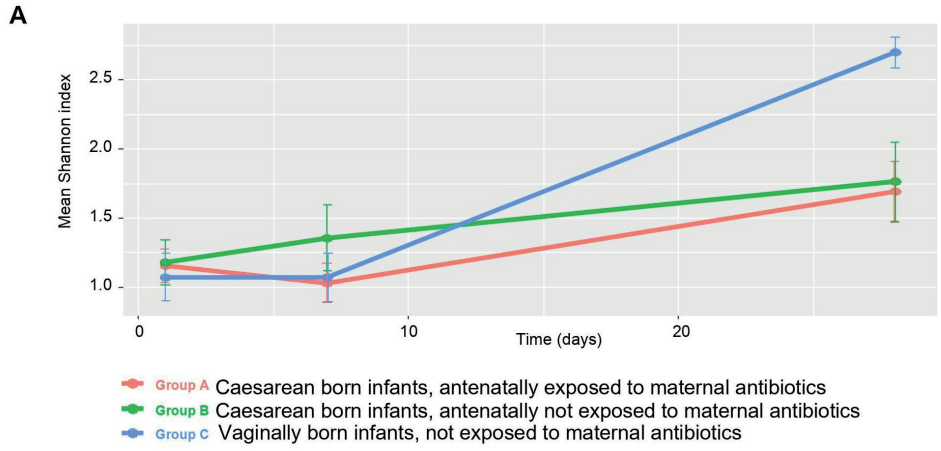
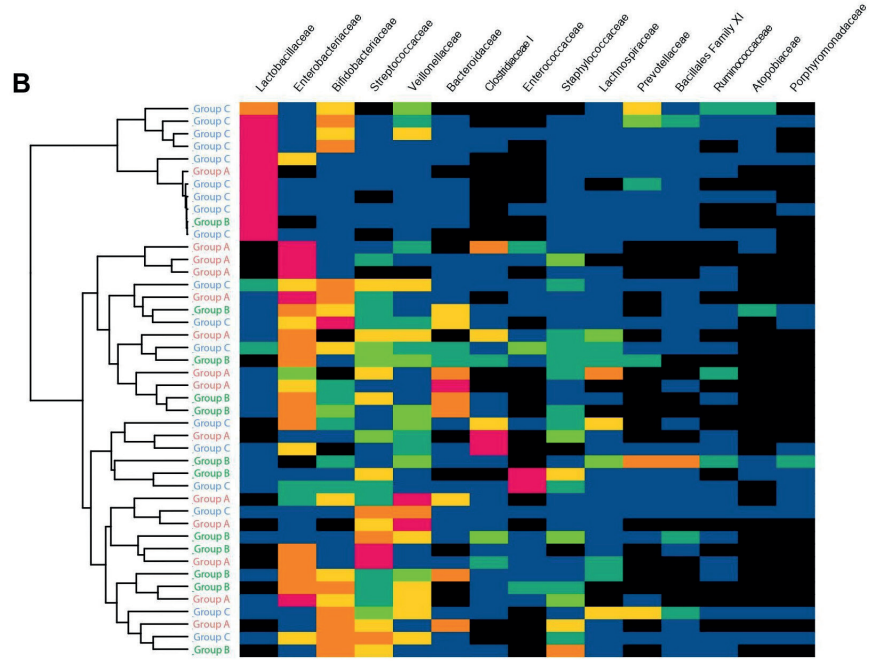
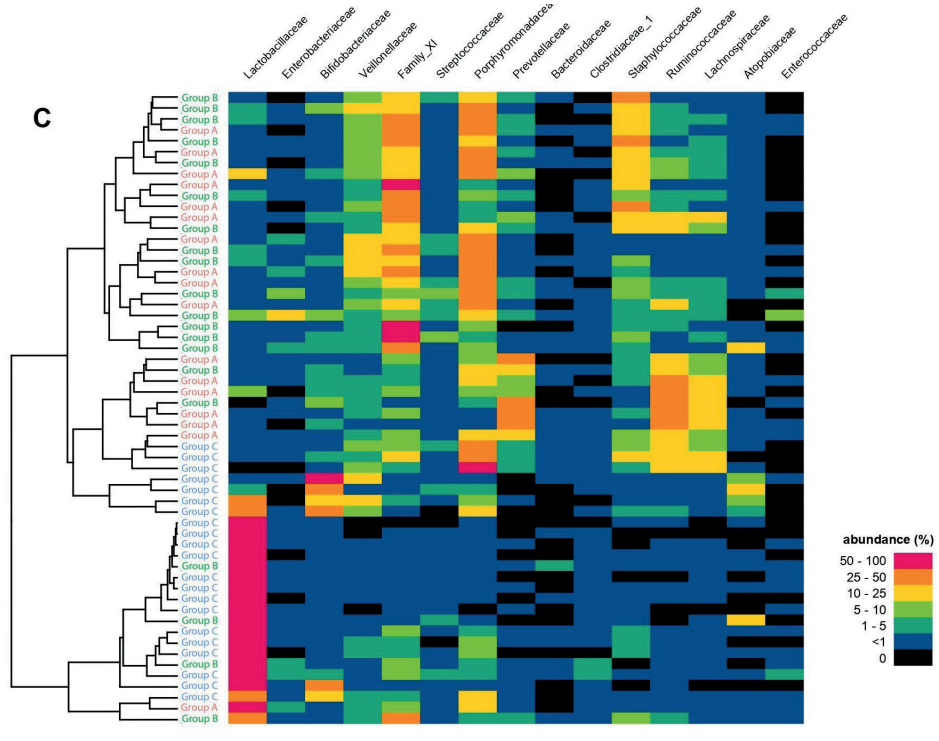


Figure 2. Mean Shannon diversity indices and taxonomic composition of the microbiota

2A) Mean Shannon diversity indices calculated from the taxonomic assignments (genus level) of the 16S rRNA gene sequence analyses of faecal samples collected at one, seven and 28 days postpartum from infants of mothers delivering via cesarean section who received prophylactic antibiotics either before skin incision (group A: antenatally antibiotic exposed infants) or after cord clamp (group B: antenatally antibiotic unexposed infants). Faecal samples were also collected from a third group of vaginally born infants (group C). Samples were analysed by 16S rRNA gene sequencing. At day 1 and 7 no significant difference was present between infants from all three groups. At day seven mean Shannon diversity was 1.03 in group A and 1.36 in group B (p=0.23). At day 28 Shannon diversity index of vaginally born infants was significantly higher compared to both cesarean groups (p<0.001).

2B and C) Left side dendrogram shows results of unsupervised cluster analysis of the taxonomic assignments (genus level) based on Bray-Curtis dissimilarity. Samples collected from vaginally born infant (group C) cluster to the exclusion of samples collected from caesarean section born infants (group A and B). Right side; taxonomic composition of the microbiota demonstrated in a heat map of individual samples collected at day 7 (B) and day 28 (C) depicting the relative abundance (%) of the 15 most abundant bacterial families.





Cefuroxime cord blood levels

In 17 of 20 included infants of group A umbilical cord blood was analysed to determine cefuroxime levels. Two samples were excluded since two mothers received prophylactic clindamycin because of a suspected cefuroxime allergy and in one case the blood sample was collected incorrectly. The median cefuroxime level of the analysed samples was 13.7 mg/L (interquartile range 11.2-17.8 mg/L), which is above the minimal inhibitory concentration (MIC) of most bacterial species.[46]

Questionnaire 3 years after birth

No differences were observed in the health status at the age of three years between the three groups (Online Supplemental Table 2).

Discussion

In this RCT, the effect of timing of maternal prophylactic antibiotic administration during CS on the microbiome and health state of infants up to three years of age was evaluated. Moreover, the findings were compared with a control group of vaginally born infants during the same time period. This study confirmed previous findings that CS delivery in general leads to a profound hit on the initial microbial colonisation. Our data suggest that maternal antibiotic administration prior to CS does not lead to a 'second hit' on the already compromised microbiome in CS born infants.

The rate of infants born by CS continues to increase worldwide. Currently, reported rates vary from around a quarter to more than half of all infants.[47] In this study, besides a decreased diversity, the abundance of numerous phyla, genera and species was significantly affected by a CS delivery. The main aberrations found in the microbiota of CS born infants included a decreased abundance of members belonging to genera *Bacteroides* and *Bifidobacterium* and an increased abundance of Proteobacteria and enterococci during the first month of life. These findings are largely in line with previous studies demonstrating a decreased abundance of *Bacteroidetes*[48], decreased diversity[49] and an increase in opportunistic pathogens, mainly including enterococci, in CS born infants.[48,49] Knowledge about the development and impact of perinatal factors on species level is still limited. We confirmed findings by Saturio et al. (2021) that CS born infants have a decreased abundance of *B. bifidum* and *B. catenalutum*, but we did not find evidence of increased abundance in other *Bifidobacterium* species such as *B. adolescentis* and *B. animalis*.[50] The abundance of numerous species, mainly of the mentioned genera and phyla, was significantly affected by the route of delivery.

Alterations in microbiota colonisation have previously been associated with disturbed development of the immune system and long-term complications.[4-6] Bifidobacteria and *Bacteroides*, for example, are considered to confer positive health benefits in general on the host.[51,52] B*ifidobacteria* produce acetate and lactate which act as a barrier against enteropathogenic infections. Delayed colonisation with bifidobacteria has been associated with a decreased number of memory B-cells later in infancy and with immune dysregulations,[53] and an consequently with increased risk for multiple non-communicable diseases later in life.[51] *Bacteroides* also influence immune development, and depletion of this genus in infancy could negatively impact T-cell response. Proteobacteria comprise multiple known human pathogens. An increase in the abundance of Proteobacteria is seen in numerous clinical conditions. Furthermore, a microbiota depleted of *Bacteroides* with increased abundance of Proteobacteria during infancy has been associated with long-term complications including impaired neurocognitive development.[54]

Besides CS itself, it has been shown that postnatal antibiotics impact the abundance keystone microbial taxa.[55] Antibiotic exposure early in life decreases the diversity, the abundance of *Bacteroides* and *Bifidobacterium* species and increases the abundance of Enterobacteriaceae.[56] Currently, it is unknown whether the effects of maternal IAP resemble effects of postnatal antibiotics on the microbiota in CS born infants, and further increases the risk for microbiota-related long-term health complications. In vaginally born infants, maternal IAP has been shown to decrease the diversity and abundance of *Bacteroidetes* and bifidobacteria and to increase the abundance of Proteobacteria,[15,16] which might increase the risk for negative long-term health outcomes.[4-6] It might be counter-intuitive to assume negative effects of maternal IAP are only present in vaginally born infants and not in CS born infants. This is the first RCT evaluating effects on infant microbiota colonisation of exposure to maternal IAP during CS in a randomised design using metagenomics. Despite the high concentrations of cefuroxime measured in the umbilical cord and the fact that numerous species of the human gut microbiota are susceptible to cefuroxime,[57,58] we showed that intrauterine exposure to antibiotics does not result in a 'second hit' on the already compromised microbiome in CS born infants.

Only one previous RCT investigated the effect of timing of antibiotic administration during CS on the infant microbiota using 16S rRNA gene sequencing.[59] In that study, the effect on the infant microbiota was measured after ten days and nine months. In line with our findings, no differences were demonstrated in the taxonomic composition at ten days postnatally, but a significantly decreased microbial species richness was found in intrauterine antibiotic exposed infants after nine months. Besides 16S rRNA sequencing, we analysed samples using WMS. Both methods

are substantially different and can yield quantitatively and qualitatively different results.[60-64] The advantage of WMS is that it provides direct information about the presence or absence of specific microbial functions such as antibiotic resistance.[60-64] Since it has been demonstrated that perinatal factors could influence the abundance of different species of the same genera in opposite directions (e.g. an increase in *B. bifidum* and a simultaneous decrease in *B. adolescentis* following CS), the importance of analysis on species level, possible with WMS, is emphasized.[50] Previous studies showed only a weak correlation between amplicon sequenced data and WMS sequencing data and this may explain why we observed differences in results between both methods. Discrepancies between the 16S and WMS datasets might further be explained by PCR primer bias.[65] Since both methods have their own advantages and are therefore considered as complementary, it is considered useful to analyse samples parallel with both techniques.[60-64]

Besides combined 16S rRNA amplicon sequencing and WMS sequencing, which allows taxonomic analysis up to species level and analysis of functional genes and antibiotic resistance genes, other strengths of this study include the randomised controlled study design for the CS group and inclusion of the vaginally born group. Application of strict in- and exclusion criteria limited the risk of bias and long follow up period provides insight on long-term microbiota development. The cefuroxime cord blood concentrations in exposed neonates provided valuable information on the degree of antibiotic exposure. Despite the short exposure period of 30 minutes, a median concentration of 13,7 mg/L could be found in the umbilical cord, which is above the MIC of most bacterial species.[46,57,58] Limitations of our trial include the relatively small sample size, hampering to draw firm conclusions regarding long-term health outcomes.

A reduction of maternal infectious morbidities was the reason for revising the recommendation regarding the timing of IAP in the NICE guidelines.[7,12] Women receiving antibiotics prior to CS are affected in 3.9%, predominantly by endometritis and wound infections, compared to 6.9% of women receiving antibiotics after cord clamp (risk ratio: 0.57 and number needed to treat: 33.3).[12] Importantly, effects on neonatal gut microbial colonisation and long-term effects associated with antibiotic exposure have not been investigated before implementation of these adjusted guidelines. Notably, the majority of eligible parents preferred to be treated according to the previous NICE guidelines, considering the uncertain risk of antibiotic exposure more important than the proven protective effects on risk of maternal infection. Here, we have for the first time shown that adhering to the current NICE guidelines does not seem to significantly impact the infant faecal microbiome up to three years of age. Future studies should confirm the hypothesis that antenatal antibiotic exposure in CS indeed does not influence long-term health outcomes, like asthma, allergy and obesity.[5] These studies could further reduce the uncertainty and doubts of parents and clinicians whether the beneficial protective effects for mother by the guideline adjustment do not lead to negative long-term consequences for the child and justify the guideline adjustment.

Conclusions

We confirmed that early-life microbiome development is strongly affected by mode of delivery. In this RCT, we observed that maternal antibiotic administration before onset of the CS according to the current guidelines, does not seem to further impact the compromised microbiota development in CS born infants. Disturbances in microbial colonisation have previously been associated with a disturbed priming of the immune system, even when these microbial disturbances are restored later in life. Since around 30 million infants are born via CS yearly[66], it is important that prospective studies, including a larger number of inclusions validate our observation that antenatally antibiotic exposure in CS born infants does not seem to impact long-term health outcome.

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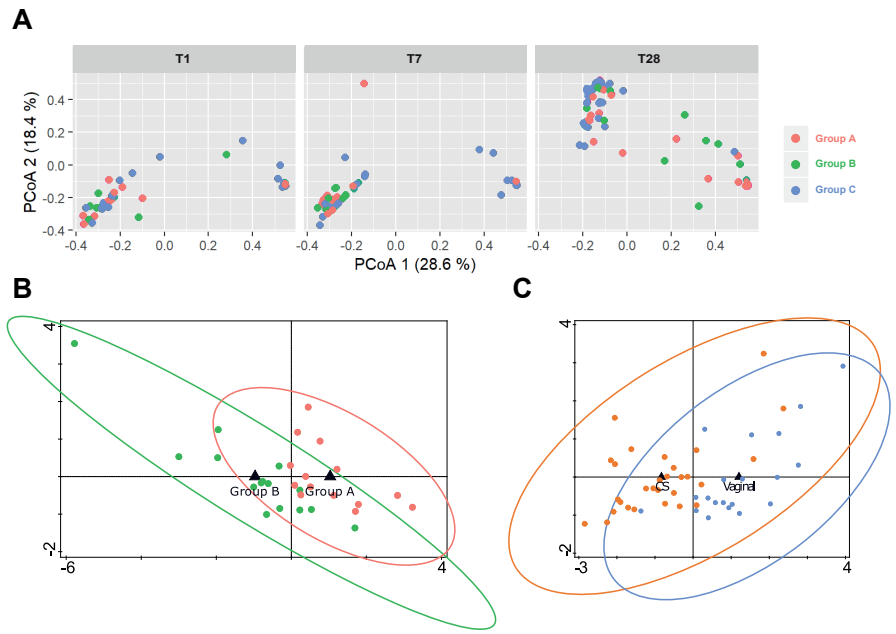
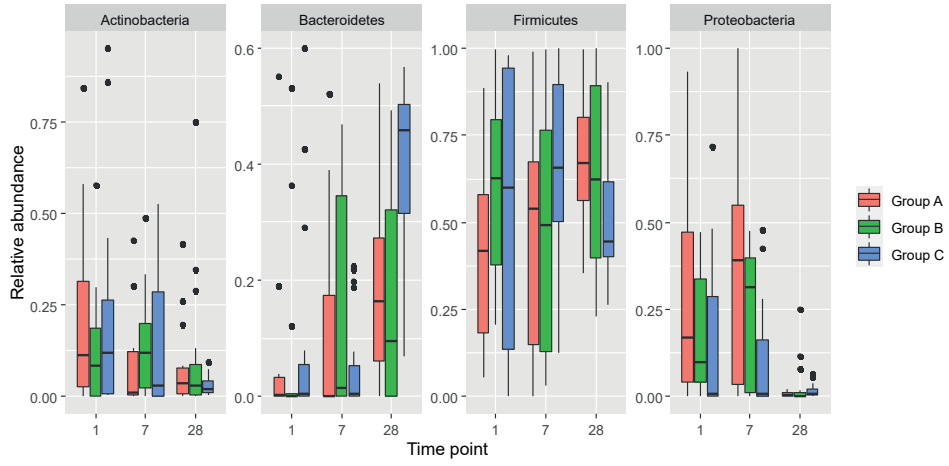
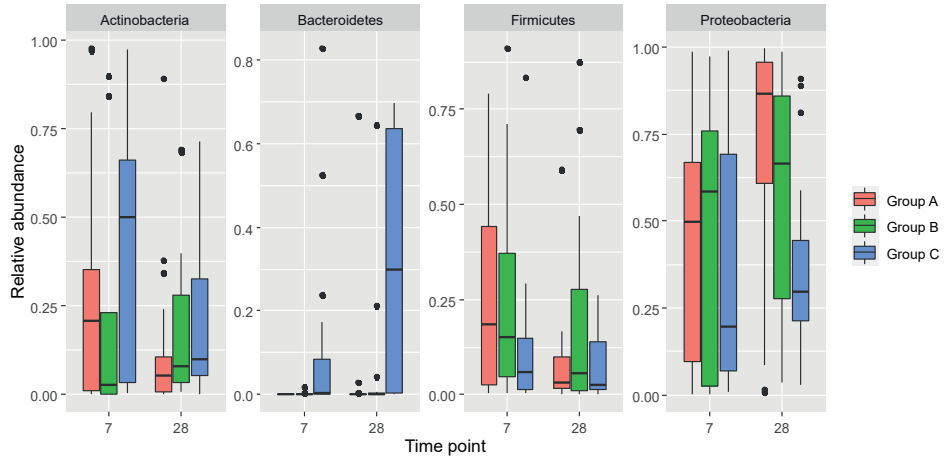
Supplementary files



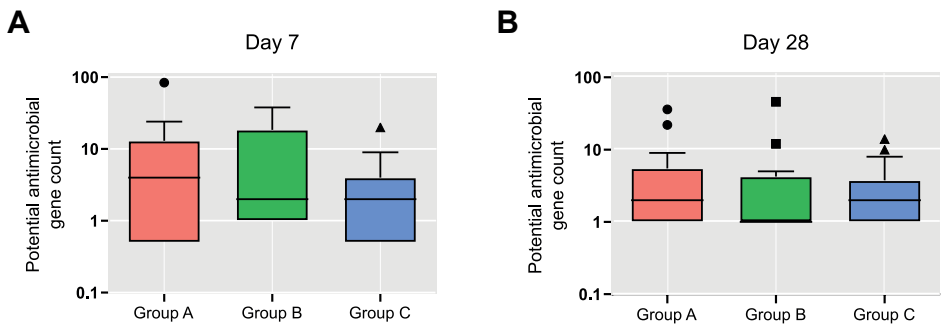
Supplemental Figure 1: Principal coordinates analysis (PCoA) on genus level of faecal samples collected at day one, seven and 28 and three years postpartum analysed by 16S rRNA gene sequencing. No clear difference were present between caesarean born infants whose mother received prophylactic antibiotics either before skin incision (group A: antenatally antibiotic exposed infants) or after umbilical cord clamping (group B: antenatally antibiotic unexposed infants) at day one, seven and 28. At day 28 samples from the vaginal control group (group C) clustered together, whereas both caesarean groups did not (1A). No differences were found between both caesarean groups (1B) nor between caesarean born infants and vaginally born infants (1C) at three years of age.



Supplemental Figure 2: Relative abundance of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria in faecal samples obtained at one, seven and 28 days analysed by 16S rRNA gene sequencing. No differences were found between caesarean born infants whose mother received prophylactic antibiotics either before skin incision (group A: antenatally antibiotic exposed infants) or after umbilical cord clamping (group B: antenatally antibiotic unexposed infants). The microbiota of vaginally born infants (group C) harboured a decreased abundance of Firmicutes on day 28 (p=0.001). In vaginally born infants a higher abundance of Bacteroidetes was observed at day 28 compared to caesarean born infants (p<0.001).



Supplemental Figure 3: Relative abundance of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria in faecal samples obtained at seven and 28 days analysed by whole metagenome shotgun sequencing. No differences were observed between caesarean born infants whose mother received prophylactic antibiotics either before skin incision (group A: antenatally antibiotic exposed infants) or after umbilical cord clamping (group B: antenatally antibiotic unexposed infants). The microbiota of vaginally born infants (group C) consisted of a higher abundance of Bacteroidetes at day seven and 28 (p=0,008 and p<0.001 respectively).



Supplemental Figure 4: Abundance of potential antimicrobial resistance genes. No significant differences were found in the potentential antimicrobial resistance genes between caesarean born infants ant vaginally born infants, nor between caesarean born infants whose mother received prophylactic antibiotics either before skin incision (group A: antenatally antibiotic exposed infants) or after umbilical cord clamping (group B: antenatally antibiotic unexposed infants).

Chapter 4

Association between duration of early empiric antibiotics and necrotizing enterocolitis and late-onset sepsis in preterm infants: a multicenter cohort study

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European Journal of Pediatrics 2022 Oct; 181(10): 3715-3724

Abstract

Purpose: The threshold for empiric antibiotics for suspicion of early-onset sepsis (EOS) is low in preterm infants. Antibiotics' effects on short term outcomes have recently been debated. We aimed at exploring the extent of early empiric antibiotic exposure (EEAE) in preterm infants and the association between the duration of EEAE with necrotizing enterocolitis (NEC) and late-onset sepsis (LOS) within different EEAE groups.

Methods: EEAE practice for suspicion of EOS was evaluated in all included infants (gestational age <30 weeks) born in 9 centers in the Netherlands and Belgium between Oct.2014-Jan.2019. EEAE association with NEC and LOS development was analyzed by multivariate regression.

Results: After excluding 56 EOS cases, 1259 infants were included. A total of 1122 infants (89.1%) were exposed to empirical antibiotics for the suspicion of EOS of whom 802(63.7%) had short (≤72h) and 320(25.4%) prolonged EEAE (>72h). Infants with EEAE ≤72h had a lower incidence of NEC compared to both infants without EEAE (adjusted odds ratio (aOR) 0.39; 95% confidence interval (CI) [0.19–0.80]; *p*=0.01) and with prolonged EEAE (>72h) (aOR[95%CI]: 0.58[0.35–0.96]; p=0.03). With every additional day of EEAE, LOS incidence decreased (aOR[95%CI]: 0.90[0.85-0.97]; p=0.003).

Conclusions: Almost 90% of preterm infants who have negative blood culture results in the first 72h of life, are exposed to EEAE under suspicion of EOS. One fourth has prolonged EEAE. Duration of EEAE was differently associated with NEC and LOS incidence. The effects of antibiotics, and potentially induced microbial dysbiosis related to development of NEC and LOS, should further be explored.

Introduction

Neonatal sepsis remains one of the leading causes of morbidity and mortality at the neonatal intensive care unit (NICU) [1]. Given the high burden associated with delayed treatment of early-onset sepsis (EOS), threshold for empiric initiation of antibiotics is low in preterm infants [2]. Consequently, over 75% of very low birthweight (VLBW; birth weight <1500 g) infants are empirically exposed to antibiotics [3]. Empirical therapy is usually discontinued upon negative blood culture results after 48-72 hours. However, as blood culture has a low sensitivity, the course is often prolonged out of fear of undertreating clinical sepsis [2,4].

Potential adverse effects of antibiotic exposure include antibiotic-resistance and dysregulation of microbial gut colonization by decreasing the diversity and promoting overgrowth of potential pathogens [5]. Specifically at neonatal age, early empiric antibiotic exposure (EEAE) has been suggested to increase the risk of long-term adverse effects, such as development of metabolic and auto-immune disorders [5]. On the short-term, it has been demonstrated in VLBW infants that every additional day of antibiotic exposure is associated with worse composite outcome of multiple adverse events, including necrotizing enterocolitis (NEC) and late-onset sepsis (LOS) [6]. However, these findings have recently been questioned by observational and animal model studies, suggesting a mitigating effect of antibiotics on NEC [7,8]. In murine models, antibiotics decrease bloodstream infections, potentially by delaying colonization, lowering the bacterial load at the level of the intestinal mucosa and the load of invasive microorganisms at the epithelial border [9].

This hypothesis is supported by two recent case-control studies performed by our group, showing that antibiotic exposure was associated with decreased odds of gram-positive LOS and, when initiated directly postpartum, with decreased odds of NEC [10,11]. Neither study, however, focused specifically on EEAE for EOS suspicion and both were prone to confounding by indication, as antibiotic treatment and extension thereof could depend on clinical factors, which are also associated with NEC and LOS.

In the current larger multicenter cohort study, we aim to explore clinical characteristics associated with (prolongation of) EEAE and investigate the association between the duration of EEAE with NEC and LOS.

Materials and Methods

Study design and participants

This study was embedded in an ongoing prospective multicenter preterm cohort study in nine participating NICUs in the Netherlands and Belgium, with the primary objective of identifying novel non-invasive biomarkers, as well as clinical risk factors, for LOS and NEC in the first 28 days of life [12]. Consequently, included participants have, in part, been described in previous case-control studies investigating fecal biomarkers and a wide range of risk factors for LOS and NEC [10,11]. In our current study, we included all infants born before 30 weeks of gestation between October 2014 and July 2019 whose parents provided informed consent (Ethical Board permission A2020.190). Antibiotics for risk or suspicion of EOS were started by the attending physician in standard dosage and administered parenterally, according to the NICE guideline on *Antibiotics for early-onset neonatal infection* [13]. None of the participating centers routinely prescribed probiotics in the study period.

We excluded infants with major congenital malformations, including gastrointestinal malformation, such as anal or intestinal atresia and Hirschsprung's disease [10,11]. Additionally, in accordance with previous research, we excluded infants with culture-proven EOS and infants demised in the first week of life, irrespective of the cause of death [6,14,15]. Infants with culture-proven EOS were excluded since they require prolonged treatment with antibiotics, thus not being treated empirically. Finally, inaccessibility to patient record data on antibiotic exposure and morbidity was an additional exclusion criterion.

Definitions

EEAE was defined as antibiotic exposure started within the first 72 hours of life under the suspicion of EOS, but in the absence of a positive blood and, if applicable, cerebrospinal fluid culture. Duration was counted per started 24 hours. Common antibiotic practice per center for suspicion of EOS with included number of participants is presented in **Supplementary Table 1**. Subjects were categorized based on EEAE duration: 1) no EEAE; 2) short EEAE (≤72h); or 3) prolonged EEAE (>72h). The cut-off point of 72 hours was chosen in agreement with common clinical practice, where empiric antibiotic therapy is often discontinued within 48-72 hours in case clinical and biochemical correlates for sepsis are missing [16].

Infants were classified as NEC cases, when diagnosed with NEC stage IIA or higher, according to the modified Bell's staging criteria [17]. All infants with NEC were independently reviewed by two experts (TM, HN) for classification. In case of discrepancy, infants were reevaluated until agreement was reached. All neonatal LOS episodes, defined as blood culture-proven sepsis with onset beyond the first 72 hours and within the first 28 days, were analyzed and classified (**Supplementary Table 2**) [18,19]. Infants could be classified as both NEC and LOS cases if they met the criteria for both.

Feeding practice was subcategorized as done previously, consisting of three categories: 1) human milk (HM), either own mother's milk (MM) or donor milk (DM), 2) formula feeding (FM), 3) combination of HM and FM (**Table S2**) [11]. The highest C-reactive protein level within 72h after birth was recorded. Inotropic medication and type of ventilation support were registered between 48-72h after birth, as the decision whether to prolong empirical antibiotics is made at that moment. Standard demographic and clinical data were collected. Additional definitions of clinical and demographic characteristics are depicted in **Table S2**.

Statistical analysis

Statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS) version 26.0 (IBM, Armonk, NY, USA). Continuous demographic and clinical characteristics were depicted, depending on normality, as either mean and standard deviation (SD) or median and interquartile range [IQR] for the three groups of interest: 1) no EEAE, 2) short (≤72h) and 3) prolonged (>72h) EEAE. Where appropriate, continuous data were analyzed by parametric one-way ANOVA, or non-parametric Kruskal-Wallis tests. Normal distribution of continuous data was assessed visually. Categorical data were analyzed by Pearson's chi-squared test. Two-sided p-values of <0.05 were considered statistically significant.

Associations between EEAE and incidence of NEC and LOS were analyzed by univariate and multivariate logistic regression methods with EEAE as a dichotomous variable (unexposed versus exposed infants). Secondly, duration of EEAE was analyzed both as a categorical variable (no EEAE vs. short (≤72h) vs. prolonged (>72h EEAE), and as a continuous variable (EEAE in number of calendar days).

In the multivariate models, odds ratio's (OR) were adjusted for confounding variables previously associated with NEC and LOS development [11,20]: center of birth, gestational age, birthweight percentiles, gender, mode of delivery, invasive ventilation and/or inotropic medication use at day two of life, and type of enteral feeding. For LOS, five-minute Apgar score and duration of parenteral feeding were added. Results from the logistic regression were reported as OR and adjusted OR (aOR), along with the respective 95% confidence interval (95% CI). Subgroup analyses for coagulase negative staphylococcus (CoNS) and non-CoNS sepsis was additionally performed.

A post-hoc uni- and multivariate analysis was performed to assess odds for LOS and non-CoNS LOS after exclusion of all LOS cases who were diagnosed before postnatal age of 7 days. Although the most common definition of LOS is sepsis with onset ≥72 h of life, some clinicians, as well as several studies, define LOS as sepsis beyond the first week of life [19,21]. With this analysis we aimed at ensuring comparability of our methods with those studies.

Results

A total of 1490 infants born before 30 weeks of gestation were screened for eligibility between October 2014 and January 2019, of whom 231 were excluded. The main reasons for exclusion were lack of informed consent (n=159) and culture-proven EOS (n=56). Additional motives for exclusion are depicted in **Figure 1**.

Of the 1259 included infants with negative blood culture results from the first 72h of life, 1122 (89%) had EEAE for the suspicion of EOS, of whom 802 (64%) had short EEAE (≤72h) and 320 (25%) prolonged EEAE (**Fig. 1).** Prolonged EAEE ranged between 19 and 44%, depending on the center of birth (**Table S1**).

Baseline characteristics are depicted in **Table 1**. Infants without EEAE were more often born by caesarean section and were smaller for gestational age (SGA), while infants with prolonged EEAE were invasively ventilated, needed inotropic medication and had an increased CRP level (≥10mg/dl) more often than the other groups.

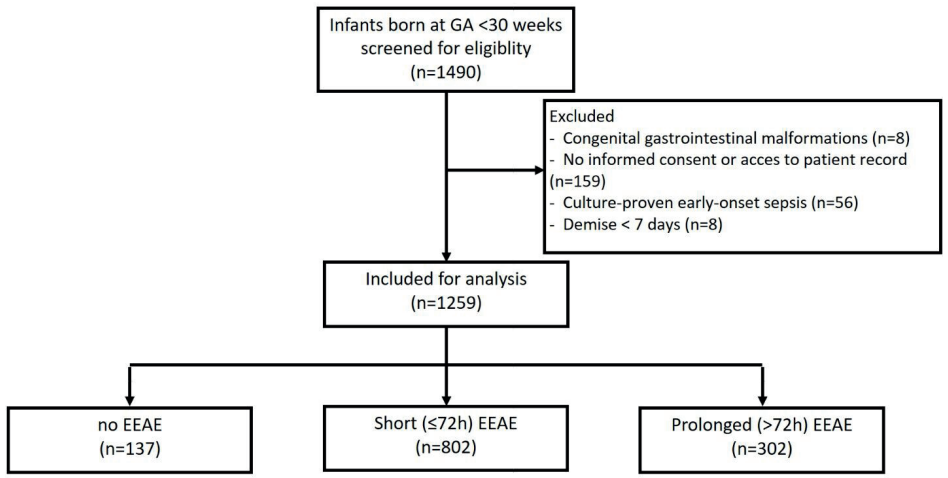


Figure 1. Flow chart of patient inclusions. EEAE: early empiric antibiotic exposure; GA: gestational age; h: hours.

In the first 28 days of life, NEC occurred in 107 infants (8.4%), of whom 40 needed surgical intervention. LOS was diagnosed in 421 (33.4%) neonates, of which 192 caused by a non-CoNS pathogen. Median age of onset of NEC was comparable between EEAE groups, while LOS occurred at a later age with increasing EEAE duration (**Fig. 2A** and **2B**, resp., **Table 1**). Incidence of NEC and LOS by EEAE duration are represented graphically in **Supplementary Figure 1A-C** and **1D-E**, respectively.

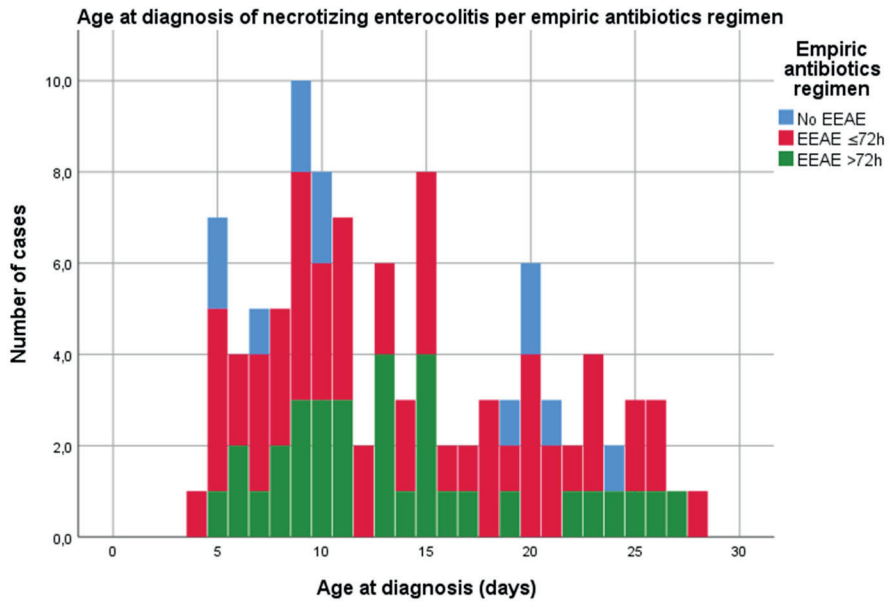
When corrected for confounding factors, odds of NEC were lower in infants with any EEAE, compared to no EEAE (aOR 0.47; 95%CI 0.23–0.96; p=0.04). Short (≤72h) EEAE was associated with lower odds of developing NEC, compared to both no EEAE (aOR 0.39; 95%CI 0.19–0.80; p=0.01) and prolonged (>72h) EEAE (aOR 0.58; 95%CI 0.35–0.96; p=0.03) (**Table 3**). EEAE duration as a continuous variable could not be analyzed in relation to NEC incidence as the linearity assumption for logistic regression analysis was not met, regardless of data transformation or non-linear term addition.

Table 1. Demographic and clinical characteristics of all subjects categorized according to early empiric antibiotic exposure

	No EEAE (n=137)	Short EEAE (n=802)	Prolonged EEAE (n=320)	p-value
Gestational age, *weeks + days* (median [IQR])	28+6 [27+6 - 29+3]	27+6 [26+1 - 28+6]	27+1 [25+6 - 28+4]	<0.001
Birth weight, *gram*, mean (SD)	1001 (280)	1055 (262)	940 (261)	<0.001
Birthweight, *z-score*, mean (SD)	-0.48 (0.94)	0.24 (0.80)	-0.02 (0.90)	<0.001
SGA, n (%)	27 (20)	41 (5)	31 (10)	<0.001
Gender, *female*, n (%)	80 (58)	364 (46)	143 (45)	0.02
Delivery mode, *vaginal*, n (%)	14 (10)	439 (55)	145 (46)	<0.001
Singleton, n (%)	110 (80)	534 (66)	210 (66)	0.002
Invasive ventilation at 48-72h of life, n (%)	16 (12)	168 (21)	149 (47)	<0.001
Inotropic medication at 48-72h of life, n (%)	0 (0)	25 (3)	37 (12)	<0.001
Enteral feeding type				0.01
Human milk, n (%)	80 (65)	562 (74)	232 (78)	
Formula milk, n (%)	16 (13)	100 (13)	22 (7)	
Combination, n (%)	28 (23)	101 (13)	43 (14)	
Parental feeding, *days* (median [IQR])	9 [6 - 11]	9 [7 - 11]	10 [8 - 11]	<0.001
EEAE duration, *days* (median [IQR])	N/A	3 [2 - 3]	7 [6-8]	<0.001
Highest CRP within first 72h of life				<0.001
≥ 10 mg/L, n (%)	1 (1%)	37 (5%)	103 (32%)	
missing values, n (%)	58 (42%)	78 (10%)	35 (11%)	
Age of NEC onset, *days* (median [IQR])	11 [9 - 20]	13 [9 - 20]	14 [10 - 18]	0.84
Age of LOS onset, *days* (median [IQR])	6 [4 - 10]	9 [6 - 12]	11 [9 - 15]	<0.001

AB: antibiotics; CRP: C-reactive protein; EEAE: early empiric antibiotic exposure; GA: gestational age; LOS: late-onset sepsis; N/A: not applicable; NEC: necrotizing enterocolitis; PPROM: premature prolonged rupture of membranes; NICU: neonatal intensive care unit; SGA: small for gestational age. Data are summarized as mean and standard deviation (SD) or number and percentage (%), unless stated otherwise.

A



B

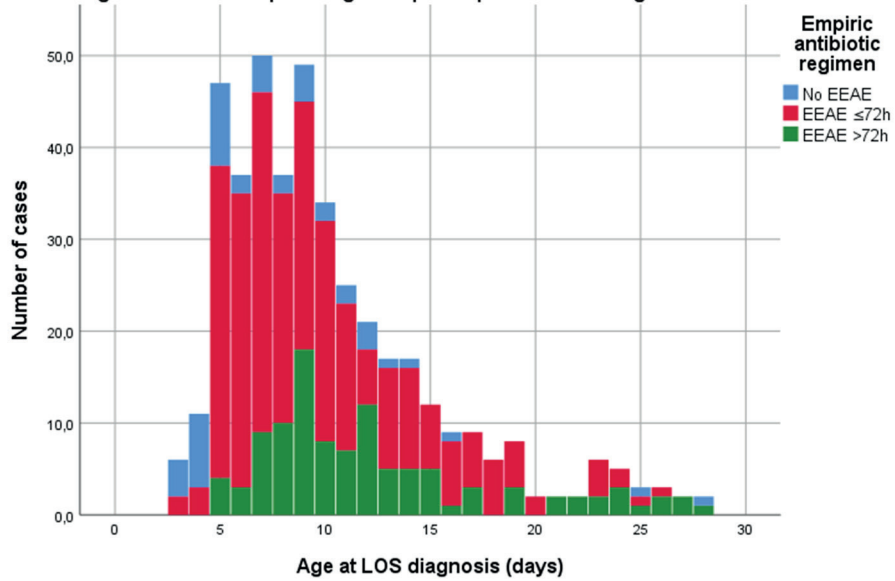


Figure 2. Stacked bar chart of incidence of A) NEC and B) LOS in the first 28 days of life, by EEAE category. EEAE, early empiric antibiotic exposure; LOS, late-onset sepsis, NEC, necrotizing enterocolitis

LOS was diagnosed in 421 of the 1259 infants (33.4%). Median onset of LOS differed significantly between EEAE groups (**Table 1**). **Table 2** demonstrates the incidences of LOS subtypes, based on causative pathogens and type of LOS. No differences were found in overall LOS incidence between infants with and without EEAE **(Table 3)**. However, EEAE was associated with a lower incidence of non-CoNS LOS, compared to non-exposure to antibiotics (aOR 0.49; 95%CI 0.25–0.96; p=0.04) **(Table S3)**. Only prolonged EEAE, but not short EEAE, was associated with lower non-CoNS LOS incidence, compared no EEAE (aOR 0.35; 95%CI 0.16–0.74; p=0.007) (**Supplementary Table 3**).

Table 2. NEC and LOS cases shown for infants without, short (≤72h) or prolonged (>72h) early empiric antibiotic exposure.

	No EEAE (n=137)	Short EEAE (n=802)	Prolonged EEAE (n=320)
NEC, n (%)	13 (9.5)	60 (7.4)	34 (10.6)
Surgical NEC, n (%)	4 (3)	23 (3)	13 (4)
LOS, all pathogens, n (%)	45 (32.8)	266 (33.2)	110 (34.4)
1) CoNS LOS, n (%)	26 (19.0)	158 (19.7)	68 (21.3)
2) All non-CoNS pathogens, n (%)	22 (16.1)	122 (15.3)	50 (15.6)
2a) Gram positive LOS, n (%)	14 (10.2)	49 (6.1)	21 (6.6)
2b) Gram negative LOS, n (%)	10 (7.3)	78 (9.7)	30 (9.4)

AB: antibiotics; CoNS: coagulase negative; EEAE: early empiric antibiotic exposure; h: hours; NEC: necrotizing enterocolitis; LOS: late-onset sepsis.

Table 3. Odds ratio of late-onset sepsis per causing pathogen between different duration of early empiric antibiotic exposure

		OR [95%CI]	p-value	Adjusted OR[a] [95%CI]	p-value
NEC					
	Any EEAE vs. non EEAE	0.85 [0.46 - 1.57]	0.61	0.47 [0.23-0.96]	0.04*
	Short EEAE vs. no EEAE	0.70 [0.37-1.32]	0.27	0.39 [0.19-0.80]	0.01*
	Prolonged EEAE vs. no EEAE	1.25 [0.64-2.40]	0.52	0.65 [0.30-1.41]	0.28
	Prolonged EEAE vs. short EEAE	1.78 [1.15-2.75]	0.01	2.56 [1.25-5.26]	0.03*
LOS, all pathogens					
	Any EEAE vs. non EEAE	1.03 (0.71-1.50)	0.88	0.78 (0.47-1.28)	0.32
	Short EEAE vs. no EEAE	1.02 (0.69-1.49)	0.94	0.83 (0.50-1.38)	0.47
	Prolonged EEAE vs. no EEAE	1.07 (0.70-1.64)	0.75	0.62 (0.35-1.10)	0.10
	Prolonged EEAE vs. short EEAE	1.43 (0.80-1.39)	0.70	0.75 (0.35-1.07)	0.11
	EEAE duration (days)	0.97 (0.92-1.02)	0.19	0.90 (0.85-0.97)	0.003**

* P<0.05; **P<0.01

[a]Adjusted for Center, Mode of delivery, Gender, Birth weight percentile, Gestational age, Apgar score 5 min, days of parenteral feeding, invasive ventilation support and/or inotropic medication use
95%CI, 95% confidence interval; CoNS: coagulase-negative staphylococci; EEAE: early empiric antibiotic exposure; LOS: late-onset sepsis; NEC: necrotizing enterocolitis; OR: odds ratio.

When antibiotic exposure was analyzed as a continuous variable (number of days of exposure), a lower LOS incidence was found for every additional day of EEAE (aOR 0.90; 95%CI 0.85–0.97; p=0.003). This negative association with duration of empirical antibiotic exposure was observed in all subcategories of LOS (**Table 3; Supplementary Table 3**).

Post-hoc analysis was performed solely on sepsis cases diagnosed beyond the first week. As analyzed by univariate logistic regression, prolonged EEAE was associated with higher odds for LOS, compared to both short and no EEAE. When corrected for confounding factors, this association could not be observed (**Supplementary Table 4**).

Discussion/conclusion

The continuation of early empiric antibiotics despite negative blood culture results, and its effect on short term outcomes, is debated [5,7-9]. In this prospective multicenter cohort study, we observed that the vast majority of preterm infants are empirically exposed to antibiotics directly after birth. In about one quarter of infants, antibiotics were continued empirically beyond 72h, despite negative cultures. Infants with prolonged EEAE were of lower gestational age and were more often intubated, receiving inotropic medication and had higher CRP values in the first 72h of life. They, however, had lower adjusted odds of developing LOS, compared to infants without EEAE. The group without EEAE, moreover, had higher adjusted odds of developing NEC, relative to the short EEAE group, but similar adjusted odds of NEC compared to infants with prolonged EEAE.

Similar to our findings, several studies have reported an increased risk for NEC with prolonged EEAE, compared to short EEAE [22-24]. Contrary, the recent NEOMUNE study including 2831 VLBW infants did not demonstrate a significant difference in NEC incidence in the short antibiotic exposure (≤72h) group versus the prolonged exposure (>72h) group: 4.3% vs. 3.7% [7]. However, they did report a lower NEC incidence (3.9%) following any early antibiotic exposure in comparison to non-exposed infants (9%) (OR 0.25, 95% 0.12-0.47 p<0.001). Notably, the study population consisted of over 90% of infants receiving antibiotic treatment, of whom the majority received prolonged antibiotic treatment (>72h), as opposed to our cohort, in which a short course was more common. Moreover, there was a disproportionally large amount of infants born small for gestational age (SGA) and/or by caesarean section in the group of infants without EEAE, both of which are known risk factors for NEC [25]. Even though the outcome was statistically corrected

for this potential confounding by indication, residual confounding may still be present. This limitation could not be avoided in our current study.

Other studies including sufficiently large groups of preterm infants not exposed to antibiotics are scarce, but our findings are further corroborated by experimental studies on preterm piglets, showing that no EEAE was associated with a higher incidence of NEC compared to EEAE [8]. EEAE resulted in increased mucosal integrity and decreased inflammatory responses, suggesting a potential protective mechanisms of early antibiotics exposure on the preterm gut trough immune modulation related to early gut microbiota colonization [8]. It is hypothesized that this protective mechanism could result from a delay in intestinal colonization with potential pathogens [7]. Because of this delayed colonization of pathogenic bacteria, the intestinal immune defense system might be stimulated towards postnatal adaptation [8,9]. However, this potential beneficial effect might be negated by prolonged EEAE, as this might provoke NEC by perturbed microbial colonization [26].

One small RCT including 22 preterm infants supports the hypothesis of a protective role of short EEAE, as a more favorable microbial composition was found in infants who were randomized to 48h of antibiotic treatment versus no EEAE [27]. Kim *et al.* reported an increased abundance of *Actinobacteriota (*formerly *Actinobacteria)*, which was largely contributed by *Bifidobacteriaceae*, in the EEAE group [27], a family previously associated with a decreased risk of NEC [28]. Notably, increased *Actinobacteriota* have also been associated with NEC in other studies, however in combination with significantly decreased abundance of *Bifidobacteriaceae*. The REASON trial, a small RCT comparing a short course of antibiotics to no antibiotics, did not show a difference in microbiota between the treatment and control arm and concluded that difference in microbiota was largely attributable to feeding type [29].

The potential protective role of EEAE for LOS that is suggested by our results, and those of el Manouni el Hassani *et al.* [11], are not supported by current literature on humans. Kuppala *et al*., e.g., reported a positive association between every additional day of antibiotic exposure and LOS incidence in a preterm cohort [21]. Their study design, however, differed in terms of follow-up period – 120 days, compared to 28 days in the current study – and in terms of study population – infants developing LOS in the first week of life, were excluded by the research group [21]. In the current study, a post-hoc analysis was performed excluding sepsis cases with onset <7 days. In line with Kuppala's *et al*. results, unadjusted odds for LOS were lower for the non-EEAE group, compared to the prolonged EEAE group. After adjustments for confounding factors, there was no association between duration of EEAE and LOS incidence. In our opinion, the exclusion of infants developing LOS in the first week

of life, might be subjected to bias, especially given that more than half of the infants who developed LOS in our non-EEAE group, were diagnosed within the first week of life (median age of LOS onset 6 days). As the median age of LOS onset in the short and prolonged EEAE group was 9 and 11 days, respectively, exclusion of all LOS cases would proportionately exclude more infants with LOS in the non-EEAE group, thus underestimating LOS onset in this group. This could, however, not entirely explain the difference in results as two larger studies including 587 and 4039 infants respectively, which did include early LOS cases during the first week of life, also found a higher LOS incidence with increasing antibiotics administration [14,30].

Although both NEC and (non-CoNS) LOS are preceded by intestinal dysbiosis [31,32], the contrast between NEC and LOS incidence in association with EEAE might suggest a different pathophysiology regarding gut microbiota related immune responses. Despite the fact that antibiotic administration could stimulate immune maturation [33], this might not be equally relevant for different diseases and should further be explored.

The current observational study has several strengths, including the multi-center design, the large cohort size and prospective collection of detailed data on daily basis, allowing adjustment for relevant clinical and demographic factors. This also allowed us to study NEC and LOS separately and not as a combined outcome as was previously done in some studies [14,34]. The categorization of participants based on antibiotic duration allowed to identify non-linear associations between duration of antibiotic exposure and NEC.

This study has several limitations, next to those characteristic for observational studies. Despite that several differences in baseline characteristics were corrected for in the multivariate analysis, there remains a risk of residual confounding of unidentified factors. Furthermore, obstetrical data could not be accessed, missing data on pre-eclampsia, umbilical cord blood flow and intrapartum antibiotic treatment, potentially leading to underestimation of the infants' antibiotic exposure. Registration was discontinued after the 28th day of life, which could have led to missing some LOS cases. As the first LOS episode usually occurs within the first weeks of life, we hypothesized that the number of missed cases would be limited [35].

Further research on EEAE and health effects is warranted. Future perspectives include larger RCTs aiming at unravelling the effects of EEAE in low-risk infants for EOS. For example, results from the NICU Antibiotics and Outcomes (NANO) trial (ClinicalTrials.gov identifier: NCT03997266), are needed to identify the suggested (protective) effect of empirical antibiotics for NEC and LOS and to identify the optimal duration of

empirical antibiotics. Interaction of antibiotics with other factors influencing the early gut colonization and immunity should be investigated. It remains to be elucidated whether current strategies against NEC, e.g. enteral feeding with human milk and the use of probiotics, have a synergistic preventive effect when combined with (short) EEAE or whether EEAE might rather be more helpful in a subgroup receiving formula feeding [36]. Studies should additionally take a broad spectrum of potential short- and long-term adverse events into account [37,38]. In parallel, microbiota studies, preferably by metagenomics analysis, should be performed in infants receiving different lengths of empirical antibiotics to assess short- and long-term effects on intestinal colonization. In the future, these insights could allow for targeted microbiota-based preventive strategies in an optimally selected population and time-window for improving development of the immature gut [9].

Despite our finding, we believe that providing more antibiotics than currently advised, e.g. a standard short-term administration of empiric antibiotics (48-72h) instead of watchful waiting without antibiotics in case of low risk of early onset sepsis, should not be advised. First, the plethora of potential antibiotic-related adverse events, such as increased antibiotic resistance and other short- and long-term effects should be further investigated [5,39]. Current guidelines on antibiotic stewardship should be followed until results on RCTs assessing the effects of EEAE, such as the abovementioned NANO trial, are published. Empirical antibiotics should only be started when there is substantial suspicion or high risk on EOS and discontinued as soon a deemed safe (in absence of positive blood culture and reassuring clinical picture).

In conclusion, in this multicenter cohort, almost 90% of preterm infants with negative postnatal blood cultures was exposed to empirical antibiotics for suspicion of EOS. Twenty-five percent had prolonged (>72h) empirical exposure. A short (≤72h) empirical course of antibiotics was associated with a decreased risk for NEC compared to no antibiotics and a prolonged antibiotic course. On the other hand, prolonged EEAE was associated with a decreased risk for LOS in the first 28 days of life, compared to no antibiotics. Potential antibiotic-induced changes in microbiome composition and function and their association with NEC and LOS development should be explored in future studies.

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Supplementary files

Supplementary Table 1. Level of care, antibiotic protocol for EOS and inclusions per EEAE duration per participating center.

Inclusion Center	Level of care	Antibiotic for suspicion of EOS	No EEAE (n, %)	Short (≤72h) EEAE (n, %)	Prolonged (>72h) EEAE (n, %)
1	Level IV NICU	Benzylpenicillin + gentamicin	23 (10)	166 (71)	45 (19)
2	Level IV NICU	Benzylpenicillin + amikacin	16 (8)	120 (63)	56 (29)
3	Level III NICU	Amoxicillin + gentamicin	49 (21)	137 (60)	43 (19)
4	Level IV NICU	Amoxicillin + gentamicin	8 (12)	46 (67)	15 (22)
5	Level III NICU	Amoxicillin + ceftazidim	10 (7)	84 (61)	43 (31)
6	Level IV NICU	Amoxicillin + amikacin	12 (11)	70 (65)	26 (24)
7	Level IV NICU	Amoxicillin + gentamicin	6 (8)	45 (57)	28 (35)
8	Level IV NICU	Amoxicillin – clavulanic acid + gentamicin	9 (5)	113 (68)	44 (27)
9	Level IV NICU	Benzylpenicillin + gentamicin	4 (9)	21 (47)	20 (44)

EOS, early-onset sepsis; EEAE, early empiric antibiotic exposure; NICU neonatal intensive care unit

Supplementary Table 2. Definitions and classification of demographics

Feeding practice	
Formula feeding	Enteral feeding volume consisting of 50-100% formula milk
Full human milk feeding	Enteral feeding volume consisting of 80-100% human milk (own mother's or donor milk)
Combined feeding	Enteral feeding volume consisting of <50% formula milk AND <80% human milk
Days of parenteral feeding	Total number of postnatal days until either 120 ml/kg of enteral feeding and/or two days without parenteral feeding (amino acids and/or lipids) was reached.
Late-onset sepsis (LOS)	
Culture-proven late-onset sepsis (LOS)	A clinical suspicion of sepsis, as reported by the treating physician, combined with a positive blood culture after the third day of life (≥72h).
Contaminated (CoNS) blood culture	Reported as such by the treating physician, followed by immediate antibiotics cessation AND/OR CoNS-positive culture with remaining low C-reactive protein (CRP) levels (<10 mg/L) AND/OR CoNS was involved in a polymicrobial culture
LOS pathogen classification	Coagulase negative Staphylococci (CoNS) non-CoNS Gram-negative pathogens Gram-positive pathogens
Other demographics	
Small for gestational age (SGA)	Birthweight <10th percentile, according to the Fenton birthweight calculator [1]

[1] Fenton, T.R. and J.H. Kim, A systematic review and meta-analysis to revise the Fenton growth chart for preterm infants. BMC Pediatr, 2013. 13: p. 59.

Supplementary Table 3. Odds ratio of late-onset sepsis per causing pathogen between different duration of early empiric antibiotic exposure

Analysis		OR [95%CI]	p-value	Adjusted OR[a] [95%CI]	p-value
1) CoNS LOS					
	Any EEAE vs. non EEAE	1.07 [0.68-1.70]	0.77	1.04 [0.56-1.95]	0.89
	Short EEAE vs. no EEAE	1.04 [0.65-1.67]	0.86	1.12 [0.60-2.09]	0.73
	Prolonged EEAE vs. no EEAE	1.15 [0.69-1.92]	0.60	0.89 [0.44-1.78]	0.73
	Prolonged EEAE vs. short EEAE	1.10 [0.79-1.52]	0.57	0.79 [0.53-1.19]	0.27
	EEAE duration (days)	0.96 [0.90-1.02]	0.15	0.92 [0.86-1.00]	0.04*
2) All non-CoNS pathogens					
	Any EEAE vs. non EEAE	0.96 [0.59-1.58]	0.89	0.49 [0.25-0.96]	0.04*
	Short EEAE vs. no EEAE	0.95 [0.57-1.58]	0.85	0.54 [0.28-1.05]	0.07
	Prolonged EEAE vs. no EEAE	1.00 [0.57-1.74]	0.99	0.35 [0.16-0.74]	0.01**
	Prolonged EEAE vs. short EEAE	1.05 [0.73-1.51]	0.81	0.64 [0.39-1.06]	0.08
	EEAE duration (days)	0.99 [0.93-1.05]	0.70	0.88 [0.80-0.96]	0.01**
2a) Gram positive LOS					
	Any EEAE vs. non EEAE	0.62 [0.33-1.14]	0.12	0.38 [0.17-0.85]	0.02*
	Short EEAE vs. no EEAE	0.60 [0.32-1.13]	0.12	0.43 [0.19-0.97]	0.04*
	Prolonged EEAE vs. no EEAE	0.66 [0.321.35]	0.25	0.27 [0.10-0.71]	0.01**
	Prolonged EEAE vs. short EEAE	1.09 [0.64-1.87]	0.74	0.62 [0.31-1.26]	0.19
	EEAE duration (days)	0.98 [0.90-1.07]	0.62	0.87 [0.77-1.00]	0.04*
2b) Gram negative LOS					
	Any EEAE vs. non EEAE	1.33 [0.67-2.64]	0.41	0.56 [0.22-1.42]	0.22
	Short EEAE vs. no EEAE	1.34 [0.67-2.68]	0.41	0.62 [0.24-1.58]	0.32
	Prolonged EEAE vs. no EEAE	1.31 [0.61-2.80]	0.48	0.36 [0.12-1.01]	0.05
	Prolonged EEAE vs. short EEAE	0.98 [0.63-1.54]	0.94	0.57 [0.30-1.09]	0.09
	EEAE duration (days)	0.98 [0.91-1.06]	0.59	0.85 [0.75-0.97]	0.02*

* P<0.05; **P<0.01

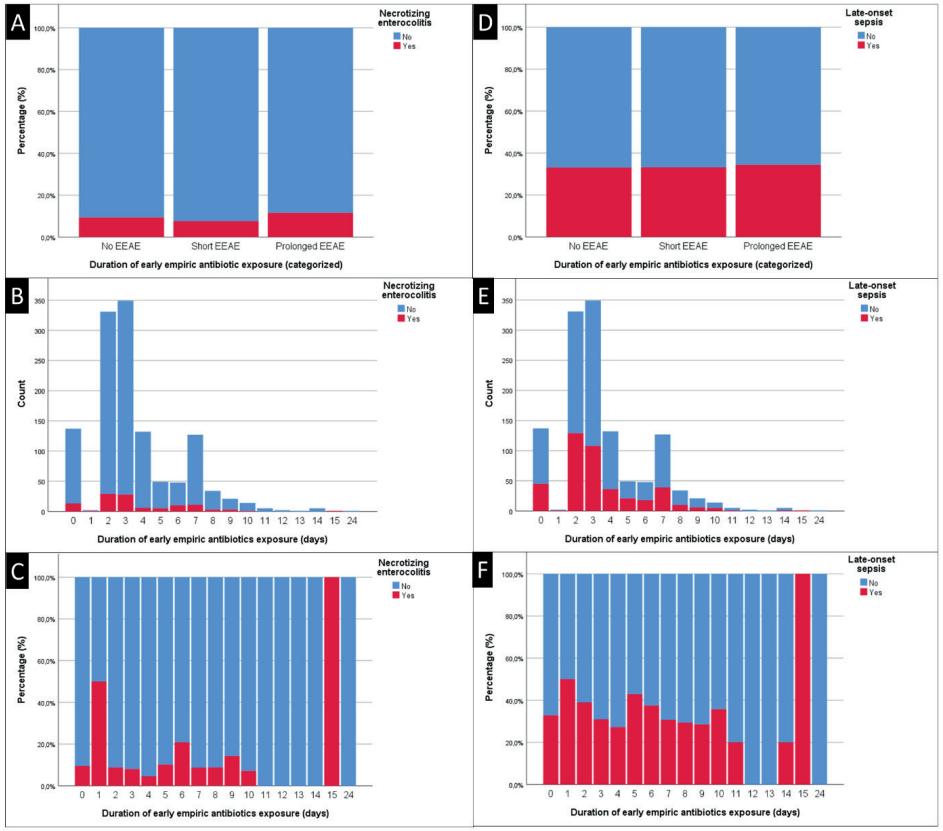
[a] Adjusted for Center, Mode of delivery, Gender, Birth weight percentile, Gestational age, Apgar score 5 min, days of parenteral feeding, invasive ventilation support and/or inotropic medication use
95%CI, 95% confidence interval; CoNS: coagulase-negative staphylococci; EEAE: early empiric antibiotic exposure; LOS: late-onset sepsis; NEC: necrotizing enterocolitis; OR: odds ratio. Data is summarized as odds ratio (95% confidence interval).

Supplementary Table 4. Odds ratio of late-onset sepsis per causing pathogen between different duration of early empiric antibiotic exposure

Analysis		OR [95%CI]	p-value	Adjusted OR[a] [95%CI]	p-value
1) All LOS with onset at age ≥7 days (n=323, 28%)					
	Any EEAE vs. non EEAE	1.50 [0.99-2.57]	0.06	1.04 [0.60-1.80]	0.89
	Short EEAE vs. no EEAE	1.46 [0.90-2.37]	0.13	0.97 [0.56-1.68]	0.92
	Prolonged EEAE vs. no EEAE	1.94 [1.16-3.25]	0.01*	0.93 [0.52-1.68]	0.82
	Prolonged EEAE vs. short EEAE	1.33 [1.00-1.75]	0.05	0.96 [0.69-1.33]	0.80
	EEAE duration (days)	1.03 [0.98-1.08]	0.22	0.97 [0.91-1.03]	0.29
2) All non-CoNS pathogens with onset at age ≥7 days (n=151, 13%)					
	Any EEAE vs. non EEAE	1.93 [0.95-3.92]	0.07	1.11 [0.48-2.53]	0.81
	Short EEAE vs. no EEAE	1.82 [0.81-3.73]	0.10	1.18 [0.51-2.72]	0.70
	Prolonged EEAE vs. no EEAE	2.22 [1.04-4.72]	0.04*	0.94 [0.38-2.30]	0.89
	Prolonged EEAE vs. short EEAE	1.22 [0.82-1.79]	0.32	0.79 [0.50-1.27]	0.34
	EEAE duration (days)	1.05 [0.99-1.12]	0.11	0.99 [0.91-1.07]	0.71

* P<0.05

[a] Adjusted for Center, Mode of delivery, Gender, Birth weight percentile, Gestational age, Apgar score 5 min, days of parenteral feeding, invasive ventilation support and/or inotropic medication use
95%CI, 95% confidence interval; CoNS: coagulase-negative staphylococci; EEAE: early empiric antibiotic exposure; LOS: late-onset sepsis; NEC: necrotizing enterocolitis; OR: odds ratio.



Supplementary Figure 1. Incidence of necrotizing enterocolitis (A-C) and late-onset sepsis (D-E) by duration of early empiric antibiotics administration. A+D) Relative incidence (percentage) of cases per category of EEAE; B+E) Absolute incidence per amount of days of EEAE; C+F) Relative incidence per amount of days of EEAE.

Data used for this graph are not adjusted for confounding factors and trends might differ from trends observed by performing multivariate regression analysis.
EEAE, early empiric antibiotics exposure

Chapter 5

Umbilical cord blood culture in neonatal early-onset sepsis: a systematic review and meta-analysis

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Pediatric Research 2022 Aug; 92(2): 362-372

Abstract

Background: Peripheral blood culture (PBC) is considered the gold standard for diagnosis of neonatal early-onset sepsis (EOS), but its diagnostic value can be questioned. We aimed to systematically asses the diagnostic test accuracy (DTA) of umbilical cord blood culture (UCBC) for EOS.

Methods: A systematic literature search was performed in PubMed, Embase, Web of Science and the Cochrane Library. Studies performing UCBC for the diagnosis of EOS were included.

Results: A total of 1.908 articles were screened of which 17 were included. Incidences of positive PBC and UCBC were low in all studies. There was a large heterogeneity in the consistency between positive PBC and UCBC outcomes. PBC had a pooled sensitivity of 20.4% (95%CI 0.0–40.9) and specificity of 100.0% (95%CI 100.0–100.0) compared to 42.6% (95%CI 12.7–72.4%) and 97.8% (95%CI 93.1–100.0) of UCBC for clinical EOS, defined as clinical sepsis regardless of PBC outcomes.

Conclusion: This systematic review shows that compared to PBC, UCBC has higher sensitivity and comparable specificity for clinical EOS and might be considered as diagnostic test for EOS. Due to the limited number of studies, low incidences of EOS cases and the imperfect reference standards for EOS, results should be interpreted cautiously.

Introduction

Neonatal early-onset sepsis (EOS), defined as sepsis occurring within 72 hours after birth, has high morbidity and mortality.[1] The overall incidence of EOS is 0.1% and increases in certain subgroups, such as very low birthweight (VLBW) infants (birthweight < 1500 g) or infants born after a gestational age <28 weeks to 1.4% and 1.8% respectively.[2] Diagnosis of EOS is challenging given the subtle and non-specific signs and symptoms. Since timely commencement of antibiotics could prevent sepsis-related morbidity and mortality, the threshold to initiate empiric antibiotic therapy before diagnostic confirmation is low.[3,4] Consequently, 5% of all late preterm and term infants and up to 75% of VLBW infants are exposed to antibiotics empirically for suspected EOS.[3,4] Exposure to antibiotics early in life increases risk of antibiotic-resistance and impacts microbial gut colonization by decreasing its diversity and increasing the abundance of pathogens.[5] This may increase the risk of both immediate and long-term adverse effects, such as growth retardation and auto-immune disorders.[5-9] In order to reduce the risk for sepsis related morbidity and mortality on one hand, and to prevent overtreatment with antibiotics on the other hand, a diagnostic test with high sensitivity and specificity is needed.

The currently considered gold standard for EOS diagnosis is a bacterial blood culture drawn from a peripheral vein.[10] The exact sensitivity of a peripheral blood culture (PBC) for EOS is unknown, however, clinicians have questioned the accuracy since cultures obtained from patients with clinical illness often remain sterile. The sensitivity of a PBC decreases with sample volume, whilst collecting an adequate blood volume from neonates can be challenging .[11] Furthermore, maternal intrapartum antibiotic use might further decrease the sensitivity, although advances in blood culture techniques limit this risk nowadays.[12,13] Besides, PBCs typically require phlebotomy which is associated with pain[1,10] and it contributes to iatrogenic anemia, especially in VLBW infants.[14]

The use of umbilical cord blood culture (UCBC) has been suggested as an alternative diagnostic test if EOS is suspected at the time of birth. Collection of umbilical cord blood is not painful, it is technically easy to perform and sufficient sample volume can be obtained circumventing the risk for iatrogenic anemia.[15] However, studies on the diagnostic accuracy of UCBC compared to PBC included low sample sizes and the results are conflicting. To date, no systematic review nor meta-analyses has been performed. Therefore, we aimed to systematically identify, appraise and evaluate the diagnostic test accuracy (DTA) of UCBC for the diagnosis of EOS compared to PBC including a meta-analysis.

Methods

Study objectives

To investigate the primary aim of this review, we first compared results of UCBC as index test directly with results of the gold standard, PBC, as reference test. Second, because of uncertainty about the true sensitivity of either tests, we compared the results of UCBC and PBC as separate index tests with the previous papers' definition of clinical EOS as reference. For this comparison only studies with paired UCBC and PBC were included. Third, the DTA of PBC and UCBC combined as index test (if one or both tests were positive, the outcome was regarded positive) for clinically diagnosed sepsis was evaluated.

Protocol and registration

The protocol for this systematic review was registered prospectively with Prospero (ID-number CRD42021238106). The manuscript was written in accordance with the Preferred reporting items for systematic review and meta-analysis of diagnostic test accuracy studies (PRISMA-DTA) checklist.[16]

Study eligibility criteria

Studies investigating the diagnostic accuracy of UCBC for EOS were eligible. Studies comparing the accuracy of UCBC with either PBC proven or clinically diagnosed EOS as the gold standard were included. Since there is currently no uniform definition of clinically diagnosed EOS, we did not include a strict definition.[17] Articles including conventional and/or non-conventional culture techniques were included. Animal studies and case reports were excluded. If no full-text was available, full-text was requested from the author. If original authors did not respond, studies were excluded. No date or language restrictions were applied.

Information sources and search strategy

A literature search was performed based on the PRISMA-statement.[16] To identify eligible studies, systematic searches were performed in collaboration with a medical information specialist in the bibliographic databases PubMed, Embase, Web of Science (Core Collection) and Wiley/Cochrane Library from inception up to January 21, 2021. The following terms were used (including synonyms and closely related words) as index terms or free-text words: "Neonates", "Early-onset sepsis" and "Umbilical cord blood". The full search strategies for all databases can be found in the Online Supplemental.

Study selection and data collection

After removal of duplicates, two reviewers (TD and DV) independently screened all potentially relevant titles and abstracts for eligibility. The full text of the selected articles was obtained for further review of the eligibility criteria. Differences in judgement were resolved through a consensus procedure. Data from the included articles was extract by the two reviewers (TD and DV) and verified by the other authors. Articles found through references and other sources were also included if eligible. The following data was extracted if available: year of study, country, study design including study setting, in- and exclusion criteria, characteristics of study population, number of participants, incidence of culture proven and clinically diagnosed EOS, cultured pathogens, definition of clinically diagnosed sepsis, DTA of UCBC for PBC, DTA of both UCBC and PBC for clinically diagnosed EOS, maternal intrapartum antibiotic use and collection technique of umbilical cord blood.

Risk of bias and quality assessment

Two reviewers (TD and DV) independently evaluated the methodological quality and the risk of bias of the articles included in the final analysis, using the QUADAS-2, a tool for the quality assessment of diagnostic accuracy studies.[18]

Meta-analysis

The true and false positive and negative values for each individual study were entered into RevMan Version 5.4.1.[19] This software was used to create forest plots and summary receiver operating characteristics (sROC). Subsequently, a bivariate random effects model[20] was used to estimate the pooled summary sensitivity and specificity including 95% confidence intervals. This was done using Proc NLMIXED in SAS version 9.4.[21,22] If no variance in sensitivity or specificity was observed between the studies, the delta method was used to calculate confidence intervals.[23] The calculated parameter estimates were imported to RevMan to visualize the calculated summary operation points in the sROC.

Forest plots and sROCs were visually inspected to identify heterogeneity. We planned to explore potential sources of heterogeneity such as the incidence of culture proven and/or clinically diagnosed EOS, number of inclusions, year of publication, gestational age, volume of blood used for UCBC and the reporting of well-defined protocol for sterilization of the umbilical cord. If sufficient studies were available, these potential sources were added to the model as a covariate.

Results

Study selection

The literature search generated a total of 3.830 references: 983 in PubMed, 1.709 in Embase, 944 in Web of Science, and 194 in the Cochrane Library. Three additional articles were identified through other sources. After removing duplicates, 1.908 references remained. The abstract and titles of these articles were screened, excluding 1.856 studies. The full text of the remaining 52 articles were further checked for eligibility. A total of 33 were excluded based on the in- and exclusion criteria. The other 19 articles (17 on conventional culture, 2 on non-conventional molecular cultures) were included in this systematic review. The flow chart of the search and selection process is presented in Figure 1.

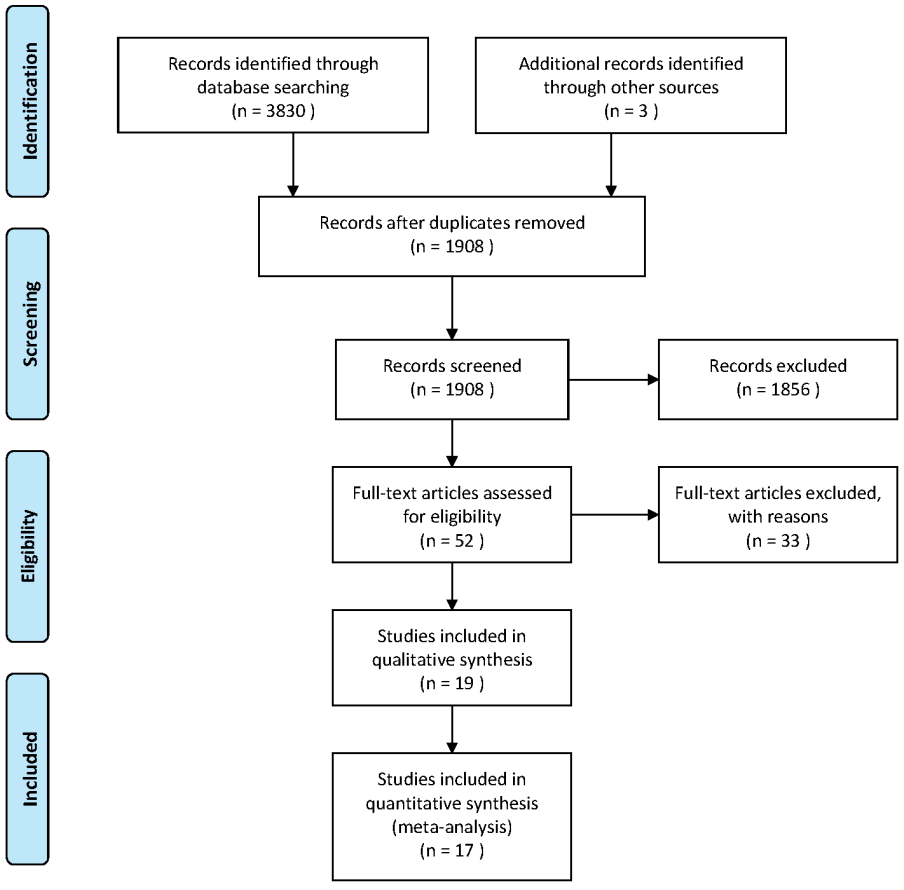

Figure 1. Flow diagram of study selection

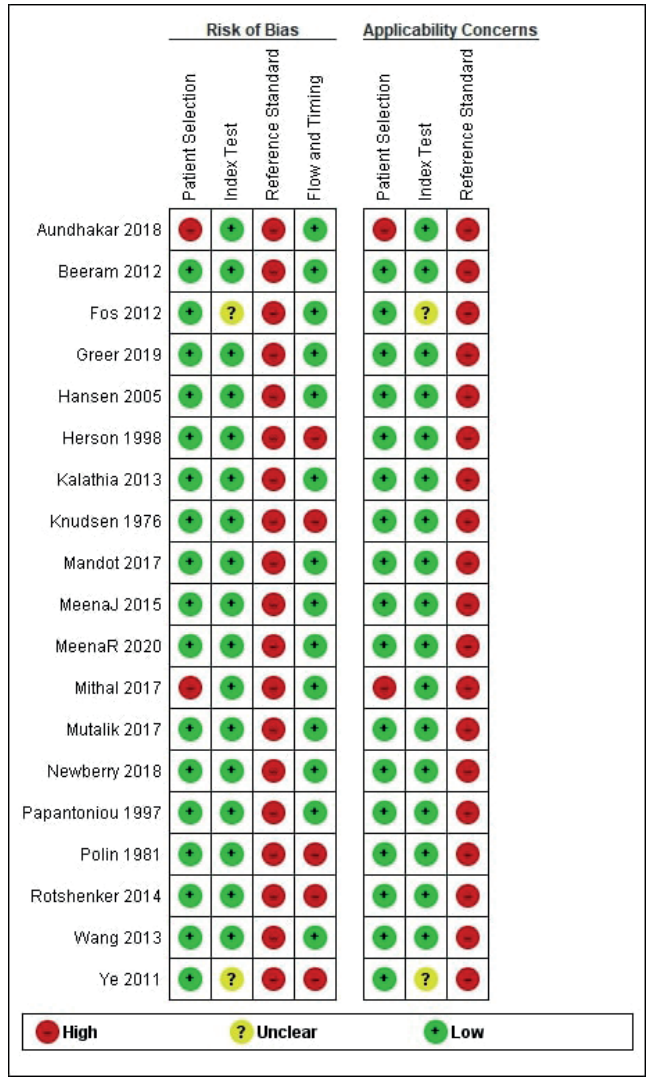

Figure 2. Summary of risk of bias and applicability concerns: Judgement of authors about each of the four domains in the QUADAS-2 for every included study.

Study characteristics

The selected studies included a total of 2.385 infants, with sample sizes ranging from 30 up to 323 participants. Publication dates of included studies ranged from 1976 to 2020. One case-control study[24] and 18 observational cohort studies were included.[25-42] From the latter, one included all admitted infants[32] and the other 17 included only infants at higher risk of EOS based on the presence of one or more risk factors.[25-31,33-42] One study included only term born infants,[25] four only preterm born infants[24,26-28] and the other studies included both term and preterm born infants.[29-42]

Umbilical cord blood samples were collected directly after birth and peripheral blood samples as soon as possible postpartum, but before the initiation of antibiotics in all studies. Not all studies collected a paired sample of cord blood and peripheral blood from every individual participant. Umbilical cord blood and peripheral blood was collected from 2.152 and 1.519 infants for conventional culture, respectively. The two studies on molecular culturing techniques included a total of 123 infants and collected paired cord blood and peripheral blood of all 123 infants. Characteristics of included studies and the main outcomes are described in Table 1.

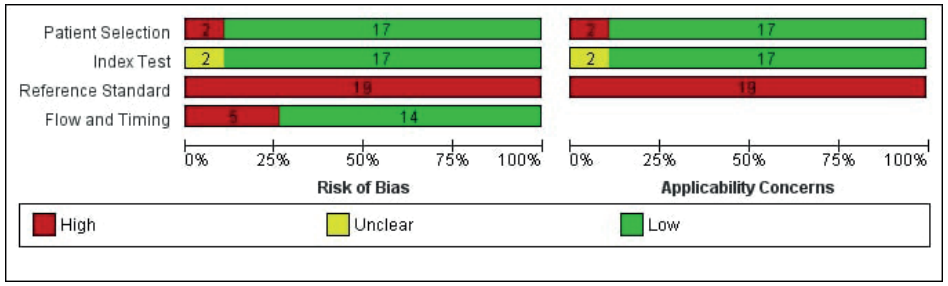

Figure 3. Risk of bias and applicability concerns graph: Judgement of authors about each of the four domains in the QUADAS-2 presented as percentages across included studies. Each bar shows the number of studies in each category.

Risk of bias and quality of evidence

The risk of bias due to patient selection was regarded as low. All cohort studies included a consecutive number of patients, based on predetermined eligibility criteria. Two studies excluded patients with contaminated cultures,[24,29] possibly introducing bias in patient selection. None of the studies reported whether the results for the index test and reference test were interpret blinded from the other test. However, due to the standard laboratory protocols and evident results from blood cultures, it was deemed unlikely that this introduced bias. Risk of bias for two studies in the domain of the reference standard was unclear, since it was not reported if umbilical cord blood was collected under sterile conditions.[31,42] Due to the study objectives, all studies used either PBC and/or clinically diagnosed sepsis (defined as the presence of a set of clinical symptoms and/or laboratory values indicating the presence of sepsis) as a reference standard. Since both PBC and clinically diagnosed sepsis are imperfect reference standard to detect EOS,[1] this might affect the validity of results. Therefore, risk of bias and applicability concerns were estimated to be high in all studies for the reference standard. It was estimated that the flow and timing of the participants did not introduce bias in most studies. Few studies, however, were unable to collect paired samples from both umbilical cord blood and peripheral blood from all infants, which might introduce

partial verification bias.[33,35,40-42] A summary of the risk of bias for individual studies and overall summary of the risk of bias per domain is respectively demonstrated in Figure 2 and 3. In general, studies were qualitatively well performed. However, due to low sample sizes and low incidence of EOS cases in combination with the imperfect reference standards, the overall quality of evidence was regarded as low.

Umbilical cord blood culture results compared to peripheral blood culture results

A total of 13 studies, including a total of 1.213 patients, compared the outcomes of conventional UCBC with paired PBC as the gold standard.[25-27,29-32,34,36-40] Most studies showed a high rate of negative PBC and UCBC, resulting in high specificity of UCBC for PBC. The number of patients with a positive PBC was low and the reported sensitivity of UCBC for PBC showed considerable heterogeneity across the different studies (Figure 4). Meta-analysis of the study results showed a pooled sensitivity of 75.0% (95% CI 44.1-91.9) and specificity of 91.3% (95% CI 83.4-95.6) of UCBC for PBC. Supplemental Table 1 demonstrates the cultured micro-organisms in both PBC and UCBC.

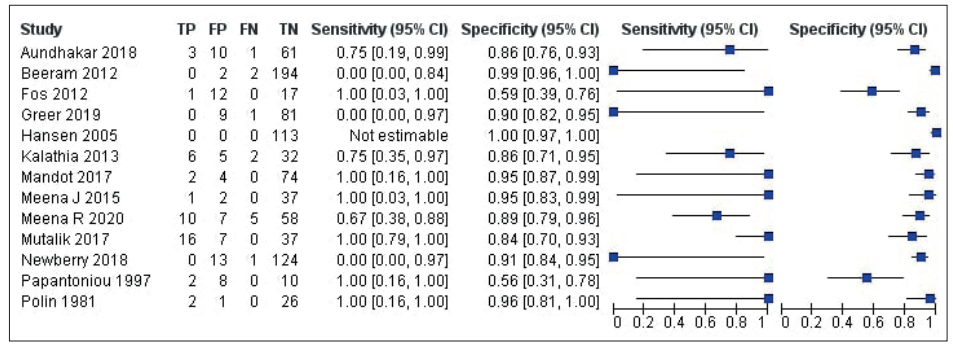

Figure 4. Forest plot of umbilical cord blood culture consistency with paired peripheral blood culture results. umbilical cord blood culture had a pooled sensitivity of 44.1% (95% CI 75.0-91.9) and specificity of 91.3% (95% CI 83.4-95.6) for peripheral blood culture results. CI: confidence interval; FP: false positive; FN: false negative; TN: true negative; TP: true positive.

Diagnostic test accuracy for clinically diagnosed sepsis

The definition of false positive (contamination) culture results differed between the studies. A positive culture was determined as false positives based on the cultured pathogen,[27,32] laboratory values,[25,26,29,36,37] clinical symptoms[40] or a combination of these factors.[30,31,34,38,39] A total of 17 studies reported true positive and false positive rates for UCBC (range 0% – 24% and 0% – 12%, respectively).[25-27,29-42] From these studies, 13 also reported these rates for PBC (range 0% – 27% and 0% – 27%, respectively).[25,26,29,30,32-38,40,41] Supplemental Table 1 demonstrates the

number contaminated cultures for PBC and for UCBC including the cultured micro-organisms. Sensitivity and specificity could not be calculated in all studies, because true and false negative values were not reported. A total of 8 studies reported the number of true and false negative outcomes for UCBC (Figure 5).[26,29,31,35-37,39,42] Four of these studies also collected paired samples for PBC and reported the DTA of both tests for clinically diagnosed EOS.[26,29,36,37] In these four studies, clinical diagnosis of sepsis was defined as the presence of two or more risk factors for EOS in combination with two or more laboratory values indicating sepsis according to Evidence Based Practice guideline on the Management of Neonatal Sepsis by the National Neonatology Forum.[43,44] The summary operation points from the meta-analyses of these four studies demonstrated a pooled sensitivity of 20.4% (95% CI 0.0 – 40.9) and specificity of 100.0% (95% CI 100.0 – 100.0) for PBC to detect a clinical diagnosis of EOS. The meta-analysis for UCBC including the four studies collecting paired samples from the cord and a peripheral vein, yielded a pooled sensitivity of 42.6% (95% CI 12.7 – 72.4%) and specificity of 97.8% (95% CI 93.1 – 100.0) for clinical EOS as demonstrated in the sROC plot in Figure 6. The DTA for PBC and UCBC combined as one index test (if one or both cultures were positive, the outcome was regarded as positive) yielded a pooled sensitivity and specificity of 44.0% (95% CI 20.5 – 70.5) and 97.8 (95% CI 89.6 – 99.6) for clinical EOS, respectively.

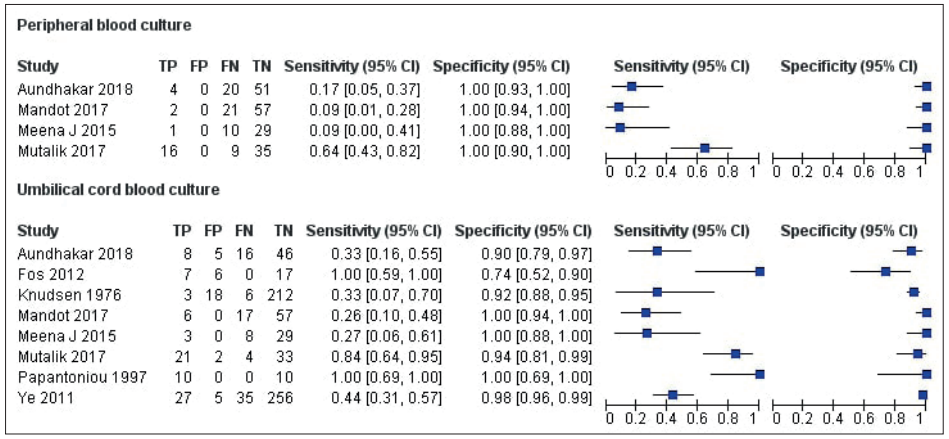

Figure 5. Forest plot of peripheral blood culture and umbilical cord blood culture for diagnosis of clinically diagnosed sepsis. CI: confidence interval; FP: false positive; FN: false negative; TN: true negative; TP: true positive.

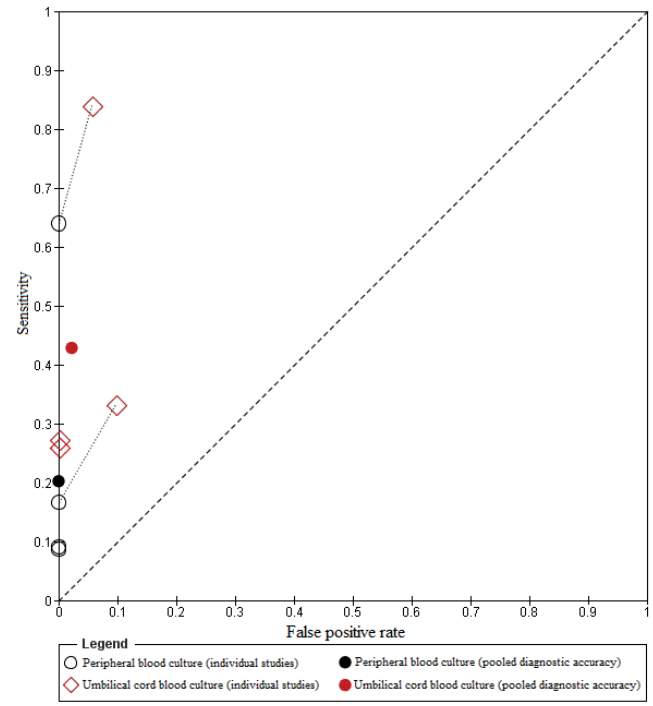

Figure 6. Summary receiver operating characteristic plot of the diagnostic test accuracy of peripheral blood culture and the diagnostic test accuracy of umbilical cord blood culture for diagnosis of clinically diagnosed early-onset sepsis. Only studies collecting paired blood samples from the umbilical cord and a peripheral vein are included. The solid circles represent the pooled sensitivity and specificity for each test.

Due to the low number of studies, the low numbers of inclusions in the individual studies and the following wide 95% CI, statistical comparison of the summary operation points of the sensitivity and specificity was not possible. There was large heterogeneity in the year of publication, number of EOS cases and region of conduction between the studies. Given the low number of studies reporting the DTA of both PBC and UCBC for clinical EOS, we were also unable to statistically assess the influence of these sources of heterogeneity on the results of the meta-analyses. Since only two studies with low sample sizes determined the DTA of non-conventional molecular culturing techniques using cord blood for EOS including different techniques (Sanger sequencing and 16S rRNA gene sequencing),[24,28] we decided to not include them in a meta-analysis.

Discussion

This is the first systematic review investigating the DTA of UCBC for diagnosing neonatal EOS at the time of birth. The currently used gold standard for diagnosing EOS, a conventional PBC, is a painful procedure for the infant and it is often a challenge for the attending clinicians to obtain an adequate sample volume. Besides it contributes to the risk for iatrogenic anemia, especially in VLBW infants. UCBC circumvents above mentioned challenges and risks, but the DTA of UCBC for EOS has not been studied thoroughly before. We demonstrated that, compared to the DTA of PBC, UCBC has a higher sensitivity (20 versus 43 percent) and comparable specificity (100 versus 97 percent) for clinically diagnosed EOS.

In the majority of infants with negative PBC, also a negative UCBC was found. We demonstrated a pooled specificity of 91.3% and a varying, but lower pooled sensitivity of 75.0% of UCBC for paired PBC outcomes. However, it is known that PBC results for the diagnosis of EOS can be false negative, especially when an inadequate sample volume is obtained, impairing the sensitivity.[45-47] This demonstrates the necessity to evaluate the accuracy of new diagnostic tests for EOS using 'clinically diagnosed EOS' besides 'culture proven EOS' as target outcome. In our study, the pooled sensitivity for clinically diagnosed EOS of UCBC was higher compared to PBC. Combining both UCBC and PBC as one index test, did not further increase the sensitivity.

Due to the risk for iatrogenic anemia it is not feasible to collect a large amount of neonatal peripheral blood.[14]. However, often the bacterial load in blood of septic neonates is low[48,49] and consequently, a larger sample volume is required for adequate sensitivity of blood culture in this population. One of the advantages of UCBC over PBC includes the opportunity to collect larger sampling volume more easily,[15] which might explain the increase in sensitivity. Meanwhile, collecting blood from an unsterile umbilical cord may introduce the risk for contamination and false positive results, possibly decreasing the specificity. One study reported a false positive rate for UCBC of 26.1%[31], but definition for contamination, (sterile) collecting technique, nor a false positive rate for PBC were not reported. Two studies reported no false positives for UCBC [36,37]. The other four studies reported a false positive rate ranging between 1.9 and 9.8% [26,29,35,42], of whom two did not report on their (sterile) collection technique [35,42]. The four studies included in the meta-analyses for clinically diagnosed EOS did use well-defined protocols for cord sterilizing prior to collection of cord blood samples,[26,29,36,37] thereby reducing risk of contamination as demonstrated by the low pooled false positive rate of 2.2% (i.e. pooled specificity of 97.8%).

Strengths and limitations

Outcomes of individual studies with small number of EOS cases lack power and random errors may have a large influence, especially on the sensitivity. This is the first meta-analysis, pooling the results of these small studies. Given the imperfect gold standard (PBC), it is valuable not only to compare the results of UCBC with those of paired PBC, but also to compare the accuracy of both tests for clinically diagnosed EOS.

There are also some limitations that need to be addressed. First, due to the sparse available data on paired samples of PBC with UCBC for clinically diagnosed sepsis, only four studies with a limited number of participants were included in the meta-analysis comparing the DTA of UCBC with the DTA of PBC for clinical EOS. Second, a clinical diagnosis of sepsis was defined as the presence of two or more risk factors in combination with two or more laboratory values indicating EOS in these four studies. This imperfect reference standard for EOS might have classified infants without bacterial or fungal sepsis as clinical sepsis cases and consequently the sensitivity of both tests might have been underestimated. Third, there was large heterogeneity in the year and country of publication and the study populations. Besides, not all studies reported if umbilical cord blood was obtained under sterile conditions, possibly influencing the DTA.

Currently, there is an enormous overtreatment with antibiotics in newborns with a suspicion or increased risk for EOS due to a lack of accurate tests. Withholding antibiotics in non-septic infants could prevent antibiotic related adverse events.[1,3,4] Based on the pooled sensitivity of 43% of an UCBC, it might be unlikely that clinicians will discontinue antibiotics in case of a negative UCBC, while a strong clinical suspicion for EOS exists. However, the increased sensitivity in combination with low risk for false positives (i.e. high specificity) will guide clinicians for pathogen specific targeted therapy more often when using UCBC. Sensitivity of both conventional PBC and UCBC conventional culture might be impaired by low bacterial load and intrapartum maternal antibiotic use, although the risk on the latter is decreasing nowadays by the use of specialized culture media removing antibiotics from the sample.[12] Since non-conventional molecular cultures, can also detect and amplify DNA of dead bacteria and may detect bacterial DNA even with lower bacterial loads in a sample, these techniques may further increase sensitivity. We identified only two studies were investigating the accuracy of different non-conventional culturing techniques,[24,28] limiting the possibility to draw conclusions.

Given the low cumulative number of EOS events in the meta-analysis, the limited number of studies investigating the accuracy of both conventional UCBC as well as PBC for clinically diagnosed EOS and the heterogeneity between studies in country

and year of publication, the results from the meta-analyses should be interpret cautiously. Larger prospective studies, including higher numbers of EOS cases are warranted. These studies should collect paired samples of the umbilical cord and a peripheral vein from the same infant and define the target outcome clinically diagnosed sepsis according to internationally accepted and validated methods, such as proposed by Vergnano (2016).[50] When implementing UCBC in clinical care, a (slight) increase in the false positive rate cannot be excluded and unnecessary prolongation of antibiotics in false positive cases should be taken into account. As demonstrated for PBC,[51] quality improvement initiatives might reduce the risk for false positives and might improve adoption of UCBC in future studies and in clinical care. These initiatives include staff education on aseptic collecting techniques and the preparation and availability of pre-made collection kits in the delivery room. If these strategies improve the diagnostic accuracy of UCBC for EOS needs to be assessed in future studies. Whether rapid culture-independent molecular diagnostic procedures such as PCR-based techniques can further increase the sensitivity for EOS diagnosis using umbilical cord blood also needs to be elucidated in future studies.

In conclusion, this systematic review demonstrated that UCBC has higher sensitivity and comparable specificity for clinical EOS, compared to PBC. Considering the larger blood volume that can be obtained from the umbilical cord via a painless procedure, the low risk of iatrogenic anemia, and low risk of false positives, UCBC might be considered as reference test in the diagnosis of EOS. However, given the limitations of the current available studies, future high quality studies on the accuracy of UCBC for EOS diagnosis are needed to validate these findings.

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Chapter 6

Can presepsin be of value in reducing unnecessary antibiotic exposure after birth?

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Antibiotics 2023 Apr; 12(4): 695

Abstract

Background: Due to a lack of rapid, accurate diagnostic tools for early-onset neonatal sepsis (EOS) at initial suspicion, infants often start unnecessarily with antibiotics directly after birth. We aimed to determine the diagnostic accuracy of presepsin for EOS before antibiotic initiation and investigate whether presepsin can be used to guide clinicians whether or not to start antibiotics.

Methods: In this multicenter prospective observational cohort study, all infants that started on antibiotics for an EOS suspicion were consecutively included. Presepsin concentrations were determined in blood samples collected at initial EOS suspicion (t=0). Next to this, samples were collected at 3, 6, 12 and 24 hours after initial EOS suspicion and from the umbilical cord directly after birth. The diagnostic accuracy of presepsin was calculated.

Results: A total of 333 infants were included of which 169 were born preterm. We included 65 term and 15 preterm EOS cases. At initial EOS suspicion the area under the curve (AUC) was 0.60 (95% confidence interval (CI) 0.50-0.70) in term born infants compared to 0.84 (95% CI 0.73-0.95) in preterm infants. A cut-off value of 645 pg/mL resulted in a sensitivity of 100% and specificity of 54% in preterm infants. Presepsin concentrations in cord blood and other time-points did not differ significantly compared to concentrations at initial EOS suspicion.

Conclusion: Presepsin is a biomarker with acceptable diagnostic accuracy for EOS (culture-proven and clinical) in preterm infants and might be of value in reducing antibiotic exposure after birth when appended to current EOS guidelines. However, the small number of EOS cases limits us to draw firm conclusions. Further research should be performed to evaluate whether appending a presepsin-guided step to current EOS guidelines leads to a safe decrease in antibiotic overtreatment and antibiotic related morbidity.

Introduction

Sepsis is one of the leading causes of neonatal morbidity and mortality.[1] Accurate and rapid diagnosis of early-onset neonatal sepsis (EOS), defined as sepsis onset within 72 hours of life, remains problematic mainly due to the non-specific signs and symptoms, and lack of reliable, timely diagnostic tools. In the Netherlands the national EOS guideline is used for the decision to start empirical antibiotics after birth. This guideline is comparable to the NICE guideline and follows a risk based approach including maternal and neonatal risk factors with a low threshold for the start of empirical antibiotic treatment.[2] Consequently, up to 58 times higher number of newborns receive antibiotic therapy for suspected EOS compared to the number of newborns with a positive blood culture.[3] Once started, antibiotic treatment is continued in about 30% of newborns despite a negative blood culture.[4,5]

This unnecessary antibiotic exposure increases antibiotic resistance, leads to aberrations in microbial colonization, and increases the risk for necrotizing enterocolitis in preterm infants and long-term complications such as asthma and obesity.[6,7] To diminish these complications, a strategy to safely reduce unnecessary antibiotic exposure in uninfected infants is urgently needed. Adding an early and accurate biomarker to the existing EOS guideline could be such a strategy. The diagnostic value of biomarkers used in daily care like C-reactive protein, procalcitonin and different interleukins have been studied for this purpose, but all lack sufficient accuracy at initial EOS suspicion.[8] In contrast, the biomarker presepsin (soluble CD14 subtype) seems to be promising for this purpose as concentrations increase rapidly after infection onset.[9,10]

After binding of bacterial ligands to the cell surface of monocytes and macrophages, CD14 is shedding from the cell surface and is subject to proteolysis.[11,12] This leads to release of various fragments and finally generation of a small soluble peptide structure (64 amino acids, 13kDa) named soluble CD14 subtype (sCD14-ST) or presepsin.[13] Reference ranges of presepsin in healthy infants have been determined, with conflicting results on possible differences between term and preterm born infants and influence of clinical characteristics and way of delivery.[14-16] Previous diagnostic studies on the diagnostic accuracy of presepsin for EOS in newborns have methodological flaws and a clear cut-off value with a high negative predicting value is consequently still lacking.[17,18]

Therefore, the primary aim of this multicenter prospective observational cohort study was to assess the diagnostic accuracy of presepsin directly after birth in all infants suspected for EOS and investigate whether presepsin can be used to guide

clinicians whether or not to start antibiotics. The secondary aim was to evaluate presepsin concentrations over time, as concentrations in EOS cases might change.[10]

Materials and Methods

Participants

In this multicenter prospective observational cohort study, all infants that started with antibiotics within the first 72 hours based on the Dutch EOS guideline were eligible for participation.[2] In the Dutch EOS guideline maternal and neonatal risk factors for EOS are categorized as red flags or minor criteria. In the presence of 1 red flag or ≥ 2 minor criteria it is advised to draw a peripheral blood culture and initiate antibiotics for a EOS suspicion. [2,19] Infants were included if both parents gave written informed consent. Infants were not eligible in case of a confirmed congenital infection (toxoplasmosis, rubella, cytomegalovirus infection, syphilis and herpes). Participants were consecutively recruited in one level III center (Emma Children's Hospital) and in one level II center with two locations (OLVG East and West) between August 2018 and June 2021. The study protocol was approved by the medical ethical committee (WO 18.020).

Antibiotic treatment was discontinued after 36 hours in case of a negative blood culture, reassuring clinical condition with no clinical indicators of possible infection. Infants that received antibiotics for ≥ 5 days in combination with growth of potentially pathogenic micro-organism in the blood culture were classified as culture-proven EOS. Infants that continued on antibiotics for ≥ 5 days for suspected EOS based on the clinician's judgement and having CRP levels ≥ 10 mg/l, but with negative blood cultures result were classified as clinical EOS. All other participants not meeting the criteria for culture-proven or clinical EOS were considered uninfected controls. Treatment and classification of participants as EOS cases or as controls was done blinded from the presepsin measurements.

Study samples

Combined with blood collection for standard care, 0.2 ml of blood was obtained before initiation of antibiotics directly after birth at initial EOS suspicion (t=0) and 3, 6, 12 and 24h afterwards in ethylenediaminetetraacetic acid (EDTA) tubes. If it was prenatally known that the infant would start on empirical antibiotics and be eligible for participation, a blood sample of the umbilical cord was collected as well. Blood was centrifuged at 2000g for 10 minutes at 18 °C. Plasma was extracted and stored at -80 °C until further handling.

After completion of participant recruitment, samples were thawed and presepsin levels were measured blinded by a rapid chemiluminescent enzyme immunoassay on the PATHFAST immunoanalyzer (Mitsubishi Chemical Medience corporation, Tokyo, Japan) according to the manufacturer's protocol using 100 *µl* plasma. If <100 *µl* plasma was available, samples were diluted with sodium chloride.

Statistical analysis

Baseline characteristics are presented descriptively. As reference ranges differ between term and preterm infants,[10,14-16] analyses were performed for term and preterm born infants separately. Presepsin concentrations directly after birth at initial EOS suspicion (t=0) were compared between EOS cases and uninfected controls using Mann-Whitney U-test. The receiver operating characteristic (ROC) curve was analyzed and the area under the curve (AUC) was calculated. The Youden's index was determined and the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined at this cut-off. Furthermore, the cut-off point with maximum sensitivity of 100% and the highest possible specificity was calculated in order to determine a cut-off value at which point no infected EOS cases would be missed. The 95% confidence intervals (CI) were calculated around the diagnostic accuracy measures. Subgroup analyses were performed for preterm born infants with gestational age <32 weeks, for infants with gestational age between 32 and 37 weeks and for the two different recruiting sites.

To evaluate whether presepsin concentration in the umbilical cord differed from the concentration in the first neonatal sample collected postpartum, the Wilcoxon signed rank test was used. Mixed model analysis was performed to evaluate whether presepsin concentrations changed during the first 24h after antibiotic initiation. Two-tailed P-values of <0.05 were considered statistically significant. Statistical analyses were performed in IBM Statistical Product and Service Solutions (SPSS) for Windows Version 28 (*IBM Corp*., Armonk, NY, USA) and R version 4.0.3.

Results

Participant inclusions

A total of 398 participants were eligible for inclusion, of whom parents of 65 infants did not consent to participation. Baseline characteristics of the 333 included infants are given in Table 1. Median time from birth to collection of the first postnatal sample (t=0) was 2.0 hours (interquartile range (IQR) 1.1 - 5.5).

A total of 65 term born infants and fifteen preterm born infants were classified as EOS cases. In all infants a blood culture was collected. Three EOS cases were culture-proven cases (0.9%). All three isolated bacterial pathogens were *Streptococcus agalactiae*. CRP concentrations during the first 48 hours after initial EOS suspicion were higher in both term and preterm born EOS cases (median: 45.1 mg/l (IQR: 33.2 – 64.6) and 65 mg/l (IQR: 44.9-81.8), respectively) compared to controls (median 5.6 mg/l (IQR: 2.1-17.0) and 1.1 mg/l (IQR: 0.6-4.0), respectively).

Table 1. Baseline characteristics

Clinical Values	Control (n=253)	Case (n=80)
Gestational age, median [IQR], weeks + days	36^{+0} [30^{+1} – 39^{+5}]	40^{+0} [37^{+6} – 41^{+0}]
Gestational age 32^{+0} to 36^{+6} weeks, n (%)	73 (29)	8 (10)
Gestational age < 32^{+0} weeks, n (%)	81 (32)	7 (9)
Birthweight, median [IQR], grams	2518 [1143-3416]	3405 [2909-3796]
Female sex, n (%)	111 (44)	35 (44)
Vaginal delivery, n (%)	181 (72)	54 (68)
Maternal age, mean (SD), years	32.7 (4.8)	33.1 (4.7)
Admission in level III center, n (%)	112 (44)	15 (19)
Septic mother (red flag), n (%)	9 (4)	8 (10)
Infection twin (red flag) , n (%)	1 (0.4)	0 (0)
Invasive GBS previous child, n (%)	0 (0)	1 (1)
Maternal GBS, n (%)	29 (12)	4 (5)
PROM [a], n (%)	43 (17)	19 (24)
PPROM [b], n (%)	53 (21)	9 (12)
Spontaneous premature birth, n (%)	106 (42)	10 (13)
Maternal fever > 38°C, n (%)	40 (16)	29 (36)
Maternal intrapartum antibiotics, n (%)	160 (63)	48 (60)
Neonatal red flag clinical symptom, n (%)	19 (8)	18 (23)
Well appearing, n (%)	70 (28)	15 (19)

[a] PROM defined as rupture of membranes > 24 hours before labor onset after a pregnancy of ≥ 37 weeks

[b] PPROM defined as rupture of membranes > 18 hours before labor onset after a pregnancy of < 37 weeks

IQR: interquartile range; PROM: premature rupture of membranes; PPROM: preterm premature rupture of membranes SD: standard deviation

Diagnostic accuracy of presepsin in term born infants

Presepsin concentrations were significantly higher in EOS cases compared to controls directly after birth in term born infants (p=0.04). The ROC curve at this time point is presented in Figure 1 and the AUC was 0.60 (95%CI [0.50-0.70]). The

Youden's index was highest at a cut-off of 874 pg/ml with 46% (95%CI [0.32-0.61]) sensitivity, 74% (95%CI [63-83]) specificity, a PPV of 53% (95%CI [38-69]) and NPV of 68% (95%CI [57-78]). At a cut-off of 307 pg/ml, sensitivity was 100% (95%CI [93-100]) but specificity was decreased to 2% (95%CI [0-7]).

Cord blood concentrations did not differ from concentrations in the first postnatal sample in EOS cases (p=0.77) and controls (p=0.11). Mixed model analysis demonstrated no significant changes in presepsin concentration over time in both EOS cases and controls (p=0.14 and p=0.46 respectively; Fig. 2A). The AUC using cord blood samples and at other time-points are demonstrated in Supplemental Table 1.

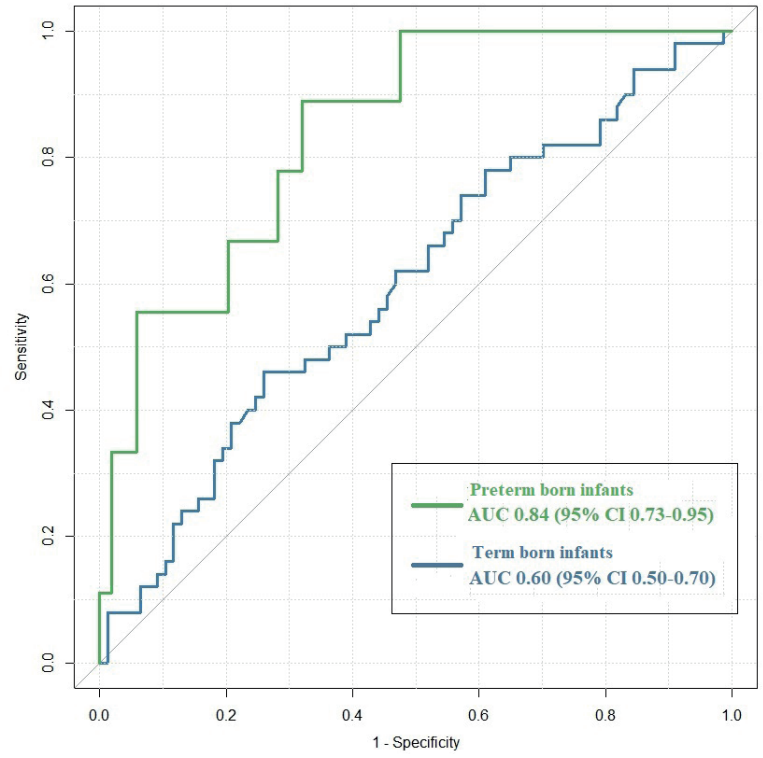

Figure 1. Receiver operating characteristic (ROC) curves for presepsin concentrations before antibiotic initiation at initial sepsis suspicion (t=0) differentiating between early-onset neonatal sepsis cases and uninfected controls in term born infants (blue) and preterm born infants (blue). Area under the curve (AUC) values were 0.60 (95% CI: 0.50-0.70) 0.84 (95% CI: 0.73-0.95) respectively.

In the secondary center 58 term EOS cases were recruited compared to 7 in the tertiary center. At initial EOS suspicion, the AUC of term born participants recruited in the secondary center was 0.56 (95%CI [0.44-0.67]) and 0.82 (95% CI [0.65-0.99]) in the tertiary center.

Diagnostic accuracy of presepsin in preterm born infants

Presepsin concentrations were significantly higher in EOS cases compared to controls directly after birth (t=0) in preterm infants (p<0.001). The ROC curve at initial EOS suspicion is presented in Figure 1 (AUC: 0.84; 95%CI [0.73-0.95]). The Youden's index was highest at a cut-off of 855 pg/ml. Sensitivity was 87% (95%CI [60-98]) and specificity was 68% (95%CI [58-77]), with a PPV and NPV of 28% (95%CI [16-43]) and 97% (95%CI [90-100]) respectively. A sensitivity of 100% (95%CI [78-100]) was reached with a specificity of 54% (95%CI [44-64]) at a cut-off value of 645 pg/ml.

Also in preterm born infants, no differences were found between cord blood concentrations and concentrations in the first postnatal samples in EOS cases and controls (p=0.12 and p=0.14 respectively). No significant changes in presepsin concentration were found over time in EOS cases (p=0.92) nor in controls (p=0.67) (Fig. 2B). The AUC in cord blood sampels and the other time points are shown in Supplemental Table 2.

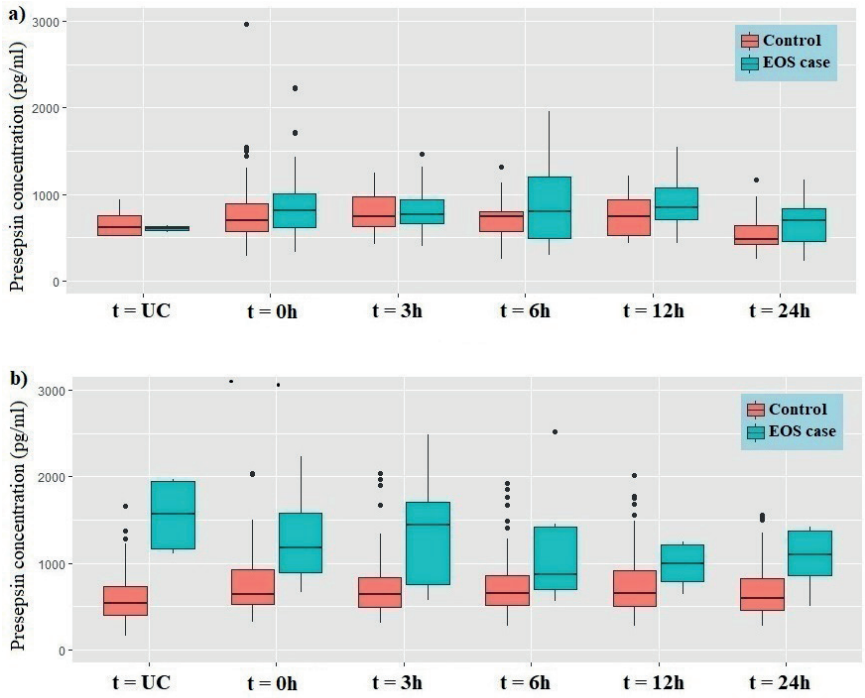

Figure 2. Boxplots of presepsin concentrations (pg/ml) before antibiotic initiation at initial sepsis suspicion (t=0) and the other time points for early-onset neonatal sepsis cases (blue) and uninfected controls (Red) in term born infants (a) and preterm born infants (b). EOS = early onset sepsis; h = hour; UC = umbilical cord blood.

A total of 7 preterm EOS cases were recruited in the secondary center and the other 8 in the tertiary center. Directly after birth the AUC was 0. 75 (95%CI [0.54-0.95]) in the secondary care center and 0.92 (95% CI 0.83-1.00) in the tertiary care center in preterm infants. In preterm infants with GA < 32 weeks, the AUC was 0.98 (95%CI [0.94-1.00]) and 0.73 (95%CI [0.56-0.89]) in preterm infants with GA between 32 and 37 weeks (Supplemental Table 3).

Discussion/Conclusion

In this prospective cohort study, we evaluated the diagnostic accuracy of presepsin for culture-proven and culture-negative EOS in a cohort of infants with an indication for empirical antibiotics based on the Dutch EOS guideline. The results of this study show that presepsin is a biomarker with acceptable diagnostics accuracy for EOS in preterm infants and can be of value in reducing antibiotic exposure after birth when appended to the Dutch EOS guideline. In term born infants the diagnostic accuracy was low.

The majority of studies on presepsin in neonatal sepsis included both EOS and LOS cases. [10,17,18] However, differences in presepsin concentrations between EOS and LOS cases and differences in reference ranges with increasing postnatal age underline the importance of studying them as separate entities.[14,20] To our knowledge, only five previous studies reported diagnostic accuracy measures of presepsin specifically for EOS.[13,21-24] None of these studies, however, included all patients with suspicion of EOS consecutively. These studies were either case-control studies, comparing culture-proven EOS cases to healthy controls without suspicion of EOS, or excluded patients with possible and/or culture-negative EOS from their analysis. Both approaches lead to bias and overestimation of the AUC. Besides, a different population is included via these approaches compared to the population this biomarker is intended to be use for in clinical practice, namely all infants with EOS suspicion. Consequently, these flaws limit the possibility to generalize applicability of previous results to clinical practice.[25-27]

The peripheral blood culture is still used as gold standard for diagnosing EOS, but its diagnostic accuracy has been questioned since cultures obtained from infants with clinical illness or increased inflammatory markers often remain sterile. Whether a prolonged antibiotic therapy is indicated in these infants is still subject of discussion. Due to the lack of accurate diagnostic tools for EOS and absence of consensus definition for clinical EOS clinicians often (up to 30%) decide to continue antibiotic treatment despite a negative blood culture.[4,5] In our cohort almost 25%

of all infants received a prolonged antibiotic therapy underlining the urgency of an international accepted consensus definition in order to prevent unnecessary antibiotic exposure.[28] Before implementing a biomarker in clinical care, it is pivotal to study the diagnostic accuracy in the population reflecting clinical practice including both culture-positive and culture-negative EOS. In contrast to previous studies, we therefore did consecutively include all infants with suspicion of EOS and defined cases as both culture-negative or culture-proven EOS and compared results with infants in which EOS was ruled out. In preterm infants the diagnostic accuracy of presepsin remained acceptable. This implies that if presepsin would be measured before initiating antibiotic a high sensitivity could be achieved when using a relative low cut-off value of 645 pg/ml and thus no culture-proven nor clinical EOS cases would be missed.[22] At the same time, specificity will still be reasonable and antibiotics could thus be withheld in a large part of uninfected infants with EOS suspicion that would have started empirically on antibiotics with current guidelines. Before appending a presepsin-guided step to current guidelines further research should be performed to evaluate whether implementation would indeed lead to a safe decrease in antibiotic prescriptions in preterm infants shortly after birth.

Results of our study show conflicting results regarding the diagnostic accuracy of presepsin in term versus preterm infants. This difference is not completely elucidated as this was not found in a recently performed meta-analysis.[10] Classification bias in term infants might be an explanation as we found a higher percentage of EOS cases than expected in term infants (65/164; 39%) and higher compared to preterm infants (15/169; 9%). Due to lack of consensus definition for EOS, one could hypothesize that part of uninfected term born control infants were misclassified as EOS cases. This might be a consequence of difference in rationale for antibiotic initiation as preterm infants are more often started on antibiotics based solely on risk factors in the absence of strong clinical suspicion for EOS and may have led to underestimation of the AUC in term born infants. Future studies consecutively including all infants suspected for EOS, with predefined definitions for culture-negative EOS, are warranted to determine whether classification bias affected our results in term born infants, or whether presepsin might not be an accurate biomarker for culture-negative EOS in term born infants.

Since collection of blood directly after birth can be challenging, especially in low-birthweight infants,[29,30] we evaluated the correlation of presepsin concentrations in umbilical cord blood and neonatal plasma samples from a peripheral vein within two hours after birth. Presepsin concentrations in umbilical cord blood in our cohort were comparable to concentrations in neonatal samples taken, as previously reported.[14] In line with our findings, a previous study reported that the discriminative ability of umbilical cord blood presepsin is high, as presepsin concentration were higher in cord blood of all 76 preterm EOS cases (range 1442-3988 pg/ml) compared to the 212 preterm controls (range 116-326 pg/ml) in that study.[31] Therefore, non-invasive collected umbilical cord blood might be used for presepsin measurement if there is a prenatal EOS suspicion.

Presepsin concentrations may be affected by other factors than EOS, such as the route of delivery and the presence of respiratory distress syndrome (RDS). The main goal of a new EOS biomarker is discriminate between EOS cases requiring antibiotics and uninfected controls in the population of all infants with EOS suspicion. It is most important not to miss any of the EOS cases, but still have high specificity so you can withhold antibiotics in uninfected cases simultaneously. We demonstrated that this is possible using a relative low-cut off value and factors such as RDS to not significantly impact the discriminative ability of presepsin.

Strengths of this study include the large sample size, making it possible to perform analyses stratified on gestational age. Furthermore, all infants started antibiotics for suspected EOS were recruited consecutively and included in the analysis, so bias is minimized and our results provide a realistic view on the potential of this biomarker in the clinical practice.[26] The longitudinal collection of samples, including umbilical cord blood, provided valuable information on the course of presepsin during the first 24 hours in infected and uninfected infants.

Limitations of this study and other studies on biomarkers for neonatal sepsis, include the lack of a consensus case definition for EOS, increasing the risk for classification bias. Furthermore, we did not compare the diagnostic accuracy of presepsin with other biomarkers such as CRP, PCT and IL-6, but other studies and meta-analysis demonstrated a higher accuracy of presepsin compared to these other biomarkers at initial EOS suspicion.[32,33] The small number of EOS cases (culture proven and clinical EOS) is another limitation leading to wide confidence intervals of the sensitivity and specificity.

In conclusion, presepsin is a biomarker with acceptable diagnostic accuracy for EOS (culture-proven and clinical) in preterm infants and might be of value in reducing antibiotic exposure after birth when appended to current EOS guidelines. However, the small number of EOS cases limits us to draw firm conclusions. Presepsin can be measured in umbilical cord blood with results comparable to samples taken directly after birth. Further research should be performed to evaluate whether appending a presepsin-guided step to current EOS guidelines leads to a safe decrease in antibiotic overtreatment and antibiotic related morbidity.

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Chapter 7

Potential of Molecular Culture in Early-onset Neonatal Sepsis Diagnosis: a Proof of Principle Study

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Microorganisms 2023 Apr; 11(4); 960

ABSTRACT

Background

Delay in time-to-positivity of a peripheral bacterial culture (PBC), the gold standard for early-onset neonatal sepsis (EOS) diagnosis, has resulted in overuse of antibiotics. Here, we evaluate the potential of the rapid Molecular Culture (MC) assay for quick EOS diagnosis.

Methods

In the first part of this study, known positive and spiked blood samples were used to assess the performance of MC. In the in vivo clinical study, the second part of this study, all infants receiving antibiotics for suspicion of EOS were consecutively included. At initial EOS suspicion, a blood sample was collected for PBC and MC.

Results

MC was able to detect bacteria present in low concentrations in the spiked samples. In the clinical study, MC was positive in one infants with clinical EOS (*Enterococcus faecalis*), which was not detected by PBC and in two infants without clinical sepsis (*Streptococcus mitis* and multiple species), referred to as contamination. The other 37 samples were negative both by MC and PBC. MC seems to be able to detect bacteria even when the bacterial load is low.

Discussion

The majority of MC and PBC results were comparable and the risk for contamination and false positive MC results seems to be limited. Since MC can generate results within 4 hours following sampling compared to 36-72 hours in PBC, MC may have potential to replace conventional PBC in EOS diagnostics in order to guide clinicians when to discontinue antibiotic therapy several hours after birth.

Keywords: Early-onset sepsis; Neonates; Molecular culture; Diagnosis; IS-pro

INTRODUCTION

Early-onset neonatal sepsis (EOS), defined as sepsis within the first 72 hours of life, has high morbidity and mortality.[1,2] The gold standard for EOS diagnosis is a conventional peripheral blood culture (PBC), but time to positivity is commonly up to 36-72 hours and thus is of no value to rule out EOS at time of initial presentation.[3] Since delay in treatment of EOS may lead to rapid deterioration or even death, antibiotics are often initiated empirically awaiting PBC results. Roughly 5% of all newborns and over 85% of neonates with a gestational age < 30 weeks are exposed to antibiotics empirically directly after birth,[4-6] while the incidence of culture-proven EOS is only 0.1 – 1.2%.[7-9] In the vast majority of infants that are started on antibiotics empirically, treatment is thus discontinued after 36-72 hours if the PBC remains negative. Besides increasing the risk for multidrug resistant infections, this overexposure to antibiotics early in life leads to aberrations in microbial colonization, increasing the risk for adverse long-term outcomes such as asthma and obesity.[10] Besides, both infants and their mothers need to be hospitalized, often separated from each other, leading to increased unnecessary hospital costs.

To reduce unnecessary hospitalizations and antibiotic treatment in neonatology intensive care units, it is pivotal that rapid diagnostic tools with a high negative predictive value become available to exclude EOS faster.[11] Molecular techniques that directly detect bacterial DNA might circumvent delay of PBC by providing rapid results. Currently, the available quality of evidence for application of molecular techniques in EOS is moderate to low for all studied techniques such as qPCR and 16S rRNA sequencing, due to inconsistency and imprecision of results.[11] Disadvantage of qPCR testing includes restrictions based on a limited number of microbial targets based on the selected PCR panel. Drawback of unrestricted sequencing techniques include high costs, delay in reporting up to one or more days, lack of standardization and complexity of the procedure.[12] A novel broad-scope molecular technique with capacities to circumvent this delay is the Molecular Culture (MC; inBiome, Amsterdam, the Netherlands) assay. MC is an unrestricted PCR based technique that detects and identifies bacterial DNA via the 16S-23S rRNA gene interspace regions, of which the length signature combined with small sequence polymorphisms is specific for microbial species.[13,14] This unrestricted technique allows for identification of all bacteria up to species level and generates results within 4 hours. Previous studies in adults comparing MC with results of conventional culturing in samples from abscesses and empyema are very promising, demonstrating that MC detected bacteria in 100% of conventional culture-positive samples. Additionally, MC could detect clinically relevant pathogens that were missed by conventional culture.[14] The sensitivity of conventional PBC for diagnosis

of sepsis in neonates is being questioned. In contrast to conventional PBC, MC may detect bacteria in blood even when bacterial load is low, is not influenced by previous antibiotic exposure and is able to detect species uncultivatable by PBC.[14] Therefore, it is hypothesized that MC may detect more relevant pathogens in infants with suspicion for EOS compared to a conventional PBC and the main limitation is expected to be the risk for contamination. However, studies investigating the risk for false positive MC results and its potential as diagnostic test in blood samples from infants suspected of sepsis are lacking. Therefore, we aimed to assess the ability of MC to detect bacteria in vitro using spiked samples, in clinical samples that were previously shown to be positive and to investigate the risk for false positive results and its potential in cord blood and peripheral blood in a clinical cohort of neonates suspected of sepsis.

METHODS

Part one: Positive blood samples and spiking experiments

To test the efficacy of the MC method on bacterial DNA isolated from blood samples, we used samples that were previously collected and processed for molecular detection of bacteria by a panel of specific qPCRs (MARS study).[15] In this study, the Polaris method was used to enrich bacterial DNA in 5ml of blood for improved downstream detection. All methods have been described previously.[16] A total of 15 samples were selected which had previously been found positive for nine different pathogens with either a high load (Ct<30) or a low load (Ct>30). DNA was used in the MC assay (inbiome, Amsterdam, the Netherlands) according to the manufacturer's instructions. Resulting loads as expressed in Log2 Relative Fluorescence Units (RFU) were compared to Ct values.

To test the performance of the Polaris method on small volumes of blood, we spiked 1ml aliquots of blood from a healthy volunteer with three different bacterial species. *Staphylococcus haemolyticus, Escherichia coli* and *Proteus mirabilis*, as representative Gram positive and Gram negative bacterial species were grown overnight on blood agar. From these colonies, a suspension was made in PBS of 0.5 McFarland. These suspensions were diluted tenfold in PBS, after which 10µl of each dilution was added to 6ml of blood. The spiked blood was split into six portions of 1ml. Three of these were pre-processed according to the Polaris protocol as described previously after which automated DNA extraction was performed on the EasyMAG machine (BioMerieux, Marcy l'Etoile, France).[16] Three were directly processed with the EasyMAG machine (see below).

Part two: Clinical study in infants with suspicion for early-onset sepsis

In the second part of this study, we performed a clinical study using samples collected from infants with EOS suspicion. In this prospective observational study, we consecutively included all infants starting on antibiotics within the first 72 hours of life for suspicion of EOS. Participants were recruited in a level 2 center with two locations (OLVG East and West) between July 2020 and June 2021. Prescription of antibiotics for EOS suspicion was done according to the Dutch guideline. In this guideline, maternal risk factors and neonatal risk factors or symptoms of EOS are categorized as red flags or minor criteria.[4] In the presence of 1 red flag and/or ≥ 2 minor criteria, it is advised to initiate antibiotics empirically for suspicion of EOS. The study protocol was approved by the medical ethical committee of the MEC-U (WO 18.020). All parents gave written informed consent. Infants were not eligible in case of a confirmed congenital infection (toxoplasmosis, rubella, cytomegalovirus, syphilis and herpes).

Discontinuation of empiric antibiotics after 36-72 hours was considered in case of a negative PBC and when the clinical condition was reassuring in combination with repeated low C-reactive protein (CRP) concentrations. Infants with a positive PBC for a micro-organisms considered as a true pathogen were classified as culture-proven EOS cases. Culture-negative infants who, according to the judgement of the treating physician, continued with antibiotics for ≥ 5 days and having CRP levels ≥ 10 mg/L were defined as clinical EOS cases. All other participants were classified as uninfected infants. Classification of participants as EOS cases or as uninfected infants was done blinded from the MC results.

Simultaneously to blood collected for conventional PBC at initial EOS suspicion, 1.0 ml of blood was obtained in an ethylenediaminetetraacetic acid (EDTA) tube from term born infants. We decided not to collect peripheral blood for MC from preterm infants, as this may increase the risk for iatrogenic anemia due to their low circulating blood volume.[17] If it was prenatally known that the infant would start on antibiotics directly after birth and thus would be eligible for participation, an additional blood sample was collected from the umbilical cord from both term and preterm born infants. These samples were collected in a standardized manner after sterilization of the umbilical cord as previously described.[18] Directly after collection, the blood was stored at -80 °C until further handling.

Sample handling processing

All participant samples and half of the spiked samples were pre-processed with the Polaris method as described previously, after which DNA extraction was

performed on the EasyMAG machine (BioMérieux) with the Specific A protocol as described by the manufacturer.[16] DNA was eluted in 70µl. The MC analyses were performed according to a previously published protocol by the manufacturer.[13] Identified pathogens by MC were identified and quantified with the online analysis platform Antoni (inBiome). Bacteria found in clinical samples were classified as contamination or as clinically relevant by two independent experts (TdM, DB), blinded from the other participant characteristics and PBC results.

Statistical analysis

Baseline characteristics are presented descriptively for EOS cases and uninfected controls separately. Continuous data was presented as means (standard deviation) or median (interquartile range) depending on the normality of the distribution. Categorical data was presented as the number (percentage). Results of MC were compared with results of the conventional PBC for (clinical) EOS cases and uninfected infants. Statistical analyses were performed in R version 4.0.3.

RESULTS

Part one: Positive and spiked blood samples

In 14 of 15 known positive blood samples, the correct pathogen was detected and identified with MC. The sample in which MC did not detect anything was a sample with a low load of *S. aureus* (Ct 37,6). All comparisons are shown in table 1. To investigate the relation between the MC load and the Ct values as found by the qPCR panel, a linear regression analysis was performed between Ct values and Log2 transformed Relative Fluorescence Units of the MC. Log2 transformation was done as Ct value should also be seen as a log2 scale, as it represents measurements of the doubling cycles of PCR. Regression showed a good correlation between Ct values and MC load, with an R^2 of 0,78 with an associated p value of $2,92 \times 10^{-5}$ (figure 1).

Spiked samples were tested as blood volumes available for diagnostics from infants suspected for EOS are typically low (1ml). As the Polaris method has been designed to enrich microbial DNA in larger volumes of blood, we tested whether this method would have additional value in these small volumes of blood. The test was performed on three replicates on three different bacterial species, *Staphylococcus haemolyticus*, Proteus mirabilis and Escherichia coli. Pre-processing with the Polaris method showed a strong and comparable increase in measured load for all three bacterial species tested (8.4 fold for *S.haemolyticus*, 8,3 fold for *P.mirabilis* and 7,6 fold *for E.coli*), see figure 2.

Table 1. Detection of different bacterial species with either a high (Ct<30) or a low (Ct>30) load isolated from blood with Polaris pre treatment.

	High		Low	
	Ct	MC load	Ct	MC load
Enterococcus faecalis	24,89	199345	35,55	8548
Enterococcus faecium	28,46	114870	36,05	11143
Escherichia coli	26,77	134370	34,03	27040
Klebsiella pneumoniae	21,75	413780	33,84	33867
Morganella morganii			32,55	13174
Pseudomonas aeruginosa	29,57	67570	37,68	500
Salmonella enteritidis	27,33	100043		
Staphylococcus aureus	31,1	123076	37,64	0
Streptococcus pneumoniae	27,71	75570		

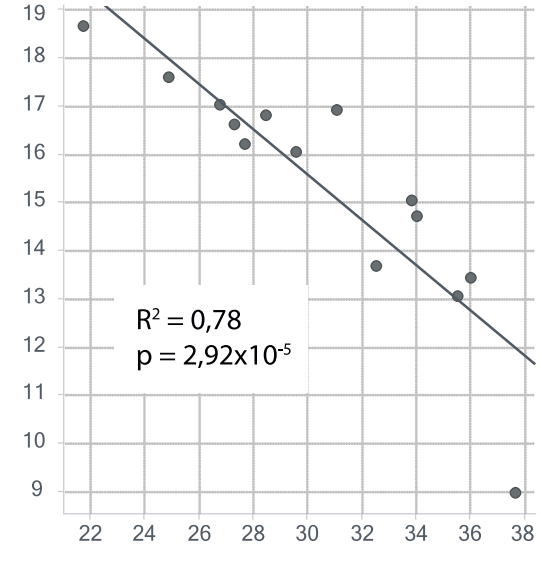

Figure 1. A linear correlation can be seen between Ct values as measured by specific qPCR and MC load, as measured by Log2 transformed Relative Fluorescence Units (RFU) ($R^2 = 0,78$, $p = 2,92 \times 10^{-5}$).

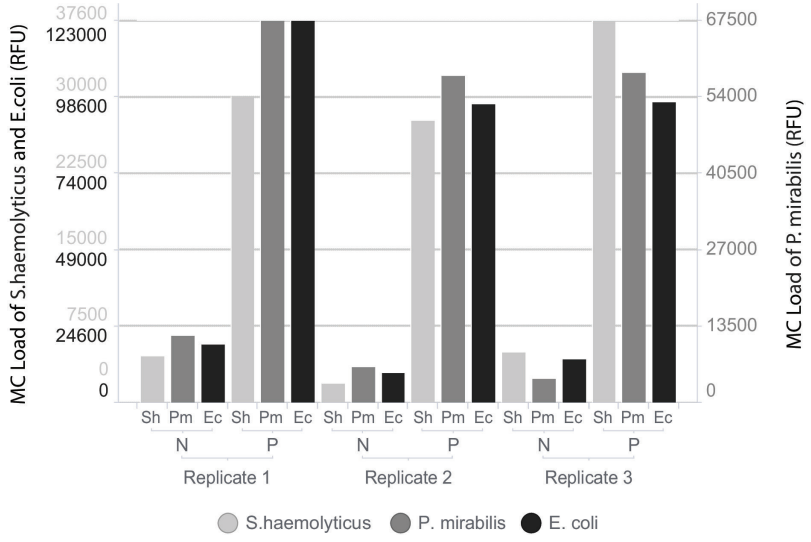

Figure 2. Comparison of DNA extraction with (P) or without (N) Polaris pre-treatment. The test was performed on three replicates on three different bacterial species, Staphylococcus haemolyticus (Sh), Proteus mirabilis (Pm) and Escherichia coli (Ec). Molecular Culture loads are expressed in Relative Fluorescence Units (RFU). Adding Polaris pre-treatment resulted in significantly increased detected loads.

Part two: Clinical study in infants with suspicion for early-onset sepsis

A total of 38 eligible participants starting on antibiotics for a suspicion of EOS were included. From all participants a PBC was performed at initial sepsis evaluation and before start of antibiotics. None of the participants were classified as culture-proven EOS, 17 infants (44.7%) as clinical EOS cases and 21 (55.3%) as uninfected infants. Of the 38 included participants, four cord blood samples and 36 peripheral samples were collected for MC analysis. From two participants (one clinical EOS case and one uninfected infant) both cord blood and peripheral blood samples were collected. In two participants (both uninfected infants), only cord blood was collected. Baseline characteristics are given in Table 2.

None of the infants were exposed to antibiotics before collection of samples for both PBC and MC. PBCs were negative in all 38 participants. MC was positive in three of 40 (7.5%) samples. All three positive samples were peripheral neonatal samples. In one infant classified as clinical EOS, *Enterococcus faecalis* was identified by MC. In one participant classified as an uninfected infant *Streptococcus mitis* was detected and in another uninfected infant MC showed multiple species (*Sneathia vaginalis*, *Prevotella bivia*, *Phocaeicola dorei* and *Bacteroides fragilis*). No umbilical cord

blood samples were collected from these three participants. In the other 37 of 40 (92.5%) MC samples, results were negative and thus comparable to PBC results. The MC was negative in all four cord blood samples.

Table 1. Baseline characteristics

	Controls (n=21)	Clinical EOS cases (n=17)
Gestational age, median [IQR], weeks + days	38^{+1} [36^{+0} - 40^{+6}]	40^{+2} [38^{+6} - 41^{+1}]
Birthweight, median [IQR], grams	3300 [2697 - 3835]	3676 [3353 - 4126]
Female Gender, n (%)	9 (43%)	4 (24%)
Vaginal delivery, n (%)	6 (29%)	8 (47%)
C-reactive protein, median [IQR], mg/l	6,8 [1,7 - 17,0]	48,0 [32,5 - 70,0]
Maternal age, mean (sd), years	32,0 [30,0 - 34,0]	34,0 [29,3 - 35,5]
5 minute Apgar score, median [IQR]	10 [10 - 10]	9 [7-10]
Maternal fever, n (%)*	9 (43%)	10 (59%)
Maternal GBS colonization, n (%)	6 (29%)	1 (6%)
PROM, n (%)**	14 (67%)	8 (47%)
Maternal IAP, n (%)	10 (48%)	7 (41%)
Well-appearing at inclusion, n (%)***	11 (52%)	1 (6%)

*maternal fever defined as intrapartum temperature >38 °C
**PROM defined as rupture of membranes > 18 hours before labor onset after a pregnancy of < 37 weeks and >24 hours after a pregnancy of ≥ 37 weeks
***Asymptomatic infants without (non-specific) clinical signs such as tachypnea, dyspnea temperature instability starting on antibiotics solely based on maternal risk factors for early-onset neonatal sepsis
GBS: Group B Streptococcus; IAP: Intrapartum antibiotic prophylaxis; IQR: Interquartile range; PROM: Premature rupture of membranes; sd: Standard deviation

DISCUSSION

In this prospective cohort study, we demonstrated the applicability of MC to detect bacteria in blood. Furthermore, we demonstrated that pre-processing with the Polaris method showed improved detection of bacteria, even in low volumes of blood. Furthermore, we investigated the risk for false positive results and potential as diagnostic test in a cohort of infants suspected for EOS. Bacteria were detectable by MC in spiked and known positive samples, even when present in low concentration. All conventional PBCs of included infants were negative and MC results were similar in 92.5% of samples. MC detected *Enterococcus faecalis* in one clinical EOS case, which was missed by PBC, and was positive in two uninfected infants, which are suspected to be false positive.

Diagnostic tools with rapid turnaround time and a high negative predictive value are needed to safely decrease antibiotic overuse in unaffected infants suspected of EOS. The past decades molecular techniques have become available for identification of bacterial DNA, such as real time PCR, 16S rRNA gene sequencing and MC.[11,19] qPCR techniques are restricted by the used panel, so it only detects a pre-defined set of bacteria.[20] Unrestricted techniques such as 16S sequencing are costly and have a reporting delay of one to several days.[12] MC, on the other hand is a unrestricted technique that allows for identification of all bacteria to the species level and generates results within 4 hours. In contrast to conventional PBC, this molecular technique is not influenced by maternal intrapartum antibiotic prophylaxis and is able to detect species uncultivatable by PBC.[14] Consequently, the sensitivity of MC for EOS might be higher compared to a conventional PBC. On the other hand, this sensitive method also increases the risk for false positive results. Here we demonstrated that MC is able to detect bacteria present in low loads using spiked and known positive samples.

In our study including infants with EOS suspicion, all conventional PBC results were negative. MC results were comparable to the PBC in the majority of blood samples. Besides, MC allowed for detection of *Enterococcus faecalis* in one clinical EOS case, which had negative PBC. Notably, *Enterococcus faecalis* is a micro-organism which is difficult to detect using conventional techniques,[21,22] illustrating the limited sensitivity of standard PBC. The risk for false positive MC results in peripheral blood and cord blood of neonates suspected for EOS seemed to be limited, as only two other samples were positive by MC.

Discrepancies between MC results and PBC results can be explained by a number of factors. First, MC can detect certain types of bacteria, both true pathogens and contaminants, that are unable to grow in PBC medium due to fastidious growth requirements.[22,23] This is shown by the positive samples of one clinical EOS case (*Enterococcus faecalis*) and two positive samples from uninfected infants is this cohort. Based on the detected bacteria in uninfected controls (*Streptococcus mitis* and a sample with multiple species associated with vaginal and rectal microbiota), however, these bacteria have been considered to be contaminants. Besides, PBC results may be false negative in case of low bacterial loads and previous antibiotic exposure. Furthermore, both tests are at increased risk for false negative results in case of limited and inadequate sampled blood volume, consequently leading to discrepant results.

New diagnostic tests can either replace the original test, be applied as triage test before the current test or applied as add-on test to the existing standard.[24] Based on

the fast turnaround time of the MC and the potential ability to predict negative PBC results, it might be suitable to replace the conventional culture in the current EOS guidelines. This could guide clinicians to discontinue antibiotics in case of negative MC if clinical condition and other laboratory measures are reassuring within 4 hours, instead of after 36-72 hours when using PBC. This would decrease the duration of unnecessary antibiotic exposure, reduce unnecessary hospitalization and costs and lead to improvement of microbiota related short- and long-term outcomes. As there were no positive PBCs in our cohort, we were unable to investigate whether the MC will detect all cultured bacteria by PBC in infants, as demonstrated in a previous study in adults. Here, we demonstrated that the risk for false positive MC results seems to be limited. Before clinical application, the value of MC needs to be validated in larger cohorts including culture-positive EOS cases.

Collecting blood for a PBC in infants can be challenging and is a painful procedure. A limited volume is often sampled due to risk for iatrogenic anemia in infants,[25-27] but this may increase the risk for false negative results. Collection of blood from the umbilical cord allows sampling of a larger volume, which increases the sensitivity of a blood culture.[28] Previous standard operating manuals have been designed for sterile collection of cord blood.[18] The four cord blood samples collected in this study all had negative MC results. Due to the limited number of cord blood samples, future research needed to validate that cord blood is of added value for molecular bacterial culturing in EOS diagnostics.

Strengths of this study include the pre-clinical testing of the efficacy of the MC to detect bacteria in known positive and spiked blood samples and the evaluation of the added value of a preprocessing technique that specifically enriches bacterial DNA. The prospective, consecutive inclusions of patients allows generalization of results to clinical practice. Furthermore, results of the MC were interpreted blindly from PBC results and other participant's data. Limitations of this study include the lack of culture-positive EOS cases, hampering the opportunity to investigate whether MC can also predict a positive PBC. Furthermore, clinicians were trained to collect samples sterile, but samples might still have been contaminated during collection or during the analysis. Finally, the sample size of this cohort was relatively small and limited blood volume available in infants might have impacted the results of the MC. To further investigate the potential of MC for EOS diagnosis, we are planning to perform a larger study. We also aim to include samples from older infants, children and adults to determine whether this technique may be suitable for sepsis diagnosis in other populations.

Conclusion

MC was able to detect bacteria in low bacterial concentrations in positive and spiked samples. This is the first study to investigate the risk for false positive MC results and the potential of MC as diagnostic test in neonates suspected of sepsis. All PBC results and the majority of MC results were negative too, the risk for false positive MC results seems to be limited. MC allowed for detection of *Enterococcus faecalis* in one clinical EOS case, which was missed by PBC, and two positive tests in uninfected infants, considered to be contamination. Since MC can generate results within 4 hours following sampling, compared to 36-72 hours in PBC, MC may guide clinicians faster to discontinue antibiotic therapy in case of a negative MC test and reassuring clinical condition of the infant. Future prospective studies are needed in larger cohorts containing culture-positive EOS cases to evaluate the accuracy of the rapid MC technique for EOS diagnosis, avoiding the delay characterizing PBC. This could dramatically reduce antibiotic overuse at neonatology wards.

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Chapter 8

Multispecies Probiotic for the Prevention of Antibiotic-Associated Diarrhea in Children: A Randomized Clinical Trial

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JAMA Pediatrics 2022 Sep; 176(9): 860-866.

Abstract

Importance: The efficacy of multispecies probiotic formulations in the prevention of antibiotic-associated diarrhea (AAD) remains unclear.

Objective: To assess the effect of a multispecies probiotic on the risk of AAD in children.

Design, setting, and participants: This randomized, quadruple-blind, placebo-controlled trial was conducted from February 2018 to May 2021 in a multicenter, mixed setting (inpatients and outpatients). Patients were followed up throughout the intervention period. Eligibility criteria included age 3 months to 18 years, recruitment within 24 hours following initiation of broad-spectrum systemic antibiotics, and signed informed consent. In total, 646 eligible patients were approached and 350 patients took part in the trial.

Interventions: A multispecies probiotic consisting of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis*W51, *Lactobacillus acidophilus* W37, *L acidophilus* W55, *Lacticaseibacillus paracasei* W20, *Lactiplantibacillus plantarum* W62, *Lacticaseibacillus rhamnosus* W71, and *Ligilactobacillus salivarius* W24, for a total dose of 10 billion colony-forming units daily, for the duration of antibiotic treatment and for 7 days after.

Main outcomes and measures: The primary outcome was AAD, defined as 3 or more loose or watery stools per day in a 24-hour period, caused either by *Clostridioides difficile* or of otherwise unexplained etiology, after testing for common diarrheal pathogens. The secondary outcomes included diarrhea regardless of the etiology, diarrhea duration, and predefined diarrhea complications.

Results: A total of 350 children (192 boys and 158 girls; mean [range] age, 50 [3-212] months) were randomized and 313 were included in the intention-to-treat analysis. Compared with placebo (n = 155), the probiotic (n = 158) had no effect on risk of AAD (relative risk [RR], 0.81; 95% CI, 0.49-1.33). However, children in the probiotic group had a lower risk of diarrhea regardless of the etiology (RR, 0.65; 95% CI, 0.44-0.94). No differences were observed between the groups for most of the secondary outcomes, including adverse events.

Conclusions and relevance: A multispecies probiotic did not reduce the risk of AAD in children when analyzed according to the most stringent definition. However, it reduced the overall risk of diarrhea during and for 7 days after antibiotic treatment. Our study also shows that the AAD definition has a significant effect on clinical trial results and their interpretation.

Introduction

Antibiotic-associated diarrhea (AAD) is a common complication of antibiotic treatment.[1,2] Several different definitions of AAD have been proposed, including "diarrhea that occurs in relation to antibiotic treatment with the exclusion of other etiologies."[3,4] In clinical practice and in most clinical trials, microbiological tests are not routinely performed to exclude an infectious origin of AAD, confirming its etiology.[5] AAD is considered to result from gut dysbiosis by antibiotics, which may provoke overgrowth of specific pathogens, most prominently *Clostridioides difficile*, and lead to altered function of the microbiota.[6,7]

The most thoroughly studied preventive intervention for AAD is the administration of probiotics, defined as "live microorganisms, that when administered in adequate amounts, confer a health benefit on the host."[8] According to a 2019 Cochrane review,[2] probiotics as a group have a moderate protective effect on the prevention of pediatric AAD. The results of individual studies in this review varied depending on the dose of probiotic, with higher doses of 5 billion colony-forming units (CFU) or more per day demonstrating a better effect. Among the 33 included studies, only 6 randomized clinical trials (RCTs) of limited size investigated combinations of more than 3 probiotic strains, with varied results. [9-14] Thus, the effect of multispecies probiotic supplementation on AAD incidence in children remains in question. In adult patients, one of the previously studied multispecies probiotics consisted of 9 bacterial species.[15,16] In the current study, we aimed to assess the efficacy of a comparable multispecies probiotic mixture in the prevention of AAD in a pediatric population.

Methods

Study Design

A parallel-group, randomized, quadruple-blind placebo controlled RCT was conducted in pediatric clinical and outpatient wards of 3 Dutch and 2 Polish hospitals (Supplement Table 1). The study was prospectively registered in ClinicalTrials.gov database (NCT03334604), and the protocol was published in a peer-reviewed journal.[17] Consolidated Standards of Reporting Trials (CONSORT) guidelines were followed for reporting trial results.[18]

Ethics

The study was approved by the Bioethics Committees of the Medical University of Warsaw (KB/198/2017) and Amsterdam UMC (2019.227). Written informed consent was obtained by the parents or the legal guardians of all participants. During the study, 2 changes in the study protocol were introduced in response to an unsatisfactory inclusion rate. First, recruitment in additional centers was started, as planned in the study protocol. Second, the lower age limit of the participants was adjusted from 6 months to 3 months.

Participants

Eligibility criteria included age from 3 months to 18 years, recruitment within 24 hours following initiation of broad-spectrum oral or intravenous antibiotic therapy, and signed informed consent. The exclusion criteria were as follows: use of antibiotics within the previous 4 weeks; use of probiotics, proton pump inhibitors, laxatives, or antidiarrheal drugs within the previous 2 weeks; severe infection or life-threatening illness at recruitment (i.e., indicated or probable admission to an intensive care unit); preexisting diarrhea within the previous 4 weeks based on patient's or caregiver's report; severe chronic disease (e.g., cancer, inflammatory bowel disease, short-bowel syndrome); diagnosed primary or secondary immune deficiency; required tube-feeding; exclusive breastfeeding; and known allergy or hypersensitivity to any component of the study product.

Randomization and Masking

A block randomization in blocks of 4 was performed centrally in a 1:1 ratio by Winclove Probiotics B.V. with use of a computer random-sequence generator, by a person not otherwise involved in the study. The randomization lists were stored in sealed, opaque envelopes at the study centers. The participants, caregivers, and all investigators, including data collectors and outcomes assessors, were blinded until the primary data analysis was performed. Probiotic and placebo were packed identically and had the same appearance, taste, and smell.

Procedures and Interventions

The parents were instructed to administer 2 sachets of the study product daily to their children for the duration of antibiotic treatment and for 7 days after, up to a maximum of 17 days, starting within 24 hours of the first antibiotic dose. The multispecies probiotic (Ecologic AAD 612; Winclove Probiotics B.V.) contained 8 bacterial strains: *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W37,*Lacidophilus* W55, *Lacticaseibacillus paracasei* W20, *Lactiplantibacillus plantarum* W62, *Lacticaseibacillus rhamnosus* W71,and *Ligilactobacillus salivarius* W24 (formerly known as *Lactobacillus salivarius* W24), for a total dose of 5 billion CFU per sachet (10 billion CFU daily).

The data on outcomes were collected using study diaries during antibiotic treatment and for 7 additional days. The consistency was reported according to the Amsterdam Infant Stool Scale (AISS)[19] or Bristol Stool Form Scale (BSFS),[20] depending on participant's age. In case of diarrhea occurrence, the participants' caregivers were requested to provide stool samples for testing for rotavirus, adenovirus, and norovirus by immunoassay; *Campylobacter species, Salmonella species, Shigella species,* and *Yersinia species* by isolation from stool cultures; and *C difficile* in children older than 1 year by detection of glutamate dehydrogenase in conjunction with toxins A and B with immunoassay. Additionally, stool samples for microbiota and metabolomics analysis were collected from a subset of patients at 4 time points: at baseline, on the day of antibiotic discontinuation, at the end of the intervention period, and 1 month after the intervention period. The results of microbiota and metabolomics analysis will be reported in a separate publication.

Outcome Measures

The primary outcome measure was AAD, defined as 3 or more loose or watery stools (a score of A on the AISS or 5-7 on the BSFS) per day in a 24-hour period, caused either by *C difficile* or of otherwise unexplained etiology, after testing for common, predefined diarrheal pathogens. Secondary outcomes included diarrhea, defined as 3 or more loose or watery stools per day in a 24-hour period regardless of the etiology, mild AAD, defined as 2 or more loose or watery stools per day for a minimum of a 24-hour period caused by *C difficile* or of otherwise unexplained etiology, severe AAD defined as 3 or more loose or watery stools per day for a minimum of a 48-hour period caused by *C difficile* or of otherwise unexplained etiology, diarrhea duration, defined as the interval until normalization of stool consistency according to the BSFS (1, 2, 3, or 4) or AISS (B, C, or D) and the presence of normal stools for 48 hours, diarrhea caused by *C difficile,* discontinuation of the antibiotic treatment owing to diarrhea, hospitalization caused by diarrhea, need for intravenous rehydration owing to diarrhea, and adverse events.

Sample Size Calculation

Based on the pooled risks of AAD determined from the previous studies conducted at the Medical University of Warsaw,[21,22] as well as those reported in a Cochrane review,[2] we expected that the incidence of AAD would be 16% among children receiving placebo. To detect a difference of 11% between the arms at a 5% significance level and with 80% power, we determined that 350 participants (175 in each arm) were needed assuming potential loss to follow-up of 20%.

Statistical Analysis

Descriptive statistics were used to present the participants' characteristics. For the dichotomous outcomes, relative risk (RR) was calculated with 95% CIs, along with number needed to benefit (NNTB), if appropriate. Presented *P* values were derived from $\chi 2$ test or Fisher exact test where appropriate. For the continuous outcome, Man Whitney *U* test was performed. All of the statistical tests were 2-tailed and performed with a 5% level of significance. The primary outcome was also analyzed by logistic regression, controlling for 5 prespecified potential risk factors for AAD (age, sex, antibiotic type, duration of antibiotic treatment, and duration of hospital stay). Intention-to-treat (ITT) analysis was performed on the available participants. Owing to the completeness of our baseline data, no imputation methods were used in ITT analysis.[23] Sensitivity analyses with plausible assumptions regarding patients lost to follow-up as described by Akl et al.[24] were performed. Additionally, per-protocol analysis was performed on the participants who ingested at least 75% of the study formula based on caregivers' reports and the counting of unused sachets. For the all of the calculations, StatsDirect, version 3.3.5 (StatsDirect Ltd) was used.

Result

Between February 2018 and May 2021, 350 participants (192 boys and 158 girls; median age: 28 months; mean [range] age, 50 [3-212] months) were consecutively enrolled. Among them, 202 participants were included in Poland and 148 in the Netherlands. Available case analysis was carried out in 313 participants and per-protocol analysis in 229 compliant participants (Figure 1). Participants' characteristics were comparable between the 2 groups (Table 1). Patients from the Netherlands differed from the Polish patients mainly in terms of class of used antibiotics, antibiotic administration route, and setting. Also, loss to follow-up frequency in Poland was almost 4 times higher than in the Netherlands (15.1% vs 4.1%, respectively) (Supplemental Table 2). The characteristics of the patients lost to follow-up were similar in the placebo and probiotic groups (Supplement Table 3) and similar to characteristics of the remaining study participants (Table 1).

Table 1. Characteristics of participants

	Placebo (n=174)	Probiotic (n=176)	Total (n=350)
Median age in months (range)	27 (3 to 204)	32 (3 to 212)	28 (3 to 212)
Sex			
Female, n(%)	76 (43.7)	82 (46.6)	158 (45.1)
Male, n(%)	98 (56.3)	94 (53.4)	192 (54.9)
Setting			
Inpatient, n(%)	135 (77.6)	136 (77.3)	271 (77.4)
Outpatient, n(%)	39 (22.4)	40 (22.7)	79 (22.6)
Reason for antibiotic treatment			
Lower respiratory tract infection, n(%)	54 (31)	56 (31.8)	110 (31.4)
Upper respiratory tract infection, n(%)	52 (29.9)	49 (27.8)	101 (28.9)
Urinary tract infection, n(%)	35 (20.1)	24 (13.6)	59 (16.9)
Skin infection, n(%)	8 (4.6)	16 (9.1)	24 (6.9)
Lymphadenitis, n(%)	6 (3.4)	7 (4)	13 (3.7)
Nervous system infection, n(%)	3 (1.7)	4 (2.3)	7 (2)
Gastrointestinal infection, n(%)	5 (2.9)	5 (2.8)	10 (2.9)
Joint infection, n(%)	3 (1.7)	2 (1.1)	5 (1.4)
Other, n(%)	8 (4.6)	13 (7.4)	21 (6)
Antibiotic administration route			
Only oral, n(%)	71 (40.8)	73 (41.5)	144 (41.1)
Only intravenous, n(%)	25 (14.4)	28 (15.9)	53 (15.1)
Intravenous followed by oral, n(%)	78 (44.8)	75 (42.6)	153 (43.7)
Antibiotic type			
2nd generation cephalosporin, n(%)	25 (14.4)	26 (14.8)	51 (14.6)
3rd generation cephalosporin, n(%)	33 (19)	36 (20.5)	69 (19.7)
Aminopenicillin, n(%)	69 (39.7)	71 (40.3)	140 (40)
Amoxicillin+clavulanic acid, n(%)	67 (38.5)	55 (31.3)	122 (34.9)
Clindamycin, n(%)	14 (8)	17 (9.7)	31 (8.9)
Cloxacillin/flucloxacillin, n(%)	0	6 (3.4)	6 (1.7)
Gentamicin, n(%)	1 (0.6)	3 (1.7)	4 (1.1)
Other, n(%)	6 (3.4)	6 (3.4)	12 (3.4)
Two concomitant antibiotics, n(%)	15 (8.6)	24 (13.6)	39 (11.1)
Change of antibiotic class, n(%)	26 (14.9)	20 (11.4)	46 (13.1)
Median treatment duration days (range)	10 (2 to 21)	10 (1 to 36)	10 (1 to 36)
Median hospital stay duration (range)	5 (1 to 35)	5 (1 to 45)	5 (1 to 45)

Among 83 patients who developed diarrhea, stools from 10 children tested positive for rotavirus, 3 for norovirus, 1 for adenovirus, and 1 for *Salmonella enterica;* 6 patients in the probiotic group and 11 patients in the placebo group did not provide a stool sample for the etiology testing. The reasons for the stool sampling failures were difficulties in communicating with patients after discharge from the hospital. All of these patients were not qualified as AAD cases for the primary outcome measure. In the ITT analysis (Table 2), AAD incidence was comparable between the probiotic and placebo groups (23 of 158 [14.6%] vs 28 of 155 [18.1%,] respectively; RR, 0.81; 95% CI, 0.49-1.33). The frequency of AAD according to the alternative definitions (mild, severe) was also similar between both study groups. The patients in the probiotic group had a significantly lower risk of developing diarrhea than those in the placebo group when analyzed regardless of its etiology (33 of 158 [20.9%] vs 50 of 155 [32.3%], respectively; RR, 0.65; 95% CI, 0.44-0.94; NNTB = 9; 95% CI, 5-64; *P* = .02); they were also less likely to require intravenous rehydration owing to diarrhea (0 of 158 [0%] vs 5 of 155 [3.2%], respectively; NNTB = 32; 95%CI, 14-125; *P* = .03).We found no significant difference between the groups in the other outcomes. Effect sizes in the per-protocol analysis were similar to the ones observed in the ITT analysis; however, because of a smaller sample size, they were not statistically significant (Supplement Table 4).

To investigate whether the country-related differences might have had an effect on the results, we performed a subgroup analysis. The effect sizes for AAD, diarrhea, and diarrhea duration were similar in Poland and in the Netherlands, and only small differences were observed in the effect sizes for mild AAD and severe AAD outcomes between the countries. None of these differences between groups were statistically significant (Supplement Table 5).

To examine which subgroup(s) of patients contributed to the difference between the effect sizes for AAD and diarrhea outcomes, we performed sensitivity analyses with modified outcomes: (1) patients with AAD combined with the patients with diarrhea who did not provide a stool sample, (2) infectious diarrhea with the exclusion of *C difficile* diarrhea, and (3) infectious diarrhea caused by specific pathogens (Supplement Table 6). For all of these outcomes, the effect size was larger than that for the AAD outcome, especially for rotaviral diarrhea (RR, 0.11; 95% CI, 0.02-0.65; NNTB = 19; 95% CI, 10-63; *P* = .01). In the sensitivity analysis with plausible assumptions about missing data, the effect size for the diarrhea outcome was either no longer significant, of borderline significance, or statistically significant depending on the assumed risk of diarrhea among patients lost to follow-up (Supplement Table 6). In the logistic regression, AAD was associated with younger age and diarrhea was associated with allocation to the placebo group, younger age, and use of amoxicillin with clavulanic acid (Supplement Table 7).

Table 2. Main results of the available case analysis

Outcome	Probiotic group no. of events (%)	Placebo group no. of events (%)	Relative risk (95% CI)	Absolute risk reduction (%)	NNTB[a] (95% CI)
AAD	23 (14.6)	28 (18.1)	0.81 (0.49 to 1.33)	3.5	n/a
Severe AAD	18 (11.4)	19 (12.3)	0.93 (0.51 to 1.69)	0.9	n/a
Mild AAD	40 (25.3)	38 (24.5)	1.03 (0.7 to 1.52)	-0.8	n/a
Diarrhea	33 (20.9)	50 (32.3)	0.65 (0.44 to 0.94)[b]	11.4	9 (5 to 64)[b]
C. difficile diarrhea	1 (0.6)	3 (1.9)	0.33 (0.05 to 2.26)	1.3	n/a
Hospitalization due to diarrhea	1 (0.6)	2 (1.3)	0.49 (0.06 to 3.71)	0.7	n/a
Antibiotic cessation due to diarrhea	0 (0)	0 (0)	n/a	0	n/a
Intravenous rehydration due to diarrhea	0 (0)	5 (3.2)	n/a	3.2	32 (14 to 125)[b]
Adverse events[c]	16 (10.1)	10 (6.5)	1.57 (0.75 to 3.3)	-3.6	n/a
	Probiotic group median (IQR)	Placebo group median (IQR)	Median difference (95% CI)		
Diarrhea duration in days	5 (3-7)	4 (3-7)	0 (-1 to 1)		

[a]number needed to benefit
[b]result statistically significant
[c]Including: readmission to hospital due to reasons other than diarrhea (5 in probiotic group, 4 in placebo group), rash (2 in probiotic group, 3 in placebo group), vomiting (3 in probiotic group, 1 in placebo group), gag reflex (2 in probiotic group) abdominal pain (3 in probiotic group, 2 in placebo group), trace of blood in the stool (1 in probiotic group).

Discussion

In this RCT, a multispecies probiotic did not significantly reduce the risk of AAD when analyzed according to the most stringent definition. However, the participants in the probiotic group had a significantly lower overall risk of diarrhea during the antibiotic treatment and 7 days after when the groups were analyzed regardless of diarrhea etiology. The studied probiotic did not demonstrate a beneficial effect on most other secondary outcomes, with the exception of the need for intravenous rehydration due to diarrhea, which was less common in the probiotic group. In the per-protocol analysis, the results were similar to those in the ITT analysis. Our results did not change after an adjustment for potential AAD risk factors.

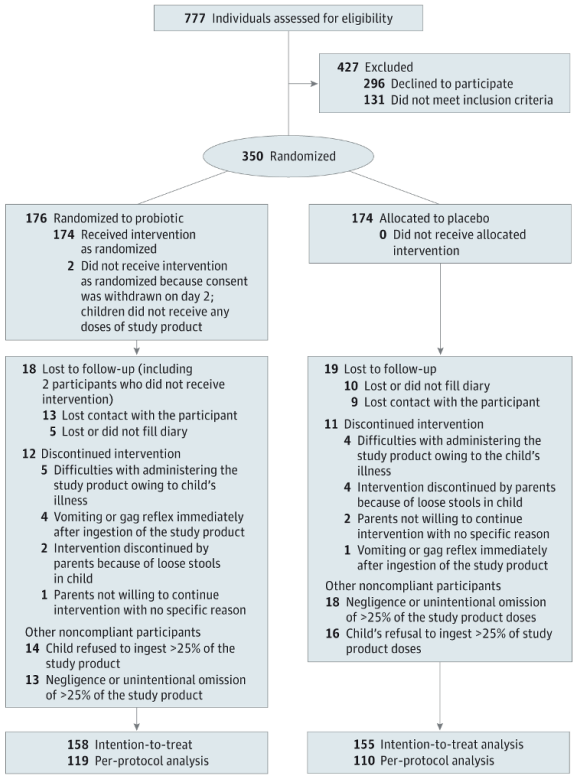

Figure 1. CONSORT 2010 Flow Diagram.

It remains unclear why the studied probiotic had no significant effect on the AAD outcome, despite its beneficial effect in the prevention of diarrhea when analyzed regardless of the etiology. One could speculate that a trial involving a larger group might have shown significant results for the primary outcome. Nevertheless, considering the satisfactory incidence of AAD in the placebo group, our study was adequately powered to detect a clinically significant difference in this outcome and even more than adequately powered for assessing the diarrhea outcome. In the sensitivity analyses, we investigated which subgroup(s) of patients contributed to this difference in outcome effect sizes to the highest extent. We found that the effect was highest for viral gastroenteritis, especially caused by rotavirus. Another significant result, i.e., the number of children requiring intravenous rehydration due to diarrhea, was also related to this finding, as all of these patients received intravenous fluids owing to rotavirus infection. There is evidence supporting a role of the microbiota in rotavirus infection, [25,26] as well as for a preventive effect of certain probiotics.[27] One could speculate that our study detected a similar effect of the studied probiotic on diarrhea caused by rotavirus. However, caution is needed when interpreting this finding, as this trial was not designed to answer this specific

research question. Moreover, since the participants were not tested for the presence of diarrheal pathogens at baseline, some of them might have already been within the incubation period of infectious diarrhea on hospital admission.

In our study, we used a rather stringent definition of AAD, which allowed us to differentiate between clinically relevant conditions and clinically unimportant changes in the consistency of stools. It also considered the most common etiology of diarrhea related to antibiotic administration and assumed that common nosocomial infections, such as norovirus or rotavirus gastroenteritis,[28,29] are not directly associated with antibiotic treatment. However, the definitions of AAD in published studies vary, and in many studies it was similar to the definition of diarrhea, as applied in current study. To illustrate, a 2020 review found that microbiological tests were not performed to identify AAD outcomes in 28 of 33 previous studies on probiotic supplementation during antibiotic treatment in children.[5] While this approach may pose a question as to whether the researchers really measured AAD or rather diarrhea during antibiotic treatment regardless of the etiology, it also represents a much more pragmatic point of view. Etiology testing is not routinely recommended for cases of acute diarrhea in children,[30] and for both the patient and the physician, what caused the diarrhea may not be relevant as long as the preventive intervention is effective.

Why the effect sizes in the ITT analysis were similar to those observed in the per-protocol analysis is unclear. This finding may reflect misclassification of compliance data, as it was collected only by indirect methods, i.e., study diaries and counting of unused sachets. Another possible explanation is that the studied probiotic is effective even if not taken regularly. Additionally, participants deemed as overall noncompliant might have been compliant during a specific time period crucial for diarrhea, e.g., during the first days of antibiotic therapy.

Strengths and Limitations

Our study had a number of strengths. To our knowledge, this is the largest trial investigating the effect of a probiotic containing more than 3 species of microorganisms on the incidence of AAD in children. The number of participants is almost 3 times higher than that in the second largest study of which we are aware.[11] It was designed with an intent to answer an unambiguous research question with a choice of clearly predefined outcomes. The study was conducted in settings of international cooperation, which enabled verification of the collected data by comparison between the different populations and recruitment centers. However, there are also some limitations. Loss to follow-up was relatively high, which is reflected by the range of uncertainty demonstrated in analyses with plausible assumptions about missing data. To search for indications of imbalances between

the trial arms owing to selective missing data,[31] we investigated the number and characteristics of participants lost to follow-up in both arms. We found them to be comparable with each other, as well as with the rest of the study participants. We also compared the outcome data between the Polish and the Dutch participants, who differed greatly in terms of loss to follow-up, and we found mostly similar effect sizes. We assume that the missing data were unlikely to have introduced a significant bias to our study; nevertheless, no method of testing can rule out such a possibility completely.[32] As mentioned, there was a puzzling difference between loss to follow-up in Poland and in the Netherlands. All but 4 of the participants were recruited and followed-up by 3 researchers (J.Ł., T.D., and T.d.M.) who were in a regular contact with each other to standardize the study conduct. Therefore, this difference may be explained by country-specific attitudes of patients and overlooked differences in the researchers' practice. Another study limitation is a potential misclassification between the AAD and diarrhea outcomes, owing to the limited diagnostic accuracy of immunoassay tests,[33] the limited number of diarrheal pathogens tested, and the number of patients who failed to provide stool samples. Additionally, the limited study follow-up duration might have led to an omission of some diarrhea cases occurring later than a week after antibiotic cessation.[7]

Conclusions

The multispecies probiotic used in this trial did not reduce the risk of AAD when analyzed according to the most stringent definition. However, we found a beneficial effect of the formulation on the overall risk of diarrhea during and 7 days after antibiotic therapy (NNTB = 9). The latter outcome corresponds well with the standard approach to AAD in clinical practice. Therefore, the use of the studied probiotic may be considered for diarrhea prevention during antibiotic treatment in children. Our study also shows that the AAD outcome definition has a significant effect on clinical trial results and their interpretation.

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Supplementary tables

Supplementary Table 1. Recruitment centres.

Location	Number of the included participants
Amsterdam UMC, location VUmc De Boelelaan 1117 Amsterdam, NL	14
Amsterdam UMC, location AMC Meibergdreef 9, 1105 Amsterdam, NL	59
OLVG location East Oosterpark 9, 1092 Amsterdam, NL	31
OLVG location West Jan Tooropstraat 164, 1061 Amsterdam, NL	44
University Clinical Center of the Medical University of Warsaw, Żwirki i Wigury 63A, 02091 Warsaw, PL	198
St. Jadwiga Śląska Hospital Prusicka 53-55, 55100 Trzebnica, PL	4

Supplementary Table 2. Patient characteristics depending on the country of recruitment

Clinical values	Poland	The Netherlands
Total	202	148
Lost to follow-up, n(%)	31 (15.1)	6 (4.1)
Compliant participants, n(%)	128 (63.4)	101 (68.2)
Median age in months (range)	27 (3-212)	32 (3-204)
Sex		
Female, n(%)	100 (49.5)	58 (39.2)
Male, n(%)	102 (50.5)	90 (60.8)
Setting		
Inpatient, n(%)	200 (99)	71 (48)
Outpatient, n(%)	2 (1)	77 (52)
Reason for antibiotic treatment		
Lower respiratory tract infection, n(%)	62 (30.7)	48 (32.4)
Upper respiratory tract infection, n(%)	83 (41.1)	18 (12.2)
Urinary tract infection, n(%)	27 (13.4)	32 (21.6)
Skin infection, n(%)	3 (1.5)	21 (14.2)
Lymphadenitis, n(%)	9 (4.5)	4 (2.7)
Nervous system infection, n(%)	2 (1)	5 (3.4)
Gastrointestinal infection, n(%)	3 (1.5)	7 (4.7)
Joint infection, n(%)	1 (0.5)	4 (2.7)
Other, n(%)	12 (5.9)	9 (6.1)
Antibiotic administration route		
Only oral, n(%)	31 (15.3)	113 (76.4)
Only intravenous, n(%)	43 (21.3)	10 (6.8)
Intravenous followed by oral , n(%)	128 (63.4)	25 (16.9)
Antibiotic type		
2nd generation cephalosporin, n(%)	48 (23.8)	3 (2)
3rd generation cephalosporin, n(%)	51 (25.2)	18 (12.2)
Aminopenicillin, n(%)	90 (44.6)	50 (33.8)
Amoxicillin+clavulanic acid, n(%)	36 (17.8)	86 (58.1)
Clindamycin, n(%)	29 (14.4)	2 (1.4)
Cloxacillin/flucloxacillin, n(%)	2 (1)	4 (2.7)
Gentamicin, n(%)	0	4 (2.7)
Other, n(%)	5 (2.5)	7 (4.7)
Two concomitant antibiotics, n(%)	31 (15.3)	8 (5.4)
Change of antibiotic class n(%)	28 (13.9)	18 (12.2)
Median treatment duration days (range)	10 (1-21)	7 (2-36)
Median hospital stay duration (range)	5 (2-21)	4 (1-45)

Supplementary Table 3. Characteristics of patients lost to follow-up

Clinical values	Placebo	Probiotic
Total	19	18
Median age in months (range)	26 (3-144)	25 (6-161)
Sex		
Female, n(%)	9 (47)	9 (50)
Male, n(%)	10 (53)	9 (50)
Setting		
Inpatient, n(%)	16 (84)	17 (94)
Outpatient, n(%)	3 (16)	1 (6)
Reason for antibiotic treatment		
Lower respiratory tract infection, n(%)	10 (53)	6 (33)
Upper respiratory tract infection, n(%)	5 (26)	7 (39)
Urinary tract infection, n(%)	1(5)	2 (11)
Nervous system infection, n(%)	1 (5)	-
Lymphadenitis	-	1 (6)
Other, n(%)	2 (10)	2 (11)
Antibiotic type		
2nd generation cephalosporin, n(%)	3 (16)	5 (28)
3rd generation cephalosporin, n(%)	2 (11)	2 (11)
Aminopenicillin, n(%)	10 (53)	9 (50)
Amoxicillin+clavulanic acid, n(%)	4 (21)	2 (11)
Clindamycin, n(%)	4(21)	4 (22)
Two concomitant antibiotics, n(%)	4 (21)	4 (22)
Median treatment duration days (range)	10 (5-21)	10 (3-14)
Median hospital stay duration (range)	4 (3-14)	4 (2-9)

Supplementary Table 4. Results of the per protocol analysis including 119 patients in probiotic group and 110 patients in placebo group.

Outcome	Probiotic group no. of events (%)	Placebo group no. of events (%)	Relative Risk (95% CI)
AAD	16 (13.4)	18 (16.4)	0.82 (0.45 to 1.52)
Severe AAD	13 (10.9)	12 (10. 9)	1 (0.49 to 2.07)
Mild AAD	29 (24.4)	25 (22.7)	1.07 (0.67 to 1.71)
Diarrhea	20 (16.8)	27 (24.5)	0.68 (0.41 to 1.14)
C. difficile diarrhea	1 (0.84)	2 (1.8)	0.46 (0.06 to 3.49)
Hospitalization due to diarrhoea	0 (0)	1 (0.9)	n/a
Antibiotic cessation due to diarrhea	0 (0)	0 (0)	n/a
Intravenous rehydration due to diarrhea	0 (0)	1 (0.9)	n/a
Adverse events			
Readmission to the hospital	3 (2.5)	1 (0.9)	2.77 (0.29. 26.27)
Abdominal pain	3 (2.5)	0 (0)	n/a
Vomiting	2 (1.7)	0 (0)	n/a
Rash	1 (0.84)	0 (0)	n/a
Trace of blood in the stool	1 (0.84)	0 (0)	n/a
	Probiotic group median (IQR)	**Placebo group median (IQR)**	**Median difference (95% CI)**
Diarrhea duration in days	3 (3-5.75)	4 (3-6)	1 (-1 to 2)

Supplementary Table 5. Available case analysis by the country of recruitment.

Available case analysis - Poland (probiotic n = 84, placebo n= 87)

Outcome	Probiotic group no. of events	Placebo group no. of events	Relative Risk (95% CI)
AAD	13	16	0.84 (0.44 to 1.62)
Severe AAD	8	7	1.18 (0.46 to 3.02)
Mild AAD	21	25	0.87 (0.53 to 1.42)
Diarrhoea	18	28	0.67 (0.4 to 1.1)
C. difficile diarrhea	1	2	0.52 (0.07 to 3.89)
Hospitalization	0	2	n/a
Antibiotic cessation	0	0	n/a
Intravenous rehydration	0	5	n/a
Adverse events[a]	10	5	2.07 (0.77 to 5.61)
	Probiotic group median (IQR)	Placebo group median (IQR)	Median difference (95% CI)
Diarrhea duration	3 (2 to 5,5)	4 (3 to 6)	1 (-1 to 2)

[a]Including: rash (2), readmission to the hospital (2), vomiting (1) in the placebo group and vomiting (3), rash (2), readmission to the hospital (1), gag reflex (2), trace of blood in the stool (1), abdominal pain (1) in the probiotic group.

Available case analysis - The Netherlands (probiotic n = 74, placebo n= 68)

Outcome	Probiotic group no. of events	Placebo group no. of events	Relative Risk (95% CI)
AAD	10	12	0.77 (0.36 to 1.63)
Severe AAD	10	12	0.77 (0.36 to 1.63)
Mild AAD	19	13	1.34 (0.73 to 2.5)
Diarrhoea	15	22	0.63 (0.36 to 1.09)
C. difficile diarrhea	0	1	n/a
Hospitalisation	1	0	n/a
Antibiotic cessation	0	0	n/a
Intravenous rehydration	0	0	n/a
Adverse events[a]	6	5	1.03 (0.37 to 3.28)
	Probiotic group median (IQR)	**Placebo group median (IQR)**	**Median difference (95% CI)**
Diarrhea duration	5 (3-12)	6 (4-7)	0 (-2 to 3)

[a]Including: readmission to the hospital (4), abdominal pain (2) in probiotic group and readmission to the hospital (2), abdominal pain (2), rash (1) in placebo group.

Supplementary Table 6. Sensitivity analyses

Outcome	Probiotic group no. Of events (%)	Placebo group no. of events (%)	Relative Risk (95% CI)
AAD cases + diarrhea cases where the testing for pathogens was not performed	29 (18.4)	39 (25.2)	0.73 (0.48 to 1.11)
Infectious diarrhea excluding *C. difficile* diarrhoea	4 (2.5)	11 (7.1)	0.36 (0.12 to 1.04)
Rotaviral diarrhoea	1 (0.6)	9 (5.8)	0.11 (0.2 to 0.65)[a]
Norovirus diarrhea	3 (1.9)	0 (0)	n/a
Adenovirus diarrhea	0 (0)	1 (0.6)	n/a
Salmonella diarrhea	0 (0)	1 (0.6)	n/a
Diarrhea: plausible assumption[c] 5:1	51 (29)	56 (32.2)	0.9 (0.66 to 1.23)
Diarrhea: plausible assumption[c] 2:1	41 (23.3)	56 (32.2)	0.72 (0.51 to 1.02)
Diarrhea: plausible assumption[c] 1,5:1	39 (22.2)	56 (32.2)	0.69 (0.48 to 0.97)[b]
AAD: plausible assumption[c] 5:1	36 (20.5)	31 (17.8)	1.15 (0.75 to 1.77)
AAD: plausible assumption[c] 1:1	26 (14.8)	31 (17.8)	0.83 (0.52 to 1.33)

[a]p=0.01

[b]p=0.04

[c]Explanation of plausible assumption: we performed a sensitivity analysis assuming that the incidence of events among participants lost to follow-up is equal to, or higher by a specific ratio relative to the observed event incidence among participants followed up, For example, 'plausible assumption 5:1' means that we assumed the incidence of diarrhea among missing patients in the probiotic group to be 5 times higher than that in the probiotic group patients who were followed-up, and the incidence of diarrhea among missing patients in the placebo group to be equal to the incidence of diarrhea in the placebo group patients who were followed up.

Supplementary Table 7. Results of logistic regression analysis.

A. Logistic regression – AAD outcome

Predictor	Model with covariates		
	Odds Ratio	**95% CI**	**p**
Allocation to probiotic group	0.8	0.42 to 1.52	0.49
Age in months	0.99	0.98 to 1	0.006
Male sex	0.94	0.49 to 1.81	0.85
2nd gen. cephalosporin	0.83	0.24 to 2.91	0.78
3rd gen. cephalosporin	2.02	0.72 to 5.7	0.18
Aminopenicillin	0.76	0.24 to 2.45	0.65
Amoxicillin with clavulanic acid	2.07	0.68 to 6.31	0.2
Clindamycin	0.61	0.17 to 2.23	0.45
Other antibiotic	0.49	0.1 to 2.57	0.4
Intravenous antibiotic	1.36	0.40 to 4.62	0.62
Oral antibiotic	0.62	0.26 to 1.49	0.29
Hospital stay duration	1.04	0.97 to 1.12	0.26
Antibiotic treatment duration	1.05	0.96 to 1.14	0.28
	Model without covariates		
	Odds Ratio	95% CI	p
Allocation to probiotic group	0.77	0.42 to 1.41	0.4

B. Logistic regression – Diarrhea outcome

Predictor	Model with covariates		
	Odds Ratio	**95% CI**	**p**
Allocation to probiotic group	0.55	0.32 to 0.96	0.04
Age in months	0.99	0.98 to 0.99	<0.001
Male sex	1.05	0.60 to 1.82	0.86
2nd gen. cephalosporin	1.75	0.59 to 5.15	0.31
3rd gen. cephalosporin	2.44	0.98 to 6.05	0.05
Aminopenicillin	1.43	0.52 to 3.93	0.48
Amoxicillin with clavulanic acid	2.63	1 to 6.9	0.05
Clindamycin	0.72	0.23 to 2.24	0.57
Other antibiotic	1.65	0.45 to 6.02	0.45
Intravenous antibiotic	2.37	0.83 to 6.81	0.11
Oral antibiotic	0.78	0.38 to 1.61	0.5
Hospital stay duration in days	1.02	0.95 to 1.09	0.65
Antibiotic treatment duration in days	1	0.92 to 1.08	0.98
	Model without covariates		
	Odds Ratio	95% CI	p
Allocation to probiotic group	0.55	0.33 to 0.92	0.02

Chapter 9

Longitudinal effects of a multispecies probiotic formulation on antibiotic-induced microbial aberrations in children: a randomized clinical trial

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Manuscript in preparation

ABSTRACT

Background: The use of probiotics in children is often considered during antibiotic treatment to prevent antibiotic associated diarrhea (AAD). However, the underlying mechanistic effects of multispecies probiotics on antibiotic-induced microbiota aberrations remain unclear. Aim of this study was to longitudinally assess the effect of multispecies probiotics on the gut microbiota in children receiving antibiotics.

Methods: This study was embedded in a randomized controlled trial (RCT) with the primary aim to assess the effect of the probiotic supplementation on prevention of AAD (NCT03334604). In total, 350 children receiving broad-spectrum antibiotics were included and received either a multispecies probiotic formulation containing eight different strains or placebo on daily base during antibiotic treatment and the seven subsequent days. Subjects were requested to collect fecal stool samples to study effects on the microbiota at four time-points: (1) first stool following inclusion; (2) last day of antibiotic use; (3) last day of the study intervention and (4) one month after termination of the study intervention. Samples were analyzed by 16S rRNA gene sequencing. Alpha- and beta-diversity as well as relative abundance were compared between the placebo and probiotic arm.

Results: In total 94 of 350 children, of whom 47 received probiotics and 47 placebo, collected at least two stool samples for microbiota analysis. Alpha diversity did not differ between the two groups at the first three time-points, but Shannon diversity (p=0.028) and Inverse Simpson (p=0.040) were higher in the placebo group at the fourth time-point. Beta diversity indices did not differ significantly between the two groups at any of the time-points. The microbiota of probiotic supplemented children was characterized by a higher abundance of the supplemented genera *Ligilactobacillus* (p=0.007), *Lactiplantibacillus* (p=0.007) and *Lactobacillus* (p=0.009) at the second and third time-point, compared to the placebo group. In the placebo group, an increased abundance of Proteobacteria (p=0.049) and *Eggerthella* (p=0.012) was found at the second and third time-point, respectively.

Conclusion: The abundance of three out of five supplemented probiotic genera was increased during probiotic supplementation. This effect disappeared one month after cessation of probiotic supplementation. Future studies, also focusing on the microbial function, are needed to assess whether these transient effects on taxonomic composition and effects on diversity have a mechanistic role in the protection against antibiotic induced side effects like AAD.

INTRODUCTION

Antibiotics are one of the most frequently prescribed drugs worldwide and its use in children has increased over the last decades.[1,2] Currently, antibiotic prescription rates range between 0.5 – 1.6 courses per child-year in western countries.[3] Exposure to antibiotics has been described to result in a decreased diversity and a decreased abundance of commensal micro-organisms with a concurrent increased abundance of pathogens in the gut microbiota.[4,5] The early life gut microbiota plays an important role in multiple physiologic processes including priming and development of the immune system and digestion.[6] Consequently, antibiotic-induced dysbiosis, especially during early childhood, has been shown to negatively impact health outcomes on the long-term, such as obesity, asthma, Crohn's disease and type 1 diabetes.[7,8] On short-term, the most prevalent side effect is antibiotic-associated diarrhea (AAD).[4] As prescription of antibiotics cannot always be avoided, it is pivotal to study interventions that could prevent, mitigate or quickly restore antibiotic-induced microbial alterations and clinical side effects in children.

The most thoroughly studied intervention to prevent clinical side effects of antibiotics in children are probiotics, defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'.[9] Recently, we demonstrated in a randomized controlled trial that in antibiotic exposed children supplementation of multispecies probiotics led to a decreased risk of diarrhea, defined as ≥3 loose stools within 24 hours.[10] It is hypothesized that parallel supplementation of probiotics during antibiotic therapy protects against such antibiotic-induced side effects.[11,12] However, the presumed underlying protective mechanisms of probiotics including its mitigating effects on antibiotic-induced microbiota aberrations has not been studied in children.[11] Therefore, we aimed to longitudinally assess the effect of multispecies probiotics on the microbiota composition in children receiving antibiotics.

METHODS

Study design

We conducted a parallel-group, randomized, double-blind, placebo-controlled trial in pediatric clinical and outpatient wards of three Dutch and two Polish hospitals (NCT03334604).[13] The primary aim of the trial was to assess the effect of multispecies probiotics on the incidence of AAD, which results were reported previously.[10] This RCT had two arms comparing a placebo group with a probiotic supplemented group. We obtained fecal samples from these children to longitudinally describe the

effects of multispecies probiotics on the gastrointestinal microbiota composition in children receiving antibiotics.

Participants

All children aged 3 months to 18 years starting on broad-spectrum oral or intravenous antibiotics were eligible for participation. Children were eligible if recruited within 24 hours following initiation of antibiotics. Children were only included if the child or parents collected two or more fecal samples and if children were compliant to the study protocol. Children were considered compliant if they received over 75% of the recommended doses of the study product. Exclusion criteria were described previously.[10] The study was approved by the Bioethics Committees of the Medical University of Warsaw (KB/198/2017) and Amsterdam UMC (2019.227). Written informed consent was obtained from all children and/or parents.

Procedures & Interventions

Children received either probiotics or placebo twice a day for the duration of antibiotic treatment and the seven subsequent days, up to a maximum of 17 days, starting within 24 hours of the first antibiotic dose. The multispecies probiotic (Ecologic AAD 612, Winclove Probiotics B.V., the Netherlands) contained eight bacterial strains: *Bifidobacterium bifidum* W23, *B. lactis* W51, *Lactobacillus acidophilus* W37, *L. acidophilus* W55, *Lacticaseibacillus paracasei* W20 (formerly known as *Lactobacillus paracasei* W20) , *Lactiplantibacillus plantarum* W62 (formerly known as *Lactobacillus plantarum* W62), *Lacticaseibacillus rhamnosus* W71 (formerly known as *Lactobacillus rhamnosus* W71) and *Ligilactobacillus salivarius* W24 (formerly known as *Lactobacillus salivarius* W24), for a total dose of 5 billion CFU per sachet (10 billion CFU daily).

Fecal samples were collected at four time points: (1) first stool following inclusion; (2) on the last day of antibiotic treatment; (3) on the last day of the placebo or probiotic supplementation and (4) one month after termination of placebo or probiotic supplementation. Dutch participant collected fecal samples in sterile containers (Stuhlgefäß 10 mL, Frickenhausen, Germany) that were immediately frozen after collection. Samples collected at home were picked up at home by one of the researchers and frozenly transported to the hospital where the samples were stored at -20°C. Polish participants collected stool samples in a OMNIgene•GUT container (Omnitek, Canada) containing a DNA stabilization buffer and were send to the hospital where samples were immediately stored at -20°C.

Sample handling

Samples were analyzed in the Laboratory of the Wageningen University & Research (Wageningen, the Netherlands) using procedures described previously.[14] Briefly 250 µg of each fecal sample was homogenized using bead beating and then DNA was extracted with the Maxwell® 16 system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed to amplify the V4 hypervariable regions of the bacterial 16S rRNA gene using barcoded primers 515F (5''GTGCCAGCMGCCGCGGTAA') and 806R (5''GTGCCAGCMGCCGCGGTAA'). Six libraries were constructed by pooling 70 uniquely barcoded samples per library. Quality control was assessed by adding negative controls and artificial mock communities to libraries. Amplicon mixture was sequenced using HiSeq2000 platform. Data processing was carried out using NG'Tax framework with default settings.[15] Taxonomic assignment of ASVs was performed using SILVA_138.1_ reference database. All laboratory analysis were performed blinded.

Microbiome data analysis

All analyses were performed using R software (version 4.2.1) and the *microbiome*, *phyloseq*[16] and *vegan*[17] packages. All samples with read count lower than the negative controls were excluded from further analysis. The contaminant and rare taxa were filtered by removing all taxa that were not assigned to any phylum. Only taxa with abundance over 0.25% in at least one sample were left in the dataset.[18] The median number of reads per sample for the 16S rRNA gene amplicon dataset was 175,933 (range 2,273 – 2,106,395). In total, 1471 different ASVs and 180 genera were identified.

Alpha-diversity indices (Shannon, inverse Simpson and the number of observed taxa) were calculated for each sample on ASV level prior to filtering out rare taxa. Beta-diversity was assessed separately at each of the four time-points using PCoA method with Bray-Curtis distance on ASV taxonomic level. All analyses of gut microbiota composition were performed on the basis of the relative abundances of the taxa.

Statistical analysis

Descriptive statistics were used to present the participants' baseline characteristics of the two groups. Continuous baseline characteristics were compared using the Student's T test or Man Whitney's U test depending on the normality of the data's distribution. Dichotomous characteristics were compared using the Fisher's exact test. All of the statistical tests were two-tailed and performed with a 5% level of significance. *Two-tailed P-values of <0.05 were considered statistically significant.*

Differences in the relative abundance of bacteria on phylum, family and genus taxonomic level between the probiotic group and the placebo group were cross-sectionally analyzed for all four time-points using Mann–Whitney *U*-test. Differences in relative abundance of taxa between each time-point within each study group was assessed using Kruskall-Wallis test with Dunn test as post-hoc. All p values were corrected using the false discovery rate (FDR) approach. Permutational analysis of variance (PERMANOVA) was used to test whether the bacterial composition were related to study group at each time-point and whether there was an interaction between time-point and study group. The plots were prepared using the *ggplot2* and *microViz* packages.

RESULTS

From the 350 children, 94 children (47 probiotics, 47 placebo) were compliant to the study protocol and collected at least 2 stool samples between February 2018 and May 2021 (Supplemental Figure 1). Participants' characteristics were comparable between the two groups (Table 1).

Cross-sectional microbial differences between placebo and probiotic arm

Cross-sectional comparison between the placebo and probiotic group revealed no differences in Shannon diversity and inverse Simpson in the first three time-points. The Shannon diversity was higher in the placebo group at the fourth time-point (p=0.028; Figure 1B). Also the inverse Simpson was higher in the placebo group at the fourth time-point (p=0.040; Figure 1D). Cross sectional analysis of the beta diversity showed no difference between the placebo group and probiotic group, at all four time-points (Figure 2).

Regarding the taxonomic composition of the microbiota at phylum level, Proteobacteria had a higher relative abundance in the placebo group compared to the probiotic group at the second time-point (p=0.049; Figure 3 and supplemental figure 2). At family level, *Lactobacillaceae* were more abundant in the probiotic group at the third time-point (p=0.015; Supplemental Figure 3). An overview of all observed phyla and families along with adjusted p-values for comparison of the relative abundance between the two groups is given in supplemental dataset 1 and 2, respectively.

At genus level, significant differences were found between the two groups in 17 different taxa spread across the four time-points (Supplemental Figure 4). At the first time-point, an increased abundance of species belonging to the genus *Enterococcus*

(p=0.032) was found with a concurrent decrease in *Paraprevotella* (p=0.049) in the placebo group. The abundance of species belonging to *Coprococcus* (p=0.012) and *Paraprevotella* (p=0.037) was lower in the placebo group at the second time-point. At the third time-point, a higher abundance was observed in the genus *Eggerthella* (p=0.012) and *Odoribacter* (p=0.039) in the placebo arm. At one month follow-up, an increased abundance in *Akkermansia* (p=0.043) and *Lachnospiraceae* (p=0.046) was observed in the placebo group. Regarding the genera present in the supplemented probiotic formulation, an increased abundance was found in *Lactiplantibacillus* and *Ligilactobacillus* at the second (p=0.030; p=0.008) and third time-points (p=0.007; p=0.007) in the probiotic group, respectively. Also an increased abundance was found in the genus *Lactobacillus* at the third time-point (p=0.010). No differences in bifidobacteria was found between the two groups at any of the four time-points. No significant differences were found in the supplemented genera at one month follow-up. An overview of all observed genera including in the placebo and probiotic group is given in supplemental dataset 3 along with adjusted p-values.

Table 1. Baseline characteristics

	Placebo (n=47)	Probiotic (n=47)	p-value
Age, median [IQR], years	1,6 [1,0-3,3]	2,0 [0,8-8,4]	0.71
Female sex, n (%)	18 (38)	18 (38)	1.00
Dutch, n (%)	36 (77)	40 (85)	0.30
Inpatient, n(%)	21 (45)	23 (49)	0.68
Hospital stay, median [IQR], days	4 [2-5]	3 [2-8]	0.71
Reason for antibiotic treatment			
Lower respiratory tract infection, n(%)	11 (23)	15 (32)	0.40
Urinary tract infection, n(%)	14 (30	8 (17)	0.14
Other, n(%)	22 (47)	24 (51)	0.68
Antibiotic administration route			
Only oral, n(%)	33 (70)	33 (70)	
Only intravenous, n(%)	2 (4)	5 (11)	0.24
Intravenous followed by oral, n(%)	12	9 (19)	0.46
Antibiotic type			
2nd generation cephalosporin, n(%)	4 (9)	1 (2)	0.17
3rd generation cephalosporin, n(%)	3 (6)	5 (11)	0.46
Aminopenicillin, n(%)	14 (30)	17 (36)	0.51
Amoxicillin+clavulanic acid, n(%)	29 (62)	26 (55)	0.53
Other, n(%)	2 (4)	5 (11)	0.24
Two concomitant antibiotics, n(%)	1 (2)	3 (6)	0.31
Change of antibiotic class, n(%)	4 (9)	4 (9)	1.00
Treatment duration, median [IQR], days	7 [5-10]	8 [7-10]	0.13

Longitudinal microbial changes over time in both arms

At baseline, the median Shannon diversity index was 2.71 (IQR 1.17) in the placebo group and 2.99 (IQR 1.47) in the probiotic group. The Shannon diversity index at the fourth time-point was higher compared to the other three time-points in the placebo group (p=0.006). Also the inverse Simpson was higher at the fourth time-point group compared to the first and second time-points in the placebo group (p=0.002).This was not observed in the probiotic group, where the Shannon diversity (p=1.00) and inverse Simpson (p=1.00) did not change significantly over time (p=1.00; Figure 1A and 1C).

The interaction term between the time-points and the two groups was statistically significant when analyzing the beta-diversity (p=0.001, adj R^2 = 4.4%), indicating that the beta diversity changed differently over time in the placebo group compared to the probiotic group. The beta diversity was significantly associated with time-point (p = 0.006, adj R^2 = 4.15%), but only in the placebo group (Figure 4). Samples taken at the end of the antibiotic treatment (time-point 2) were significantly further along the second coordinate axis than the samples taken from time-point 3 and 4 in this group. Since PERMANOVA results investigating association between beta diversity and time-point in probiotic group were not statistically significant (p=0.073) we did not compare coordinates for axes in this group.

Regarding the taxonomic composition at phylum level, the relative abundance of Firmicutes increased over time in the placebo arm (p=0.015). At the fourth time-point, the abundance of Firmicutes was higher compared to the first (p=0.003) and second time-point (p=0.016) and the abundance at the third time-point was higher compared to the second time-point (p=0.033) in this group. Proteobacteria were significantly increased at the second time-point compared to the third (p=0..004) and fourth time-point (p<0.001) in the placebo arm. Other phyla did not change significantly over time in the placebo group. In the probiotic group, no changes over time were observed in any of the phyla (Supplemental Figure 5). Adjusted p-values for comparison between all four time-points in the placebo and probiotic group are shown in supplemental dataset 4 and 5 respectively.

Also at family level, statistically significant changes over time were observed in both arms. At the third and fourth time-point, *Clostridiaceae* were increased compared to the first (p<0.001; p<0.001) and second time-point (p<0.001; p<0.001) in the placebo group. This was also observed for *Lachnospiraceae* (p=0.014; p=0.001 and p=0.011; p=0.011 respectively). *Enterobacteriaceae* (p=0.028) and were increased compared to the first and second time-point compared to the last time-point (p=p=0.038; p=0.021; Supplemental Figure 6). Furthermore, the relative abundance of eight other families changed over time. These differences over time along with adjusted p-values are demonstrated in supplemental dataset 6. In the probiotic arm, the abundance of *Lachnospiraceae* was higher at the fourth time-point compared to the first (p=0.013) and second time-point (p=0.008). Furthermore, the abundance of *Pasteurellaceae* (p=0.017) and *Coriobacteriaceae* (p=0.049) was significantly increased at the fourth time-point with a concurrent decrease in *Actinomycetaceae* (p=0.043) in the probiotic group (Supplemental Figure 6 and supplemental dataset 7). In both the placebo and

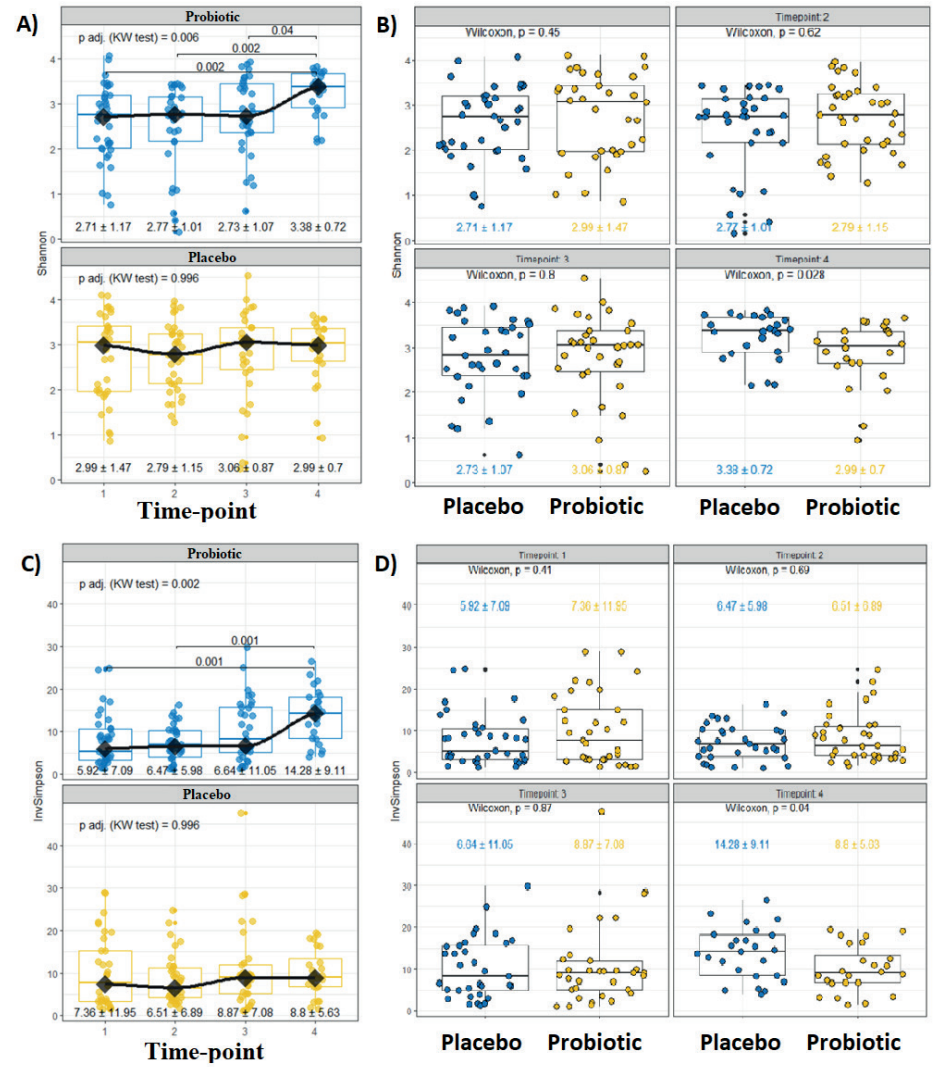

Figure 1. Alpha divseristy indiced (Shannon diversity and inverse Simpson). The Shannon diversity index (1A) and inverse Simpson (1C) were higher at the fourth time-point compared to the other three time-points in the placebo group. This was not observed in the probiotic group. The Shannon diversity (1B) and inverse Simpson (1D) were higher in the placebo group compared to the probiotic group at the fourth time-point (p=0.028 and p=0.040).

probiotic group, the relative abundance of numerous genera changes significantly over time. In the placebo group, the abundance of 31 genera changed over time compared to 14 genera in the probiotic group, as demonstrated in Supplemental Figure 7 and in supplemental dataset 8 and 9 respectively.

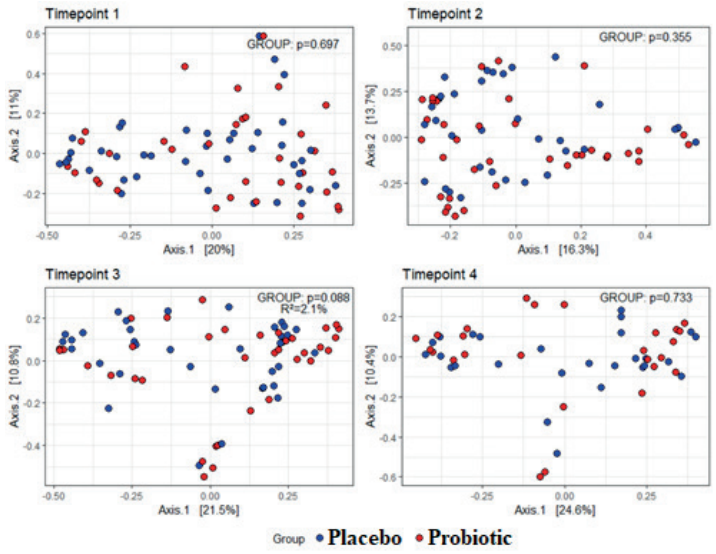

Figure 2. Cross sectional analysis of the beta diversity at each of the four time-points using PCoA method with Bray-Curtis distance on ASV taxonomic level. No differences between the placebo group and probiotic group were observed at all four time-points.

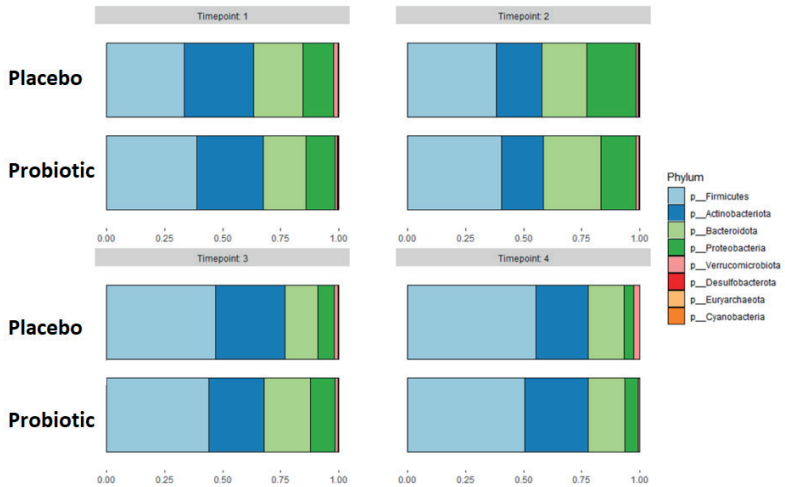

Figure 3. Relative abundance of observed phyla in the placebo and probiotic group at all four time-points. Proteobacteria had a higher relative abundance in the placebo group compared to the probiotic group at the second time-point (p=0.049).

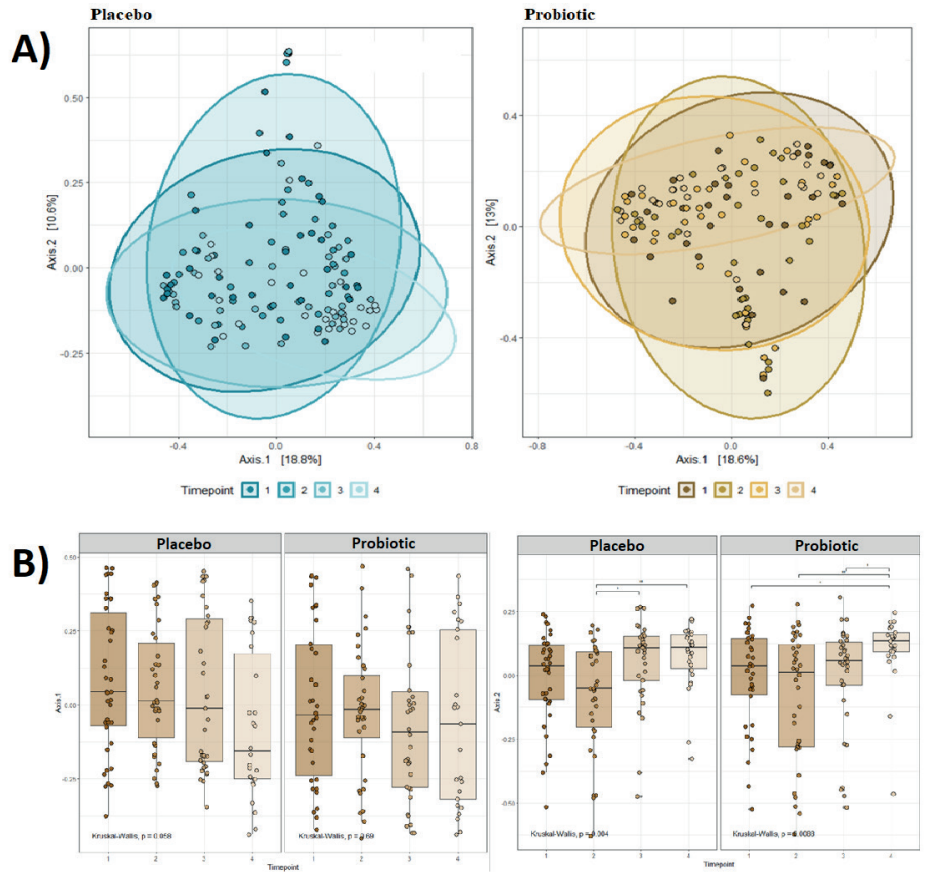

Figure 4. Analysis of the beta diversity at each of the four time-points using PCoA method with Bray-Curtis distance on ASV taxonomic level. The interaction term between the time-points and the two groups was statistically significant when analyzing the beta-diversity (p=0.001, adj R2 = 4.4%), indicating that the beta diversity changed differently over time in the placebo group compared to the probiotic group. The beta diversity was significantly associated with time-point (p = 0.006, adj R2 = 4.15%), but only in the placebo group. Samples at the second time-point 2 were significantly further along the second coordinate axis than the samples taken from time-point 3 and 4 in this group.

DISCUSSION

In this RCT we investigated the effect of probiotic supplementation on antibiotic induced microbiota aberrations in children. Alpha diversity did not differ between the two groups during the intervention period, but Shannon diversity and Inverse Simpson were higher in the placebo group at the fourth time-point. We observed a higher abundance of *Lactiplantibacillus Ligilactobacillus*, *Lactobacillus* and Firmicutes in the probiotic group during the probiotic supplementation period, while *Eggerthella* and Proteobacteria were more abundant in the placebo group during the intervention period. These alterations were transient as at one month follow-up, these differences were not observed between both groups anymore.

Probiotics are one of the most thoroughly studied interventions to prevent antibiotic related side effects such as AAD.[9] Recently, we demonstrated that the probiotic formulation used in this trial reduces the risk of diarrhea, defined as ≥ 3 loose stools within 24 hours, in children receiving antibiotics.[10] It is hypothesized that probiotics mitigate antibiotic induced gut microbiota aberrations and consequently decrease antibiotic related side effects. However, mechanistic evidence is limited, particularly in children.[11,12] In a study including adult participants receiving a seven-day antibiotic course and either a comparable probiotic formulation consisting of bifidobacteria, lactobacilli and streptococci for four weeks (n=8), or no probiotics (n=7), a higher abundance of bifidobacteria and lactobacilli was found only during probiotic supplementation, which is in line with our observations.[19] Contrary to our findings, they observed differences between probiotic supplemented and non-supplemented arm for a prolonged period. Microbiota of participants receiving probiotics did not return to their baseline levels within the five-month study period, while this was observed in the non-probiotic arm.[19] Another placebo controlled trial in antibiotic exposed adults (n=136), supplementing the intervention arm with L. *paracasei* and L. *rhamnosus* for 28 days, including the 14 day antibiotic treatment, also showed that probiotic supplementation resulted in increased abundance of the supplemented probiotics during supplementation, in line with our results. Besides, they found a reduced degree of antibiotic induced aberrations and earlier restoration within 28 days after antibiotic cessation, which was not clearly observed in our study.[20]

Several other studies on the effects of probiotic supplementation during antibiotic treatment on the gut microbiota have shown conflicting results regarding diversity indices, microbiota composition and recovery time.[11] It has to be noted, however, that these studies included different study populations as adults or neonates, other types, doses and duration of probiotics and antibiotics and stool samples were collected at different time-points and analyzed by different analytical methods.[11] These differences limit the possibility to reliably compare results of these studies with our data. To our knowledge, this is the first study to investigate the effect of probiotics on the microbiota composition during antibiotic treatment in a pediatric population.

In contrast to some studies in adults,[11] we did not observe an increased abundance of bifidobacteria in probiotic supplemented children. In order for orally supplemented probiotics to reach the intestine, they need to survive gastric acid and bile acids and be able to colonize the gut. Bifidobacteria in general have low acid tolerance, and are strictly anaerobic and will die quickly in an aerobic environment.[21,22] Although the supplemented strains were carefully selected, based on their ability to survive in the intestines, and were sealed in a vacuum packaging, bifidobacteria may still have failed to survive due to aerobic conditions in the mouth, gut or in the package or due to low gastric pH levels.[23]

The supplemented genera *Ligilactobacillus, Lactiplantibacillus* and *Lactobacillus*, were, as expected, found in higher abundance in probiotic supplemented infants. These genera all are members of the lactic acid bacteria, are aerotolerant and express urease allowing to survive low pH levels in the stomach. This makes them able to survive the intestines in active form.[22,24].

Besides colonization of the supplemented probiotic strains, administration of probiotics may result in a broad range of changes in the taxonomic composition and function of the microbiota.[25] We observed, for example, an increased abundance of Proteobacteria in the placebo group. Consequently, probiotics may hypothetically have the potential to prevent antibiotic associated side effects such as diarrhea.[11] Antibiotics may lead to decreased intestinal epithelium function leading to a leaky gut and increased risk for diarrhea.[26] *Lactobacillus* may prevent antibiotic induced epithelium dysfunction and stimulate the gut barrier integrity.[11] Besides, antibiotic exposure leads to microbiota aberrations, accumulation of carbohydrates and consequently to reduced levels of short chain fatty acids (SCFAs). As SCFAs promote the absorption of water from the colon, a decrease in SCFA provoke diarrhea.[11] In probiotic supplemented infants we also observed a increase in *Coprococcus*, with a concurrent decrease in *Eggerthela*. As *Coprococcus* and the different lactic acid bacteria play an important role in the digestion of carbohydrates into SCFA, increased abundance of these taxa may lead to increased SCFA concentrations. This will stimulate water absorption and decrease risk for antibiotic induced diarrhea.[11] Increased levels of SCFAs were found after *Lactobacillus* supplementation in adults and animal models.[27,28] Studies in antibiotic exposed children receiving probiotics on such metabolites are lacking. Seen the limited evidence, future mechanistic studies focusing on the microbiota function are warranted to elucidate the exact working

mechanisms of probiotics. This may elucidate the optimal types, combination, dosing and duration of probiotics. These studies should also focus on long-term health outcomes of probiotic exposure, as this has not been studied.

Strengths of study include the randomized, placebo-controlled design of the study, allowing to compare probiotics exposed subjects to controls, and standardized collection of a relatively large amount of samples. Besides, it is the first study focusing on the longitudinal effects of multispecies probiotics in antibiotic exposed children.

A limitation of this study is that in the majority of cases baseline stool samples were collected after ingestion of the first dose of antibiotics since, from a clinical perspective, it was obviously not feasible to postpone start of antibiotics after sampling of the first stool sample. The first antibiotic dose may consequently have affected the microbiota composition measured in the baseline sample. This may explain why the diversity indices did not change during the intervention period. Furthermore, not all children recruited in the initial trial focusing on AAD incidence were included in this part of the study, as not all participants collected at least two stool samples and were compliant to the study protocol. There was a broad age range of children included in our study and different types of antibiotics were prescribed, potentially impacting the results. Lastly, only 16S rRNA gene sequencing was performed to study the microbiota composition, metabolomics analysis will be performed on collected samples, allowing to obtain insight in microbial function rather than only composition.

In conclusion, we observed a transient increased abundance of the supplemented genera *Ligilactobacillus, Lactiplantibacillus* and *Lactobacillus* during probiotic supplementation, but not at one month follow-up. Proteobacteria were transiently increased in the children non-exposed to probiotics. Alpha and beta diversity was not different during probiotic supplementation, but both Shannon diversity and inverse Simpson were increased in the placebo arm at one month follow-up. Future studies, also focusing on the function of the microbiota, are needed to assess whether observed transient effects on taxonomic composition and effects on diversity have a mechanistic role in protection against antibiotic induced side effects, including AAD.

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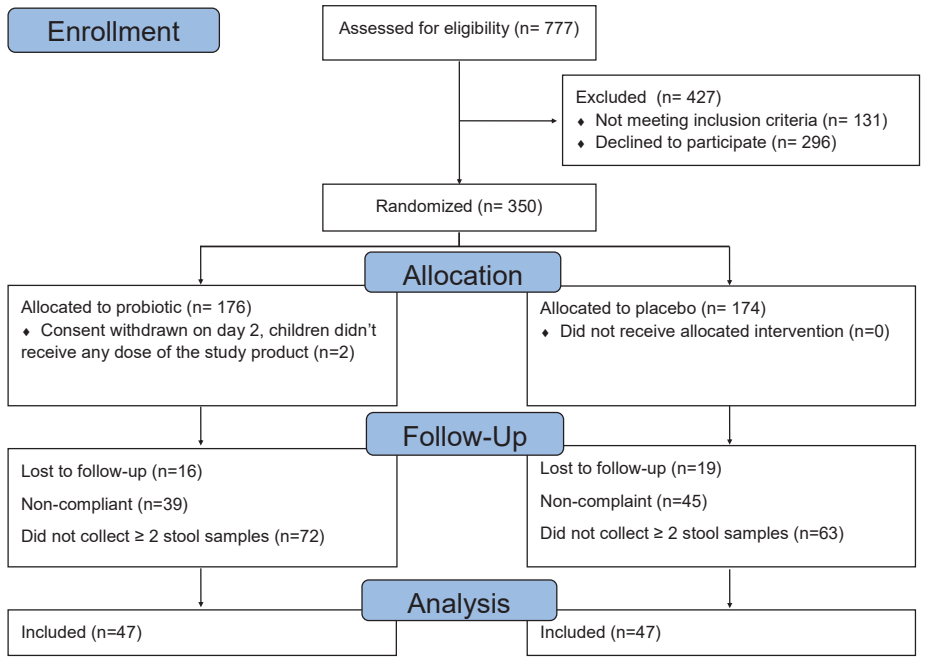
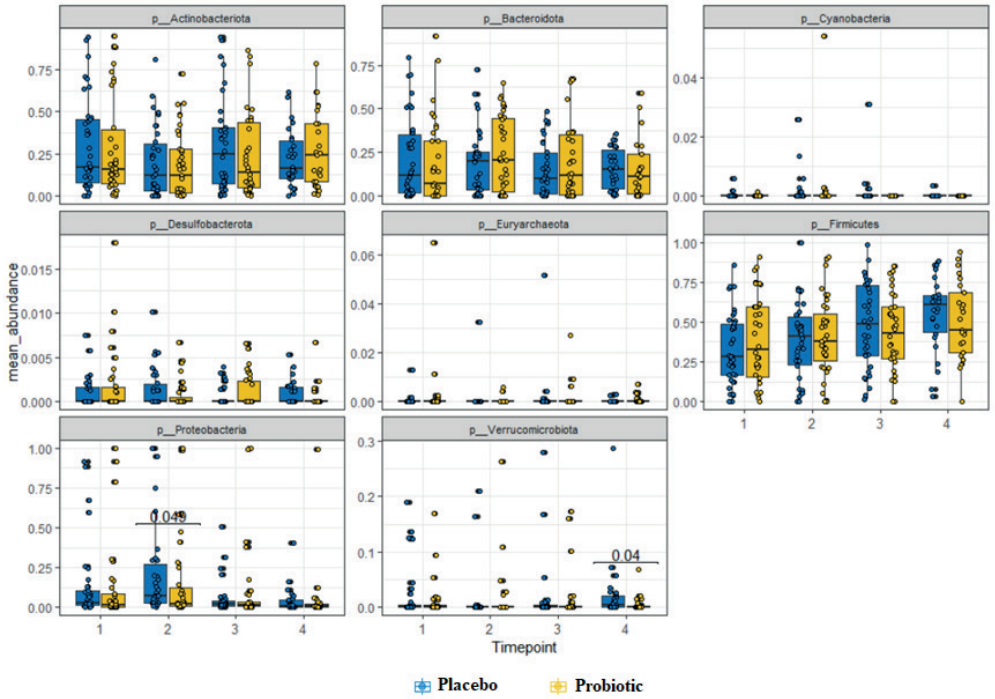
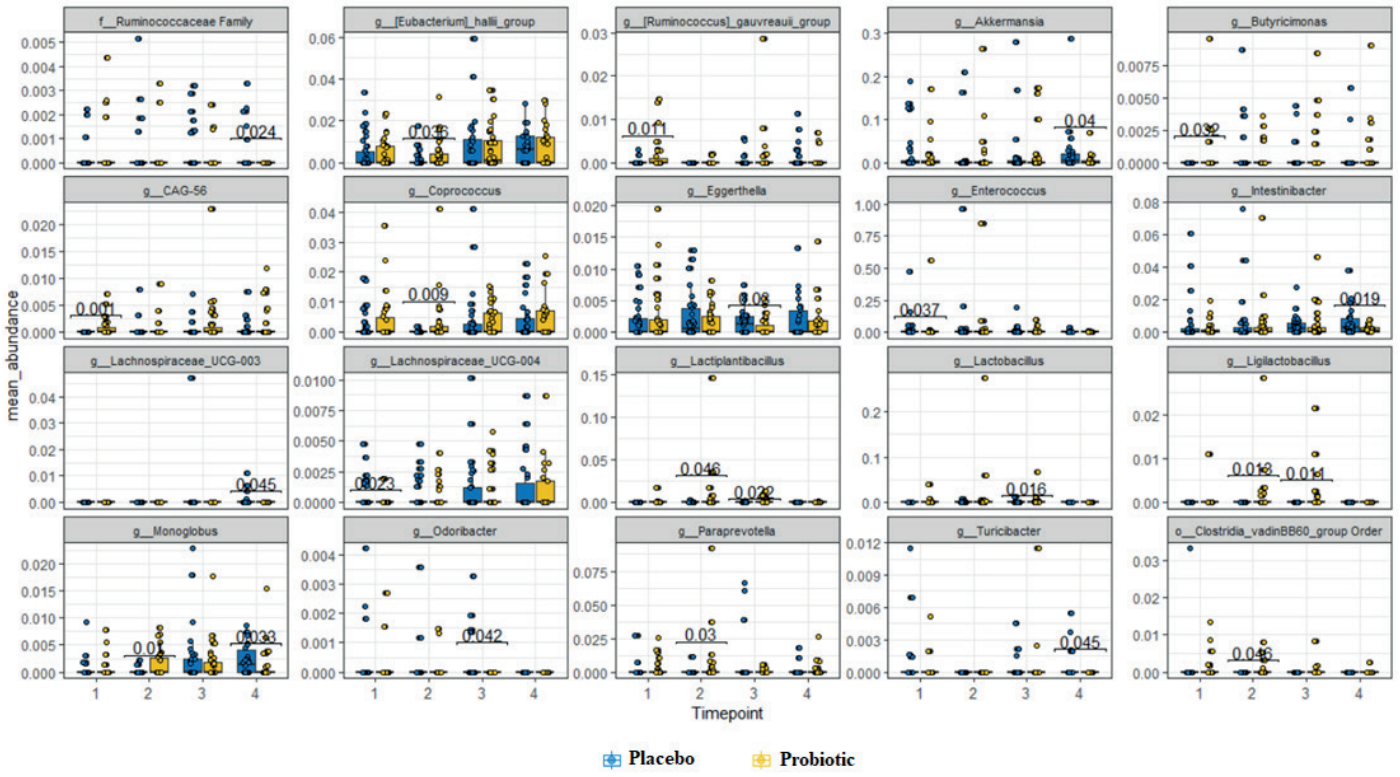


Supplemental figure 1. Flow chart of participant inclusions

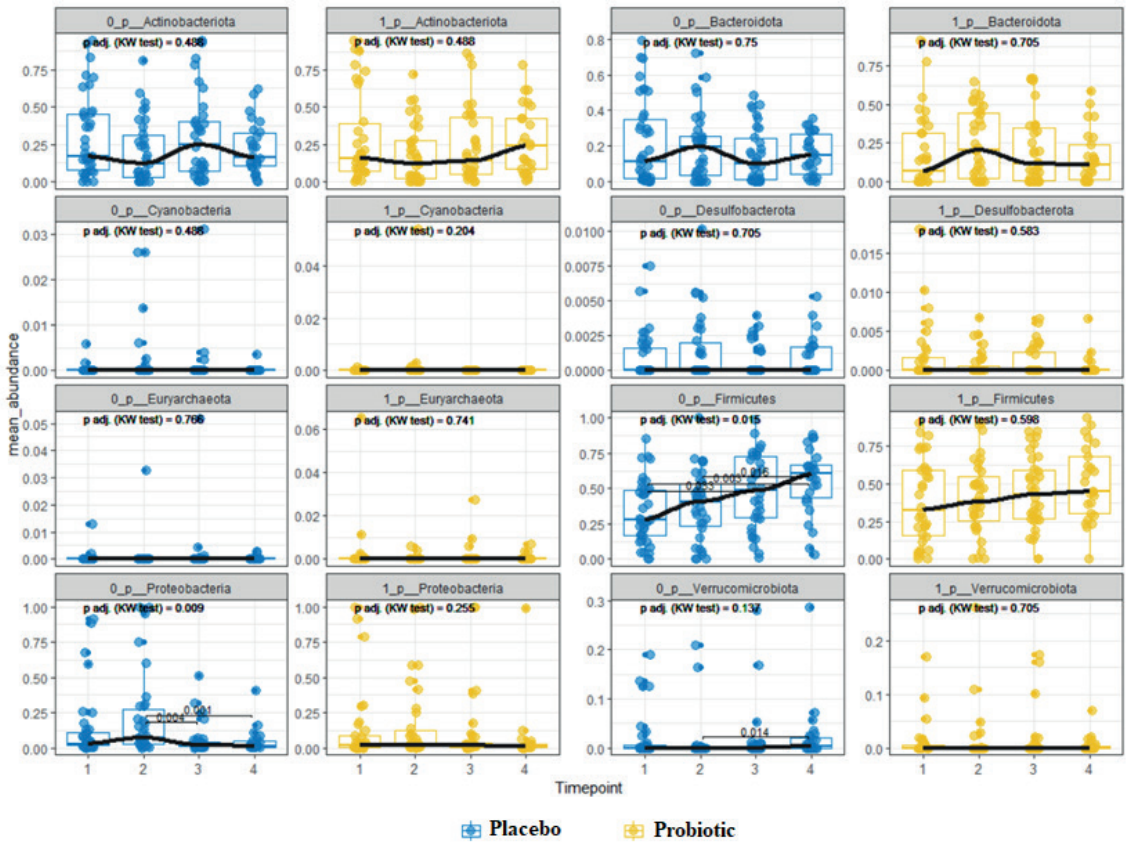


Supplemental figure 2. Relative abundance of observed phyla in the placebo and probiotic group at all four time-points. Proteobacteria had a higher relative abundance in the placebo group compared to the probiotic group at the second time-point (p=0.049).

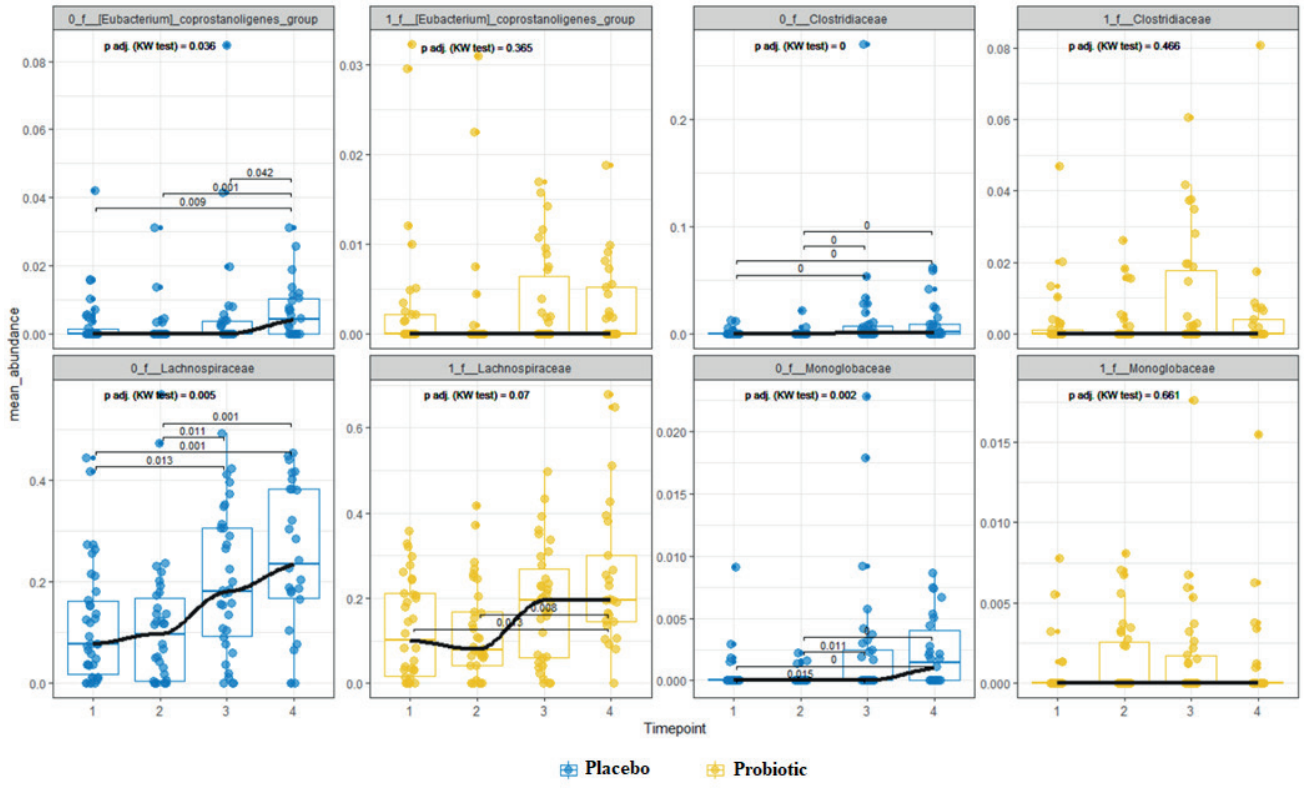
Supplemental figure 3. Relative abundance of families with significantly different relative abundance between the placebo and probiotic group.



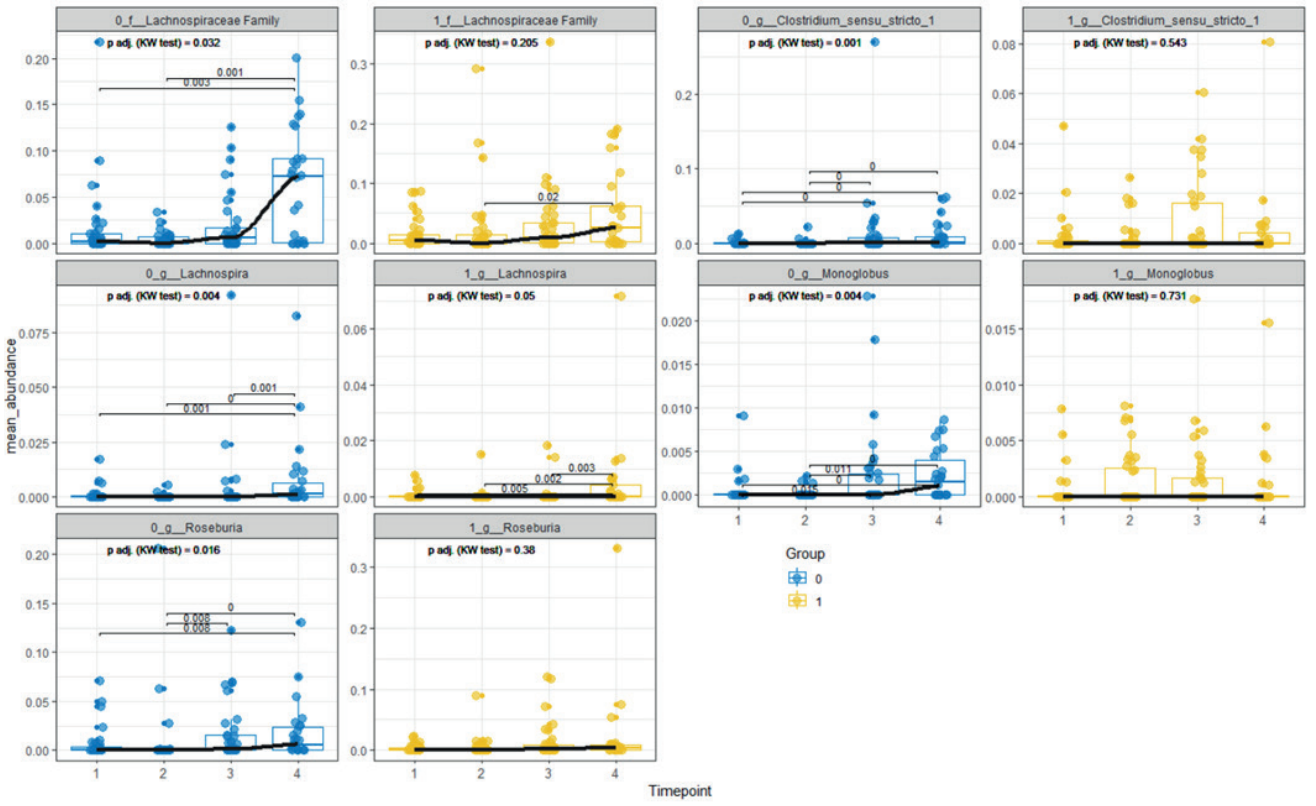
Supplemental figure 4. Relative abundance of genera with significantly different relative abundance between the placebo and probiotic group.



Supplemental figure 5. Relative abundance of observed phyla over time within the placebo and probiotic group. In the placebo group, the relative abundance of Proteobacteria, Firmicutes and Verrucomicrobiota changed significantly over time. In the probiotic group, no significant changes over time were observed.



Supplemental figure 6. The relative abundance of families that changed significantly over time.



Supplemental figure 7. The relative abundance of genera that changed significantly over time.

Chapter 10

General Discussion and Future Perspectives

Up to 40% of infants are prenatally exposed to maternal prescribed intrapartum antibiotic prophylaxis (IAP).[1,2] The use of maternal IAP has increased in the past decades due to adjustments of international guidelines.[3,4] Besides, 50% of children in the Netherlands are prescribed at least one course of antibiotics in the first 4 years of life.[5,6] Postnatally, antibiotics are often prescribed for presumed bacterial infections on neonatal and pediatric wards, but in approximately 30% of these patients bacterial infection is not proven.[7-11]

The last decades knowledge on negative consequences following antibiotic exposure early in life has increased,[5,9-11] emphasizing the need for reduction of unnecessary prescription of antibiotics. Negative consequences of early antibiotic exposure include an increase in antibiotic resistance and aberrations in gastrointestinal microbial colonization, which is linked to an increased risk of numerous diseases such as asthma, eczema and obesity.[12-14] In this thesis, we focused on effects of antibiotic exposure early in life on the microbiota composition and function and on health outcomes (part I). Furthermore, we have evaluated potential strategies aiming at a safe reduction of antibiotic use in infants (part II) and the potential of probiotics to reduce antibiotic related side effects in children (part III). In this chapter we highlight and discuss the most important findings. Moreover, we present recommendations for future studies and implications for clinical practice.

PART I: Effects of perinatal antibiotic exposure on microbiota colonization and health outcomes in infants

Adjustment in guidelines to prevent neonatal *Group B Streptococcus* (GBS) infections and maternal perinatal infections has led to an increased use of maternal IAP.[3,4] In **Chapter 2** we systematically searched for studies investigating effects of IAP on the infant microbiota and health outcomes. Especially in vaginally born infants, IAP seemed to significantly impact the colonization of beneficial bacteria such as *Bacteroides* and *Bifidobacterium,* as demonstrated by several observational studies. Notably, data on effects of IAP in caesarean section (CS) born infants and effects on health outcomes are lacking. *Bacteroides* and bifidobacteria are considered to confer positive health benefits in general on the host, like protection against pathogens.[15,16] Delayed colonization with bifidobacteria has been associated with a decreased number of memory B-cells later in infancy and with immune dysregulations, and consequently with increased risk for multiple non-communicable diseases later in life.[15,17] *Bacteroides* also influence immune development, and depletion of this genus in infancy could possibly negatively impact T-cell response.[17,18] Consequently, concerns on aforementioned adverse events of antibiotic exposure were raised when the National Institute for Health and Care Excellence (NICE) changed their recommendation on the timing of IAP to the mother

during CS. In the revised guideline it is recommended to administer IAP 30 minutes prior to the CS, instead of after clamping of the umbilical cord, unintentionally exposing the infant to antibiotics at birth.[3] This policy has shown to reduce the incidence of maternal post-operative infections from 7 to 4%, but effects on the infant microbiota and long-term health remained unstudied.[19] In **chapter 3** we aimed to study the effect of this guideline adjustment on infant microbiota colonization. Here, we demonstrated that microbiota colonization was strongly affected by mode of delivery. Compared to vaginally born infants, the abundance of beneficial bacterial taxa such as *Bacteroidetes* and *Bifidobacterium* was decreased in CS born infants in the first month of life. This effect had disappeared at the age of 3. Contrary to the effects of IAP in vaginally born infants, in our randomized controlled trial (RCT), we did not find evidence that exposure to IAP due to the guideline adjustment further impacts the compromised microbiota in CS born infants. Recently, the first large scaled retrospective study was performed in the UK that did not demonstrate any effect of timing of IAP during CS on long-term health outcomes such as asthma, eczema and obesity.[20] So, with current evidence, the NICE guideline adjustment on timing of antibiotics during CS seems to be beneficial for the mother, but does not seem to impact infant microbiota and infant health.

As mentioned before, up to 40% of mothers receive antibiotics during pregnancy and yearly 30 million infants are born via CS. Our findings that exposure to IAP in vaginally born infants (**chapter 2**) and route of delivery (**chapter 3**) strongly impact microbiota colonization should be validated in larger, preferably randomized trials in order to limit the impact of confounding factors. Seen the scarcity of studies focusing on health outcomes, these studies should also collect data on immune status and on short- and long-term health of these children.

Besides exposure to IAP during birth, antibiotic exposure directly postpartum for a suspicion of early-onset sepsis (EOS) contributes to a great amount of antibiotic exposure early in life. In the Netherlands, around 5% of all newborns are exposed to antibiotics under suspicion of EOS. With current diagnostic tools, EOS can currently only be ruled out after 36-72 hours following negative peripheral blood culture (PBC) in combination with reassuring clinical condition and laboratory values as C-reactive protein (CRP).[21] As delay in treatment initiation of EOS may have dramatic consequences, guidelines advice to administer empiric antibiotics in all infants suspected for EOS for at least 36-72 hours if blood culture turns out negative. This leads to an enormous number of unnecessary antibiotic exposed infants, with a number needed to treat of 100.[4,22,23] Concerns about effects of increasing use of perinatal antibiotic exposure on infant health outcome are described in **chapter 4**. In this large national cohort study including culture-negative preterm infants born with

a gestational age <30 weeks from 9 NICUs, roughly 90% was exposed to antibiotics for a suspicion of EOS directly after birth. In the vast majority (802/1122; 71.5%) antibiotics were discontinued within 72 hours. In this study, we found that infants with short antibiotic exposure (<72 hours) were at lower risk for necrotizing enterocolitis (NEC) compared to both infants with prolonged (> 72 hours) antibiotic exposure (OR 0.58) and infant without antibiotic exposure (OR 0.39). Previous literature on effects of early antibiotic exposure on the incidence of NEC is conflicting.[24] We hypothesize that short antibiotic exposure prevents the colonization of facultative pathogenic anaerobes such as *Streptococcus* and *Enterococcus* species, but allows beneficial strict anaerobic genera such as *Bifidobacterium* and *Bacteroides* to colonize the infant gut after termination of antibiotic treatment contrary to prolonged antibiotic exposure, potentially also inhibiting colonization of beneficial bacteria the first week of life. In line with this hypothesis, one small RCT comparing microbiota of preterm infants with short or no antibiotic exposure found a more favorable microbial composition with increased abundance of *Bifidobacteriaceae* in infants with short antibiotic exposure,[25] potentially decreasing the risk for NEC.[26] Prolonged antibiotic exposure, on the other hand, might protect against late-onset sepsis development during antibiotic exposure but may lead to more profound dysbiosis of commensal gut bacteria, potentially increasing the risk for NEC[27]. To test this hypothesis, we have now planned to perform microbiota analysis on collected stool samples of all these infants and to evaluate the effects of duration of antibiotics on microbial colonization. Furthermore, randomized trials such as the NICU Antibiotics and Outcomes (NANO) trial (ClinicalTrials.gov identifier: NCT03997266) are needed to study the effects of antibiotics in preterm infants, limiting the effect of bias characterizing observational studies.

PART II: Strategies aiming to safely reduce unnecessary empirical antibiotic exposure for early-onset sepsis suspicion

A lack of rapid and accurate diagnostic tools at initial EOS suspicion have led to a high number of unnecessary empirical antibiotic use shortly after birth. Because results of a peripheral blood culture (PBC) can only be interpreted reliably after 36-72 hours, all infants with EOS suspicion are empirically exposed to antibiotics awaiting PBC results, as delay in treatment initiation may lead to rapid clinical deterioration. It is estimated that up to 1.400 infants are empirically exposed to antibiotics for every culture-proven EOS case in well-appearing infants from mothers with risk factors for EOS,[28,29] impacting microbiota colonization and potentially affecting health outcomes.[9-11] Besides, this also leads to unnecessary hospitalization for infants and caregivers, with high costs, painful procedures for the infant and sometimes separation from parents, negatively impacting parental attachment and physical and emotional development.[30,31] A rapid diagnostic test with high accuracy

at initial EOS suspicion is therefore urgently needed, in order to guide quickly clinicians which infants need antibiotics and which not in case of EOS suspicion. In **Chapter 6** and **Chapter 7** we studied the potential of presepsin and Molecular Culture (MC) for this purpose, respectively. Presepsin is expressed as CD-14 subtype on antigen presenting cells and immediately released after binding to bacteria, and consequently expected to increase earlier compared to other biomarkers such as C-reactive protein and procalcitonin. Previous studies on the diagnostic accuracy of presepsin, however, combined EOS and LOS cases, despite difference in reference ranges.[32,33] Only a few studies reported the diagnostic accuracy specifically for EOS, but these were all characterized by methodological flaws.[34-38] In our study, presepsin could discriminate EOS cases from uninfected controls with high accuracy in preterm infants (area under the curve (AUC) of 0.84). In term born infants the AUC was 0.60. In preterm infants 15/169 (8.9%) were classified as EOS cases compared to 65/164 (39.6%) in term born infants. To date, there is no consensus definition for clinical EOS, which is a limitation for studies on EOS diagnostics. We hypothesized that misclassification of uninfected controls as EOS cases in term born infants led to this difference in AUC between term and preterm infants, since the number of EOS cases in term born infants was higher than expected. As presepsin seems to be an accurate biomarker directly at initial EOS suspicion in preterm infants, in contrast to biomarkers as CRP and procalcitonin, presepsin may guide clinicians when and when not to initiate antibiotics in case of EOS suspicion. Implementation of presepsin could consequently reduce the amount of unnecessary antibiotic exposure. Before presepsin could be implemented in clinical are, however, an RCT should be performed to investigate whether implementation of presepsin is feasible and would indeed lead to a decrease in unnecessary antibiotic exposure, without an increase in EOS related morbidity and mortality.

In our proof-of-principle study on the potential of MC (**chapter 7**), this technique identified the same bacteria in 14 out of 15 known positive blood samples. Besides, the MC was able to detect bacteria even when present in low concentrations is positive spiked samples. In our clinical cohort of infants with EOS suspicion, test results were similar in 92.5% of infants, while MC allowed for detection of *Enterococcus faecalis* in one clinical EOS case, which was missed by PBC, and 2 positive tests in uninfected infants, which were suspected to be contamination.

Sensitivity of a PBC can be impaired by limited sampled blood volume, low bacterial loads and previous antibiotic exposure.[39] In contrast to conventional PBC, this molecular technique is not influenced by maternal intrapartum antibiotic prophylaxis and is able to detect species uncultivatable by PBC.[40] Therefore, the sensitivity of MC for EOS is expected to be higher compared to a conventional PBC, demonstrated by

the identified bacteria using MC which were missed by conventional PBC. As there were no positive PBCs in our cohort, we were unable to investigate whether the MC will detect all cultured bacteria by PBC in infants, as demonstrated in a previous study in adults. Besides, as this was a proof-of-principle study, the cohort was relatively small. Before clinical application, the value of MC needs to be validated in a large clinical cohort, also including culture-positive EOS cases.

Seen the limited sensitivity of a PBC due to aforementioned factors, antibiotic treatment is often continued for >72 hours, despite negative culture results as demonstrated in chapter 4 where antibiotics were continued in 320 of 1122 culture-negative infants (28.5%). As umbilical cord blood is easier to obtain and a larger volume can be sampled,[41] it was hypothesized that the sensitivity of an umbilical cord blood culture (UCBC) would be higher compared to the sensitivity of a PBC. In **chapter 5** we systematically searched, evaluated and appraised evidence about the diagnostic accuracy of UCBC for EOS compared to PBC. A limited number of studies were found that compared the diagnostic accuracy of both tests for (clinical) EOS. These studies demonstrated that sensitivity of UCBC seems to be higher (42.6%) compared to the sensitivity of PBC (20.4%) for clinical EOS. Most studies, however, compared the outcomes of PBC with UCBC outcomes, without investigating the accuracy for clinical EOS. In case of discrepancy in results between UCBC and PBC, it is unknown whether the PBC was false negative or the UCBC was false positive. Larger observational studies including a predefined definition of clinical EOS are therefore needed to investigate and compare the diagnostic accuracy of UCBC and PBC.

Part III Role of probiotics in preventing antibiotic related side effects and microbial aberrations in children

Also later in life during childhood, antibiotic exposure is common. In Europe, between 0.5 and 1.6 antibiotic courses are prescribed per child-year.[5] The most frequent adverse event of antibiotic exposure in children is antibiotic-associated diarrhea (AAD).[42,43] It is assumed that AAD results from aberrations in the microbiota leading to overgrowth of pathogens and metabolic imbalances.[44,45] The most studied intervention to prevent AAD are probiotics.[46] It is hypothesized that probiotics limit and/or prevent disruption of the commensal microbiota and consequently prevent AAD. Besides, the use of probiotics is thought to decrease the amount of undigested carbohydrates and increases the levels short-chain fatty acids (SCFAs) in the colon.[47] This decreases the risk for AAD, as undigested carbohydrates increase the osmotic load and attack water and SCFAs stimulate the absorption of water.[47] The effect of multispecies probiotics on the incidence of AAD in children, however, is understudied. Also, the underlying mechanisms of probiotics and the effects

on the microbiota are limited. In **chapter 8** and **chapter 9**, we therefore aimed to investigate the effect of a multispecies probiotic on the incidence of AAD and the microbiota composition, respectively. As there is no consensus on the definition of AAD, we used different AAD definitions.[48,49] In children receiving probiotics, we found a reduction of 35% in AAD incidence, defined as diarrhea regardless of the etiology and thus not including microbial tests to rule out bacterial or viral infection as possible cause of the diarrhea. According to the more stringent definition, excluding children with diarrhea due to common bacterial or viral pathogens, we did not find a significant effect of probiotics on AAD incidence. The former definition of pediatric AAD is the most widely used. To illustrate this, 28 / 33 trials included in a recent Cochrane review, did not include etiology tests in their AAD definition.[50] As this approach is also in line with the approach in clinical practice, we concluded that probiotics could be considered during prescription of antibiotics. One of the reasons for differences in the effect of probiotics on the different AAD definitions is the fact that in the placebo group more viral pathogens, especially rotavirus, was found. There is evidence supporting a role of the gut microbiota in rotavirus infections as well as for a preventive effect of certain probiotics.[51-53] One could speculate that our study detected a similar effect of the studied probiotic on diarrhea caused by rotavirus. However, caution is needed when interpreting this finding, as our trial was not designed to answer this specific research question. Future studies are warranted to elucidate the possible role of probiotics in rotaviral diarrhea. Besides, further research is needed to elucidate the optimal doses, combination of species and duration of treatment before probiotics can be implemented in clinical practice to prevent AAD.

To test the hypothesis that probiotics limit and/or prevent disruption of the commensal microbiota, we investigated the effect of probiotic supplementation on antibiotic induced microbiota aberrations using fecal samples of children included in our trial on AAD incidence. We observed a higher abundance of three of five supplemented genera (*Lactiplantibacillus Ligilactobacillus*, *Lactobacillus*) in the probiotic group during probiotic supplementation. Besides, an increased abundance of Firmicutes was observed in the probiotic group, while *Eggerthella* and Proteobacteria were more abundant in the placebo group during probiotic supplementation. These alterations were transient as these differences were no longer present at one month follow-up. To date, studies on effects of probiotics on antibiotic induced microbiota aberrations in children are lacking. In a study including adult volunteers receiving ciprofloxacin and metrodinazol for seven days and either a comparable probiotic formulation consisting of bifidobacteria, lactobacilli and streptococci for four weeks (n=8), or no probiotics (n=7), a higher abundance of bifidobacteria and lactobacilli was found only during probiotic supplementation, which is in line with

our observations.[54] Contrary to our findings, they observed differences between probiotic supplemented and non-supplemented arms for a prolonged period. Microbiota of participants receiving probiotics did not return to their baseline samples within the five-month study period, while this was observed in the non-probiotic arm.[54] Another placebo controlled trial in adults receiving clarithromycin and amoxicillin for eradication of helicobacter pylori (n=136), supplementing the intervention arm with L. *paracasei* and L. *rhamnosus* for 28 days, including the 14 day antibiotic treatment, also showed that probiotic supplementation resulted in increased abundance of the supplemented probiotics during supplementation, in line with our results. Besides, they observed a reduced degree of antibiotic induced aberrations and earlier restoration within 28 days after antibiotic cessation, which was not observed in our study.[55] Several other studies on the effects of probiotic supplementation during antibiotic treatment on the gut microbiota included only adults or neonates, other types, doses and duration of probiotics and antibiotics and stool samples were collected at different time-points and analyzed by different analytical methods.[47] These differences limit the possibility to reliably compare results of these studies with our data.

The supplemented genera *Ligilactobacillus, Lactiplantibacillus* and *Lactobacillus* were found in higher abundance in the probiotic arm. These general are members of the lactic acid bacteria, are aerotolerant and express urease allowing to survive low pH levels in the stomach. This makes them able to survive the intestines in active form.[56,57] *Lactobacillus* species may prevent antibiotic induced epithelium dysfunction and stimulate the gut barrier integrity.[47] Besides, antibiotic exposure leads to microbiota aberrations, accumulation of carbohydrates and consequently to reduced levels of short chain fatty acids (SCFAs). As SCFAs promote the absorption of water from the colon, a decrease in SCFA provoke diarrhea.[47] As the lactic acid bacteria that were increased in the probiotic group play an important role in the digestion of carbohydrates into SCFA, increased abundance of these taxa may lead to increased SCFA concentrations. This will stimulate water absorption and decrease risk for antibiotic induced diarrhea.[47] Increased levels of SCFAs were found after *Lactobacillus* supplementation in adults and animal models.[58,59] Studies in antibiotic exposed children receiving probiotics on such metabolites are lacking. Seen the limited evidence, future mechanistic studies focusing on the microbiota function are warranted to elucidate the exact working mechanisms of probiotics. These studies may demonstrate whether the observed transient microbial effects have a mechanistic role in protection against antibiotic induced side effects, including AAD. We plan to perform metabolomics analysis on collected samples, allowing to obtain insight in microbial function rather than only composition. This may further elucidate the optimal types, combination, dosing and duration of probiotics. Future studies should also focus on long-term health outcomes of probiotic exposure, as this has not been studied.

Future perspectives

What may be clear from this thesis, is that infants and children are frequently exposed to antibiotics, which are often prescribed unnecessarily. As antibiotic exposure may have serious consequences for the microbiota and the risk for impact health outcomes such as NEC, asthma and obesity, it is pivotal to keep unnecessary antibiotic exposure to a minimum and improve strategies aiming at prevention of antibiotic-related side effects. In future, more personalized approaches are needed to determine which women and infants will benefit from IAP to reduce the amount of perinatal antibiotic exposure. More accurate diagnostic tools for EOS will likely further reduce the amount of unnecessary perinatal antibiotic exposure. Besides the studied diagnostic tools for EOS in this thesis, other biomarkers in maternal blood, cord blood and neonatal blood are currently being studied. It is expected that a multivariable approach using a combination of biomarkers, rapid culture techniques, risk factors and the clinical condition of the infant will provide the most accurate and quick diagnosis and predict which infants need antibiotics and which do not.

If perinatal antibiotic exposure and aberrations in microbial colonization cannot be averted, interventions preventing microbial-related side effects are needed. Currently, multiple feeding strategies such as the use of specific probiotic strains are being studied to prevent microbial aberrations and microbial-related side effects. In premature infants, the use of probiotics has been demonstrated to reduce the incidence of NEC. Future trials are however needed to determine whether infants exposed to antibiotics perinatally will also benefit from this intervention. Other strategies being studied to prevent microbial aberrations and microbial-related include maternal vaginal or rectal seeding. In CS born infants, it has been demonstrated that this quickly restores microbial aberration, but it is unknown if it is safe and effective after perinatal antibiotic exposure. Also for antibiotic exposure later in life, it is unknown what the best strategy is to prevent antibiotic-related side effects. We showed that probiotic use reduced the amount of diarrhea in children. However, future studies are needed to determine the most effective duration, dose and combination of probiotics.

In summary, antibiotic exposure in infants and children is frequent and unnecessary antibiotic exposure cannot always be averted. In this thesis we aimed to describe effects of perinatal antibiotic exposure, provide tools to improve the diagnosis of infections in order to reduce the amount of unnecessary antibiotic exposure and

study the efficacy of probiotic use in children exposed to antibiotics. Future research building on these data will hopefully further improve the care and outcomes for infants and children with (suspected) infections.

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Chapter 11

English Summary

In this thesis, we aimed to 1) describe effects of perinatal antibiotic exposure on the microbiota development and on health outcomes, 2) study strategies aiming at improvement in diagnosis of early-onset sepsis (EOS) and consequently a safe reduction in antibiotic overtreatment and 3) investigate whether probiotics can reduce antibiotic-induced side effect.

PART I: Effects of perinatal antibiotic exposure on infant microbiota and health outcomes

The microbiota is essential for our health and aberrations early in life increase risk for long-term outcomes such as asthma, allergies and obesity. Past decades, however, the use of maternal intrapartum antibiotic prophylaxis (IAP) has increased due to adjustments of international guidelines. In **Chapter 2** we systematically reviewed all data available on the influence of maternal IAP on the infant microbiota colonization and on health effects. We found that the use of IAP during vaginal delivery results in aberrations on microbial colonization. We observed a decreased diversity, decreased abundance of beneficial bacterial taxa such as bifidobacteria and Bacteroides with a concurrent increase in Proteobacteria. Studies investigating caesarean section (CS) born infants and studies on health outcomes were lacking. Therefore, we studied the effect of antibiotic exposure during CS in **Chapter 3**. Yearly, 30 million infants are born via CS, which are now all exposed to broad spectrum antibiotics at birth due to the guideline adjustments. Previously it was advised to administer IAP after clamping of the umbilical cord, but it is currently recommended to give these antibiotics 30 minutes prior to the CS, also exposing the infant to these antibiotics. This has led to a 3% reduction in post-operative maternal infections, but effects on the infant gut microbiota colonization were unknown. In our randomized controlled trial (RCT), we randomized 40 pregnant women to receive antibiotics either 30 minutes prior to CS or after clamping of the umbilical cord. Beside, we recruited 23 women delivering vaginally as a control group. We demonstrated that delivery via CS has a profound impact on the infant microbiota, but the timing of maternal antibiotic administration during CS did not further impact the microbiota of CS born infants.

Due to lack of rapid and accurate diagnostic tools for early-onset sepsis (EOS), infants are often exposed to antibiotics for a suspicion of EOS, awaiting results of the peripheral blood culture (PBC). To date, there are no accurate diagnostic tools to exclude EOS at initial suspicion. Current gold standard for EOS, a PBC generates a result 36-72 hours following sampling. As delay in antibiotic initiation may have dramatic consequences, many infants are empirically started on antibiotics for at least 36-72 awaiting PBC outcomes. Consequently, 5% of all newborns and over 80% of preterm infants (gestational age <30 weeks) are empirically exposed to

antibiotics, while the incidence of culture-proven EOS is only 0.1-0.5%. In a large observational study including 1257 preterm infants with gestational age below 30 weeks (**Chapter 4**), we demonstrated that a short course of antibiotics (36-72 hours) was associated with a decreased risk for necrotizing enterocolitis (NEC) compared to no or a prolonged antibiotic course. Besides, we observed a decreased odds for late-onset sepsis (LOS) for every additional day of antibiotic exposure.

PART II: improvement of early-onset sepsis diagnosis

Currently, there are no quick accurate tests to rule out EOS at initial suspicion. It is pivotal that strategies become available that can guide clinicians directly when to start and when to withhold antibiotics in case of EOS suspicion, in order to decrease antibiotic overtreatment of uninfected infants. In **Chapter 6** we studies whether presepsin is suitable for this goal in the largest observational study on presepsin so far. We found a high accuracy of 0.84 in preterm infants at initial EOS suspicion before initiation of empiric antibiotics. In term born infants, the accuracy was low (0.60). Presepsin may be suitable for clinical practice in preterm infants. This could potentially decrease the antibiotic overtreatment in this population. It is recommended to perform a RCT to study whether presepsin-guided antibiotic stewardship would indeed lead to a reduction in antibiotic overtreatment, without withholding antibiotics in infected EOS cases.

Last years, rapid molecular culture techniques have become available that can generate results much faster compared to PBC. One of these techniques is the Molecular Culture (MC), which is able to identify bacteria in a blood sample within 4 hours. In **Chapter 7** we studied whether MC was able to identify bacteria cultured by PBC and to identify the potential of MC in EOS diagnosis. Out of 15 selected blood samples that were positive by PBC, MC identified the same bacteria in 14 samples. In positive spiked blood samples, MC was able to detect bacteria even when the bacterial load was low. In 40 samples from a clinical cohort of infants with suspicion of EOS, both MC and PBC were negative in 92.5% of samples. MC was positive in one clinical EOS case for *Enterococcus faecalis*. Besides, MC was positive in two uninfected control patients, potentially due to contamination. MC may thus facilitate quick culture results within 4 hours and potentially replace PBC. In order to replace the PBC, one needs to demonstrate that the MC is able to detect all bacteria that are cultured by PBC. In other words, MC need to have a very high sensitivity and negative predictive value for the PBC results. Unfortunately, none of the infants in our clinical cohort had a positive PBC, and we were thus unable to demonstrate this. Future larger studies, including culture-positive infants are therefore warranted to further investigate whether the MC should replace PBC.

For current gold standard for EOS, a PBC, blood need to be drawn from a peripheral vein. This is a difficult and painful procedure and increases the risk for iatrogenic anemia, especially in very low-birthweight (VLBW) infants. Besides, if inadequate volume is samples, the risk for false negative PBC outcomes increases. Therefore, we performed a systematic review to study whether blood from the umbilical cord can also be used for culturing (**Chapter 5**). The collection of blood from the umbilical cord is easier, there is no risk for iatrogenic anemia and a larger volume can be sampled. We demonstrated that the accuracy of an umbilical cord blood culture (UCBC) seems to be comparable to PBC. However, studies were limited and had methodologic flaws. So, future research to validate these observations are needed.

PART III: role of probiotics during antibiotic therapy

In case of a bacterial infection in children, antibiotics are often indispensable. To date, it is unknown how to prevent or reduce the unwanted side effects of antibiotics. The most studies interventions are probiotics, but solid evidence in children is lacking. Therefore, we performed a blinded RCT including children receiving broad-spectrum antibiotics and randomized them to receive either a placebo product or probiotics. In **Chapter 8** and **9** we describe the effects of probiotic supplementation on the incidence of antibiotic-associated diarrhea (AAD) and effects on antibiotic-induced microbial aberrations respectively. We found that the risk for diarrhoea, regardless of its etiology, was significantly reduced in the probiotic group (relative risk 0.65). According to our more stringent definition of AAD, excluding diarrhoea caused by known pathogens as rota- noro- and adenovirus or salmonella, Shigella, Yersinia and Campylobacter spp. (SSYC), there was no statistically significant difference. As the former definition is in line with clinical practice, where it is not common to perform etiology tests in case of diarrhoea during antibiotic therapy, we concluded that probiotics may be considered during antibiotic treatment to reduce the risk for diarrhoea.

When we studied the microbiota of children included in our RCT, we found that three of five supplemented probiotic genera were more abundant during the intervention period in the probiotic group. This effect disappeared one month after cessation of probiotic supplementation. These genera play a role in the digestion of carbohydrates, and produce short chain fatty acids (SCFAs). As SCFAs promote the absorption of water from the colon, a decrease in SCFA may provoke diarrhea. In our study, we only investigated the microbiota, omitting its function. Future studies, also focusing on the microbial function, are needed to assess whether these transient effects on taxonomic composition and effects on diversity have a mechanistic role in the protection against antibiotic induced side effects like AAD.

Chapter 12

Nederlandse samenvatting

In dit proefschrift hebben we 1) onderzocht wat de effecten van perinatale blootstelling aan antibiotica op de ontwikkeling van het microbioom op gezondheidsuitkomsten zijn, 2) strategieën bestudeerd die gericht zijn op een meer accurate diagnose van early-onset sepsis EOS en daarmee een veilige vermindering van overbehandeling met antibiotica en 3) onderzocht of probiotica bijwerkingen en schade aan het microbioom ten gevolge van antibiotica kan verminderen.

DEEL I: Effecten van perinatale blootstelling aan antibiotica op het microbioom en gezondheidsuitkomsten

Het microbioom is essentieel voor onze gezondheid en afwijkingen op jonge leeftijd verhogen het risico op langetermijngevolgen zoals astma, allergieën en obesitas. De afgelopen decennia is het gebruik van maternaal intrapartum antibioticaprofylaxe (IAP) echter toegenomen door veranderingen in internationale richtlijnen. In **hoofdstuk 2** hebben we systematisch alle beschikbare literatuur beoordeeld over de invloed van maternale IAP op de kolonisatie van het microbioom bij neonaten en de invloed op gezondheidseffecten. We vonden dat het gebruik van IAP tijdens een vaginale bevalling leidt tot afwijkingen in het microbioom. We observeerden een lagere diversiteit, lager aantal commensale bacteriële taxa zoals bifidobacteria en Bacteroides met een gelijktijdige toename van Proteobacteria. Studies naar kinderen geboren via een keizersnede (CS) en studies naar gezondheidsresultaten ontbraken. Daarom hebben we in **hoofdstuk 3** het effect van antibioticablootstelling tijdens CS onderzocht. Jaarlijks worden 30 miljoen neonaten geboren via CS, die allemaal worden blootgesteld aan breedspectrum antibiotica als gevolg van de aanpassing van de richtlijn. Voorheen werd geadviseerd om IAP toe te dienen na het afklemmen van de navelstreng, maar momenteel wordt aangeraden om deze antibiotica 30 minuten voor de CS toe te dienen, waardoor het kind ook aan deze antibiotica wordt blootgesteld. Dit heeft geleid tot een vermindering van 3% in postoperatieve maternale infecties, maar de effecten op de kolonisatie van het darm microbioom van het kind waren onbekend. In onze gerandomiseerde gecontroleerde studie (RCT) hebben we 40 zwangere vrouwen gerandomiseerd om antibiotica te krijgen ofwel 30 minuten voorafgaand aan CS of na het afklemmen van de navelstreng. Daarnaast werden 23 vrouwen die vaginaal bevielen geïncludeerd als controle groep. We hebben aangetoond dat bevalling via CS een grote invloed heeft op het microbioom van de neonaat, maar dat de timing van de toediening van antibiotica aan de moeder tijdens CS geen verdere invloed heeft op het microbioom van CS geboren neonaten.

Door het gebrek aan snelle en nauwkeurige diagnostische testen voor early onset sepsis (EOS), worden neonaten vaak blootgesteld aan antibiotica voor een verdenking op EOS, in afwachting van de resultaten van de perifere bloedkweek

(PBC). Tot op heden zijn er geen nauwkeurige diagnostische testen om EOS direct bij de eerste verdenking hierop uit te sluiten. De huidige gouden standaard voor EOS, een PBC geeft pas na 36-72 uur een resultaat. Aangezien vertraging met het starten van antibiotica dramatische gevolgen kan hebben, worden bij veel neonaten empirisch gestart met antibiotica gedurende ten minste 36-72 in afwachting van PBC uitslag. Momenteel worden hierdoor 5% van alle pasgeborenen en meer dan 80% van de te vroeg geboren neonaten met zwangerschapsduur <30 weken empirisch blootgesteld aan antibiotica, terwijl de incidentie een bloedkweek bewezen EOS slechts 0,1-0,5% is. In een grote observationele studie met 1257 premature neonaten met een zwangerschapsduur van minder dan 30 weken (**hoofdstuk 4**), hebben we aangetoond dat een korte antibioticakuur (36-72 uur) geassocieerd was met een verminderd risico op necrotiserende enterocolitis (NEC) in vergelijking met geen of een langdurige antibioticakuur. Bovendien zagen we een lagere kans op late-onset sepsis (LOS) voor elke extra dag dat antibiotica werd gegeven.

DEEL II: verbetering in het diagnosticeren van early-opnset sepsis

Momenteel zijn er geen snelle, nauwkeurige tests om EOS bij een eerste verdenking uit te sluiten. Het is van cruciaal belang dat er diagnostische tests beschikbaar komen die direct kunnen aangeven of antibiotica gestart moet worden of niet in geval van verdenking op EOS, om hiermee overbehandeling met antibiotica van niet-geïnfecteerde neonaten te verminderen. In **hoofdstuk 6** onderzochten we of presepsin geschikt is voor dit doel. We vonden een hoge accuratesse van 0.84 bij premature neonaten bij de initiële verdenking op EOS vóór de start van empirische antibiotica. Bij a terme neonaten was de nauwkeurigheid laag (0.60). Presepsin is mogelijk geschikt voor de klinische praktijk bij premature neonaten. Dit zou mogelijk de overbehandeling met antibiotica in deze populatie kunnen verminderen. Het wordt aanbevolen om een RCT uit te voeren om te onderzoeken of een presepsin gestuurd antibioticabeleid inderdaad zou leiden tot een vermindering van overbehandeling met antibiotica, zonder antibiotica onthouden neonaten met EOS.

De laatste jaren zijn er snelle moleculaire kweektechnieken beschikbaar gekomen die veel sneller resultaten kunnen genereren in vergelijking met een PBC. Een van deze technieken is de Molecular Culture (MC), die in staat is om binnen 4 uur bacteriën in een bloedmonster aan te tonen. In **hoofdstuk 7** hebben we onderzocht of MC in staat was om bacteriën te identificeren die gekweekt waren door PBC en hebben we de potentie van MC voor EOS diagnose onderzocht. Van de 15 geselecteerde bloedmonsters die positief waren met een PBC, identificeerde MC dezelfde bacteriën in 14 monsters. In bloedmonsters met toegevoegde bacteriën kon MC deze bacteriën detecteren, zelfs als de bacteriële load laag was. In een klinisch cohort met 40 samples van neonaten met een verdenking op EOS, waren zowel MC

als PBC negatief in 92,5% van stede samples. MC was positief in één neonaten met klinische EOS voor *Enterococcus faecalis*. Bovendien was MC positief bij twee niet-geïnfecteerde controle patiënten, mogelijk als gevolg van contaminatie. MC kan dus snelle kweekresultaten genereren binnen 4 uur en mogelijk de PBC vervangen. Om de PBC te vervangen moet men aantonen dat de MC in staat is om alle bacteriën die door PBC worden gekweekt te detecteren. Met andere woorden, MC moet een zeer hoge sensitiviteit en negatief voorspellende waarde hebben voor de PBC resultaten. Helaas had geen van de neonaten in ons klinische cohort een positieve PBC en konden we dit dus niet aantonen. Toekomstige grotere studies, positieve PBC's zijn daarom nodig om verder te onderzoeken of de MC de PBC zou moeten vervangen.

Voor de huidige gouden standaard voor EOS, een PBC, moet bloed worden afgenomen uit een perifere vene. Dit is een moeilijke en pijnlijke procedure en verhoogt het risico op iatrogene anemie, vooral bij neonaten met een zeer laag geboortegewicht (VLBW). Bovendien, als er onvoldoende materiaal is afgenomen, neemt het risico op fout-negatieve PBC uitslagen toe. Daarom hebben we een systematische review uitgevoerd om te onderzoeken of bloed uit de navelstreng ook gebruikt kan worden voor een bloedkweek (**hoofdstuk 5**). Het afnemen van bloed uit de navelstreng is eenvoudiger, er is geen risico op iatrogene anemie en er kan een groter volume worden afgenomen. We hebben aangetoond dat de accuratesse van een navelstrengbloedkweek (UCBC) vergelijkbaar lijkt met PBC. Er waren echter een beperkt aantal uitgevoerde studies en hadden methodologische gebreken. Er is dus toekomstig onderzoek nodig om dit te valideren.

DEEL III: rol van probiotica tijdens antibiotica therapie

Bij een bacteriële infectie bij kinderen zijn antibiotica vaak onmisbaar. Tot op heden is het onbekend hoe de ongewenste bijwerkingen van antibiotica kunnen worden voorkomen of verminderd. De meeste onderzoeken met dit doel zijn door gebruik te maken van probiotica, maar hard bewijs bij kinderen ontbreekt. Daarom hebben we een geblindeerde RCT uitgevoerd met kinderen die breedspectrum antibiotica kregen en gerandomiseerd om een placeboproduct of probiotica te ontvangen. In **hoofdstuk 8** en **9** beschrijven we respectievelijk de effecten van probiotica suppletie op de incidentie van antibiotica-geassocieerde diarree (AAD) en effecten op antibiotica-geïnduceerde microbioom afwijkingen. We vonden dat het risico op diarree, ongeacht de etiologie, significant lager was in de probiotica groep (relatief risico 0.65). Volgens een strengere definitie van AAD, exclusief diarree veroorzaakt door bekende pathogenen als rotanoro- en adenovirus of salmonella, Shigella, Yersinia en Campylobacter spp. (SSYC), was er geen statistisch significant verschil. Aangezien de eerstgenoemde definitie in overeenstemming is met de klinische praktijk, waar het niet gebruikelijk is om etiologietesten uit te voeren in geval van

diarree tijdens behandeling met antibiotica, concludeerden we dat het gebruik van probiotica overwogen kan worden tijdens behandeling met antibiotica om het risico op diarree te verminderen.

Bij het bestuderen van het microbioom van kinderen die in onze RCT waren geincludeerd, ontdekten we dat drie van de vijf gecomplementeerde probiotica stammen meer aanwezig waren tijdens de interventieperiode in de probiotica groep. Dit effect verdween een maand na het stoppen van de probiotica suppletie. Deze bacteriële stammen spelen een rol bij de vertering van koolhydraten en produceren korte-keten-vetzuren (SCFA's). Aangezien SCFA's de opname van water uit de dikke darm bevorderen, kan een afname van SCFA leiden tot diarree. In onze studie hebben we alleen het microbioom onderzocht en niet naar de functie ervan gekeken. Toekomstige studies, ook gericht op de microbiële functie, zijn daarom nodig om te beoordelen of deze voorbijgaande effecten op taxonomische samenstelling en effecten op diversiteit een mechanistische rol spelen bij de bescherming tegen bijwerkingen veroorzaakt door antibiotica zoals AAD.

Chapter 13

Appendices

PhD Portfolio

Name PhD student:	Thomas Harry Dierikx
PhD period:	September 2019 – February 2023
Name PhD supervisors:	Prof. dr. M.A. Benninga Prof. dr. A.H.L.C. van Kaam Dr. T.G.J. de Meij Dr. D.H. Visser

1. PhD training

General courses	Year	Workload (Hours/ECTS)
BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2019	1.5 ECTS

Specific courses	Year	Workload (Hours/ECTS)
Practical Biostatistics (e-learning)	2020	1.4 ECTS
E-learning 'zoeken voor een CAT maken'	2019	0.1 ECTS
Harvard course: 'The Health Effects of Climate Change'	2020	1.0 ECTS
Writing a scientific paper (e-course)	2021	1.5 ECTS
Advanced topics in biostatistics	2021	2.1 ECTS
Bioinformatics	2021	1.1 ECTS
Laboratory Safety (e-learning)	2021	0.3 ECTS
Master Evidence Based Practice in Clinical Health (Registration as Epidemiologist)	2020-2022	97 ECTS

Seminars, workshops and master classes	Year	Workload (Hours/ECTS)
Masterclass AKS	2021	0.4
Workshop Pitch GROWTH *ElroyCOM*	2021	0.4
Diner Pensant – yearly evening seminar on pediatric gastroenterology	2019-2023	1.0

Presentations	Year	Workload (Hours/ECTS)
Amsterdam Kindersymposium 2020	2020	0.25
Poster presentation ESPID 2021	2021	0.25
Affiliatiedag Kindergeneeskunde 2019	2019	0.25
Amsterdam Kindersymposium 2021	2021	0.25
ESPGHAN 2021	2021	0.25
BVK 2022	2022	0.25
Microbiome R&D and Business Collaboration Forum	2022	0.25
EAPS 2022	2022	0.25
AKS Lunch symposium 2023	2023	0.25
AR&D retreat 2022	2022	0.25

(Inter)national conferences	Year	Workload (Hours/ECTS)
Amsterdam Kinder Symposium 2019	2019	0.25
Harm Oberweis symposium 2019	2019	0.25
Gut day AMC 2019	2019	0.25
AG&M retreat 2020	2020	1.0
AG&M retreat 2022	2022	1.0
NVK 2020	2020	0.25
EPGS Meest gestelde vragen aan de kindergastro-enteroloog	2023	1.0

Other	Year	Workload (Hours/ECTS)
Journal clubs / watch 2019 – 2023	2019-2023	2.0
Organizing committee member of the AKS	2021-2023	3.0

2. Teaching

Lecturing	Year	Workload (Hours/ECTS)
Lecture Minor Bachelor Medicine students 2019	2019	0.3
Lecture Minor Bachelor Medicine students 2020	2020	0.3
Lecture Minor Bachelor Medicine students 2021	2021	0.3
Lecture Minor Bachelor Medicine students 2022	2022	0.3
Lecture Minor Bachelor Medicine students 2023	2023	0.3

Tutoring, Mentoring and supervising	Year	Workload (Hours/ECTS)
Supervision internship/thesis Bachelor students K. de Boer B3 Medicine E. Klinkenberg B3 Medicine M. Hoofdman B3 Biomedical sciences Y. Owusu B2 Biomedical sciences E. Hardijzer B3 Biomedical sciences	2019-2023	2.5
Superivison internship/thesis Master students N. Gülmez M1 Medicine J. Szkodon M1 Bioinformatics S. Habets M3 Medicine J. Groen M3 Medicine S. Hulsmann M3 Medicine C. Vervenne M3 Medicine	2019-2023	6.0
Supervising CAT medical Master students	2019-2023	2.0
Supervising PRE-university students A. van der Rijt and T. de Regt 2020 P. Smits, F. Wakkerman and F. Romeyn 2019	2019-2020	1.0

3. Parameters of Esteem

Grants	Year
Zeldzame Ziekte Fonds (ZZF) grant	2019
ForWis(h)dom grant	2020
Vaillaint Fonds grant	2020
Fonds Gezond Geboren grant	2021
Janivo grant	2020
Reggenborgh grant	2022
AR&D Travel grant	2022

Awards and Prizes	year
AKS top 6 best abstracts	2022

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Dierikx, T.H., Łukasik, J., Malinowska, A., Besseling - van der Vaart, I., Belzer, C., Szajewska, H., de Meij, T.G.J., Multispecies Probiotic in AAD Study Group. Longitudinal effects of a multispecies probiotic formulation on antibioticinduced microbial aberrations in children: a randomized clinical trial [Manuscript in preparation]

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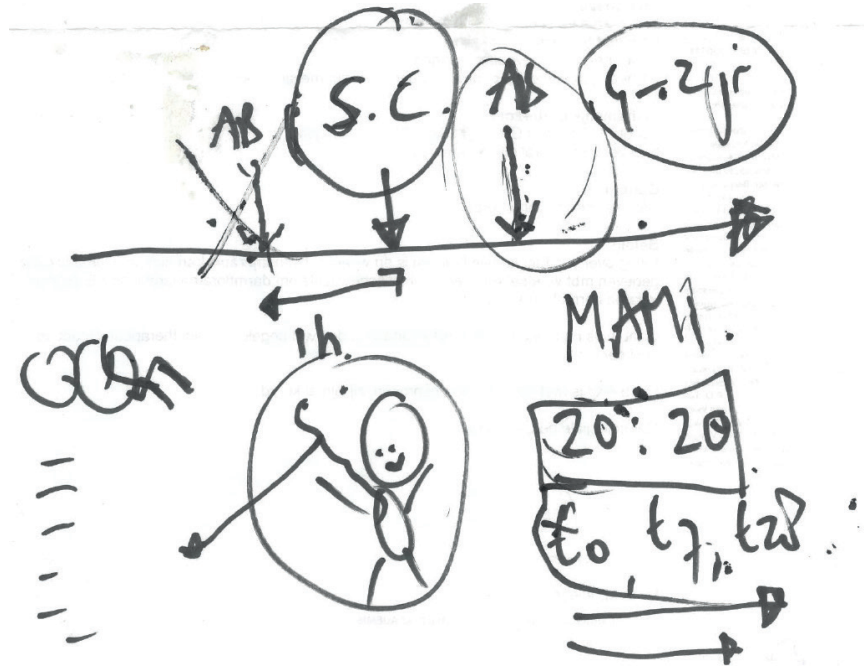
Over de auteur

Thomas Dierikx is als oudste uit een gezin van twee geboren op 22 september 1995 te Amsterdam. Alhier groeide hij op en rondde hij in 2013 zijn middelbare school af aan het Vossius Gymnasium. Daarna startte hij met zijn opleiding geneeskunde aan de Vrije Universiteit welke hij in 2019 voltooide. Tijdens deze opleiding groeide zijn interesse voor de kindergeneeskunde en sloot hij zijn opleiding tot basisarts af bij de Kinder-MDL als wetenschapsstudent bij Tim de Meij. Zijn belangstelling voor wetenschappelijk onderzoek nam toe en na deze wetenschappelijke stage stroomde hij in september 2019 door als promovendus. Tijdens dit promotietraject voltooide hij een universitaire Master tot epidemioloog, volgde hij diverse cursussen, presenteerde hij op (inter) nationale congressen, organiseerde tweemaal het Amsterdam Kindersymposium (AKS) en deed hij meerder subsidie aanvragen samen met zijn begeleiders, waarvan ze ook meerdere ontvingen. Momenteel woont hij samen met zijn vriendin Kayleigh in Eindhoven en is hij werkzaam als ANIOS Kindegeneeskunde in het VieCuri medisch centrum te Venlo, maar blijft hij betrokken bij de vervolgonderzoeken.

Dankwoord

En dan zijn we al bij het laatste, maar meest gelezen en dus misschien wel het belangrijkste hoofdstuk van dit boek aangekomen. Wie had ooit gedacht dat een wetenschappelijke stage 'waarin je wat ouders achter hun broek aan moest zitten om kak van hun kind te verzamelen' zou uitmonden in dit promotie boekje. Toen ik aan die wetenschappelijke stage begon, had ik dat in ieder geval niet gedacht. Het is dan ook dat dit boekje niet tot stand had gekomen zonder de hulp van een heleboel andere mensen. Daarom zou ik iedereen die dit boekje mede mogelijk heeft gemaakt, direct dan wel indirect, heel graag willen bedanken voor alle hulp en steun.

Allereerst wil ik jou bedanken **Tim**, als hoofdaanspreekpunt voor mij tijdens dit gehele promotietraject, voor je onvoorwaardelijk vertrouwen in mij en alle hulp de afgelopen jaren. Via **Luçan**, mijn begeleider tijdens de semi-arts stage, kwam ik bij jou terecht. Tijdens de semi-arts stage had ik nog geen idee waar ik onderzoek naar wilde doen. Luçan nam me mee naar het welbekende diner pensant waar Marc en jij ook zouden zijn. Hier zou ze me aan jullie voorstellen en bij jullie peilen of er wellicht een mogelijkheid was om mijn wetenschappelijke stage bij jullie bij de kinder-MDL te doen. Luçan, jou wil ik hiervoor heel erg bedanken. Zonder deze moeite was ik mogelijk heel ergens anders beland en had ik deze prachtige reis gemist. Helaas was jij, Tim, er zelf niet bij die avond omdat je in Indonesië zat, maar Marc was er wel. Na een kort gesprek zei Marc: we hebben vast ergens een plek voor je, mail Tim en mij maar dan bekijken we het wel even. En zo geschiedde, na je terugkomst vroeg je meteen of ik langs wilde komen, zodat je kon uitleggen wat voor stages je in de aanbieding had. Op jouw kamer op de 9e verdieping van het VUmc legde je uit dat ene Daan de MAMI studie had opgezet en ik het vervolg kon overnemen tijdens mijn wetenschappelijke stage. De opzet van de studie vertelde je nogal onduidelijk, het was iets met kak verzamelen van kinderen, maar gelukkig maakte je een hele verhelderende tekening, welke ik tot op de dag van vandaag heb bewaard (Figuur 1.). Jouw tekenkunsten haalde mij over om zelfs een verlengde wetenschappelijke stage te lopen. Voor ik het wist, volgens mij in mijn 2e week al, vroeg je me op gesprek en of ik interesse had in een promotietraject na deze stage. Enigszins onthuts als ik was (ik was toch pas net bezig, hoe weet hij nu al dat hij mij de komende jaren wil begeleiden) zei ik dat ik zeker interesse had in een promotietraject. En zo geschiedde. Tim, ik wil je bedanken voor alle leerzame feedback op de stukken, je altijd snelle reactie op mijn mails ondanks de drukte, maar uiteraard ook voor de leuke uitjes zoals de ZZF rally in Rotterdam.



Douwe, ook jou als copromotor en dagelijkse begeleider voor 'het neonatologie deel' van mijn boekje ben ik uiteraard heel veel dank verontschuldigd. Via via kwam ik in het OLVG terecht waar jij toevallig net op zoek was naar iemand die de kar kon trekken voor de presepsin studie. Na een gesprek samen met Tim kwamen we tot de conclusie dat dit wel eens heel mooi aan kon sluiten bij de andere studies in dit boekje. En zo werd jij mijn copromotor. Tijdens de afgelopen jaren heb ik je leren kennen als een hele fijne, vriendelijke en zeer laagdrempelig benaderbare mentor die altijd het beste met mij voort heeft. Je nam altijd de tijd om samen te zitten, samen naar de data en analyses te kijken en samen verder te denken over het vervolg. Dit maakte dat ik me altijd serieus genomen en gehoord voelde, wat de samenwerking heel prettig maakte.

Marc en **Anton**, als promotoren wil ik jullie uiteraard ook super erg bedanken. Omdat Tim en Douwe zo nauw betrokken waren, spraken wij elkaar niet dagelijks. Tijdens onze besprekingen echter, waren jullie allebei altijd super scherp, kwamen met goede ideeën en brachten nieuwe inzichten die mij weer verder hielpen. Tijdens de feedback rondes op een nieuw manuscript waren jullie allebei altijd één van de eerste die het stuk hadden gelezen en van kritische feedback hadden voorzien. Ook op andere mails reageerden jullie vrijwel altijd direct. Ook jullie wil ik bedanken dat jullie te allen tijde laagdrempelig beschikbaar waren en de bespreking altijd luchtig maakten met af en toe flauwe humor.

Naast jullie 4 als begeleiders wil ik ook alle neonatologen bedenken die betrokken zijn geweest bij de eNose studie voor de hulp bij en het mogelijk maken van alle dataverzameling. **Daniel Vijlbrief**, **Christian Hulzebos**, **Willem de Boode**, **Richard van Lingen**, **Esther d'Haens**, **Veerle Cossey**, **Boris Kramer**, **Marlou Kouwenberg – Raets**, **Mirjam van Weissenbruch** en **Chris van den Akker**: bedankt voor het mede mogelijk maken van de eNose studie! In het bijzonder wil ik hierbij **Hendrik Niemarkt** bedanken, voor alle hulp, overleggen, steun en wijze inzichten rondom de eNose studie en ander projecten waar je bij betrokken bent geweest.

Ook jou, **Sophie van der Schoor**, wil ik graag bedanken. Jij maakte het mogelijk dat zowel de presepsin studie als de probiotica studie in het OLVG konden plaats vinden. Bedankt dat je altijd tijd had voor overleg over logistieke zaken en monitor visites. **Malika Chegary** en **Henriëtte van Laerhoven**, ook jullie bedankt voor het mede mogelijk maken van deze studies in het OLVG.

Hierin wil ik graag ook alle andere **kinderartsen**, **neonatologen**, **arts-assistenten** en **verpleegkundigen** van het OLVG en Amsterdam UMC, en natuurlijk van alle deelnemende centra aan de eNose studie, die ooit een poep of bloed sample voor een van de studies heeft afgenomen vele malen bedanken. Zelfs in de avond en nachturen dachten jullie aan onze studies.

Verder wil ik natuurlijk alle honderden **kinderen** en **hun ouders** van harte bedanken die vrijwillig zo enorm veel energie en tijd hebben gestopt in het mee doen aan de studies, ook al betekende dit soms dat er diarree uit een stinkende poepluier geschept moest worden. Zonder jullie hadden alle onderzoeken uit dit proefschrift uiteraard nooit kunnen plaatsvinden.

Collega's en ex-basement kamergenoten, **Nancy**, **Jasmijn**, **Britt**, **Sofia**, **Hannah, Jip, Eva, Nathalie, Nina** en **Jacqueline**, maar ook de NICU rode luifel bombers, **Anouk**, **Juliette**, **Michelle**, **Ruud**, **Debbie**, **Irene**, **Trix**, dank dat jullie er altijd waren om te sparren over statistiek of over het leven. **Sofia** en **Daan**, jullie bedankt voor al het voorbereide werk en de vele uren die jullie hebben gestopt in het opzetten van zowel de MAMI studie als de eNose studie, waar ik de vruchten van kon plukken. **Nina**, jij bedankt voor al je wijze lab lessen. Zonder jou was het me nooit gelukt om zelfstandig 16S gene sequencing uit te voeren in het lab in Birmingham. **Andrew** and **Celina**, I want to thank you for the hospitality to analyses our stool samples in your lab and for all the help with this lab dummy that I am.

Iedereen van het **secretariaat**, en in het bijzonder **Sylvia**, jullie heel erg bedankt voor alle hulp en een luisterend oor. Als ik weer eens een ingewikkelde bestelling had, of een aanvraag voor een student, hielpen jullie me altijd uit de brand.

Uiteraard ook iedereen die deze studies financieel mede mogelijk heeft gemaakt wil ik graag bedanken. **Nutricia Research Foundation**, **Stichting Zeldzame Ziektefonds**, **Stichting ForWis(h)dom**, **Janivo Stichting**, **het Vaillant Fonds**, **Stichting Fonds Gezond Geboren** en het **Amsterdam Reproduction and Research institute**, veel dank voor alle financiële ondersteuning de afgelopen jaren.

Alle studenten die mij tijdens hun wetenschappelijke stage in meer of mindere maten hebben geholpen met mijn studies, ook jullie bedankt uiteraard. **Charlotte**, **Emma**, **Jip**, **Jolanta**, **Myron**, **Nur Eda**, **Ryan** & **Ryan**, **Sanne**, **Sarah** en **Yochabel**, bedankt!

Louis, **Vince** en **Victor**, jullie veel dank voor alle interesse in mijn schijtonderzoeken de afgelopen Jaren. Ook veel dank voor alle poepgrappen over mijn werk en voor de mogelijkheid om altijd even af te schakelen middels wat gezelligheid. **Seb**, ook jij bedankt het onvoorwaardelijk vertrouwen in mijn onderzoek kunsten en in probiotica. Als **paranimfen** wil ik jullie uiteraard ook bedanken voor alle hulp en regelwerk voor deze dag.

Lieve **familie**, **schoonfamilie**, **Opa's**, **Oma's** en **Petra**, ik wil jullie heel graag bedanken voor alle interesse en emotionele steun de afgelopen jaren. Als ik zag hoe enorm trots jullie op mij waren, kreeg ik weer een boost om verder te gaan.

Simone, kleine etterbak, ook jou kan ik natuurlijk niet vergeten. Ondanks dat je het vaak heel druk had in Groningen, was je er altijd voor me met een luisterend oor als ik dit nodig had. **Papa**, **Mama**, ik kan niet in woorden omschrijven hoe dankbaar ik ben voor alles wat jullie mij hebben geleerd en de afgelopen jaren hebben gedaan om deze prestatie mede mogelijk te maken. Van het ondersteunen in het volgen van een post initiële master, het verzorgen van een avondmaal als ik tot laat had gewerkt, tot het altijd uitlenen van de auto als ik weer eens een ontlasting sample ergens ver weg moest ophalen, niks was voor jullie te gek. Zonder jullie hulp en steun was dit nooit gelukt.

En last but not least, wil ik jou, **Kayleigh**, natuurlijk heel erg bedanken. Zonder jouw steun en hulp was me dit nooit gelukt. Ik denk dat veel mensen niet beseffen hoeveel jij hebt bijgedragen aan de totstandkoming van dit boek. Uren heb je met mee meegereden door het hele land, van Alkmaar, tot Rotterdam naar Bussum om kak samples voor de MAMI en IPAD studie op te halen. Vele malen leende je je auto

uit, zodat we samen poep konden ophalen. Ontwerpen van figuren voor in mijn manuscripten en stickeren van buisjes achter de TV deed je ook zonder te zeuren. En dat allemaal terwijl we samen een geheel huis verbouwden en jij ook nog eens als ANIOS aan het knallen was in Den Haag en Gouda. Hoewel je dat misschien niet altijd even goed door hebt, ben ik mega trots op hoe je dat allemaal voor elkaar krijgt. Lieve Kayleigh, nogmaals wil ik zeggen hoe ontzettend blij ik ben met jou aan mijn zijde, je onvoorwaardelijke liefde en alle hulp in het tot stand brengen van dit boek. Ik houd ontzettend veel van jou. Ik weet niet hoe ik dit zonder jou had kunnen doen.

Daarnaast ben ik vast en zeker nog een hele hoop mensen vergeten te bedanken die ook direct of indirect hebben bijgedragen aan de totstandkoming van dit proefschrift. Voel jij je aangesproken? Dan wil ik ook jou bij deze heel hartelijk bedanken.