Antibiotic exposure in the first week of life, microbiota development and health outcomes

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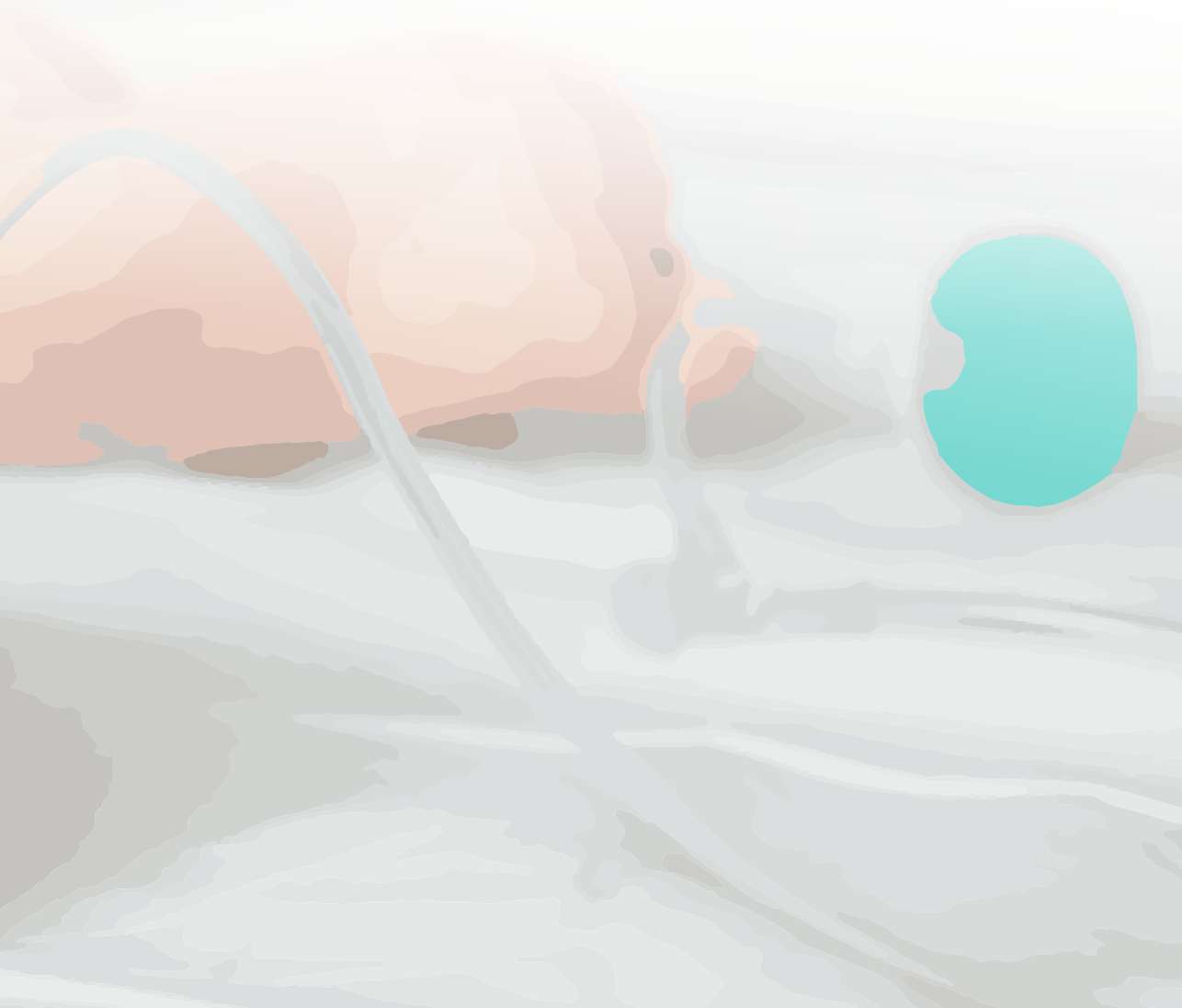
Contents

Chapter 1

General introduction, aims and outline of the thesis



First 1000 days of life

The first 1000 days of life – the period from conception to 2 years of age – is a critical period in early life (1). This prenatal and early postnatal period is characterized by a rapid maturation of the metabolic, endocrine, neural, and immune systems, developing in tandem and interdependent (1). The first 1000 days of life is therefore also referred to as the period of fetal programming: a concept that links nutritional and environmental conditions during embryonic and fetal development with the risk of diseases later in life (2). Emerging evidence suggests that the colonization of microbes in the human body during early life plays a critical role in establishing and maturing these developmental systems (1). The human body contains trillions of microbes (microbiota) and their genes (microbiome) that assemble and stabilize during the first two to three years of life (3). Disruption of the normal microbial succession may contribute to lifelong health problems (4).

The gut microbiome

The gut microbiome is a collection of all microorganisms that reside in the gastrointestinal tract and consist of bacteria, viruses, fungi, and protozoa (5, 6). Together, these microorganisms can affect many processes, like human metabolism (7, 8), inflammatory responses, and the integrity and structure of the gastrointestinal tract (7). It is therefore not surprising that the gut microbiome plays a crucial role in the development and maturation of the immune system (9). At birth, the gut microbiome is almost sterile with low diversity (10). Bacteria start to appear in the feces within hours after birth, and their numbers increase progressively during the first week of life (11). In the first two to three years of life, the gut microbiome matures according to several developmental stages with increasing diversity over time, before it stabilizes (10). Through 16S rRNA sequencing, Firmicutes and Bacteroidetes have been shown to make up approximately 92% of the human microbiome (12). The diversity at the species level differs significantly between individuals; each individual harbors at least 160 different species of the 1,000 to 1,500 prevalent species of bacteria that are found in the gut (13). The maturation of the gut microbiome is driven by exposure to microbes from maternal, environmental, and dietary sources (14) as shown in Figure 1, and can be disrupted by many factors, especially when they occur early in this developmental process. This disruption and its immune consequences are perhaps most important during the first year of life when the microbiome composition is rapidly changing (15).

Immune system and gut microbiome

The commensal gut microbiota has been shown to have a strong influence on the development of the immune system and vice versa (16). An infant's immune system is immature at birth and matures gradually during infancy (17). That the immune system is still developing is reflected, for example, by the number of upper respiratory tract infections and otitis media episodes that children experience in their first years of life (18, 19). The symbiotic relationship between the microbiota and the host is important for both. The host provides a habitat and nutrients for the microbiome while the gut microbiota supports the development and maturation of the gut immune system by providing beneficial nutrients (20, 21). Microbial colonization depends on the development of the immune system. The immune system regulates the colonization of the gut microbiome by interfering with its ability to bind the mucosa, while parts of bacterial cells and metabolites modulate the activity of the immune system (16). The rapid colonization of microbiota in the neonatal gastrointestinal tract plays a vital role in the development of the gut immune system (22). Studies in germ-free mice have shown that the lack of gut microbiota causes a significant deficiency of the immune system (23). Altering microbial communities in the gut can disturb this interplay between the immune system and the microbiome and lead to immune dysregulation and potentially disease susceptibility (24). Emerging evidence suggests that the immune influences induced by the microbiome in early life may be long-lasting, providing a "window of opportunity" for proper immune education and resistance or susceptibility to diseases later in life (25-27).

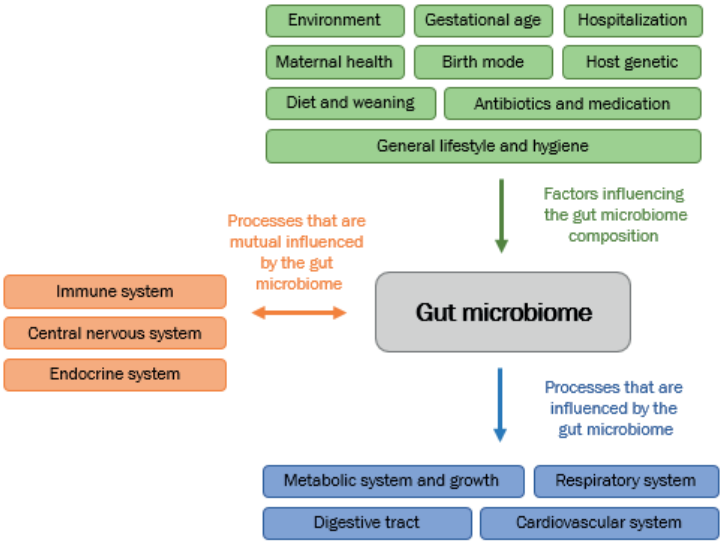


Figure 1: factors influencing the gut microbiome development and its interaction with processes in the body

The gut microbiome and antibiotics

The exact “window of opportunity” is unknown, but during the development phase, the microbiota is unstable and prone to perturbations. Many factors in early life influence microbiome development, such as maternal microbiome, birth mode, type of feeding, length of gestation, and exposure to medications (28). Disruption of the developing microbiome (dysbiosis) can impact later health (29), as it has been associated with irritable bowel syndrome, inflammatory bowel disease, autoimmune diseases, atopic diseases, and metabolic disorders (30-34). Dysbiosis, especially when it occurs immediately after birth, is likely to have serious consequences (32).

An important cause of dysbiosis are antibiotics, one of the most prescribed drugs in early infancy (35, 36). In Europe, between 2 and 16% of all newborns are exposed to antibiotics in their first week of life for suspected early-onset sepsis (EOS) (35, 37, 38). An average of 58 newborns without EOS are treated per case because the consequences of delayed treatment are significant. Even after the first week of life, antibiotics are the most prescribed drugs to children in the Netherlands (39), although the Netherlands is one of the countries with the most prudent antibiotic policy (40). However, the promise doctors make: primum no nocere, in English: first, do no harm, does not always seem to hold when it comes to antibiotic prescriptions. Antibiotics can decrease the number of bifidobacterial (41) and Bacteroidetes and increase the number of clostridia (42, 43). Not only antibiotics administrated to the infant have microbial consequences, but maternal exposure to antibiotics can affect the development of the neonatal microbiome as well (44, 45). Children born to mothers who received antibiotics intrapartum showed less colonization by bacteria belonging to the phylum Actinobacteria (including Bifidobacterium) or the genus Lactobacillus but were instead more likely to be colonized by the phyla Firmicutes and Proteobacteria (including Enterobacteriaceae) (46, 47). These changes persist during the first months of life. Maternal vaginal and fecal microbes play a role in neonatal microbiota establishment via vertical transmission (48, 49). Therefore, antibiotic exposure during pregnancy, especially at the end of a pregnancy, can alter the bacterial settlement in the unborn child (50).

Impact of antibiotics

The size of the impact of antibiotics on the gut microbiome depends on several factors. First, it is known that the gut microbiome rapidly changes in the first weeks of life (51). Therefore, the developmental stage of the gut microbiome during exposure to antibiotics is an important factor when studying the impact on the gut microbiome. Second, the route of AB administration has different effects on the gut microbiome (52-54). In the first week of life, antibiotics are mainly

administrated intravenously, while after the first week of life, antibiotic treatments are mostly prescribed orally. It has been shown previously that a combination of oral and intravenously antibiotics administration can have a larger detrimental effect on the gut microbiome than purely intravenously administration (53). Third, the spectrum of antimicrobial activity and the combination of antibiotics determines the impact on the bacterial composition of the gut microbiome (55). Broad-spectrum antibiotics have a more devastating effect on the gut microbiome compared to narrow-spectrum antibiotics (55). Furthermore, a combination of two AB (for example gentamicin and ampicillin) has been shown to have a larger detrimental effect on the gut microbiome than each antibiotic separately (55). Finally, the duration of exposure to antibiotics is of influence, a longer duration of antibiotics is associated with a more disturbed gut microbiome (56, 57).

Health problems associated with antibiotic exposure

A range of studies has demonstrated that antibiotic exposure is associated with future health problems, like atopic disorders, celiac disease, overweight and obesity, and behavioral problems (58). Since the neonate has an almost sterile gut at birth (59), hypothetically, the most profound effect of antibiotics can be expected when administered already in the first week of life. The effect of antibiotic exposure specifically in the first week of life, however, has not yet been fully elucidated. Therefore, the INCA study “INtestinal microbiota Composition after Antibiotic treatment in early life” was set up (60). The first results of this study showed that children exposed to antibiotics in their first week of life had an altered gut microbiota in the first year of life (61), a higher risk of wheezing and infantile colic (62), and an altered circulating immune marker profile at one year of age (63). In addition to the INCA study, only some studies have been performed examining antibiotic exposure specifically in the first week of life. These few studies also show that children exposed to antibiotics in their first week of life had an altered gut microbiota in the first year of life (64, 65), and had a higher risk of wheezing (66, 67), as well as showing an association with gastrointestinal disorders (68). After the first year of life, associations were found with impaired growth (69), and an increased risk of allergic rhinitis (70) and asthma (71, 72).

INCA study

The INCA study was set up in 2011 to investigate the clinical and microbial consequences of exposure to antibiotics in the first week of life. Between 2012 and 2015, term-born infants were recruited from the maternity and neonatal wards of four teaching hospitals in the Netherlands (60). All parents of term infants (≥ 36 weeks of gestational age) who stayed in

one of the four participating hospitals for at least 24 h, were approached for participation in the study. Of the 436 included infants, 151 infants were treated with a combination of broad-spectrum antibiotics because of a suspicion of EOS, and 285 infants were not exposed to antibiotics. Blood cultures were taken before antibiotic treatment was started. In case of a negative blood culture, combined with a low clinical suspicion of infection and low C-reactive protein, antibiotics were discontinued after two to three days (n=42). Otherwise, antibiotics were continued for seven days (n=109). The non-exposed infants were born in the hospital and needed clinical observation for 24–48 h for several reasons like maternal comorbidity, observation for low probability of neonatal infection, blood sugar monitoring, meconium containing amniotic fluid, or delivery by cesarean section.

First years of life

The included children were followed for the entire first year of life. At inclusion, parents filled out an online questionnaire concerning the background of the family (e.g., parental atopic diseases, household education, and the presence of siblings). Thereafter, a daily checklist was kept during the first year of life, reporting the presence of wheezing, crying, rash, and other symptoms. Furthermore, a monthly questionnaire was filled out during the first year of life concerning nutrition, antibiotic treatment, and general practitioner visits. The symptoms were verified at 1 year of age through doctors' diagnoses via the general practitioner electronic medical database using the International Classification of Primary Care (ICPC) (73). Around one year of age, a blood sample was obtained if parents gave additional informed consent (n=149). For the microbiome analyses, fecal samples were collected at nine time points (around day one and two, the first and second week, one, three, and six months of age, and one and two years).

Follow-up at 4-6 years of age

After the first year of life, a follow-up study was conducted at 4-6 years of age. Validated questionnaires were used to collect data regarding eczema, wheezing/asthma, allergic rhinitis, and functional gastrointestinal disorders (FGIDs). Furthermore, the presence of parental-reported allergies, acute otitis media (AOM), and otitis media effusion (OME) was collected. If parents gave additional informed consent, doctor's diagnoses were verified, and the pharmacy was approached to collect information about medication use.

Aim of the thesis

There is sufficient evidence showing that antibiotic treatment in early life has a detrimental effect on the gut microbiome composition. It is, however, not yet clear to what

extent the gut microbiome composition is disrupted, for how long this dysbiosis will persist, and what the effects are on the developing immune system and long-term health. Therefore, the studies presented in this thesis shed extra light on these questions.

Outline of the thesis

This thesis consists of two parts. The first part described the association between antibiotics in the first week of life and clinical health outcomes.

In **chapter 2**, we report the association between antibiotics administered in the first week of life and growth in the first year of life and compared it with the association of antibiotic courses administered later in the first year. In addition, the association between the duration of antibiotic treatment and growth in the first year of life was studied, as well as the association with different types of antibiotics.

Because the immune system is still developing in the first years of life, which is reflected, among others, in (repeated) episodes of otitis media, the association between antibiotics in the first week of life and acute otitis media and otitis media effusion in the first 4-6 years of life is described in **chapter 3**.

In **chapter 4**, a systematic review was performed examining the association between exposure to antibiotics in the first two years of life and the presence of chronic FGIDs during childhood.

In **chapter** 5, we examined if antibiotic treatment in the first week of life is associated with functional gastrointestinal disorders (FGIDs) at 4-6 years of age. The second aim of this study was to see whether we could replicate previous studies that found an association between atopic disorders and FGIDs. Since there was a relatively large group of children with infantile colic in the INCA cohort, we analysed whether infantile colic was associated with an increased prevalence of FGIDs at 4-6 years of age. Finally, we explored whether gut-associated immune markers that were significantly different at one year of age in children with and without a history of infantile colic were different for children with FGIDs at 4-6 years of age.

The aim in **chapter 6** was to determine the association between antibiotic treatment in the first week of life in term-born children and atopic disorders at 4–6 years of age.

In the second part of the thesis, we explore the relationship between antibiotics and the gut microbiome, possible ways of influencing early-life dysbiosis, and future directions.

Since the development of the microbiome in early life is important for later health and disease, we describe in **chapter 7** the impact of antibiotic exposure in the first days of life on microbiota development during the first 2.5 years of life. In addition, it was examined what the

effect of: short (2–3 days) versus long (7 days) antibiotic administration was, different types of antibiotics were, feeding and delivery mode were on the microbiome development.

To prevent long-term health complications after early life dysbiosis, we studied promising interventions in **chapters 8** and **9**. A systematic review was performed to summarize the effects of different pre-, pro-, or synbiotic supplements on the gut microbiome composition and clinical outcomes of term-born infants who were born by cesarean section or exposed to antibiotics in the first week of life.

To gain a better insight into the factors that play a role in the development of allergies for future research, a pilot study was performed in **chapter 10** to demonstrate a relatively new technique of data analysis. Here we studied predictive values for the presence of allergy at 4-6 years of age based on machine learning by using feature selection.

Chapter 11 is a general discussion of the findings of the research in this thesis, placing the results in context and comparing them with other studies. Moreover, the chapter provides directions for future research. Finally, **chapter 12** describes the English and Dutch summaries of this thesis.

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Chapter 2

Antibiotic treatment in the first week of life impacts the growth trajectory in the first year of life in term infants

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ABSTRACT

Objective: Antibiotic treatment in early life appears to increase the risk for childhood overweight and obesity. So far, the association between antibiotics administrated specifically during the first week of life and growth has not been studied. Therefore, we studied the association between growth and antibiotics, given in the first week of life and antibiotic courses later in the first year of life.

Method: A prospective observational birth cohort of 436 term infants with 151 receiving broad-spectrum antibiotics for suspected neonatal infection (AB+), and 285 healthy controls (AB-) was followed during their first year. Weight, height and additional antibiotic courses were collected monthly. A generalized-additive-mixed-effects model was used to fit the growth data. Growth curve estimation was controlled for differences in gender, gestational age, delivery mode, exclusive breastfeeding, tobacco exposure, presence of siblings and additional antibiotic courses.

Results: Weight-for-age and length-for-age increase was lower in AB+ vs AB- ($p<0.0001$), resulting in a lower weight and length increase (6.26kg (SE 0.07kg) and 25.4cm (SE 0.27cm) vs. 6.47kg (SE 0.06kg) and 26.4cm (SE 0.21cm), ($p<0.05$ and $p<0.005$ respectively) in the first year of life. Approximately 30% of the children in both groups received additional antibiotic course(s) in their first year, whereafter additional weight gain of 76g per course was observed ($p=0.0285$).

Conclusion: Decreased growth was observed after antibiotics in the first week of life, whereas increased growth was observed after later antibiotic course(s) in term born infants in the first year of life. Therefore, timing of antibiotics may determine the association with growth.

INTRODUCTION

Antibiotic (AB) exposure in early life is increasingly acknowledged for its contributing role in the development of childhood obesity (1,2). The 'microbiome induced obesity' is one of the proposed pathophysiological mechanisms for the development of obesity (3). In mice, it has been shown that AB at birth influences the gut microbiome and metabolism with sustained effects on body-composition leading to a pro-obese state (4).

A recent meta-analysis of 15 cohort studies in humans, concluded that AB treatment in early life increases the risk for childhood overweight and obesity (5). The most profound increase for both overweight and obesity were found when AB exposure occurred during pregnancy and the first six months of life. This suggests that the effect of AB on the development of obesity is influenced by the timing of the AB treatment. In addition, exposure to more than one AB course increases the risk for overweight and obesity, with a dose-response relationship (5,6). Other potential factors of influence are the type and duration of AB. Broad-spectrum ABs are thought to increase the risk for obesity more than small-spectrum AB (6,7), however, data are not consistent (8,9). Furthermore, longer duration of AB is associated with a more disturbed gut microbiome (10,11), which may increase the obesity risk.

Since the neonate has an almost sterile gut at birth, hypothetically, the most profound effect of AB can be expected when administered already in the first week of life. So far, the association between AB administration specifically during the first week of life and growth has not been studied. Therefore, we set up the INCA-study (INtestinal microbiota Composition after Antibiotic treatment in early life), in which we studied the association of AB treatment in the first week of life and the development of atopy, microbiome development and growth in the first year of life (12, 13). In this paper, we report the association between AB administered in the first week of life and growth in the first year of life and compare it with the association of AB courses administered later in the first year. In addition, the association between the duration of AB treatment and growth in the first year of life was studied, as well as the association with different AB types.

METHODS

Study design

The INCA study is a prospective birth-cohort study. The study design has been published previously (12). Between August 2012 and January 2015, term born infants (≥ 36 weeks gestational age) were recruited from the maternity- and neonatal wards of four teaching hospitals in the Netherlands.

AB treatment was at the pediatricians discretion, according to hospital protocol for suspected early onset neonatal infection, based on the Dutch guideline for early onset sepsis (14). In general, infants with suspicion of infection received a combination of broad-spectrum AB (gentamicin combined with either penicillin (AB^{Pen}), amoxicillin (AB^{AMX}) or amoxicillin/clavulanic acid (AB^{AMC})). Blood cultures were taken before AB treatment was started. In case of a negative blood culture, combined with a low clinical suspicion of infection and low c-reactive protein, antibiotics were discontinued after 2-3 days, otherwise antibiotics were continued for 7 days.

All term born infants staying in the hospital for at least 24 hours were eligible for inclusion in either AB or control group. Exclusion criteria were severe congenital malformations; severe perinatal infection needing transfer to a neonatal intensive care unit; maternal probiotic use ≤6 weeks before delivery; and insufficient knowledge of the Dutch language. Informed consent was obtained from both parents of all participating infants. The study was approved by the regional ethical board of the St. Antonius Hospital in Nieuwegein. The trial was registered in clinical trials register as NCT02536560.

Data collection

After inclusion, parents filled out an online questionnaire, mostly concerning the background of the family (e.g. environmental factors, parental smoking habits or presence of siblings). Moreover, parents filled out monthly questionnaires concerning last recorded weight and height at the healthy baby clinics, type of nutrition (breastfed and/or formula fed) and additional AB treatments. Around one year of age, children visited the outpatient clinic of the hospital for follow-up at which height and weight measurement was taken.

Statistical analysis

Baseline characteristics were analysed with the independent t-test, One-way ANOVA, or Chi-squared test as appropriate, for these analyses SPSS Statistics for windows, version 24.0. (Armonk, NY) was used. Since it is not standard care in all participating hospitals to determine the length after birth, a natural spline extrapolation was used to estimate the birth length in case it was missing.

In the analysis a generalized additive mixed effects model was used to fit the growth data (15). For each treatment group (AB vs no AB in the first week of life, subdivided by AB type and duration of AB administration) a flexible growth curve was estimated. Each individual was given a random intercept and each individual growth curve was modelled by a continuous auto-correlated process. By adding a random intercept in the generalized additive model, we control for birthweight. Furthermore, the growth curve estimation was controlled for by differences in

gender, delivery mode, number of additional AB treatments during the first year, gestational age, nutritional regime, tobacco use by the mother, whether the mother had given birth before and whether children were given probiotics. For these analyses, the package mgcv (Wood, 2006) in R version 3.3.3 (R Core Team, 2007) was used (15,16). A p-value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics

In total, 608 children were included: 196 treated with AB in the first week of life (AB+) and 412 healthy controls (AB -). As 172 children were excluded from analyses due to study withdrawal or lost to follow-up (Figure 1), final analyses were performed in 436 children (AB+ (n=151), AB- (n=285)). In AB+, all children were treated with gentamicin for 48 hours and in most children (145) this was combined with AB^{Pen} (n=89), AB^{AMC} (n=24), or AB^{AMX} (n=32). Forty-two children were treated for 2-3 days (AB2), and 109 for 7 days (AB7) of which three had a positive blood culture (two group B streptococcus (GBS) and one streptococcus oralis). Baseline characteristics were similar between AB- and AB+, except for higher gestational age (p=0.001) and birthweight (p<0.001), and active maternal smoking during pregnancy in AB+ (p=0.009) (table 1). Baseline characteristics were not different between the different AB treatment groups (Table 1).

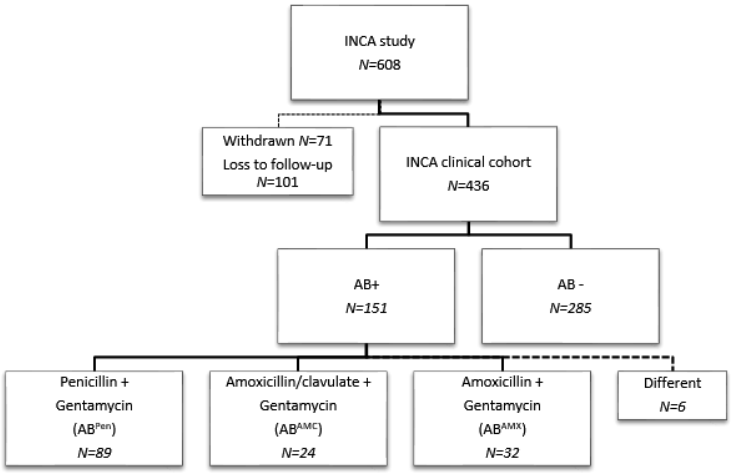


Figure 1; Inclusion flowchart. AB+ (antibiotic exposed); AB- (healthy controls). Dotted blocks show children excluded from (sub)analyses. Children were considered lost-to-follow up when data from less than three months were available. Children withdrawn were considered as such when parents actively withdrawn participation in the study.

Table 1 Baseline characteristics

	AB - **N 285**	**AB +** **N 151**	**AB-2** ***N* 42**	**AB-7** ***N* 109**	**AB^{Pen}** ***N* 89**	**AB^{AMC}** ***N* 24**	**AB^{AMX}** ***N 32***
GA mean (SD)	39.4 (1.5)	40.0 (1.3)*	39.4 (1.5)	40.3 (1.2)	40.0 (1.3)	40.4 (1.1)	40.0 (1.3)
Birthweight mean (SD)	3444 (537)	3681 (533)*	3588 (457)	3716 (558)	3644 (576)	3771 (456)	3620 (446)
Male, n (%)	152 (54)	85 (56)	19 (45)	66 (61)	48 (54)	16 (67)	15 (47)
Siblings, n (%)	122 (46)	48 (35*)	14 (37)	34 (34)	30 (39)	8 (35)	8 (25)
Additional AB courses							
1 course, n(%)	50 (18)	26 (17)	2 (5)	24 (22)	16 (18)	4 (17)	5 (16)
2 courses, n (%)	28 (10)	13 (9)	4 (10)	9 (8)	7 (8)	4 (17)	1 (3)
> 2 courses, n (%)	10 (4)	7 (5)	0 (0)	7 (6)	4 (5)	1 (4)	2 (6)
Delivery mode							
Vaginal, n (%)	187 (66)	112 (74)	29 (69)	83 (76)	67 (75)	18 (75)	24 (75)
C-section, n (%)	98 (34)	39 (26)	13 (31)	26 (24)	22 (25)	6 (25)	8 (25)
Breastfeeding exclusive							
>3 months, n (%)	104 (37)	52 (34)	15 (36)	37 (34)	33 (37)	7 (29)	10 (31)
<3 months, n (%)	116 (41)	65 (43)	14 (33)	51 (47)	34 (38)	9 (38)	19 (59)
0 months, n (%)	65 (23)	34 (23)	13 (31)	21 (19)	2 (25)	8 (33)	3 (9)
Tobacco exposure during pregnancy							
Aactive, n (%)	12 (5)	16* (12)	6 (16)	10 (10)	11 (14)	1 (4)	3 (9)
Passive, n (%)	32 (12)	23 (17)	3 (8)	20 (20)	16 (21)	5 (22)	2 (6)

AB- (controls); AB+ (antibiotic exposed, total group); AB-2 (treated for 2-3 days); AB-7 (treated for 7 days); AB^{Pen} (penicillin + gentamicin); AB^{AMC} (amoxicillin/clavulanate + gentamicin); AB^{AMX} (amoxicillin + gentamicin).GA (Gestational age) in weeks. SD (standard deviation). Birthweight in grams.*p<0.05 as compared to AB-.
Due to missing data, numbers not always add up to the total of the group, six children
were treated with different antibiotics than the three mentioned groups and therefore not included in the subgroup analyses.

The model

We considered five different response variables (weight-for-age (WFA), length-for-age (LFA), weight-for-length (WFL), weight and length). For each of the variables, the auto-correlated noise term and the random intercept were significant, suggesting a stable pattern over time within subjects but quite some variation between subjects, under the same conditions in terms of AB treatment, gestational age, and gender. No significant interaction effect between sex and AB on growth has been found, allowing for account for sex by means of an additive effect.

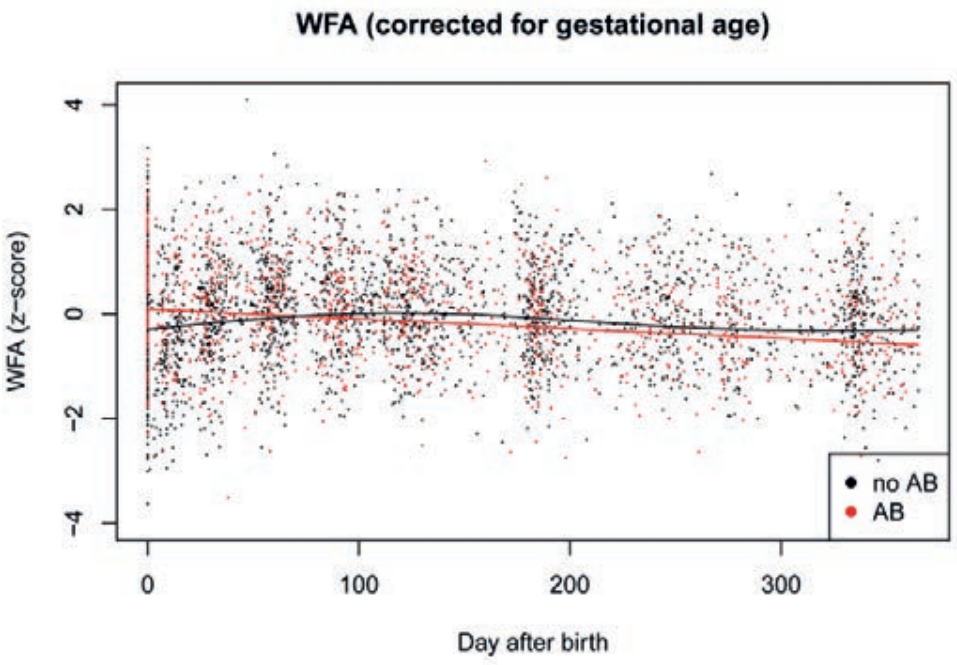
Antibiotics

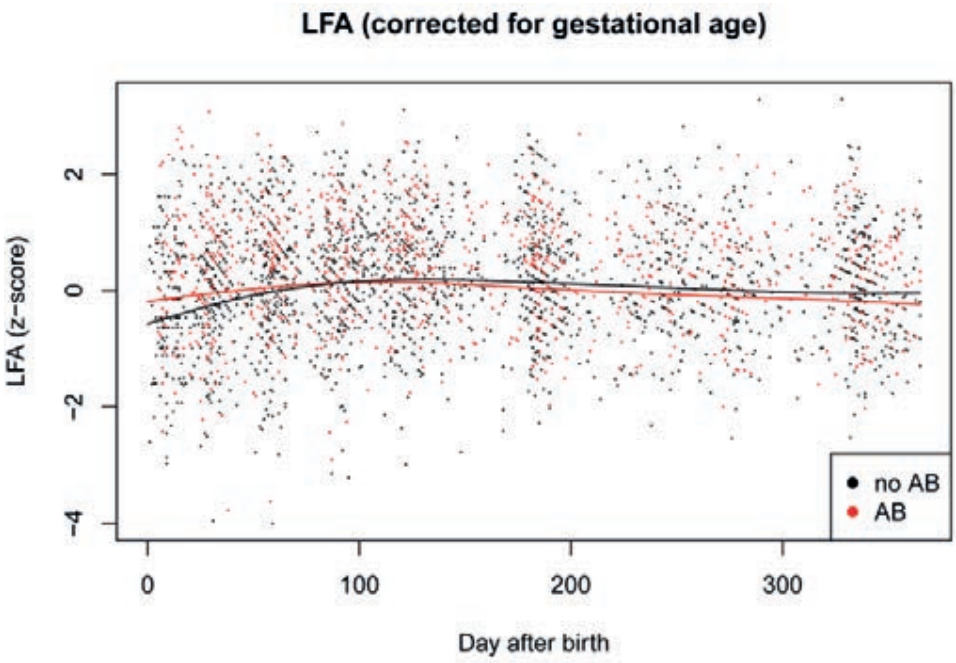
Children in AB+ were on average 180g heavier and 0.85cm longer at birth, corrected for GA differences at birth. The mean weight increase in the first three months of life was 2.45kg and 2.65kg in AB+ and AB- respectively (SE of the difference 0.086kg, p=0.0210). The mean weight increase in the first year of life in the AB+ group (6.26kg, SE 0.07kg) is significantly lower (p=0.0179) than in the AB- group (6.47kg, SE 0.06kg). Although most of the difference in absolute

weight growth between the AB- and AB+ groups takes place in the first three months, the AB+ group continues to experience a continuous reduction in weight-for-age z-score throughout the first year.

The average increase in length over the first 3 months in AB+ and AB- was 10.5cm (SE 0.27cm) and 11.4cm (SE 0.21cm), respectively (p=0.0179). The average length increase over the first year in AB+ and AB- was 25.4cm (SE 0.27cm) and 26.4cm (SE 0.21cm), respectively (p=0.0018). Around one year of age, weight and length were similar in AB+ and AB- with absolute difference in weight and length of 20g and 0.13cm [AB+ 10.0kg (SE 0.07kg), 77.2cm (SE 0.21cm), AB- 10.1kg (SE 0.06kg), 77.2cm(SE 0.19cm)].

At birth, WFA and LFA z-scores were higher in AB+ than in AB- [WFA +0.314 (SE 0.074) vs. -0.067 (SE 0.074), LFA +0.130 (SE 0.076) vs. -0.256 (SE 0.064)], but around one year of age, they were lower in AB+ than AB- [WFA -0.358 (SE 0.084) vs. -0.074 (SE 0.086), LFA +0.081 (SE 0.086) vs. +0.274 (SE 0.080)], respectively both p<0.0001 (figure 2a and 2b). The WFA and LFA lines of AB+ and AB- cross around day 100. At birth, WFL Z-scores were lower in AB- than in AB+ [WFL AB+ -0.250 (SE 0.076) vs. AB- -0.614 (SE 0.104)] but around the first birthday were similar in both groups [WFL AB+ -0.290 (SE 0.082) vs. AB- -0.310 (SE 0.121)].





Graphs of the generalized additive mixed effects model analyses with the growth curves of the cohort; Figure 2a and 2b Show Weight-For-Age (WFA) and Length-For-Age (LFA) development over the first year of life respectively. AB (treated with antibiotics), no AB (healthy controls).

Dose effect relation additional AB

After the first week of life, in the whole INCA cohort about 30% was prescribed one or more AB courses before the first birthday, equally divided over the AB+ and AB- groups. Per additional course of AB in the first year of life, irrespective of AB in the first week of life, a significant mean (SE) weight gain of 76 grams (34 gram) was observed (p=0.0285), while length increase 0.1cm (0.1cm) per course was not significant (p=0.532).

Duration of AB 2 vs 7 days

Growth in mean (SE) weight and length increase was not different between AB2 and AB7 group in the first three months (2.425kg (0.076kg) versus 2.473kg (0.065kg) and 10.3cm (0.41cm) versus 10.7cm (0.30cm)) respectively, nor in the first year of life (6.234kg (0.086kg) versus 6.262kg (0.071kg) and 25.7cm (0.41cm) versus 25.2cm (0.30cm)), respectively.

Type of antibiotics

The only significant differences were observed between AB^{AMX} and AB^{AMC} and between AB^{AMX} and AB^{Pen}. AB^{AMX} administered in the first week of life showed a smaller weight increase of on average 409 gram (SE 0.130kg) than AB^{AMC} (p= 0.001) in the first year of life. AB^{AMX} administered in the first week showed a smaller length increases of on average 1.8 cm (SE 0.53cm) than AB^{Pen} in the first three months of life and 1.3 cm (SE 0.51cm) in the first year of life (p = 0.0006 and p= 0.0101 respectively).

DISCUSSION

This is the first study examining the association between AB administered in the first week of life and the growth trajectory in the first year of life. A decreased growth was observed after AB in the first week of life compared to the AB-, whereas after each additional AB-course later in the first year of life a significant additional weight gain of 76 grams was observed, irrespective of AB in the first week of life.

Our results are partly in contrast to previously published systematic reviews, showing an increased risk for childhood obesity after AB treatment in the first 2 years of life (5,6). When these studies are evaluated in detail, it appears that especially AB exposure in the first six months of life contribute to this increased risk of overweight or obesity at 2 (9,17) or 7 (18,19) years of age. However, none of these previously published studies evaluated the association between AB and growth specifically in the neonatal period. After the first week of life, in line with existing literature, we showed an additional weight gain of 76 grams after every additional AB course in the first year of life (5,6).

Several explanations are possible for the different association between weight gain and AB given in the first week of life versus AB later in life. First, it is known that the microbiome rapidly changes in the first weeks of life (20). Therefore, the developmental stage of the microbiome at the moment of AB treatment may be an important factor in the pathophysiological mechanism of AB-induced obesity. Second, the route of AB administration may have different effects on the gut microbiome and thus different associations with weight gain (21-23). In our study, AB were administrated intravenously in the first week of life, while AB treatments later in the first year were mostly prescribed orally. It has been shown previously that a combination of oral and intravenously AB administration can have a larger detrimental effect on the gut-microbiome than purely intravenously administration (22). Therefore, it is possible that the different associations on growth, as found in our study between AB in the first week versus AB later in the first year, may not (only) have been a time-dependent factor, but influenced by the route of administration. Future studies in neonates must investigate the

difference between oral and intravenously administration (or a combination of the two) on growth and the microbiome development.

Another important difference between AB in the first week of life and AB later in the first year of life is the type and combination of AB. In our study, in the first week of life all children were treated with gentamicin for two days and a β-lactam AB for 2-7 days depending on the local hospital protocol. After the first week of life, children were treated with a single AB (mostly amoxicillin). The metabolomic function of the gut (and thus its effect on growth) is strongly affected by bacterial composition, which in turn has been shown to be affected by the type of AB (24). The combination of two AB (for example gentamicin and ampicillin) has been shown to have a larger detrimental effect on the gut-microbiome than each AB separately (24). Previous clinical studies evaluated treatment with either a single broad- or small spectrum (β-lactam) AB, macrolide or anti-metabolite agents, but found no consistent results in the effect on growth of children (8,9,25). In our study we also found differences between the types of AB given in the first week, with AB^{AMX} showed the lowest weight gain. Fecal samples, collected within the INCA study, may provide more insight in differences in the microbiome development between the AB types. Moreover, future studies will be needed to study the effects of different types of AB in the neonatal period on the developing microbiome and on growth.

Given the findings in this study, it is possible that AB in the first week of life is associated with a decreased growth rate instead of an increased growth rate as found after AB exposure later during the first year of life. Although the absolute differences in growth are small, small impairments in growth patterns in early life may impact later growth and health, such as shorter adult height, lower attained schooling, reduced adult income, and several chronic disease such as cardiovascular disorders and diabetes (26). Longer follow-up of the INCA cohort is therefore important, to evaluate the long term consequences of AB in the first week of life, and additional AB courses during the first year of life, as already it has been shown that rapid growth in early life is associated with an increased risk for obesity later in childhood and adolescence (27).

The prospective design of the INCA study with healthy term born infants as a control group is an important strength of this study. Data on growth were collected on ten time points in the first year of life. These measurements were mostly done by the healthy baby clinic, which parents visit regularly in the first year of life, resulting in standardized measurements by health care professionals. Furthermore, since data regarding additional AB courses were collected every month, there was a low chance on recall bias.

A limitation of this study is the fact that type and route of administration of AB given later in life was not similar to AB given in the first week of life, which prevents solid conclusions on the associations between growth and AB given in the first week versus AB later during the

first year of life. Moreover, we did not have data on several confounders, associated with growth in early life, such as the age of solid food introduction, antibiotic exposure during pregnancy or maternal pre-pregnancy weight and gestational weight gain. For future research, we recommend considering these factors as well.

CONCLUSION

In the INCA-cohort, we have shown less growth in infants who were treated with AB in their first week of life compared to healthy controls. In contrast, additional weight gain was observed after each additional course of AB later in the first year of life. Follow up of the INCA cohort is necessary to show if these differences in growth trajectories will continue after the first year of life and whether this will result in a different prevalence's of overweight and obesity later in childhood.

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Chapter 3

The association between exposure to antibiotics in the first week of life and later otitis media: the INCA study

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INTRODUCTION

Otitis media (OM) is one of the most common diagnoses in preschool-age children [1, 2]. About 60% of preschool children have had at least one episode of OM [3] by the time they are four years of age, most frequently acute otitis media (AOM) and otitis media with effusion (OME). Of these AOM episodes, up to 80% resolves spontaneously without antibiotics [4, 5]. Nevertheless, AOM is the most common reason for antibiotic prescriptions to children under three years of age [6, 7].

Antibiotics can have a profound effect on the gut microbiome [8]. It has been suggested that there is a critical window in early life in which alterations in the gut microbiome have important implications for later health and risk of diseases [9], possibly through its impact on the immune system [10, 11]. Growing evidence shows that the composition of the gut microbiome plays an important role in the development and regulation of the immune system, especially in early life when the microbiome and immune system develop concurrently [12]. Indeed, previous studies have shown that antibiotic exposure in the first two years of life is associated with a variety of health problems later in life, including atopic diseases [13], overweight and obesity [14], and gastrointestinal disorders such as celiac disease [15] and inflammatory bowel disease [16].

Antibiotic exposure in the first years of life also affects the microbiome composition in other parts of the body, such as the nasopharyngeal and oral microbiome [17, 18]. However, to what extent the oral or nasopharyngeal microbiome play a role in the development of OM is not clear. One study showed significant differences in the nasopharyngeal microbiome between children with recurrent AOM (rAOM) and healthy controls [19], suggesting that the nasopharyngeal microbiome plays a role in rAOM. To date, however, no studies have examined the association between early-life antibiotic exposure and subsequent AOM and OME.

The INCA study is a Dutch prospective birth cohort study in which the association between antibiotic exposure in the first week of life and later health problems is examined in children born at term. We have previously shown in this cohort that antibiotic treatment in the first week of life is associated with an increased risk of wheezing, infantile colic, poorer growth in the first year of life, and a different circulating immune marker profile at one year of age [20-22]. Follow-up of these children showed that antibiotic exposure in the first week of life is also associated with an increased risk of having an allergy and a higher risk for functional abdominal pain at 4-6 years of age [23, 24]. After antibiotic exposure, the compromised immune system may lead to an increased risk for common childhood infections such as OM. Accordingly, the aim of this study was to determine if antibiotic treatment in the first week of life in children born at term is associated with AOM and OME in the first 4-6 years of life.

METHODS

Study design

The design of the INCA study, a prospective birth-cohort, has been described previously [25]. In short, 436 infants born at term were recruited from maternity and neonatal wards of four teaching hospitals in the Netherlands between 2012 and 2015. Both infants with suspected neonatal infection, receiving a combination of broad-spectrum antibiotics (gentamicin combined with either penicillin, amoxicillin or amoxicillin/clavulanate) intravenously in their first week of life (AB+), and infants not exposed to antibiotics (AB-) were included. Before antibiotic treatment was started, a blood culture was taken. In case of a negative blood culture, combined with a low clinical suspicion of infection and low C-reactive protein, antibiotics were discontinued after 2 to 3 days (n=42). Otherwise, antibiotics were continued for 7 days (n=109).

Antibiotic exposure

To examine the association between antibiotic exposure in the first week of life and subsequent AOM or OME, exposure to antibiotics in the first week of life was collected from hospital records, as were exposure duration and antibiotic type.

Parent-reported AOM and OME

Between May 2018 and May 2019, the parents of the eligible children were approached to complete an online questionnaire. The children were then 4-6 years of age, depending on the year of inclusion. In this questionnaire, data on AOM and OME was collected based on their main symptoms according to most European guidelines [26], namely: Did your child ever have a middle ear infection (=earache/running ear/fever) for which you visited a doctor? A positive answer was defined as parent-reported AOM. This question was followed by the questions: 1. Did your child have symptoms of repeated middle ear infections (=earache/running ear/fever), and 2. Did your child experience symptoms of impaired hearing due to fluid in the middle ear? A positive answer to the first question was defined as parent-reported rAOM and to the second question as parent-reported OME. Further questions were: 3. At what age (in months) did your child have the first symptoms of a middle ear infection? and 4. Which medical specialist treated your child for the AOM/OME and what treatment was given?

Doctor-diagnosed AOM and OME

If parents gave additional informed consent, doctors' diagnoses of AOM and OME were requested using the general practitioner electronic medical database based on the International Classification of Primary Care (ICPC), code H71 for AOM and H72 for OME [27].

Potential confounders

We identified risk factors for antibiotic exposure, AOM and OME through a literature search.

Data collection in the first year of life

As described previously, data such as delivery mode and gestational age were collected from the hospital records at inclusion and parents filled out an online questionnaire concerning the background of the family (e.g., parental highest education level, presence of siblings or pets) [25]. A daily checklist was kept during the first year of life, combined with a monthly questionnaire concerning nutrition, daycare attendance, and the use of additional antibiotics.

Follow-up study

In the questionnaire at 4-6 years of age, it was asked whether the child had taken antibiotics before the second year of life. Pharmacy data were retrieved for medication use from birth until 4-6 years of age with additional informed parental consent.

Statistical analysis

Two groups were created: children exposed to antibiotics in the first week of life (AB+) and children not exposed to antibiotics (AB-). Baseline differences between these groups were analysed with the independent t-test and Chi-squared test or non-parametric test as appropriate. Both stepwise and forward multiple logistic regression analysis were performed to assess the association between antibiotic treatment in the first week of life and both parent-reported and doctor-diagnosed AOM and OME. Potential confounders were included in the regression model if the p-value was <0.1 in the univariate analysis or if the beta of the independent variable (antibiotic exposure) changed ≥10% after adding one of the following factors to the model: sex, age at follow-up, delivery mode, duration of breastfeeding, day-care attendance, presence of pets, siblings, highest parental level of education, additional antibiotic courses in the first two years of life, and allergy at 4-6 years of age. A minimum of 10 events per variable was required for the multivariate logistic regression analyses to avoid bias [28]. If the association between antibiotics and AOM or OME is significant, further analyzes would be performed into association with duration and type of antibiotics. A p-value <0.05 was used to decide whether the association between antibiotic exposure and AOM or OME could be considered as statistically significant. Analyses were performed with IBM SPSS Statistics for Windows version 26 (Armonk, NY, USA).

Ethics

Both parents of all participating children gave informed consent. The study was approved by the ethical board of the St. Antonius Hospital in Nieuwegein, the Netherlands, and was registered in the clinical trials register as NCT02536560.

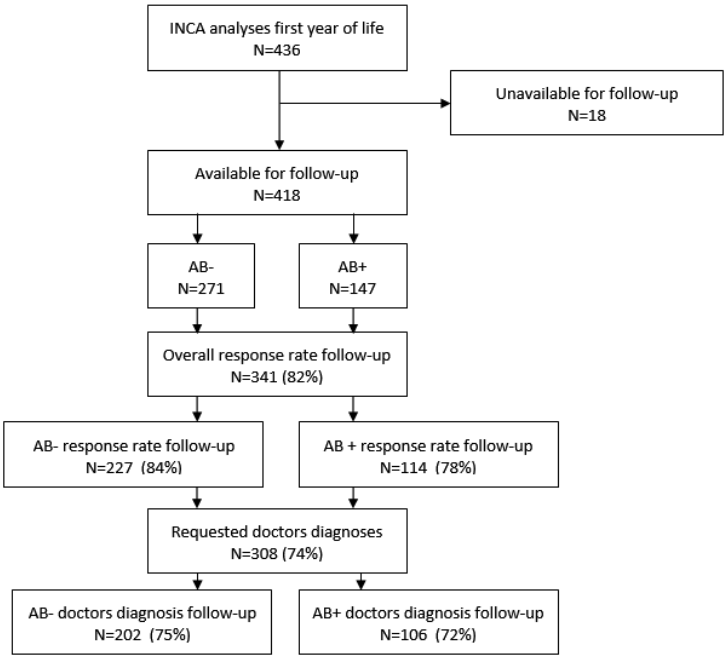

Figure 1 flowchart of follow-up.
AB+ = children born at term treated with antibiotics in the first week of life and AB- = children not exposed to antibiotics

RESULTS

Of the 436 included children at birth, 151 AB+ children were exposed to antibiotics in their first week of life. After the first year of life, 418 children were eligible for follow-up (147 AB+), of which 341 (82%) completed the questionnaire at 4-6 years of age (Figure 1). The response rate was approximately equally divided between AB+ and AB- children (114/147 [78%] vs. 227/271 [84%], p=0.118). In total, parents of 308 children (74%) gave additional informed consent for obtaining the ICPC codes from the general practitioner and parents of 296 children (71%) for obtaining a medication history from the pharmacy. Baseline characteristics between AB+ and AB- group were not different, except for age at follow-up, mode of delivery, and the number of antibiotic-free months after the first week of life in their first year (Table 1).

Pharmaceutical records showed no significant difference in prescription of systemic antibiotics in the first two years of life between AB+ and AB- (data not shown).

Table 1: baseline characteristics

	AB- N=227	AB+ N=114
Age median (IQR)*	5 (4.6-5.9)	4.7 (4.4-5.0)
Sex (male n,%)	122 (54)	65 (57)
Delivery mode (vaginal n, %)*	146 (64)	86 (75)
Duration (months) any breastfeeding (median, IQR)	4 (1-8)	2.5 (1-7)
Duration (months) exclusive breastfeeding (median, IQR)	2 (0-5)	0 (0-4)
Pets (no n,%)	84 (37)	45 (39)
Cat	59 (26)	34 (30)
Dog	37 (16)	18 (16)
Cat + dog	23 (10)	7 (6)
Other	24 (11)	10 (9)
Daycare attendance (no n,%)	73 (32)	32 (28)
<3 months	25 (11)	24 (21)
3-6 months	99 (44)	38 (33)
>6 months	30 (13)	20 (18)
Siblings Yes (%)	120 (53)	69 (61)
Missing	7 (3)	4 (4)
Highest level of education n (%)		
Low/middle	44 (19)	28 (25)
High	174 (77)	80 (70)
Unknown	9 (4)	6 (5)
Number of AB courses, after 1st week, in the first years of life (median, IQR)	0 (0-1)	0 (0-1)
AB free months, after 1st week, in the first year of life (median, IQR)*	9 (7-11)	7 (2-9)
Number of courses, after 1st week, in the first 2 years (median, IQR)	1 (1-2)	1 (1-2)
Parent-reported allergy at 4-6 years of age*	24 (11)	26 (23)
Age at first AOM diagnosis (median, IQR)	11 (6-16.5)	9.5 (6-18.5)

AB+ = children born at term treated with antibiotics in the first week of life, AB- = children not exposed to antibiotics, IQR = interquartile range, SD = standard deviation, AOM = acute otitis media
*Significant p=< 0.05 difference between AB+ and AB-

Prevalence of parent-reported and doctor-diagnosed AOM and OME

The prevalence of at least one parent-reported AOM in the first 4-6 years of life was 155/341 (45%) in the total cohort, 49% in AB+ vs. 44% in AB-. Seventy of the 341 (21%) children had rAOM, 21% AB- vs. 20% AB-. Of these 155 children with AOM, 33 children also had a diagnosis of OME. Accordingly, the prevalence of parent-reported OME was 33/341 (10%) in the whole cohort, 11% in AB+ vs. 9% in AB-.

The prevalence of doctor-diagnosed AOM was 91/308 (30%) in the total cohort, 28% in AB+ vs. 30% in AB-. Of these 91 children, 12 children also had OME and 8 children without a diagnosis of AOM had OME. Resulting in a doctor-diagnosed OME prevalence of 20/308 (6%) in

the whole cohort, 8% in AB+ vs. 5% in AB-. No differences in prevalence of AOM and OME between AB+ and AB- showed statistical significance.

Association between antibiotics in the first week of life and AOM and OME

To study the association between antibiotic exposure in the first week of life and both parent-reported and doctor-diagnosed AOM and OME, regression models were plotted (Table 2). None of the potential confounders changed the beta of antibiotic exposure in the first week of life >10% in the model, therefore only the unadjusted models were reported. No statistically significant associations were observed between antibiotic exposure and parental-reported AOM (aOR 1.248; 95% CI 0.795-1.960) and doctor-diagnosed AOM (aOR 0.912; 95% CI 0.543-1.532). Furthermore, the models for parental-reported OME (aOR 1.154; 95% CI 0.546-2.438) and doctor-diagnosed OME (aOR 1.611; 95% CI 0.646-4.019) were also not significant.

Table 2: Association between antibiotics in the first week of life and otitis media

	AB- N = 227	AB+ N = 114	OR*
Parent-reported AOM (n,%)	99 (44)	56 (49)	1.248 (0.795-1.960)
Recurrent AOM (n,%)	46 (20)	24 (21)	1.049 (0.603-1.827)
Parent-reported OME (n,%)	21 (9)	12 (11)	1.154 (0.546-2.438)
	AB- N= 202	AB+ N= 106	
Doctor diagnosed AOM (n,%)	61 (30)	30 (28)	0.912 (0.543-1.532)
Doctor diagnosed OME (n,%)	11 (5)	9 (8)	1.611 (0.646-4.019)

AB+ = children born at term treated with antibiotics in the first week of life, AB- = children not exposed to antibiotics in the first week of life. AOM = acute otitis media, OME = otitis media with effusion, (a)OR = (adjusted) odds ratio for sex and age, 95% CI = 95% confidence interval

*Potential confounders checked in and excluded from the model: sex, age at follow-up, delivery mode, duration of breastfeeding, day-care attendance, presence of pets, siblings, highest parental level of education, additional antibiotic courses in the first two years of life, and allergy at 4-6 years of age.

DISCUSSION

In this follow-up study of the INCA cohort, antibiotic treatment in the first week of life was not significantly associated with either parent-reported or doctor-diagnosed AOM and OME in the first 4-6 years of life.

To our knowledge, this is the first study examining the association between antibiotics in the first week of life and the occurrence of AOM/OME. Although the causes of OM are very diverse and associated with complex interactions of various factors, a recent review has shown that children with rAOM/OME have an aberrant immune development [29]. An aberrant

development of the immune system may be caused by early-life antibiotic treatment, as a healthy gut microbiome is essential for a normal immune maturation [10]. In the INCA study, we found that exposure to antibiotics in the first week of life led to perturbations in the fecal microbiota even up to one year of age with a decrease in Bifidobacteriaceae and an increase in Enterobacteriaceae [30]. If the immune system is affected by antibiotic exposure in early life, the incidence of common early life infections such as OM is expected to increase, which contrasts with our findings. We can only speculate why we did not find an association between antibiotic exposure in the first week of life and the presence of AOM/OME later in life.

Previous studies of exposure to antibiotics and overweight/obesity have shown that the timing of exposure influences this association and that there is a dose-response relationship [31, 32]. Furthermore, it has been shown that longer duration of antibiotic exposure is associated with a more profound disturbed gut microbiome [33, 34]. Therefore, it could be hypothesized that repeated or more prolonged courses of antibiotics may result in a more profound impact on microbiome perturbations and hence an aberrant immune development. However, it is challenging to study the association between repeated antibiotics later in the first year and OM, because OM is often the reason for the prescribed antibiotics. Another possibility is that the route of administration (parenteral vs. enteral) plays a role in the effect of antibiotics. It has been shown previously that a combination of oral and intravenously antibiotics administration can have a larger detrimental effect on the gut-microbiome than purely intravenously administration [35]. This should be examined in future research with sufficient group sizes.

A strength of this study is the high response rate of more than 80%, which resulted in a complete follow-up of 341 children. Furthermore, prospective follow-up in the first year of life, and the combination of information collected from children's general practitioners and pharmacists contributed to the reliability of the reported results.

The retrospective data collection of AOM/OME at 4-6 years of age is a limitation of the study. Most children have an episode of AOM before three years of age and OME before four years. At six years of age, it may have been several years since the child experienced complaints of AOM/OME. This could have led to recall bias. There are no validated questionnaires to collect data on parental reported AOM and OME. Therefore, we developed a questionnaire based on the main symptoms according to most European guidelines. Furthermore, not all parent-reported AOM/OME were clinically confirmed, as shown in the difference between parent-reported and doctor diagnosed prevalence. Moreover, AOM is an over-diagnosed disease, while OME is an under-diagnosed disease [36, 37]. Therefore, it is challenging to study the true association between antibiotics and OM. For future research, we recommend performing prospective follow-up of children after exposure to antibiotics in early life, confirming all

symptoms of OM by an otolaryngologist, so that the real association between AB and OM can be examined.

CONCLUSIONS

This follow-up study of the INCA cohort showed no statistically significant association between antibiotic treatment in the first week of life and both parent-reported and doctor-diagnosed AOM and OME in the first 4-6 years of life. Future studies should not only study the association with repeated antibiotic courses and OM, but also the microbiome in the ear and local immune factors to clarify if and how antibiotics induced-changes in the microbiome and hence the immune system play a role in the susceptibility for otitis media.

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Chapter 4

Early life antibiotics and childhood gastrointestinal disorders: a systematic review

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ABSTRACT

Background: In adults, there is increasing evidence for an association between antibiotic use and gastrointestinal disorders but in children, the evidence is scarce.

Objective: Assess the association between exposure to antibiotics in the first two years of life in term born children and the presence of chronic gastrointestinal disorders later in childhood.

Design: For this systematic review the MEDLINE, Embase, WHO trial register, and Web of Science were systematically searched from inception to June 8, 2020. Title and abstract screening (n=12,219), full-text screening (n=132) as well as the quality assessment with the Newcastle–Ottawa Scale were independently performed by two researchers.

Main outcome measures: The association between antibiotics and inflammatory bowel disease (n=6), eosinophilic esophagitis (n=5), celiac disease (n=6), infantile colic (n=3), functional constipation (n=2), recurrent abdominal pain, regurgitation, functional diarrhea, and infant dyschezia were examined.

Results: Twenty-two studies were included, 11 cohort and 11 case-control studies. A best evidence synthesis showed strong evidence for an association between antibiotic exposure in the first two years of life and the presence of inflammatory bowel disease, and celiac disease during childhood. Moderate evidence was found for an association with eosinophilic esophagitis and no association with functional constipation in the first year of life. There was insufficient evidence for the other studied disorders.

Conclusions: The use of antibiotics in early life may increase the risk of gastrointestinal disorders later in life. Further studies are necessary to unravel the underlying mechanisms and determine potential preventive measures. Meanwhile judicious use of antibiotics in early childhood is highly warranted.

INTRODUCTION

The incidence of pediatric gastrointestinal disorders (GI-disorders), such as pediatric inflammatory bowel disease (IBD) and celiac disease (CeD), is rising (1, 2). The increase in pediatric GI-disorders is most likely related to environmental factors and recently the focus has been on the role of the intestinal microbiome. A microbiome that has been disturbed by factors like stress, dietary change, environmental factors or drugs, can result in alterations in the immune system (3). Several studies have shown that a disturbed microbiome can be a cause or trigger of GI disorders, probably mediated by these immunological changes (4-7).

One of the drugs with the most profound effect on the microbiome are antibiotics (8). The impact of antibiotics on the microbiome depends on various factors such as type of antibiotic, dosage, and duration of exposure (8). Furthermore, age at exposure is probably also important. The gut of a newborn infant is almost sterile with a low diversity and matures according to several developmental stages with increasing diversity over time (9). The microbiome stabilizes around the age of 2 to 3 years (9). Since this developing gut microbiota plays an important role in the training of both innate and adaptive immune system, it is likely that antibiotics will have their biggest impact when administered in the first two years of life.

For the association between antibiotic use and GI disorders, that has been shown in adults, (10), there is only limited evidence in children (11). Therefore, the aim of this systematic review was to assess the association between exposure to antibiotics in the first two years of life and the presence of chronic gastrointestinal disorders during childhood.

METHODS

Study selection

This systematic review was conducted according to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses and registered in PROSPERO CRD42019132631 (12, 13). MEDLINE, Embase, WHO trial register, and Web of Science were systematically searched from inception to June 8, 2020 to identify all studies examining the association between antibiotic exposure in the first two years of life and the presence of common chronic (longer than two weeks, in order to exclude viral diarrhea) gastrointestinal disorders during the first 18 years of life. We searched for associations with IBD, eosinophilic esophagitis (EoE), CeD, irritable bowel syndrome (IBS), (functional) abdominal pain, constipation, dyspepsia, aerophagia, infantile colic, gastroesophageal reflux (GERD), regurgitation, dyschezia and chronic diarrhea.

A multi stranded search approach comprised various concept combinations of children aged 0-4 years, prognosis, gastrointestinal disorders and antibiotics. In order to reduce recall

noise and enhance search results precision we used VOS-viewer to identify terms for NOTing out irrelevant records from databases searched (14, 15). See supplementary file 1 for the full search strategies.

Patient and Public Involvement statement

As this is a systematic review of the literature, there were no patients involved in the design of the research question nor the study itself. Furthermore, for the same reason no approval for the study was required from an ethical committee.

In- and exclusion criteria

Studies were included if: 1. Antibiotics were administered between full-term birth and two years of age. 2. Study outcome was diagnosis with a chronic GI-disorder during the first 18 years of life. 3. Antibiotic use was before the diagnosis of the GI-disorder. 4. A control group was included. 5. In case multiple studies were found examining similar outcomes in one cohort, only the study with the largest cohort was included. No restrictions were placed on the time period of publication. Searches were limited to studies conducted in humans and excluded if the full text was not available in English, Dutch, German or French.

All records found in the search were exported into Rayyan after deduplication (16). Two researchers (KK and EVD) independently performed title and abstract screening as well as full-text screening. After consensus about the study selection, data were entered into a data extraction form, which included: author, year of publication, country, study design, cases, controls/cohort, population age, sample size exposed to antibiotics, age at exposure, details about classification by type of antibiotics, type of GI disorder, method of diagnosis, confounders for which corrected, and the association between exposure and outcome.

Methodological quality

To assess the risk of bias, two researchers (KK and EVD) independently assessed the methodological quality. Discrepancies were resolved by discussion until consensus was reached. The Newcastle–Ottawa Scale (NOS) was used, which has been developed to assess the quality of observational studies (17). The NOS includes different instruments for assessing case-control and cohort studies. Both scales contain a maximum of nine points and assess studies in three core areas: 1. Selection of study participants 2. Comparability of groups 3. Detection of exposure/outcome. One point for comparability of groups was given when the study controlled for the main important confounder and a second point if controlled for a second important

confounder, see supplementary file 2. Studies were rated high quality with a score of eight or higher, moderate quality with a score between five and seven, and weak quality with a score of four or less (18).

Data analyses

To synthesize the methodological quality of the studies, a commonly used best evidence synthesis was applied per disorder in which the methodological quality was considered according to the following definitions: 1. strong evidence, provided by generally consistent findings in at least two high-quality studies. 2. moderate evidence, provided by generally consistent results in one high-quality study and at least one moderate- or low-quality study, or generally consistent results in multiple moderate- or low-quality studies. 3. insufficient evidence, when less than two studies were available or inconsistent findings in multiple studies (19-21). Results were considered consistent when at least 75% of the studies showed results in the same direction.

RESULTS

Search results

Of the 14,731 retrieved records, 12,219 remained after removing duplicates. These records were screened; 132 were assessed as eligible and read in full-text of which 110 were excluded and 22 studies included in this review. Details of the selection procedure are shown in Figure 1.

Study characteristics

The included studies were published between 2010 and 2020 (table 1a-d): 11 cohort studies (22-32) and 11 case-control studies (33-43). The studies were performed in Sweden (n=4) (27, 30, 35, 36), the United States of America (USA) (n=5) (33, 34, 37, 41, 42), Italy (n=4) (22, 29, 32, 43), Denmark (n=2) (23, 31), Canada (n=2) (38, 39), and one in the United Kingdom (25), the Netherlands (26), and Finland (40). There were two international studies, one in Denmark and Norway (28), and another in Finland, Germany, Sweden and the USA (24).

The associations between antibiotics and the following GI-disorders were examined: IBD (n=6) (25, 27, 31, 38, 40, 43), EoE (n=5) (33, 34, 37, 39, 41), CeD (n=6) (22, 24, 28, 35, 36, 42), infantile colic (n=3) (23, 26, 32), functional constipation (n=2) (29, 32), recurrent abdominal pain (n=1) (30). One study examined several functional GI-disorders (FGIDs): infantile colic, functional constipation, functional diarrhea, infant dyschezia, and regurgitation (32).

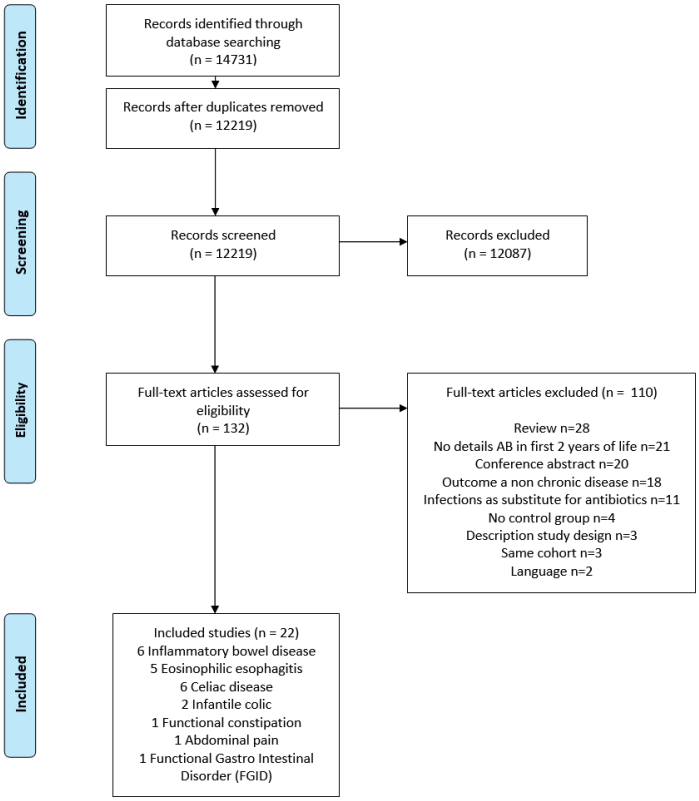

Figure 1: PRISMA flow diagram of the study selection

Exposure to antibiotics was studied in the first two years of life (n=4) (24, 30, 35, 42), the first 18 months of life (n=1) (23), the first year of life (n=13) (22, 25, 27-29, 31, 33, 34, 37-40, 43), the first six months of life (n=2) (36, 41), and the first week of life (n=2) (26, 32) (table 1a-d). Since only a few studies provided details about type of antibiotics and/or number of antibiotic treatments in the first two years of life, the associations include mostly the overall antibiotic exposure.

Table 1a study characteristics and associations with antibiotics: Inflammatory Bowel Disease

Author Year Country Design	Age diagnosis[1]/ cohort entry[2]/ study endpoint[3]	Cases / Controls or Cohort	Cases exposed/ Time exposure/ Recording details	Confounders for which corrected	Significant association	Quality score
Canova, C [43] 2020 Italy Case-control	8.8 yrs[1]	70 / 700	33 (47%) 0-12 months ATC code	• Birth order • Age mother • Apgar score at 1 minute • Birth weight • Education mother • Gestational age • Multiple birth • Season of birth	AB first six months of life childhood onset IBD • Any course aOR = 1.458, 95% CI: 0.81–2.63 • **Dose-dependent** o 2-3 courses aOR = 2.29, 95% CI: 1.01–5.24 o >4 courses aOR = 6.25, 95% CI: 1.70–23.05 Ab first 12 months of life childhood onset IBD • Any course aOR = 1.08, 95% CI 0.64–1.80 • **Dose-dependent:** >4 courses aOR = 2.92, 95% CI: 1.32–6.46	8/9 High
Hviid, A [31] 2010 Denmark Cohort	3.4 yrs[1]	117 (0.02%) (50 CD and 67 UC) / 577,627	84 (72%) 0-12 months ATC code	• Age • Calendar period • Other times since use • Other types of antibiotics	Increased risk of Crohn's disease after: AB use in the last 3 months: • 3-11 months RR = 3.32, 95% CI: 1.15-9.56 • 1 year RR = 1.53, 95% CI: .15-15.46 AB use > 3 months previously before diagnosis: • 0-2 months RR = 4.19, 95% CI: 1.64-10.68	8/9 high
Kronman, M [25] 2012 United Kingdom Cohort	Exposed 4.2 yrs[2]	748 (0.07%) / 1,072,426	436 (58%) 0-12 months Systemic AB prescriptions	• Age • Chronic granulomatous disease • IBD family • Primary sclerosing cholangitis • Sex • Socioeconomic deprivation	• Exposure was associated with a 5.5-fold increased IBD risk (aHR = 5.51, 95% CI:1.66–18.28). • **Dose-dependent:** Exposure to >2 anti-anaerobic antibiotic courses was more highly associated with IBD development than exposure to 1 or 2 courses (aHR = 4.77, 95% CI: 2.13–10.68) versus (3.33, 95% CI: 1.69–6.58). • **Type-dependent** Fluoroquinolone (aHR= 2.09, 95% CI: 1.10–3.98) and metronidazole exposure (aHR = 186.25, 95% CI: 10.86–3193.65) was significantly associated with IBD.	7/9 moderate
Örtqvist, A [27] 2018 Sweden Cohort	2 yrs[1]	95 (0.01%) 51 IBD (CD and/or UC), 20 CD & 24 UC / 827,239	IBD 43 (84,3%) CD 16 (80%) UC 20 (83.3%) 0-12 months ATC code	• Delivery mode • Education parents • Ethnicity parents • IBD parents	No significant associations (any and PcV antibiotics) or dose–response relationship were found	8/9 high
Shaw, S [38] 2010 Canada Case-control	8.4 yrs[1]	36 / 360	21 (58%) 0-12 months ATC code	• Age • Place of residence • Sex	• One or more dispensations of antibiotics was associated with 2.9 times the odds (95% CI: 1.2-7.0, P = 0.017) of having IBD. • Stratified by IBD type, only CD was significant (OR = 5.3, 95% CI: 1.6-17.4; P = 0.006). • **Dose-dependent:** association for 2-4 (OR = 2.9, 95% CI: 1.1-7.8; P = 0.039) and 5+ (OR = 5.0, 95 % CI: 1.3-18.9; P = 0.18) prescriptions.	8/9 high
Virta, L [40] 2012 Finland Case-control	CD: 9.7 yrs[3] UC: 8.5 yrs[3]	595 (233 CD, 362 UC) / 2,380	313 (52,6%) 0-12 months ATC code	• Age • Place of residence • Chronic diseases • Sex	• Use of AB overall was not significant • **Type-dependent:** phenoxymethylpenicillin was associated with an increased risk of CD. (aOR = 2.54, 95% CI: 1.3-4.98)	8/9 high

AB: antibiotics, (a)HR: (adjusted) hazard ratio, (a)OR: (adjusted) odds ratio, ATC: Anatomical Therapeutic Chemical (ATC) Classification System, CD: Crohn's disease, CI: Confidence interval , IBD: Inflammatory bowel disease, IRR: incidence rate ratio, PcV: Phenoxymethylpenicillin and UC: Ulcerative colitis.

Table 1b study characteristics and associations with antibiotics: Eosinophilic Esophagitis

Author Year Country Design	Age diagnosis	Cases / Controls	Cases exposed/ Time exposure/ Recording details	Confounders for which corrected	Significant association	Quality score
Jensen, E [33] 2013 North Carolina (USA) Case-control	Cases 11 yrs	31 / 52	22 (71%) 0-12 months Motherly reported	None	Antibiotics were associated with EoE (OR= 6, 95% CI: 1.7–20.8)	4/9 weak
Jensen, E [34] 2018 North Carolina (USA) Case-control	Cases 10.6 yrs	127 / 121	91 (72%) 0-12 months Motherly reported	• Education mother • NICU admission	Antibiotics were associated with EoE (aOR = 2.30, 95% CI: 1.21-4.38)	6/9 moderate
Radano, M [37] 2014 Massachusetts (USA) Case-control	Cases 3 yrs	25 / 74	17 (67%) 0-12 months Parental reported	• Age • Atopy • Atopy family • Sex	Antibiotics were associated with EoE (OR = 3.61, 95% CI: 1.11-11.74; P = .03)	7/9 moderate
Slae, M [39] 2015 Canada Case-control	Cases 8.6 yrs	102 / 167	60 (59%) 0-12 months Parental reported	• Breastfeeding • Birth order • Day care attendance (early) • Exposure to farm animals • Fast food consumption	Rates of antibiotic exposure were similar for cases and controls.	3/9 weak
Witmer, C [41] 2018 USA Case-control	4.2 yrs	1410 / 2,820	409 (29%) 0-6 months Pharmaceutical coding	• Age • Atopy (markers) • Delivery mode • Erythema toxicum neonatorum • Feeding problems • Infantile colic • Medication exposure • Oral candidiasis • Prematurity • Prolonged rupture/ chorioamnionitis • Reflux • Sex	The association with antibiotic exposure was statistically significant (aOR = 1.31, 95% CI: 1.10–1.56).	7/9 moderate

(a)OR = (adjusted) odds ratio, CI = confidence interval, EoE = eosinophilic esophagitis

Table 1c study characteristics and associations with antibiotics: Celiac Disease

Author Year Country Design	Age diagnosis[1]/ study endpoint[3]	Cases / Controls or Cohort	Cases exposed/ Time exposure/ Recording details	Confounders for which corrected	Significant association	Quality score
Bittker, S [42] 2019 USA Case-control	6.1 yrs[1]	332 / 241	237 (71%) 0-24 months Parental reported	• Age • Age mother (at birth) • Education mother • Ethnicity	• Antibiotic exposure is associated with susequent CeD (aOR = 1.133, 95% CI: 1.037–1.244; p= 0.007) • **Dose-dependent**: ORs increase with number of antibiotic courses	5/9 moderate
Canova, C [22] 2014 Italy Cohort	6.4 yrs[1]	1.227 CeD (0.6%) 866 confirmed* and 361 unconfirmed* / 203,557	336 (47%) 0-12 months ATC code	• Education mother (only in sensitivity analysis with pathological confirmed villous atrophy) • Sex • Year of birth	• Increased risk of developing CeD after at least 1 AB course (IRR = 1.24, 95% CI: 1.07-1.43), (IRR = 1.31, 95% CI: 1.10-1.56) for histopathologically confirmed CeD • **Dose-dependent:** risk increased with more AB courses (P-trend < 0.01). • **Type-dependent:** Cephalosporin use was strongly associated with CeD onset (IRR = 1.42, 95% CI: 1.18-1.73), (IRR = 1.51, 95% CI: 1.21-1.89) for histopathologically confirmed CeD. For first- and second-generation drugs: (IRR = 1.39, 95% CI: 1.11-1.76 and third- and fourth-generation drugs: IRR = 1.49, 95% CI: 1.14-1.95).	8/9 high
Kemppainen, K [24] 2017 Finland, Germany, Sweden and the USA Cohort	21.4 months[1]	783 (11,9%) / 6,558	Unknown 0-24 months Parental reported	• Breastfeeding • CeD genotype • Delivery mode • Maternal AB use during pregnancy • Place of residence • Probiotic use • Season of birth • Sex	• Exposure to AB was not associated with CeD. • **Dose-dependent:** 2 or more doses of macrolides within the first year of life (157 of 6558 [2.4%]) had elevated CeD risk (HR = 1.77, 95% CI: 1.18-2.66; P = .006 before but not after adjustment).	6/9 moderate
Mårild, K [35] 2013 Sweden Case-control	0-2 yrs[1]	132 celiac disease / 655 12 inflammation / 60 17 normal mucosa / 85	CeD 51 (39%) Inflammation 6 (50%) 0-24 months ATC code	• Age • Education mother • Number of outpatient visits before biopsy • Sex	Exposure to AB was associated with CeD Odds ratios for prior AB use (CeD): cases 51/132 (38.6%) controls 189/655 (28.9%) (OR = 1.58, 95% CI: 1.07-2.34)	8/9 high
Myleus, A [36] 2012 Sweden Case-control	14 months[1]	373 / 581	97 (26%) 0-6 months Parental reported	• Age • Place of residence • Sex	No significantly increased risk for celiac disease (OR = 1.2, 95% CI: 0.87-1.6; P=0.27).	7/9 moderate
Sander, S [28] 2019 Denmark and Norway Cohort	Danish: 11.6 yrs[1] Norwegian: 5.4 yrs[1]	Danish: 1427 (0.12%) / 1,168,656 Norwegian: 1919 (0.36%) / 537,457	Danish: 622 (43.6%) Norwegian: 390 (20.3%) 0-12 months ATC code	• Age mother • Associated comorbidity • Birth order • Education mother • Hospitalization with infection • Season of birth • Sex • Type 1 diabetes child and/ or mother	• Exposure to systemic AB (penicillins and extended spectrum penicillins) was positively associated with diagnosed celiac disease in both cohorts (pooled aOR = 1.26, 95% CI: 1.16–1.36) • **Dose-dependent:** between number of AB courses and risk of CeD (pooled aOR for each additional dispensed AB = 1.08, 95% CI: 1.05–1.11).	9/9 high

AB: antibiotics, aHR: adjusted hazard ratio, (a)OR: (adjusted) odds ratio, ATC: Anatomical Therapeutic Chemical (ATC) Classification System, CeD: celiac disease, CI: Confidence interval , IRR: incidence rate ratio, HR: hazard ratio.

Table 1d study characteristics and associations with antibiotics: functional gastrointestinal disorders

Author Year Country Design	Age diagnosis	Cases / Controls or Cohort	Cases exposed/ Time exposure/ Recording details	Confounders for which corrected	Significant association	Quality score
Infantile colic						
Hestbaek, L [23] 2014 Denmark Cohort	0-6 months	2183 (8,1%) / 26,983	excessive 895 (41%) extreme excessive 355 (50%) 0-6 months Motherly reported	None	At 6-month-olds, statistically significant associations between excessive crying and the use of antibiotics due to ear infections (OR = 1.47, 95% CI: 1.18–1.82) were found.	6/9 moderate
Oosterloo, B [26] 2018 The Netherlands Cohort	0-1 yr	74 (20%) / 362	33 (45%) 0-7 days Broad-spectrum AB intravenous for 2-3 days (AB2) or 7 days (AB7).	• Atopy family • Birth order • Breastfeeding • Day care attendance • Delivery mode • Education parents • Tobacco exposure	• Antibiotic treatment was an independent risk factor for infantile colic (aOR = 1.66, 95%CI: 1.00-2.77, P = .05). • Doctors-diagnosed infantile colic was higher in AB+ than in AB- (4.0% vs 0.4%; P = .014). • **Duration-dependent**: Parent-reported infantile colic was higher in AB7 compared to no antibiotics (AB-) and AB2 (24.8%, 14.4% and 14.3%, P = .048 and P = .015).	8/9 high
Salvatore, S [32] 2019 Italy Cohort	0-1 yr	265 (41.9%) / 632	141 (22.3%) 0-7 days Hospital chart and parental report	• Birth weight • Breastfeeding (at 1 month of life) • Delivery mode • Duration of hospitalization at birth • Gestational age • Neonatal complications	No association was found (OR=1.16; 95% CI: 0.79-1.70, p=0.439)	7/9 moderate
Functional constipation (FC)						
Salvatore, S [32] 2019 Italy Cohort	0-1 yr	128 (26.6%) / 632	141 (22.3%) 0-7 days Hospital charts and parental reported	• Birth weight • Breastfeeding (at 1 month of life) • Delivery mode • Duration of hospitalization at birth • Gestational age • Neonatal complications	No association was found (OR=0.77; 95%CI: 0.49-1.20, p=0.242)	7/9 moderate
Turco, R [29] 2014 Italy Cohort	0-1 yr	43 (10.7%) / 465	15 (34.8%) 0-12 months Parental reported	• Anti-inflammatory drugs or corticosteroids • Atopy & in family • Birth order • Breastfeeding & weaning • Education parents • Fever episodes before onset • FGIDs family • Nursery school age • Place of residence (>3000 citizens) • Sex	No statistically significant association was found (26% vs 19%).	8/9 high
Recurrent abdominal pain (AP)						
Uusijärvi, A [30] 2014 Sweden Cohort	12 yrs	Monthly: 231 (8,7%) Weekly: 111 (4,2%) / 2,654	Monthly 1900 (71.5%) Weekly 81 (72,9%) 0-24 months Parental reported	• Asthma at 12 years of age • Asthma at one year • Sex	Stratified analyses showed that girls, who received antibiotics during both the first and the second year of life, had an increased risk of AP at 12 years (OR = 1.65, 95% CI: 1.09–2.49).	5/9 moderate
Regurgitation, functional diarrhea and infant dyschezia						
Salvatore, S [32] 2019 Italy Cohort	0-1 yr	Regurgitation:236 (37.3%) Functional diarrhea: 24 (3.8%) Infant dyschezia: 199 (31.5%) / 632	141 (22.3%) 0-7 days Hospital charts and parental reported	• Birth weight • Breastfeeding (at 1 month of life) • Delivery mode • Duration of hospitalization at birth • Gestational age • Neonatal complications	No association was found for regurgitation (OR=1.29, 95%CI: 0.88-1.90, p=0.190), functional diarrhea (OR=0.90, 95%CI: 0.33-2.45, p=0.835), or infant dyschezia (OR=1.29, 95%CI: 0.87-1.93, p=0.205).	7/9 moderate

AB: antibiotics, AB+: infants exposed to antibiotics, AB-: unexposed infants, AB2: infants exposed for 2-3 days, AB7: infants exposed for 7 days, (a)OR: (adjusted) odds ratio, CI: Confidence interval , FC: functional constipation, recurrent abdominal pain (AP)

Quality assessment

Ten studies were of high quality (22, 26-29, 31, 35, 38, 40, 43), ten studies moderate (23-25, 30, 32, 34, 36, 37, 41, 42), and two weak (33, 39) (Table 2). Frequently observed weaknesses were a high dropout rate in the cohort studies, assessment of antibiotic exposure through parental reports, and no correction for important confounders.

Table 2 quality assessment

	Selection				Comparability		Outcome / Exposure			Score
	1.	2.	3.	4.	5.	6.	7.	8.	9.	
Cohort studies*	Representativeness	Selection	Exposure	Outcome	Most important	Second important	Assessment	Duration of follow-up	Adequacy follow-up	
Canova [22]	*	*	*	*		*	*	*	*	8/9
Hestbaek [23]	*	*	*	*				*	*	6/9
Hviid [31]	*	*	*	*		*	*	*	*	8/9
Kemppainen [24]		*		*	*	*	*	*		6/9
Kronman [25]		*	*	*	*	*	*	*		7/9
Oosterloo [26]	*	*	*	*	*	*	*	*		8/9
Örtqvist [27]	*	*	*	*	*	*	*		*	8/9
Salvatore [32]	*	*	*	*		*	*	*		7/9
Sander [28]	*	*	*	*	*	*	*	*	*	9/9
Turco [29]	*	*	*	*	*	*		*	*	8/9
Uusijärvi [30]	*	*		*				*	*	5/9
Case-Control studies**	Case definition	Cases	Controls	Definition controls	Most important	Second important	Exposure	Ascertainment	Non-Response rate	
Bittker [42]			*	*	*			*	*	5/9
Canova [43]	*	*	*	*		*	*	*	*	8/9
Jensen [33]	*	*		*				*		4/9
Jensen [34]	*	*	*	*				*	*	6/9
Mårild [35]	*	*	*	*		*	*	*	*	8/9
Myleus [36]	*	*	*	*		*		*	*	7/9
Radano [37]	*	*		*	*	*		*	*	7/9
Shaw [38]	*	*	*	*		*	*	*	*	8/9
Slae [39]	*			*				*		3/9
Virta [40]	*	*	*	*		*	*	*	*	8/9
Witmer [41]		*	*	*	*	*		*	*	7/9

*Cohort studies: 1. Representativeness of the exposed cohort, 2. Selection of the non-exposed cohort, 3. Ascertainment of exposure, 4. Demonstration that the outcome of interest was not present at start of the study, 5. Comparability of cohorts on the basis of the design or analysis most important factor, 6. Comparability of cohorts on the basis of the design or analysis second important factor, 7. Assessment of outcome 8. Was follow-up long enough for outcomes to occur and 9. Adequacy of follow up of cohort.

**Case- Control studies: 1. Is the case definition adequate? 2. Representativeness of the cases, 3. Selection of controls, 4. Definition of controls, 5. Comparability of cases and controls on the basis of the design or analysis most important factor, 6. Comparability of cases and controls on the basis of the design or analysis second important factor, 7. Ascertainment of exposure, 8. Same method of ascertainment for cases and controls and 9. Non-Response rate

Comparability: Most important confounder: IBD and CeD: presence of IBD/ CeD in 1ste degree family member, EoE: sex, colic: atopy child and/or family, functional constipation: maternal education/social economic status, abdominal pain: lactose intolerance/ cow's milk allergy.

Comparability: Second important confounder: IBD: ethnicity and/or age, EoE: presence of other atopic diseases and/or ethnicity, CeD: sex and/ or season of birth and/or the presence of other autoimmune diseases, colic: presence of GERD and/or type of feeding and/or being a first child, functional constipation: sex and/ or age, abdominal pain: anxiety/ depression/ stress in the child and/or the parents.

Inflammatory Bowel Disease

Exposure to early life antibiotics was associated with the development of IBD in five out of six studies (25, 31, 38, 40, 43) (NOS = 7,8,8,8,8), whereas no association was found in one study examining Very Early Onset (VEO) IBD, (before six years of age) (27) (NOS = 8). Three studies found a dose-response relation (25, 38, 43) and an increased risk after fluoroquinolone (25), metronidazole (25), and phenoxymethylpenicillin (40) exposure. In two studies IBD was stratified by type and only the odds ratio for Crohn's disease, but not for ulcerative colitis, was significant (38, 40). Forest plots of the main results are shown in Figure 2a.

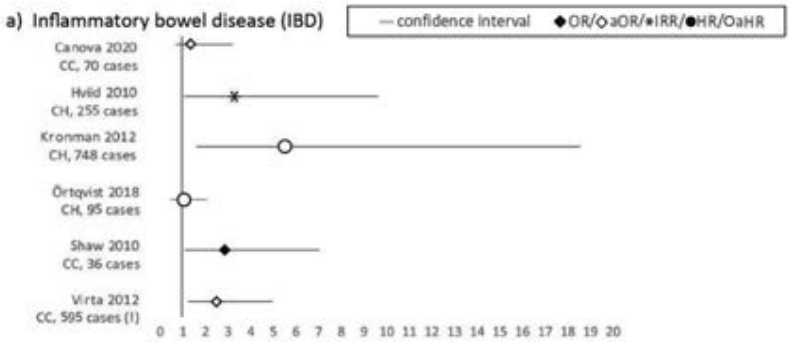

Figure 2A: forest plot of inflammatory bowel disease (IBD). CC = case control study, CH = cohort study, OR = odds ratio, aOR = adjusted odds ratio, IRR = incidence rate ratio, HR = hazard ratio, aHR = adjusted hazard ratio. (!) Virta 2012 only shows the results of the phenoxymethylpenicillin analyses, overall use of antibiotics was not significant

Eosinophilic esophagitis

In four of the five studies early life antibiotics was associated with EoE (33, 34, 37, 41) (NOS = 4,6,7,7), whereas in one study the rates of parental reported antibiotic use were similar for cases and controls (39) (NOS = 3) (Figure 2b).

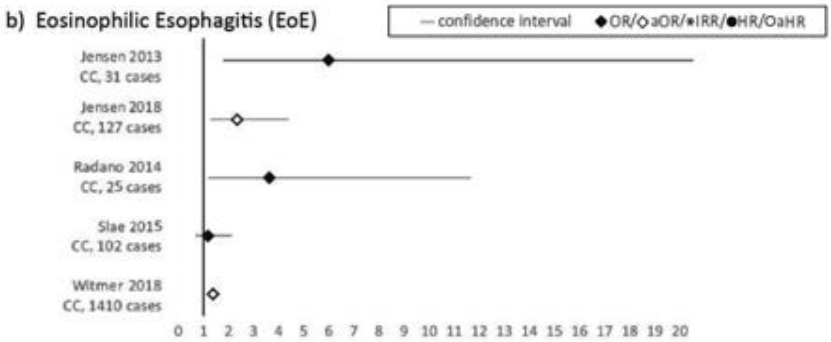

Figure 2B: forest plot of eosinophilic esophagitis (EoE). CC= case control study, CH = cohort study, (a)OR = (adjusted) odds ratio, IRR = incidence rate ratio, HR = hazard ratio, aHR = adjusted hazard ratio.

Celiac disease

In four studies, of which three had a high quality, a significant association between early life antibiotics and the presence of CeD was found (22, 28, 35, 42) (NOS = 8,9,8,5), whereas in two moderate quality studies no association was found (24, 36) (NOS = 6,7) (Figure 2c). Three studies showed a dose-response relationship between exposure to antibiotics and the risk of CeD (22, 28, 42). Furthermore, use of cephalosporin (22) and multiple courses of macrolides (24) showed a positive association with the development of CeD.

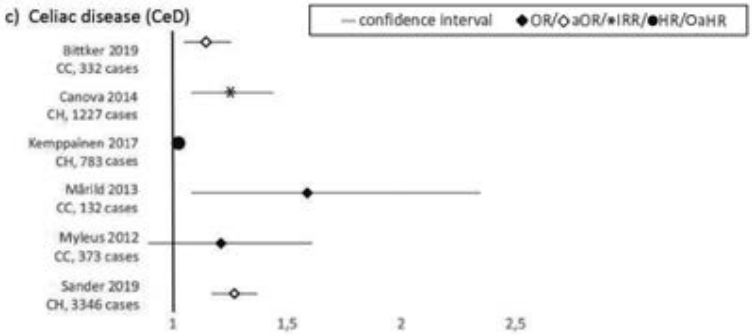

Figure 2C: forest plot for celiac disease (CeD). CC= case control study, CH = cohort study, OR = odds ratio, aOR = adjusted odds ratio, IRR = incidence rate ratio, HR = hazard ratio, aHR = adjusted hazard ratio.

Infantile colic

Two studies found a significant association between early life antibiotics and infantile colic (23, 26) (NOS = 6,8), while one study found no association (32) (NOS = 7) (Figure 2d).

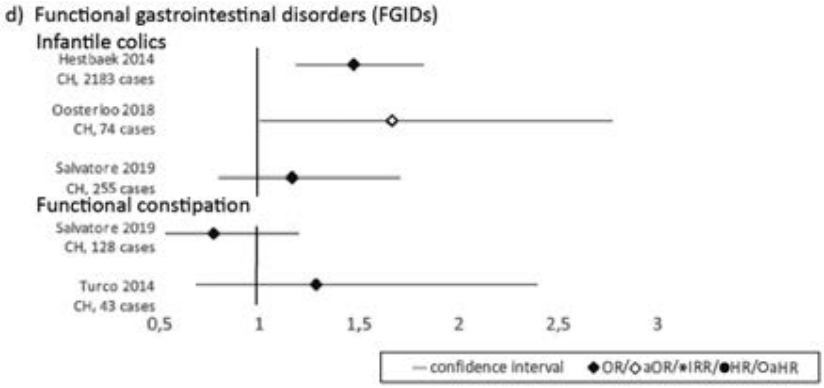

Figure 2D: forest plot for the functional gastrointestinal disorders (FGIDs) infantile colic and functional constipation. CC= case control study, CH = cohort study, OR = odds ratio, aOR = adjusted odds ratio, IRR = incidence rate ratio, HR = hazard ratio, aHR = adjusted hazard ratio.

Functional constipation

In both studies, no association was found between early life antibiotics use and functional constipation in the first year of life (29, 32) (NOS = 8,7).

Recurrent abdominal pain

The only study examining the association between antibiotics use in the first two years of life and the risk of recurrent abdominal pain (AP) at 12 years of age (30) (NOS = 5) found that only girls, but not boys, who received antibiotics in both the first and second year of life, had an increased risk of AP at 12 years.

Regurgitation, dyschezia and functional diarrhea

In one study no association was found between antibiotics in the first week of life and regurgitation, dyschezia and functional diarrhea (32) (NOS = 7).

Syntheses of individual results

Using the definitions for the best evidence synthesis, described in the method section, it can be concluded that there is strong evidence for an association of antibiotics in early life with IBD and CeD. There is moderate evidence for an association with EoE and no association with infantile constipation. The current evidence for an association between antibiotics in early life and the other studied GI-disorders is considered insufficient.

DISCUSSION

This systematic review with best evidence syntheses on the association between antibiotic exposure in the first two years of life and chronic GI disorders during childhood showed strong evidence for this association with inflammatory bowel disease and celiac disease, and moderate evidence for this association with eosinophilic esophagitis. For the other studied GI-disorders, insufficient evidence was found.

The question remains to what extent the association with IBD, EoE and CeD can be attributed to antibiotic exposure itself or to other factors such as infections and parental health seeking behavior. Infections in early life have been proposed to contribute to the development of chronic GI-disorders (44, 45) and it is difficult to differentiate between the role of infections and antibiotics which are prescribed for (suspected) infections. Furthermore, several GI-disorders like CeD can remain undiagnosed for a long time. Higher parental health seeking behavior can both lead to higher use of antibiotics and a higher chance of diagnosing the chronic

GI-disorder. Therefore, it remains unknown whether antibiotics are the true causative agent in the observed associations or whether they are intermediates in different mechanistic pathways through microbiome perturbations or changes in immune development after (suspected) infections.

Most studies found a clear association between antibiotics in early life and IBD. The study that focused on very early onset IBD (VEO-IBD), found no association between antibiotics and VEO-IBD. VEO-IBD is considered a different entity from later-onset IBD (44), since genetics play a far more important etiological role than microbial dysbiosis (45). This may explain the lack of an association with early life antibiotics.

The primary goal of antibiotic administration is to prevent detrimental effects of serious and sometimes even life-threatening infections. However, especially in early life, antibiotics are overused, since they are often prescribed for viral upper respiratory tract infections (46, 47). Given its association with the occurrence of IBD, CeD and EoE, it is highly important to prevent antibiotic overuse by strict adherence to guidelines. If antibiotics are necessary, treatment would be adjusted to minimize dysbiosis. Another possible solution is to shorten the time of antibiotic administration. Oosterloo et al. found more health issues in the first year of life after seven days compared to two days of antibiotics in the first week of life (26). Furthermore, whenever possible, narrow-spectrum antibiotics rather than broad-spectrum should be used, because these specifically reduce the capacity of pathogens to cause disease while leaving commensals unharmed (48). If adjustment of antibiotic treatment is not possible, interventions that restore or prevent dysbiosis should be considered, such as administration of pre- or probiotics, or fecal transplants (49-52).

Some limitations of this review need to be considered. As no randomized controlled trials were available, only associations but not causality can be examined. Additionally, the studied results were not evaluated for their precision and associations with wide confidence intervals can indicate uncertainty about the magnitude of the association. Hence, the results must be interpreted with caution. Furthermore, both age at exposure as well as age at diagnosis varied substantially between the studies. In addition, study outcomes were also very heterogeneous, excluding a meta-analysis. Therefore, a best evidence synthesis was applied, taking the quality of the studies into account. Furthermore, the recording of antibiotic exposure was in half of the studies parental reported, which may have led to recall bias. The antibiotics were mostly analysed as overall use, without distinguishing between types of antibiotics and therefore, it was not possible to determine associations between certain type of antibiotics and GI-disorders. Finally, for several functional gastrointestinal disorders, like IBS or GERD, only few or even no studies were found which prohibits any conclusions on these GI disorders.

One of the strengths of this review is that the search string was built and performed by an information scientist. Besides the published articles, also conference abstracts were checked for relevant studies. Furthermore, this review studies the association between antibiotics in early life and all chronic GI disorders in childhood, which provides insights in the available evidence but also shows the gap of knowledge for these associations.

For future research, it is recommended to study the association between early life antibiotics and the presence of those GI disorders that currently lack sufficient studies. Furthermore, it is necessary to gain insights in the specific effect of different types of antibiotics on the microbiome in order to optimize therapies that can prevent or counteract the detrimental effects of antibiotics in early life.

CONCLUSIONS

This systematic review shows strong evidence for an association between antibiotic exposure in the first two years of life and the presence of IBD and CeD later in childhood. For the other included GI-disorders, only moderate or insufficient evidence was found. In order to decrease the incidence of IBD and CeD, antibiotic administration in early life should be critically considered. Moreover, interventions need to be developed to restore the microbiome after unavoidable antibiotic exposure in order to prevent detrimental health consequences later in life.

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APPENDIX

Supplementary Table 1 search strategy

	Ovid MEDLINE(R) ALL <1946 to 2020 June 08> **Search date: 9 June 2020**	
#	**Searches**	**Results**
1	exp infant death/ or infant/	788526
2	(early life or infant? or infancy or toddler? or preschool* or (early adj5 (childhood or child or children or p?ediatric?)) or minors or baby or babies or kindergarten or newborn?).ab,kf,ti.	686417
3	(("0" or "1" or "2" or "3" or "4") adj1 (age? or yr? or year?)).ab.	655139
4	(("0" or "1" or "2" or "3" or "4" or "5" or "6" or "7" or "8" or "9" or "10" or "11" or "12" or "13" or "14" or "15" or "16" or "17" or "18" or "19" or "20" or "21" or "22" or "23" or "24") adj1 month?).ab.	1066665
5	or/1-4 [Ia - children 0-4 yrs]	2564903
6	((pediatric or infantile or juvenile) adj1 (functional gastrointestinal disorders or FIGD? or irritable bowel syndrome or IBS or spastic colon or inflammator* bowel or IBD or Colitis ulcerosa or ulcerative colitis or proctocolitis or colitis gravis or crohn or abdominal pain? or aerophag* or dyspepsia or indigestion or constipat* or celiac disease or gluten or appendicitis or gastritis or gastrides or enteritis or diarrh?ea? or loose stool? or liquid stool? or liquid feces or fluid stool? or colic or abdominal cramp? or (Eosinophilic adj2 Esophagitis) or Gastric Acid Reflux or Gastro Esophageal Reflux or Gastroesophageal Reflux or GERD or Esophageal Reflux or Gastro oesophageal Reflux or esophageal stenos* or esophageal stricture or Hypertrophic pyloric stenosis)).ab,kf,ti. [Ib - children 0-4 yrs]	5357
7	Gentamycins/	18247
8	(Alcomicin or Bristagen or G-Mycin or Gentacidin or Gentafair or Gentamar or Jenamicin or Ocu-Mycin or Spectro-Genta or U-gencin or U-Liang or Gentamycin? or Garamycin or Gentacycol or Gentavet or Genticin or G Myticin or GMyticin or Gentamicin or "1403-66-3").ab,kf,ti.	27205
9	or/7-8 [IIa first week exclusive use]	32706
10	(antibiotic? or erythromycin or metoclopramide).mp. [IIb]	399419
11	((meningitis or infection? or inflammat* or pneumonia or pyelonephritis) adj3 (pe?diatric or early or infant* or newborn?)).ab,kf,ti. [IIc]	48465
12	(sepsis and infant).hw.	9982
13	(sepsis adj2 early).ab,kf,ti.	1919
14	or/12-13 [IId]	11418
15	(childhood disease? and (risk or environmental factor?)).ab,kf,ti. [IIe]	360
16	exp inflammatory bowel disease/ or abdominal pain/ or aerophagy/ or dyspepsia/ or constipation/ or celiac disease/ or appendicitis/ or gastritis/ or enteritis/ or exp diarrhea/ or colic/ or Eosinophilic Esophagitis/ or Gastroesophageal Reflux/ or esophageal stenosis/	266125

17	(functional gastrointestinal disorders or FIGD? or irritable bowel syndrome or IBS or spastic colon or inflammator bowel or IBD or Colitis ulcerosa or ulcerative colitis or proctocolitis or colitis gravis or crohn or abdominal pain? or aerophag* or dyspepsia or indigestion or constipat* or celiac disease or gluten or appendicitisor gastritis or gastrides or enteritis or diarrh?ea? or loose stool? or liquid stool? or liquid feces or fluid stool? or infantile colic or abdominal cramp? or (Eosinophilic adj2 Esophagitis) or Gastric Acid Reflux or Gastro Esophageal Reflux or Gastroesophageal Reflux or GERD or Esophageal Reflux or Gastro oesophageal Reflux or esophageal stenos* or esophageal stricture).ab,kf,ti.	305723
18	Pyloric Stenosis, Hypertrophic/	654
19	(Hypertrophic pyloric stenosis or hypertrophic pylorus stenosis).ab,kf,ti.	1513
20	18 or 19	1622
21	limit 20 to yr="2015-current"	184
22	or/16-17,21 [outcomes]	424883
23	follow-up studies/ or longitudinal studies/ or retrospective studies/	1441183
24	(prognos* or predict* or course or follow-up or followup or longitudinal or retrospective or (observational adj2 stud*)).ab,kf,ti.	3775119
25	(case control or cohort study or (risk and review)).mp.	1032965
26	observational study.pt.	80055
27	or/23-26 [study design]	4954421
28	5 or 6 [Ia+b - children 0-4 yrs]	2566952
29	28 and (10 or 11) and 22 and 27	2707
30	and/9,28	4170
31	and/14,22	319
32	or/15,29-31	7477
33	(Meniere or Pouchitis or Total hip or Arthroplasty or Whipple or peritoneal dialysis or Bone? or Vertigo? or IUD? or PMMA? or SEM? or debridement or surgical site infection or ssi).ab,kf,ti. [NOTing out green]	1105473
34	(Helicobactor pylori or HIV or Newborn calf or Measle or Varicella or RCT? or Pig).ab,kf,ti. [NOTing out blue]	505926
35	(nalidixic acid? or molecular epidemiology or vitro activity or Strain? or Outbreak?).ab,kf,ti. [NOTing out red]	803243
36	(pharmacokinetic parameter or Rat or premature baby or vlbw or billirubin? or chorioamnionitis? or sisomicin or clearance or therapeutic drug monitoring or serum half life or dosage interval or experiment?).ab,kf,ti. [NOTing out yellow]	1949849
37	or/33-36	4123179
38	32 not 37 [NOTing out]	5238
39	animals/ not humans/	4672110
40	38 not 39	5096
41	("Twin cohort for the study of (pre)clinical Inflammatory Bowel Disease in the Netherlands" or NTR6681).ab,kf,ti.	0
42	40 or 41	5096

	Ovid Embase Classic+Embase <1947 to 2020 June 06> **Search date: 9 June 2020**	
#	**Searches**	**Results**
1	exp *infant/ or *infancy/ or infant.hw.	798854
2	(early life or infant? or infancy or toddler? or preschool* or (early adj5 (childhood or child or children or p?ediatric?)) or minors or baby or babies or kindergarten or newborn?).ab,kw,ti.	875279
3	(("0" or "1" or "2" or "3" or "4") adj1 (age? or yr? or year?)).ab.	1051740
4	(("0" or "1" or "2" or "3" or "4" or "5" or "6" or "7" or "8" or "9" or "10" or "11" or "12" or "13" or "14" or "15" or "16" or "17" or "18" or "19" or "20" or "21" or "22" or "23" or "24") adj1 month?).ab.	1708558
5	or/1-4 [Ia - children 0-4 yrs]	3541363
6	((pediatric or infantile or juvenile) adj1 (functional gastrointestinal disorders or FIGD? or irritable bowel syndrome or IBS or spastic colon or inflammator* bowel or IBD or Colitis ulcerosa or ulcerative colitis or proctocolitis or colitis gravis or crohn or abdominal pain? or aerophag* or dyspepsia or indigestion or constipat* or celiac disease or gluten or appendicitis or gastritis or gastrides or enteritis or diarrh?ea? or loose stool? or liquid stool? or liquid feces or fluid stool? or colic or abdominal cramp? or (Eosinophilic adj2 Esophagitis) or Gastric Acid Reflux or Gastro Esophageal Reflux or Gastroesophageal Reflux or GERD or Esophageal Reflux or Gastro oesophageal Reflux or esophageal stenos* or esophageal stricture or Hypertrophic pyloric stenosis)).ab,kw,ti. [Ib - children 0-4 yrs]	7292
7	*Gentamicin/	35017
8	(Alcomicin or Bristagen or G-Mycin or Gentacidin or Gentafair or Gentamar or Jenamicin or Ocu-Mycin or Spectro-Genta or U-gencin or U-Liang or Gentamycin? or Garamycin or Gentacycol or Gentavet or Genticin or G Myticin or GMyticin or Gentamicin or "1403-66-3").ab,kw,ti.	36468
9	"1403-66-3".rn.	104829
10	or/7-9 [IIa first week exclusive use]	113443
11	(antibiotic? or erythromycin or metoclopramide).mp. [IIb]	870330
12	((meningitis or infection? or inflammat* or pneumonia or pyelonephritis) adj3 (pe?diatric or early or infant* or newborn?)).ab,kw,ti. [IIc]	65283
13	(sepsis and infant).hw.	11891
14	(sepsis adj2 early).ab,kw,ti.	2988
15	or/13-14 [IId]	14425
16	(childhood disease? and (risk or environmental factor?)).ab,kw,ti. [IIe]	498
17	exp *inflammatory bowel disease/ or *abdominal pain/ or *aerophagia/ or *dyspepsia/ or exp *constipation/ or *celiac disease/ or *appendicitis/ or *gastritis/ or *enteritis/ or *diarrhea/ or *infantile diarrhea/ or *colic/ or *infantile colic/ or *Eosinophilic Esophagitis/ or *Gastroesophageal Reflux/ or *esophageal stenosis/	267207
18	(functional gastrointestinal disorders or FIGD? or irritable bowel syndrome or IBS or spastic colon or inflammator bowel or IBD or Colitis ulcerosa or ulcerative colitis or proctocolitis or colitis gravis or crohn or abdominal pain? or aerophag* or dyspepsia or indigestion or constipat* or celiac disease or gluten or appendicitisor gastritis or gastrides or enteritis or diarrh?ea? or loose stool? or liquid stool? or liquid feces or fluid stool? or infantile colic or abdominal cramp? or (Eosinophilic adj2 Esophagitis) or Gastric Acid Reflux	493146

	or Gastro Esophageal Reflux or Gastroesophageal Reflux or GERD or Esophageal Reflux or Gastro oesophageal Reflux or esophageal stenos* or esophageal stricture).ab,kw,ti.	
19	*hypertrophic pylorus stenosis/	1263
20	(Hypertrophic pyloric stenosis or hypertrophic pylorus stenosis).ab,kw,ti.	1940
21	19 or 20	2152
22	limit 21 to yr="2015-current"	231
23	or/17-18,22 [outcomes]	586712
24	follow up/ or longitudinal study/ or retrospective study/	2412789
25	(prognos* or predict* or course or follow-up or followup or longitudinal or retrospective or (observational adj2 stud*)).ab,kw,ti.	5528713
26	observational study.kw,ti.	27665
27	(case control or cohort study or (risk and review)).mp.	1211338
28	or/24-27 [study design]	6774290
29	5 or 6 [Ia+b - children 0-4 yrs]	3545044
30	29 and (11 or 12) and 23 and 28	5878
31	and/10,28-29	5192
32	and/15,23	576
33	or/16,30-32	11847
34	(Meniere or Pouchitis or Total hip or Arthroplasty or Whipple or peritoneal dialysis or Bone? or Vertigo? or IUD? or PMMA? or SEM? or debridement or surgical site infection or ssi).ab,kw,ti. [NOTing out green]	1532636
35	(Helicobactor pylori or HIV or Newborn calf or Measle or Varicella or RCT? or Pig).ab,kw,ti. [NOTing out blue]	664053
36	(nalidixic acid? or molecular epidemiology or vitro activity or Strain? or Outbreak?).ab,kw,ti. [NOTing out red]	962439
37	(pharmacokinetic parameter or Rat or premature baby or vlbw or billirubin? or chorioamnionitis? or sisomicin or clearance or therapeutic drug monitoring or serum half life or dosage interval or experiment?).ab,kw,ti. [NOTing out yellow]	2455192
38	or/34-37	5285640
39	33 not 38 [NOTing out]	9118
40	(animal/ or animal experiment/ or animal model/ or nonhuman/) not human/	6454629
41	39 not 40	8980
42	("Twin cohort for the study of (pre)clinical Inflammatory Bowel Disease in the Netherlands" or NTR6681).ab,kw,ti.	0
43	NTR6681.cn.	0
44	or/41-43	8980

	Web of Science Core Collection: **- SCI-EXPANDED 1975-present** **- SSCI 1975 - present** **-A&HCI 1975 - present** **- ESCI 2015 - present** **Search date: 9 June 2020**	
#	**Searches**	**results**
# 1	TS=(early life or infant or infancy or toddler or preschool or (early N4 (childhood or child or children or pediatric)) or minors or baby or babies or kindergarten or newborn)	1085229
# 2	AB=((("0" or "1" or "2" or "3" or "4") N1 (age? or yr? or year?))	1805
# 3	AB=((("0" or "1" or "2" or "3" or "4" or "5" or "6" or "7" or "8" or "9" or "10" or "11" or "12" or "13" or "14" or "15" or "16" or "17" or "18" or "19" or "20" or "21" or "22" or "23" or "24") N1 month?)	1183
# 4	TS=((pediatric or infantile or juvenile) N1 (functional gastrointestinal disorders or FIGD? or irritable bowel syndrome or IBS or spastic colon or inflammator* bowel or IBD or Colitis ulcerosa or ulcerative colitis or proctocolitis or colitis gravis or crohn or abdominal pain? or aerophag* or dyspepsia or indigestion or constipat* or celiac disease or gluten or appendicitis or gastritis or gastrides or enteritis or diarrh?ea? or loose stool? or liquid stool? or liquid feces or fluid stool? or colic or abdominal cramp? or (Eosinophilic AND Esophagitis) or Gastric Acid Reflux or Gastro Esophageal Reflux or Gastroesophageal Reflux or GERD or Esophageal Reflux or Gastro oesophageal Reflux or esophageal stenos* or esophageal stricture or Hypertrophic pyloric stenosis))	3
# 5	#4 OR #3 OR #2 OR #1	1087634
# 6	TS=antibiotic	334292
# 7	#6 AND #5	15781
# 8	TS=(Alcomicin or Bristagen or G-Mycin or Gentacidin or Gentafair or Gentamar or Jenamicin or Ocu-Mycin or Spectro-Genta or U-gencin or U-Liang or Gentamycin? or Garamycin or Gentacycol or Gentavet or Genticin or G Myticin or GMyticin or Gentamicin or "1403-66-3")	25466
# 9	#8 OR #7	40687
# 10	TS=(functional gastrointestinal disorders or FIGD? or irritable bowel syndrome or IBS or spastic colon or inflammator bowel or IBD or Colitis ulcerosa or ulcerative colitis or proctocolitis or colitis gravis or crohn or abdominal pain? or aerophag* or dyspepsia or indigestion or constipat* or celiac disease or gluten or appendicitisor gastritis or gastrides or enteritis or diarrh?ea? or loose stool? or liquid stool? or liquid feces or fluid stool? or infantile colic or abdominal cramp? or (Eosinophilic adj2 Esophagitis) or Gastric Acid Reflux or Gastro Esophageal Reflux or Gastroesophageal Reflux or GERD or Esophageal Reflux or Gastro oesophageal Reflux or esophageal stenos* or esophageal stricture)	252018
# 11	TS=(Hypertrophic pyloric stenosis or hypertrophic pylorus stenosis)	1233
# 12	#11 OR #10	253145
# 13	#12 AND #9	655

Chapter 5

Neonatal antibiotics and food allergy are associated with FGIDs at 4-6 years of age

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ABSTRACT:

Objectives: Antibiotics may contribute to the development of functional gastrointestinal disorders (FGIDs). This study aimed to determine whether antibiotics during the first week of life, infantile colic in the first year of life, gut-associated immune markers at one year of age, and allergies at 4-6 years of age in term born children were associated with a higher prevalence of FGIDs at 4-6 years of age.

Methods: A prospective observational cohort of 436 term-born infants was followed up at the age of 4-6 years; 151 received broad-spectrum antibiotics (AB+), and 285 healthy controls (AB-). Validated ROME III and ISAAC questionnaires were sent to parents of 418 available children. The independent t-test, Chi-squared test or non-parametric test, and logistic multivariate regression analyses were used.

Results: In total, 340/418 (81%) questionnaires were completed. Only the presence of functional abdominal pain was significantly higher in AB+ than AB- (4% vs. 0.4% respectively, p=0.045). Children with food allergy fulfilled significantly more often the criteria for irritable bowel syndrome (IBS) and abdominal migraine (26% vs. 9%, p=0.002 and 7% vs. 1%, p=0.043 respectively) compared to non-allergic children. No differences in FGIDs existed at the age of 4-6 years between children with and without a history of infantile colic. There were significant differences in gut-associated immune markers between children with and without FGIDs.

Conclusion: Antibiotics during the first week of life resulted in a higher risk for functional abdominal pain at 4-6 years. Furthermore, food allergy was associated with IBS and abdominal migraine at 4-6 years.

INTRODUCTION

Functional gastrointestinal disorders (FGIDs) are common disorders in children with often a major impact on their quality of life (1). FGIDs are defined as a variable combination of chronic or recurrent gastrointestinal symptoms that, after appropriate medical evaluation, cannot be attributed to any other medical condition (2). Nowadays, they are also indicated as brain-gut disorders, emphasizing the importance of both psychosocial factors and gut factors like abnormal intestinal motility, visceral hyperalgesia, and gut inflammation in the pathophysiology of FGIDs (3).

Alterations in the gut microbiome (dysbiosis) can play a role in the pathophysiology of FGIDs as well (4-6). Antibiotics are a group of drugs that can have a profound effect on the gut microbiome (7). In adults, antibiotic treatment increases the risk of developing FGIDs (8, 9), but studies in children are less conclusive. A Swedish study showed that antibiotic treatment during the first two years of life increased the risk of abdominal pain in girls at 12 years of age, but not when antibiotics were administered between 9 and 12 years old (10). An Italian study demonstrated that neonatal antibiotic treatment was associated with an increased risk of FGIDs in the first year of life, although the effect was only found in preterm born children (11). These pediatric studies suggest that the timing of antibiotics may determine the degree of dysbiosis and hence its subsequent health effects including FGIDs (7).

The gut microbiome is developing during the first two years of life and disruption of the normal developmental process may have long-term consequences (12). To study the effect of antibiotic exposure during the first week of life, the INCA study (INtestinal microbiota Composition after Antibiotic treatment in early life) was set up (13). It is a prospective cohort study of infants born at term, in which associations between antibiotic exposure in the first week of life and subsequent health problems are being studied. In the INCA study, we found that antibiotic treatment in the first week of life was associated with an increased risk of infantile colic in the first year of life (14), with a different circulating immune marker profile at one year of age (15), and with an increased risk of developing allergies at 4-6 years of age (16).

The first aim of the INCA follow-up study was to investigate if antibiotic treatment in the first week of life increases the risk of having FGIDs at the age of 4-6 years. The second aim was to examine whether findings from other studies, showing an association between atopic disorders and FGIDs, could be replicated (17, 18). Since there was a relatively large group of children with infantile colic in the INCA cohort, the third aim was to determine whether infantile colic was associated with an increased prevalence of FGIDs at 4-6 years of age. Finally, we explored whether gut-associated immune markers that were significantly different at one year

of age in children with and without a history of infantile colic were different for children with FGIDs at 4-6 years of age.

METHODS

Study design

The study design of the prospective birth-cohort INCA study has been described previously (13). In short, between 2012 and 2015, 436 term-born infants were recruited from the maternity and neonatal wards of four teaching hospitals in the Netherlands. We included 151 infants treated with a combination of broad-spectrum antibiotics in their first week of life due to suspicion for early-onset sepsis (AB+) and 285 healthy controls (AB-). All hospitals used gentamycin in combination with either penicillin, amoxicillin or amoxicillin/clavulanic acid. Blood cultures were taken before AB treatment was started. In case of a negative blood culture, combined with a low clinical suspicion of infection and low C-reactive protein, antibiotics were discontinued after 2 to 3 days (n=42). Otherwise, antibiotics were continued for seven days (n=109).

Data collection in the first year of life

At inclusion, parents filled out an online questionnaire concerning the background of the family (e.g., parental atopic diseases, household education, and presence of siblings). Thereafter, a daily checklist was kept during the first year of life, reporting the presence of wheezing, crying, and other symptoms. If parents reported excessive crying ≥ three days within a week, in the first three months of life, it was defined as infantile colic according to the ROME III criteria (19). Furthermore, a monthly questionnaire was filled out during the first year of life concerning nutrition, antibiotic treatment, and general practitioner visits for gastrointestinal symptoms. The symptoms were verified at 1 year of age through doctors' diagnoses via the general practitioner electronic medical database using the International Classification of Primary Care (ICPC) (20). Around one year of age, a blood sample was obtained if parents gave additional informed consent (n=149). After centrifugation, the serum samples were aliquoted and stored at −80◦C until further use. The method of analyzing the immune markers with a multiplex immunoassay has been described elsewhere (15). The fourteen gut-associated immune markers (IL-17F, IL-22, IL-31, IL-33, IL1R1, LIGHT, YKL-40, CXCL13, sPD1, TNF-R2, sIL-7Ra, Gal-1, Gal-9, S100A8) that differed significantly at one year of age between infants with and without infantile colic were studied for differences between children with and without FGIDs.

Follow-up study

There were 418 children (147 AB+ and 271 AB-) eligible for follow-up since 18 of the 436 children dropped out of the study after the first year of life (Figure 1). Between May 2018 and May 2019, the parents of these 418 children were approached to fill out an online questionnaire. The children were between 4 and 6 years of age at the time of follow-up, depending on the year of inclusion. Data regarding the presence of FGIDs (functional dyspepsia, irritable bowel syndrome (IBS), abdominal migraine, functional abdominal pain, functional constipation, non-retentive fecal incontinence, and aerophagia) were collected using the validated ROME III questionnaire (19). Data regarding wheezing, asthma, and eczema were collected through the validated ISAAC questionnaire (21). Furthermore, information about additional antibiotic use until the second year of life and the presence of both parental reported and doctor diagnosed allergies (food, drug, insect venom, inhalation, or contact allergy) was collected. Upper and lower respiratory disorders, gastrointestinal disorders, and allergies were verified through doctors' diagnoses using ICPC, if informed parental consent for requesting this information was obtained (20). Finally, with additional informed parental consent, the pharmacy was approached to collect information about antibiotic use after the neonatal period until 4-6 years of age.

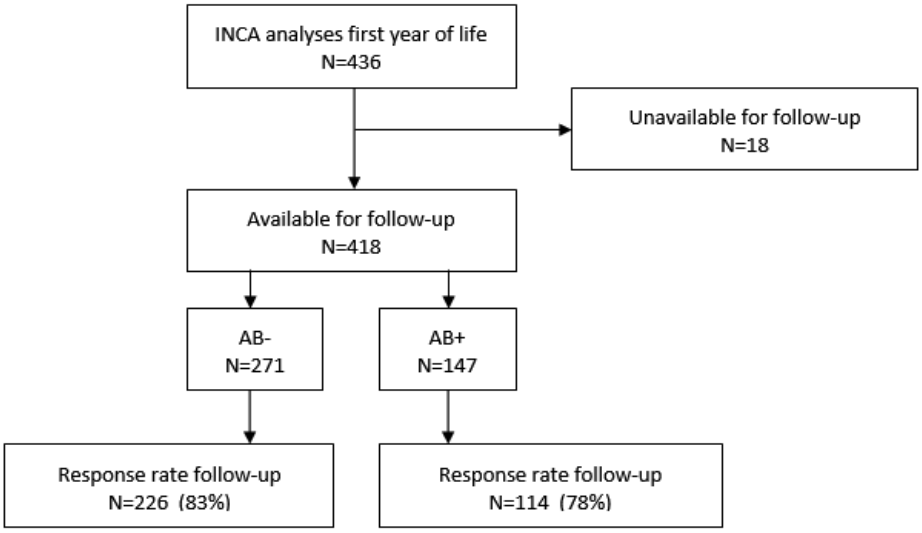

Figure 1 flowchart of follow-up.

AB+ = children born at term treated with antibiotics in the first week of life and AB- = unexposed infants.

Statistical analysis

Baseline characteristics and differences between groups were analysed using the independent t-test and Chi-squared test or non-parametric test as appropriate. If the conditions for a Chi-squared test were met, logistic regression analyses were performed and expressed as OR with 95% confidence intervals (CI), otherwise only the results of the Fisher exact test were presented. In order to prevent bias, logistic multivariate regression analyses were only performed in case there were at least 10 events per variable (22). A p-value <0.05 was considered statistically significant. Analyses were performed with IBM SPSS Statistics for Windows version 26 (Armonk, NY, USA).

Ethics

Informed consent was obtained from both parents of all participating children. The study was approved by the ethical board of the St. Antonius Hospital in Nieuwegein, the Netherlands, and was registered in the clinical trials register as NCT02536560.

RESULTS

In total, 340 of the 418 (81%) questionnaires were completed (Figure 1). The response rate was equally divided between AB+ and AB- (114/147 [78%] vs 226/271 [83%], p=0.118). Baseline characteristics between AB+ and AB- of the follow-up cohort were similar, except for age at follow-up, delivery mode, and antibiotic-free months after the first week of life in the first year of life (Table 1). Among the 149 children with a blood sample taken, there was complete follow-up of 130 (87%) children. Pharmacy records showed no significant differences in the number of children treated with additional antibiotic courses after the first week and before the age of two years between the AB+ and AB- group.

In total, 80/340 (24%) children had at least one FGID, with similar prevalence in AB+ and AB- (28% vs. 21 %, p= 0.161), aOR 1.428 (95% CI 0.837 – 2.434, p=0.191) corrected for age, delivery mode and antibiotic free months in the first year of life. IBS was the most frequently observed FGID in 39 (11%) children, followed by functional constipation in 22 (7%) children. Of all FGIDs, only the presence of functional abdominal pain was significantly higher in AB+ compared to AB- children, independent of differences at baseline (4% vs. 0,4% respectively, p=0.045) (Table 2).

Table 1 Baseline characteristics

	AB - N 226	AB + N 114
Age (median, IQR)*	5 (4.6-5.9)	4.7 (4.4-5.0)
Sex (male n,%)	121 (54)	65 (57)
Delivery mode* (vaginal, %)	146 (65)	86 (75)
Duration (months) any breastfeeding (median, IQR)	4 (1-8)	2.5 (1-7)
Duration (months) exclusive breastfeeding (median, IQR)	2 (0-5)	0 (0-4)
Siblings (yes, %)	120 (53)	69 (61)
Missing	7 (3)	4 (4)
Infantile colic in the first year of life (yes, %)	34 (15)	23 (20)
Number of AB courses, after 1st week, in the first years of life (median, IQR)	0 (0-1)	0 (0-1)
AB free months, after 1st week, in the first year of life* (median, IQR)	9 (7-11)	7 (2-9)
Number of courses, after 1st week, in the first 2 years (median, IQR)	1 (1-2)	1 (1-2)

AB+ = children born at term treated with antibiotics in the first week of life, AB- = healthy controls, IQR = interquartile range, SD = standard deviation *Significant p=< 0.05 difference between AB+ and AB-

The presence of one or more FGIDs was higher in children with parental reported food allergy compared to children without food allergy (36% vs. 22% respectively, p=0.047, Table 2), although no longer significant after correction for sex and age aOR 1.955 (95% CI 0.980 – 3.899, p=0.057). There was a significantly higher frequency of both IBS (26% and 9%, p=0.002) aOR 2.821 (95% CI 1.231-6.463, p=0.014) corrected for age, sex and presence of siblings, and abdominal migraine (7% and 1%, p=0.043) in children with a parental reported food allergy. A doctor-diagnosed food allergy (n=18) was only significantly associated with abdominal migraine (17% vs 1%, p=0.004).

The percentage of children with >1 FGID at 4-6 years was similar in children with and without infantile colic in the first year of life (26% vs. 22% respectively, OR 1.198 [95% CI 0.624-2.298, p=0.587], Table 2). Moreover, this was independent from neonatal or later antibiotic exposure aOR 1.159 (95% CI 0.602-2.232, p=0.659).

Table 2: incidence of functional gastrointestinal disorders (FGIDs) between AB- and AB+, parental reported food allergic and non-food allergic children, and between children whit and without infantile colic

	AB- N 226 N, %	AB+ N 114 N, %	P-value	Non-allergic N 298 N, %	Food allergic N 42 N, %	P-value	Colic - N 283 N, %	Colic + N 57 N, %	P-value
Any FGID	48 (21)	32 (28)	P=0.161	65 (22)	15 (36)	**P=0.047**	63 (22)	15 (26)	P=0.587
Functional dyspepsia	3 (1)	2 (2)	P=1	4 (1)	1 (2)	P=0.485	5 (2)	0 (0)	P=0.594
IBS	26 (12)	13 (11)	P=0.978	28 (9)	11 (26)	**P=0.002**	32 (11)	7 (12)	P=0.833
Abdominal migraine	5 (2)	2 (2)	P=1	4 (1)	3 (7)	**P=0.043**	6 (2)	1 (2)	P=1
Functional abdominal pain	1 (0.4)	4 (4)	**P=0.045**	5 (2)	0 (0)	P=1	5 (2)	0 (0)	P=0.594
Functional constipation	13 (6)	9 (8)	P=0.448	19 (6)	3 (7)	P=0.743	17 (6)	5 (9)	P=0.389
Non retentive fecal incontinence	3 (1)	2 (2)	P=1	4 (1)	1 (2)	P=0.485	4 (1)	1 (2)	P=1

AB+ = children born at term treated with antibiotics in the first week of life, AB- = healthy controls, colic - = children without colic in the first year of life, colic + = children with infantile colic in the first year of life. FGID = functional gastrointestinal disorder, IBS = irritable bowel syndrome. Significance tested with a X^2-test or Fisher exact test.

The gut-associated immune markers Gal-1, IL1R1, LIGHT and sPD1 were significantly lower both in children with any FGID compared to children without a FGID as well as in children

with IBS compared to children without IBS (Table 3). The marker IL-22 was significantly lower in children with any FGID compared to children without a FGID. Furthermore, the markers TNF-R2, Gal-9, CXCL13 were significantly lower in children with IBS compare to children without IBS.

Table 3: Differences between gut-associated immune markers in children with functional gastrointestinal disorders and in children with irritable bowel syndrome

	No FGID N=101 Mean (SD)*/ Median (IQR)	Any FGID N=29 Mean (SD)*/ Median (IQR)	p-value	IBS - N=114 Mean (SD)*/ Median (IQR)	IBS + N=16 Mean (SD)*/ Median (IQR)	p-value
IL-17F	17.9 (17.9-17.9)	17.9 (17.9-17.9)	0.052	17.9 (17.9-17.9)	17.9 (17.9-17.9)	0.247
IL-22	3.7 (2.6-7.8)	3.7 (1.5-3.7)	**0.043**	3.7 (2.6-7.0)	3.7 (1.2-3.7)	0.090
IL-31	24.4 (24.4-58.9)	24.4 (24.4-24.4)	0.163	24.4 (24.4-51.4)	24.4 (24.4-24.4)	0.527
IL-33	11.0 (11.0-11.0)	11.0 (11.0-11.0)	0.368	11.0 (11.0-11.0)	11.0 (11.0-11.0)	0.518
IL1R1	25.6 (9.1)*	21.7 (7.5)*	**0.033**	25.4 (8.8)*	20.0 (7.9)*	**0.021**
LIGHT	13.7 (8.1-29.9)	8.1 (5.2-14.8)	**0.020**	13.6 (8.1- 27.9)	8.1 (5.1-10.8)	**0.030**
YKL-40	13647.3 (18163.0-25104.1)	19607.9 (12827.5-25616.8)	0.795	18307.1 (13668.4 – 25234.4)	19338.1 (12637.5- 23853.7)	0.966
CXCL13	50.0 (30.2-64.7)	42.3 (25.5-53.2)	0.104	50.2 (30.6 – 63.4)	28.4 (23.0 – 48.7)	**0.012**
sPD1	642.0 (269.0)*	523.2 (228.0)*	**0.033**	635.3 (263.1)*	473.1 (232.7)*	**0.021**
TNF-R2	1227.1 (503.9)*	1148.1 (497.2)*	0.456	1241.8 (495.3)*	987.8 (500.5)*	**0.049**
sIL-7Ra	28971.5 (13478.6-46086.7)	20462.2 (4508.9-50804.1)	0.346	28761.6 (13359.6 – 46757.1)	19431.1 (2640.7- 48051.7)	0.245
Gal-1	13143.5 (5800.9)*	10514.8 (5178.4)*	**0.030**	12992.6 (5757.3)*	9454.0 (4833.2)*	**0.021**
Gal-9	2936.0 (1408.5)*	2632.7 (1015.2)*	0.282	2969.9 (1361.0)*	2144.8 (840.5)*	**0.020**
S100A8	8263.5 (4964.9-13571.0)	5926.1 (4074.8 – 11081.8)	0.314	8132.3 (4798.1 – 13775.5)	6671.9 (4215.5 – 10453.1)	0.476

FGID = functional gastrointestinal disorder, IBS = irritable bowel syndrome, IBS - = all children without IBS, IBS+ = children with IBS, SD = standard deviation, IQR = interquartile range

DISCUSSION

In this follow-up of the INCA study, exposure to antibiotics in the first week of life was associated with functional abdominal pain at 4-6 years of age. Furthermore, IBS and abdominal migraine at 4-6 years of age were significantly more present in children with a food allergy. No association between infantile colic and the development of an FGID at the age of 4-6 years was found. Finally, we found differences in gut-associated immune markers between children with and without FGIDs.

To date, few studies have examined the association between antibiotic exposure in early life and FGIDs later in life, as summarized in a recent review (23). The number of studies was too small to draw firm conclusions on any association between early life antibiotics and functional constipation, regurgitation, dyschezia, or functional diarrhea (23). One study showed an association between antibiotic exposure and recurrent abdominal pain in adolescent girls (10), which is in line with our finding that functional abdominal pain was significantly associated with antibiotic exposure in the first week of life. However, more prospective studies are necessary to elucidate the association between early life antibiotics and FGIDs later in life.

Our findings that children with a (parental reported) food allergy had more often an FGID is consistent with several other studies, showing an association between (food) allergies and FGIDs (24-28). There are several possible explanations for this association. First, food allergies may directly induce the development of FGIDs. In a recent study, oral ingestion of dietary antigens resulted in increased visceral pain via an IgE- and mast cell-dependent

mechanism (29). Another possible explanation for this association may be similarities in the pathophysiological mechanism of developing allergies and FGIDs. It is known, for example, that both allergies and FGIDs like IBS have been associated with increased intestinal permeability and even inflammatory signals (30-32). Microbiota composition can substantially influence epithelial barrier function. Commensals and probiotic bacteria were demonstrated to enhance barrier integrity (33). This suggests that early-life dysbiosis, caused by antibiotics, can play a role in developing both food allergies and FGIDs (4, 34). Further studies are necessary to elucidate if dysbiosis and the resulting increased permeability are essential for the development of both food allergies and FGIDs.

In contrast to our study, three studies examining infantile colic and FGIDs later in life found an association (18, 35, 36). Two small studies concluded that early bouts of persistent crying might be an early manifestation of abdominal pain–related FGIDs at 10 and 13 years of age (18, 36). Another, larger, retrospective study found that children with infantile colic had a higher prevalence of FGIDs at seven years of age (35). The lack of an association found in the present study could be explained by the younger age (4-6 years) at follow-up, as many FGIDs manifest later in childhood. Longer follow-up of the INCA cohort may be necessary to draw firmer conclusions whether infantile colic is a risk factor for FGIDs at an older age. Another possible explanation may be that AB-induced infantile colic has a different pathophysiological mechanism than, for example, infantile colic in children whose parents suffer from depression (37, 38).

It is remarkable that gut associated immune markers, measured at the age of 1 year, seem to be predictive for FGIDs at the age of 4-6 years, emphasizing that health problems later in life may already be triggered in early life. However, due to the explorative nature of the study, it is difficult to determine the role of changes of individual cytokines. Furthermore, there is very limited information available regarding the levels of inflammatory markers in healthy infants. Therefore, further research in larger cohorts is needed to see if these markers are indeed predictive for FGIDs later in life. However, there is no doubt that early immune signals at one year of age are at least associated with FGIDs later in life which is also true for allergies and even asthma (39).

The prospective design of this study and the high response rate (> 80%) are important strengths of this study, contributing to the reliability of the results. Furthermore, we used validated questionnaires to collect the follow-up data. A limitation of any cohort study is that during follow-up, groups become smaller. As a result, only univariate analyses could be performed prohibiting correction for potential confounding, including AB type and treatment duration. Moreover, we had no data on perinatal antibiotic exposure. Furthermore, the age at

follow-up of 4-6 years may have been a little early to diagnose FGIDs (40). The diagnosis of FGIDs was based on the questionnaires completed by the parents and their interpretation of the symptoms, which may have led to bias. Finally, at the time of follow-up, the ROME IV questionnaire was not yet validated in Dutch. Therefore, the ROME III questionnaire was used.

CONCLUSION

This follow-up of the INCA study showed that antibiotic treatment in the first week of life in children born at term was associated with functional abdominal pain at 4-6 years of age. Children with a food allergy significantly more often fulfilled Rome criteria for IBS and abdominal migraine at 4-6 years of age. No association was found between infantile colic in the first year of life and later FGIDs, independent from antibiotic exposure in the first week of life. Differences in gut-associated immune markers were observed between children with and without FGIDs. Future studies need to focus on pathophysiological mechanisms that may explain these associations.

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Chapter 6

Higher risk of allergies at 4-6 years of age after systemic antibiotics in the first week of life

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To the editor,

In humans, the first 100 days appear to be a "critical window" of colonization during which microbial communities shape immune maturation (1,2). The use of antibiotics early in life may disrupt the normal maturation process leading to adverse health outcomes such as atopic disorders (1,3-5). The effects of antibiotic exposure immediately in the first week of life have rarely been investigated, nor the differences between treatment of 2-3 days and a prolonged treatment of 5-7 days. In the INCA study, a prospective birth cohort study of 151 infants receiving broad-spectrum antibiotics in their first week of life (AB+), and 285 healthy controls (AB-), we previously showed that antibiotic treatment in the first week of life was associated with an increased risk of wheezing, infantile colic and a trend towards more allergic sensitization in the first year of life (6). The aim of this follow-up study in 418 eligible children was to determine if antibiotic treatment in the first week of life in term-born children was associated with an increase in atopic disorders at 4-6 years of age, using ISAAC questionnaires filled out by parents, ICPC codes derived from general physicians, and pharmaceutical records from local pharmacies. Detailed information regarding the subjects and methods is described in Figure 1 and the online Appendix.

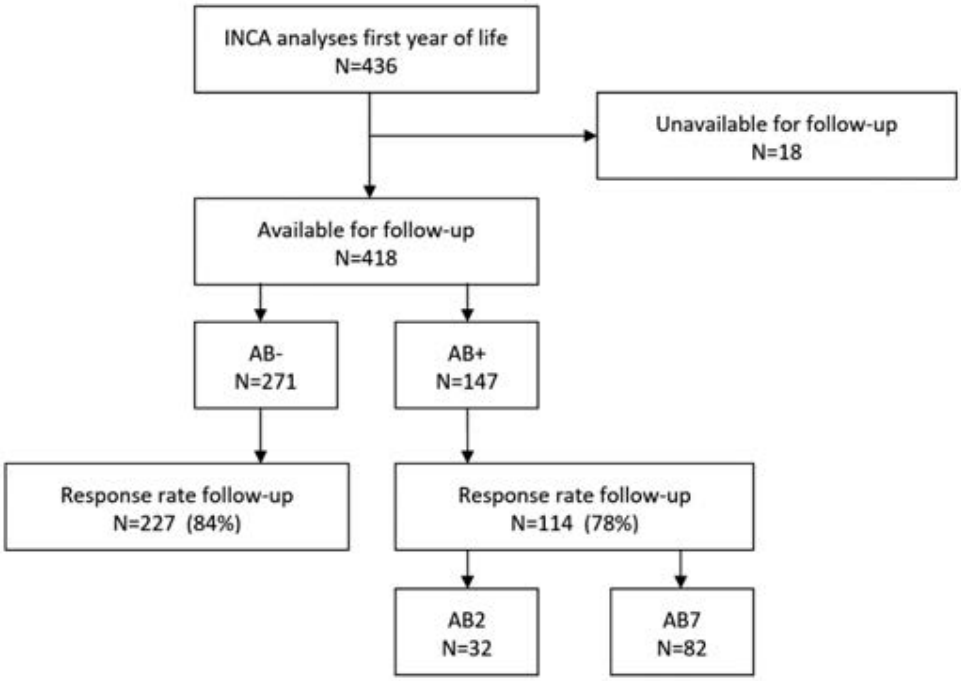

Figure 1: Flowchart of the children included in the follow-up study. AB-: no antibiotics in the first week of life; AB+: antibiotics in the first week of life, for 2-3 days (AB2) or 5-7 days (AB7).

Table 1: baseline characteristics

	AB- N=227	AB+ N=114	AB2 N=32	AB7 N=82
Age median (IQR)*	5 (4.6-5.9)	4.7 (4.4-5.0)	4.7 (4.4-5.3)	4.7 (4.4-5.0)
Sex (male n,%)	122 (54)	65 (57)	14 (44)	51 (62)
BMI mean (SD)	15.6 (1.5)	15.6 (1.4)	15.7 (1.4)	15.5 (1.4)
Delivery mode* n (%)				
Vaginal	146 (64)	86 (75)	22 (69)	64 (78)
C-Section	81 (36)	28 (25)	10 (31)	18 (22)
Breastfeeding				
Median duration (IQR)	4 (1-8)	2.5 (1-7)	2 (0-7)	3.5 (1-6)
Median duration exclusive (IQR)	2 (0-5)	0 (0-4)	0 (0-6)	0.5 (0-4)
Pets n (%)				
No	84 (37)	45 (39)	11 (34)	34 (42)
Cat	59 (26)	34 (30)	9 (28)	25 (31)
Dog	37 (16)	18 (16)	6 (19)	12 (15)
Cat + dog	23 (10)	7 (6)	3 (9)	4 (5)
Other	24 (11)	10 (9)	3 (9)	7 (9)
Daycare attendance n (%)				
No	73 (32)	32 (28)	11 (34)	21 (26)
<3 months	25 (11)	24 (21)	4 (13)	20 (24)
3-6 months	99 (44)	38 (33)	10 (31)	28 (34)
>6 months	30 (13)	20 (18)	7 (22)	13 (16)
Siblings n (%)				
Yes	120 (53)	69 (61)	18 (56)	51 (62)
No	100 (44)	41 (36)	12 (38)	29 (35)
Missing	7 (3)	4 (4)	2 (6)	2 (2)
Parental atopic disease n (%)				
Yes	141 (62)	64 (56)	21 (66)	43 (52)
No	79 (35)	46 (40)	9 (28)	37 (45)
Missing	7 (3)	4 (4)	2 (6)	2 (2)
Highest level of education n (%)				
Low/middle	44 (19)	28 (25)	10 (31)	18 (22)
High	174 (77)	80 (70)	19 (59)	61 (74)
Unknown	9 (4)	6 (5)	3 (9)	3 (4)
Number of courses in the first year of life (first week excluded) median (IQR)[1]	0 (0-1)	0 (0-1)	0 (0-0)	0 (0-1)
AB free months in the first year of life*[1] (first week excluded) median (IQR)	9 (7-11)	7 (2-9)	8 (7-10.5)	7 (2-9)
Number of courses after first week in the first 2 years[2] median (IQR)	1 (1-2)	1 (1-2)	1 (1-4)	1 (1-2)
Eczema first year of life n (%)				
Yes	79 (35)	40 (35)	12 (38)	28 (34)
No	148 (65)	74 (65)	20 (63)	54 (66)

AB-: no antibiotics in the first week of life; AB+: antibiotics in the first week of life, for 2-3 days (AB2) or 5-7 days (AB7). *Significant difference between AB+ and AB- by independent t-test or non-parametric test as appropriate. [1] Parental reported, collected prospectively every month during the first year of life. [2] Parental reported, collected retrospectively during the follow-up.

In total, 341 of 418 (82%) questionnaires were filled out (114 AB+ and 227 AB-, Table 1). Parental reported allergy was significantly higher in AB+ vs AB- children (23% vs 11% respectively, p=0.003) as was doctor-diagnosed allergy (12% vs 4% respectively, p=0.008). Confirmed food allergies were more common in AB+ children compared with AB- children (10 vs. 4% respectively, p=0.03). After correcting for sex, age, daycare attendance, and parental level of education, parental-reported allergy was clearly associated with antibiotics use in the first week of life (aOR 2.40 [95%CI 1.22-4.72, table S1]). Additional analyses showed that only 5-7 and not 2-3 days AB treatment was associated with a higher risk of parental reported allergy (aOR 2.85 [95%CI 1.37-5.91, aOR 1.47 [95%CI 0.46, 4.75], respectively]). More importantly, this effect was independent of exposure to additional antibiotics in the first two years of life (table S2). The prevalence of eczema, wheezing/asthma, or allergic rhinitis was not different between AB+ and AB- children (Table 2).

Table 2: incidence of atopic disorders

	AB- N = 227 N, %	AB+ N = 114 N, %	Significance
Parental reported allergy	24 (11)	26 (23)	**P=0.003**
Wheezing/ Asthma	51 (22)	33 (29)	P=0.206
Eczema	66 (29)	33 (29)	P=0.94
Allergic rhinitis	48 (21)	25 (22)	P=0.90
Doctor diagnosed allergy*	10 (4)**	14 (12)**	**P=0.008**
Inhalation allergy	4 (2)	6 (5)	P=0.10
Drug allergy	0 (0)	1 (1)	P=0.35
Food allergy	8 (4)	11 (10)	**P=0.03**
Hazelnut	1 (0.5)	5 (4)	**P=0.02**
Walnut	1 (0.5)	5 (4)	**P=0.02**

AB-: no antibiotics in the first week of life; AB+: antibiotics in the first week of life. Significance determined by independent t-test. *Confirmed through skin prick test, blood test and/or provocation test;
**6 children had multiple allergies (4 in AB+ and 2 in AB-)

These results suggest that very early exposure to AB in the first week of life has a higher impact on immune development than when administered later in childhood. It also emphasizes the need for judicious use of AB in neonates, especially prolonged treatment of 5-7 days. As AB in early life have a major impact on microbiota development which may lead to aberrant immune maturation (2), our findings accentuate the need for finding strategies to modify microbiome development after AB exposure to decrease the risk of allergies.

Strengths of this study are the prospective design, the high response rate (82%), and the combined information collected from doctors and pharmacists, contributing to the reliability of the reported results, which allowed us to distinguish between the effect of antibiotics within and after the first week of life. A limitation of the study is the 4-6-year follow-up, which may have been too short for diagnosing asthma and allergic rhinitis.

In conclusion, the risk of having an allergy at 4-6 years of age increased nearly 3-fold in children after antibiotic treatment for 5-7 days in their first week of life, independent of later AB treatment. These long-term adverse health effects of neonatal antibiotic use emphasize the need to implement AB stewardship programs to avoid AB overuse and reduce the duration of AB treatment where possible in the first week of life.

ACKNOWLEDGMENT
We thank Angus Dale Jones and Kitty Bach for language editing.

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APPENDIX

SUBJECTS and METHODS

Study design

The study design of the prospective birth-cohort INCA study has been described previously (1). In short, between 2012 and 2015, 436 infants born at term were recruited from maternity and neonatal wards of four teaching hospitals in the Netherlands. Both infants with suspicion of infection, receiving a combination of broad-spectrum antibiotics (n=151) in their first week of life (AB+), and 285 healthy controls (AB-) were included. Blood cultures were taken before AB treatment was started. In case of a negative blood culture, combined with a low clinical suspicion of infection and low C-reactive protein, antibiotics were discontinued after 2 to 3 days (n=42) (AB2), otherwise antibiotics were continued for 7 days (n=109) (AB7).

Data collection in the first week and first year of life

Data collection in the first week and first year of life has been described previously (1,2). In short, at inclusion, parents filled out an online questionnaire concerning the background of the family (e.g., environmental factors, parental smoking habits, family history of atopy and presence of siblings or pets). In addition, perinatal data were collected such as mode of delivery, gestational age, birthweight and type of feeding. During the first year of life, a daily checklist on the health of the child was kept combined with a monthly questionnaire concerning nutrition, parent-reported eczema and wheezing, day care attendance, antibiotic treatment, and general practitioner visits for respiratory or gastrointestinal symptoms. These symptoms were verified at one year of age by checking doctors' diagnoses using the general practitioner electronic medical database based on the International Classification of Primary Care (ICPC) 3.

Follow-up study

Of the 436 children, 18 children were unavailable for follow-up after the first year of life. Therefore, 418 children (147 AB+ and 271 AB-) were eligible for follow-up (Figure 1). Between May 2018 and May 2019, the parents of these 418 children were approached to complete an online questionnaire. The children were 4-6 years of age depending on the year of inclusion. Data regarding eczema, wheezing/asthma, and allergic rhinitis were collected using the validated ISAAC questionnaire (4). Furthermore, information about additional antibiotic use during the second year of life and the presence of allergies (food, drug, insect venom, inhalation or contact allergy) was collected. If parents reported an allergy, additional information was collected whether this was confirmed through a skin prick test, blood test and/or provocation test. If allergy was confirmed, this was defined as a doctor diagnosed allergy. Upper and lower

respiratory disorders, gastrointestinal disorders, and allergies were verified through doctors' diagnoses using ICPC, subject to obtaining informed parental consent for requesting this information (3). Furthermore, with additional informed parental consent, the pharmacy was approached to collect information about medication use from birth until 4-6 years of age.

Ethics

Informed consent was obtained from both parents of all participating children. The study was approved by the ethical board of the St. Antonius Hospital in Nieuwegein, the Netherlands and was registered in the clinical trials register as NCT02536560.

Statistical analysis

Baseline characteristics and differences between AB+ and AB- were analysed by using the independent t-test and Chi-squared test or non-parametric test as appropriate. Multiple logistic regression analysis was used to assess the association between antibiotic treatment in the first week of life and allergic disorders. The model was adjusted for sex and age at follow-up, other confounders (BMI, delivery mode, duration of breastfeeding, day-care attendance, presence of pets, siblings, family history of atopy, highest parental level of education, additional antibiotic courses in the first two years of life, and eczema in the first year of life) were included if beta change was ≥10% (table S1). The effect of duration of antibiotic treatment (AB2 vs AB7) was also analysed. Furthermore, we performed a sensitivity analysis in the group with medication history from the pharmacies, to determine the effect of antibiotic exposure after the first week but within the first 2 years of life on parental reported allergy at 4-6 years of age (Table S2). A p-value <0.05 was considered statistically significant. Analyses were performed with IBM SPSS Statistics for Windows version 26 (Armonk, NY, USA).

Table S1. logistic regression analyses for the association between antibiotic exposure in the first week of life and parental-reported allergies at 4-6 years of age (n=341)

		Model 1 OR (95%CI)	Model 2 aOR (95%CI)	Model 3 aOR (95%CI)
AB-		Reference	Reference	Reference
AB+	All	2.15 (1.14, 4.06)	2.09 (1.09, 4.02)	2.40 (1.22, 4.72)
	AB2	1.34 (0.43, 4.20)	1.44 (0.45, 4.58)	1.47 (0.46, 4.75)
	AB7	2.77 (1.25, 4.89)	2.37 (1.18, 4.77)	2.84 (1.36, 5.95)

Model 1: AB+;
Model 2: model 1 + sex+ age;
Model 3: model 2 + day-care attendance + highest parental level of education.
AB-: no antibiotics in the first week of life; AB+: antibiotics in the first week of life; AB2: 2-3 days antibiotics in the first week of life; AB7: 5-7 days antibiotics in the first week of life; (a)OR: (adjusted) odds ratio; 95% CI: 95% confidence interval.

Table S2. Sensitivity analysis: logistic regression analyses for the association between antibiotic exposure in the first week of life and parental-reported allergy at 4-6 years of age in the group with medication data (n=296).

		Model 1 OR (95%CI)	Model 2 aOR (95%CI)	Model 3 aOR (95%CI)	Model 4 aOR (95%CI)
AB-		Reference	Reference	Reference	Reference
AB+	All	1.99 (1.02, 3.86)	1.95 (0.99, 3.84)	2.26 (1.11, 4.58)	2.29 (1.13, 4.65)
	AB2	1.51 (0.47, 4.82)	1.64 (0.50, 5.38)	1.67 (0.51, 5.52)	1.69 (0.51, 5.58)
	AB7	2.16 (1.06, 4.39)	2.08 (1.00, 4.32)	2.51 (1.16, 5.42)	2.60 (1.20, 5.64)

Model 1: AB+

Model 2: model 1 + sex + age

Model 3: model 2 + day-care attendance + highest parental level of education

Model 4: model 3 + antibiotics in first 2 years

AB-: no antibiotics in the first week of life; AB+: antibiotics in the first week of life; AB2: 2-3 days antibiotics in the first week of life; AB7: 5-7 days antibiotics in the first week of life; (a)OR: (adjusted) odds ratio; 95% CI: 95% confidence interval

Additional results and Discussion

Antibiotics administered between the first week and two years of age were similar in the AB+ and AB- group (36% and 35% respectively). Furthermore, family history of atopy was similar in both groups (approximately 60%) and did not influence the statistical analyses and therefore was not included in the final models (tables S1 and S2).

An important point of the study is the generalizability. The positive family history of around 60% in both the AB group and the control group, and is comparable to other large cohort studies in the Netherlands such as the PIAMA study (5), which makes the study results applicable for the general population, not specific for a high-risk population. However, in our logistic regression analysis, family history of atopy did not influence the association between AB in the first week of life and risk of parental reported allergy and therefore was not included in the final model. We cannot comment on the question whether the proportion of children exposed to antibiotics in the first week of life was comparable to the Dutch population or other studies, because of the study design: a prospective birth cohort study of 151 infants receiving broad-spectrum antibiotics in their first week of life (AB+), and 285 healthy controls (AB-). This design with 1/3 of the children exposed to AB in the first week of life and 2/3 of the children not exposed was specifically chosen to prospectively evaluate the effect of AB in the first week of life. However, the percentage of children exposed to antibiotics in the first two years of life in another recent Dutch study was similar to that in our study (6). Overall, except for the percentage of infants exposed to antibiotics in the first week of life, the results of our study are fairly comparable to other studies.

Chapter 7

Effect of antibiotics in the first week of life on faecal microbiota development

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ABSTRACT

Background: Infants are frequently exposed to antibiotics (AB) in the first week of life for suspected bacterial infections. Little is known about the effect of AB on the developing intestinal microbiota. Therefore, we studied intestinal microbiota development with and without AB exposure in the first week of life in term born infants

Methods: We analysed the faecal microbiota from birth until 2.5 years of age by 16S rRNA gene amplicon sequencing in a cohort with 56 term born infants , exposed to AB in the first week of life (AB+) [AB for 2-3 days (AB2, n=20), AB for 7 days (AB7, n=36)], compared to 126 healthy controls (AB-). The effect of AB and duration were examined in relation to delivery and feeding mode.

Results: AB+ was associated with significantly increased relative abundance of Enterobacteriaceae at three weeks and one year and a decrease of Bifidobacteriaceae, from one week until three months of age only in vaginally delivered, but not in C-section born infants. Similar deviations were noted in AB7, but not in AB2. After AB, breastfed infants had lower relative abundance of potentially pathogenic Enterobacteriaceae compared to formula fed infants and recovered 2 weeks faster towards controls.

Conclusions: AB exposure in the first week of life alters faecal microbiota development with deviations in the relative abundance of individual taxa week until one year of age. These alterations can have long-term health consequences, which emphasizes the need for future studies aiming at restoring intestinal microbiota after AB administration.

INTRODUCTION

During the first 1000 days of life, the intestinal microbiota impacts health in later life through the interdependent development of microbiota, immune system, growth, and cognitive function (1,2). After birth, the intestinal microbiota develops rapidly, driven by exposure to microbes from maternal, environmental, and dietary sources (3). During this early development, the intestinal microbiota is unstable and susceptible to perturbations such as those caused by antibiotic (AB) exposure. These perturbations may have long-term consequences on the developing microbiota, but also on the developing immune system (4), growth (5,6), and have already been associated with increased prevalence of asthma, allergies, celiac disease, eczema, eosinophilic esophagitis, infantile colic, inflammatory bowel disease, and obesity (7–11).

In mice based studies, AB exposure in the first week of life altered microbiota composition and immune function, but gavage with mature untreated microbiota, restored the perturbation and reduced the negative health effects (12). To understand the full impact in humans and develop restoration strategies, more knowledge is needed.

Worldwide, up to 20% of all neonates are prescribed ABs because of (suspected) early-onset neonatal sepsis, although in most cases sepsis is unconfirmed, and ABs could be discontinued after 48-72 hours (13–15). More prolonged AB exposure can gradually reduce overall diversity (16) and richness (17) of the neonate's intestinal ecosystem. Additionally, AB type also determines the microbial perturbation through specific mechanisms of action and host interactions. Studies in infants have been inconclusive (17–19) but indicated types with faster recovery towards the microbiota composition of controls (20). Therefore, more comparative studies are needed between durations and types in AB regimes to optimize AB administration (21).

In this study, we investigated the microbiota development in a subset of the Intestinal Microbiota Composition after AB treatment in early life (INCA) cohort (22). The primary aim was to investigate the impact of AB exposure in the first days of life on microbiota development during the first 2.5 years of life. Secondary aims were to examine (1) short (2-3 days) vs. long (7 days) AB administration, (2) different AB types and (3) the impact of feeding and delivery mode on AB perturbation.

MATERIALS AND METHODS:

Study design

This prospective, observational study has been described previously (10,22). To study the impact of feeding mode on AB-induced deviations, we selected a subset of 182 infants with 1128 samples who were exclusively breast- (BF) or formula-fed (FF) in the first three months of

life. An overview of collected samples and their selection for analyses reported here is provided in Supplementary Figure 1. All AB+ infants received gentamicin, which was combined with penicillin (AB^{Pen}), amoxicillin (AB^{AMX}) or amoxicillin with clavulanic acid (AB^{AMC}). The INCA study was approved by the ethical board of the St. Antonius Hospital in Nieuwegein, the Netherlands (registered as NCT02536560), and informed consent was obtained from both parents.

Data and faeces collection

Baseline characteristics such as birth mode were assessed through hospital records. Feeding mode was reported monthly during the first year of life. Nine faecal samples were collected from infants (Figure 1). Until discharge from the hospital, faeces were sampled from diapers by hospital staff and immediately frozen at -20°C. Sampling continued at home by the parents, using sampling instructions and freezer storage. After one year, parents delivered the samples to the clinic after transport on ice. A final sample was taken around two years, stored in the home freezer and collected by the study nurse. At the hospital, all samples were stored at -20 °C.

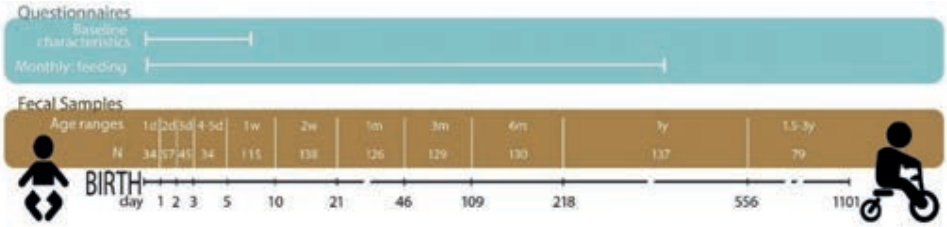

Figure 1. Overview of the collected questionnaires, faecal sampling points and the age categories based on the age (days) at sampling. N, number of infants for which samples were available for a given time point. d(ays), m(onths) and y(years) indicate the age category ranging from birth until around 2 years of age.

16S rRNA gene amplicon sequencing

DNA was extracted from a maximum of 200mg of the homogenized faecal sample at GenProbio srl (Parma, Italy) with the QIAamp DNA Stool Mini kit according to manufacturer's instructions (Qiagen Ltd, Strasse, Germany). Sequencing libraries were prepared according to the 16S Metagenomic Sequencing Library Preparation Protocol (Part No. 15044223 Rev. B—Illumina) at GenProbio srl (Parma, Italy). Minor adaptations to the published protocol (23) are noted in Supplementary file 1. Sequencing resulted in ~44934 (SD 17205) reads per sample. Data were processed using NG-Tax 2.0 on demultiplexed fastq files, using default settings (24). Taxonomy was assigned using SILVA reference database version12825. Amplicon sequence variants (ASVs) were defined as unique sequence variants. Two synthetic mock communities were sequenced as positive controls (26).

Statistical analysis of the baseline characteristics

Baseline characteristics were calculated using R 3.6.127 and tableone (28) (Table 1). Differences between groups were examine by using the Fisher exact test for the categorical variables and ANOVA for continuous variables. Not normally distributed variables were tested with the Kruskal-Wallis test and indicated by their median and inter quartile range. Bonferroni correction was performed to correct for multiple testing.

Bioinformatic and statistical analysis of the sequence data

All analyses were performed in R 3.6.127. Samples were stratified into 11 age-based and right-closed intervals for statistical analysis (Figure 1). The Jenks Natural Breaks Classification (classInt package) was used to calculate the optimal ranges (29). Because of increasing increments between sampling, the x-axis (age) was log2 transformed for visualisation.

Alpha diversity (within sample diversity) was calculated at ASV level using Picante (30) and Microbiome (31) packages and following metrics: Faith's phylogenetic diversity32, ASV richness, Shannon and Inverse Simpson. All except Shannon diversity, needed logarithmic transformation to obtain normal distribution for one-way Analysis of Covariance (ANCOVA). Consecutive analyses were corrected for the baseline characteristic that differed significantly between AB+ and AB-, and between AB2 and AB7.

Temporal trends in relative abundance were visualized using local regression with locally estimated scatterplot smoothing (LOESS) using ggplot2. These relative abundances did not meet normality requirements and were therefore compared using beta regression with BetaReg (33) per age interval. The effects of AB on the relative abundance of phyla were modelled including and excluding delivery mode, feeding mode, and additional AB exposure between one and six months. The optimal beta regression models, based on Akaike (AIC) and Bayesian information criteria (BIC), only included AB exposure in the first week of life without additional terms.

Beta diversity (between sample diversity) was calculated using pairwise Weighted (WU) (34) and Unweighted UniFrac (UU) distances (35). WU takes the relative abundance of each ASV into account, whereas UU uses presence or absence of ASVs. Pairwise UU and UW distance matrices were used to plot principle response curves (PRCs) (36). PRC analysis is a redundancy analysis for interpreting longitudinal data. It visualizes multivariate responses in a repeated observation design (36,37). The method was designed to analyze the treatment effect over time compared to controls, as it can disentangle the time-dependent effects from other possible determinants (38,39). In this study, time was displayed on the x-axis and the intestinal

microbiota development was shown compared to AB- infants as the baseline reference group. Differences between AB groups were assessed per age interval using ANOVA in the vegan package (40).

RESULTS:

Baseline characteristics of INCA cohort subset

The baseline characteristics differed between AB- and AB+ for gestational age, birth weight, and additional AB exposure between one to six months (Table 1). Birth weight z-score (birth weight corrected for gestational age), however, was comparable. AB2 differed from AB7 with regard to additional AB exposure between one to six months (5% vs. 34% respectively, p=0.019). Baseline characteristics were comparable between the AB type groups (Table S1).

Table 1. Baseline characteristics of the INCA cohort subset included in this study.

	AB-	AB+	AB2	AB7
n	126	56	20	36
Gestational age (GA), weeks [IQR]*	39.4 [38.5,40.4]	40.4 [39.4,41.1]	40.05 [39.3,41.0]	40.55 [40.1,41.2]
Birth weight, mean grams (SD) *	3478 (515)	3711 (484)	3734 (428)	3699 (518)
Birth weight for GA z-score (SD)	0.15 (1.2%)	0.39 (1%)	0.30 (1%)	0.57 (0.9%)
Sex (Female %)	58 (46%)	28 (50%)	13 (65%)	15 (42%)
Delivery mode (Vaginal %)	83 (66%)	41 (73%)	15 (75%)	26 (72%)
Maternal intra-partum AB (Yes %)*	15 (15%)	19 (40%)	6 (38%)	13 (41%)
Exclusive breastfeeding at 3m (Yes %)	47 (37%)	23 (41%)	17 (47%)	6 (30%)
Additional AB 1-6m (Yes %)*,**	10 (8.2%)	13 (24%)	1 (5%)	12 (34%)
Additional AB 7-12m (Yes %)	30 (27%)	9 (19%)	2 (10%)	7 (24%)

AB: antibiotics, AB-: unexposed infants, AB+: infants who received AB during their first week of life. Birth weight for GA z-score is calculated according to the z-score formula (59). AB2: AB exposure for 2 to 3 days in the first week of life, AB7: AB exposure for 7 days, GA: gestational age, IQR: inter quartile range, m:months, SD: standard deviation, Statistically significant differences are indicated with * $p < 0.05$ compared to AB-, ** $p < 0.05$ compared to AB2

Antibiotic-induced alterations to intestinal microbiota development

AB exposure during the first week of life did not alter microbial alpha diversity between birth and 2.5 years (Supplementary Figure 1). The temporal patterns of the four major phyla (95% of the average relative abundance) were compared with univariate analyses (Figure 2). Relative abundance of Proteobacteria was high overall, during the first months of life. AB exposure further increased this relative abundance at one month (mean 43.6% AB+, 31.5% AB-) and one year (mean 17.6% AB+, 6.5% AB-). Actinobacteria peaked around three months, but AB exposure decreased their relative abundance at one week (mean 6.3% AB+, 18.2% AB-), two weeks (mean 8.4% AB+, 24.4% AB-), one month (mean 17.5% AB+, 27.2% AB-) and three months (mean 26.5% AB+, 34.4% AB-). The average relative abundance of Bacteroidetes was stable at approximately 10% during the first year of life. Firmicutes drastically increased in relative abundance towards 2.5 years. Both were unaffected by AB.

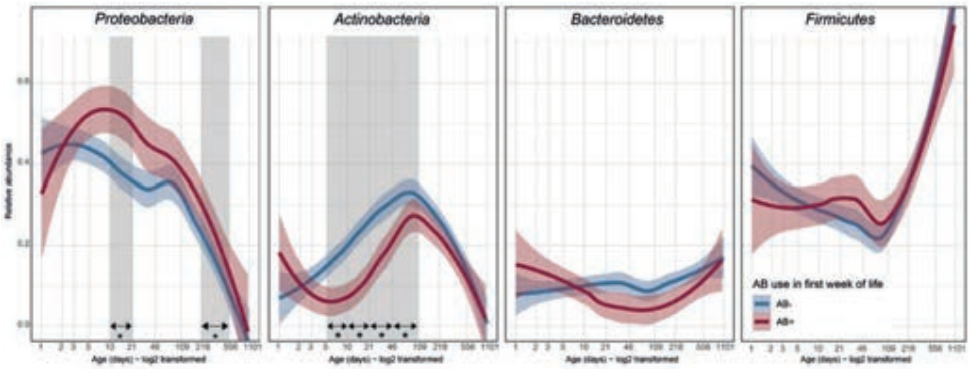

Figure 2. Temporal trajectories of the relative abundance of the four main phyla in the developing intestinal microbiota from birth to 2.5 years of age. Thick lines represent the average and the shading shows the 95% confidence interval. Difference between AB+ and AB- were calculated using beta regression for each age category. * and vertical grey shading: difference in relative abundance ($p < 0.05$), AB+: infants exposed to antibiotics during their first week of life, AB- unexposed infants, LOESS: locally estimated scatterplot smoothing.

Effect of antibiotic duration on long-term microbiota development

Based on univariate statistics, the temporal trajectories of Actinobacteria and Proteobacteria were most affected by AB. Based on UU, ABs increased several members of the Enterobacteriaceae and decreased Bifidobacteriaceae at one and two weeks (Supplementary Figure 2a). For WU, ABs impacted similar taxa at one year ($p<0.05$) (Figure 3a). These AB deviations were similar in AB2 and AB7, but only the microbiota composition of AB7 differed from AB- baseline (Figure 3b and Supplementary Figure 2b).

AB types had a different impact on the microbiota (Supplementary Figure 3a and b) with AB^{AMX} not deviating from AB- baseline and AB^{PEN} deviating at one week and one year with increased relative abundance of Enterobacteriaceae members in WU and UU. UU-based deviations between $AB^{AM}C$ and AB- baseline were limited to week one and involved different ASVs compared to AB^{PEN}. AB^{AMC} affected WU in the long-term with increased Enterobacteriaceae and Enterococcaceae at two weeks and one month but also Bifidobacterium at one week and 2.5 years.

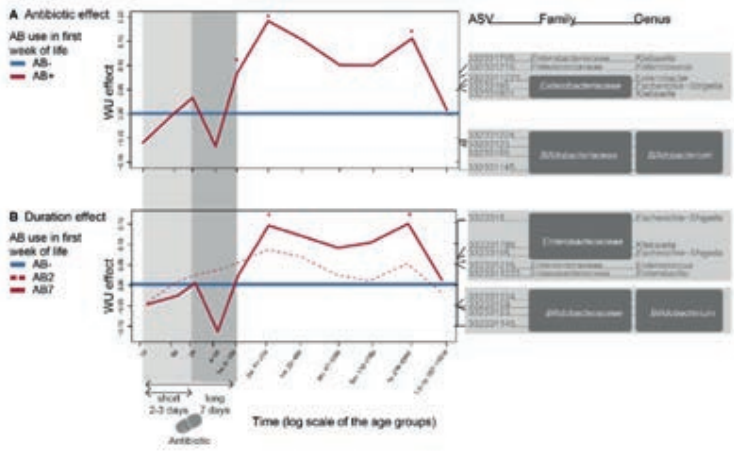

Figure 3. Weighted UniFrac (WU)-based Principal Response Curve (PRC). This analysis is a special case of Redundancy Analysis (RDA) for multivariate responses in a repeated observation design. (a) unexposed infants (AB-), were compared as a baseline to the antibiotic exposed infants (AB+). Bacterial genera shown are the main drivers of the differences between AB+ and AB-: taxa on the same side of baseline as the curve are linked to an increased relative abundance at that time point, opposite sides indicate a decrease. (b) AB- was also compared as a baseline with antibiotic durations: 2-3 (AB2) or 7 days (AB7). Significance was tested at the different time points using an ANOVA like permutation test (* = p-value < 0.05 compared to baseline AB-). Covariates that were controlled for included additional AB exposure between the age of one and six months. AB-:, AB+: also indicated within grey shading, AB2:, AB7:, ASV: Amplicon sequence variants, WU: weighted UniFrac.

Impact of delivery and feeding mode on AB-associated deviations in the faecal microbiota development

Due to the relatively low number of faecal samples in the first week per feeding and delivery type (Figure 1), effects were only reported in samples collected between one week and 2,5 years. Within AB-, microbiota deviated based on delivery mode from one week until one month (Figure 4d). In vaginally delivered infants the AB effect on the microbiota was still significant at one year with an increase of several Enterobacteriaceae, Enterococcaceae and Streptococcaceae and decrease in Bifidobacterium and Escherichia-Shigella. In contrast, no AB-mediated deviations were noted between C-section born infants.

Compared to AB- BF baseline, AB+ BF infants only deviated at two weeks, whereas AB+ FF infants showed longer deviations from the first week up to one month (Figure 4a). Because there was also a feeding effect during the first six months, the AB effect was also analysed within the separate feeding groups. AB+ was associated with decreased Bifidobacterium relative abundance in FF (one month) (Figure 4c), which occurred later than in BF infants (two weeks) (Figure 4b). In turn, AB+ was associated with increased relative abundance of Parabacteroides in BF, and with increased relative abundance of Enterobacteriaceae and Enterococcus in FF infants.

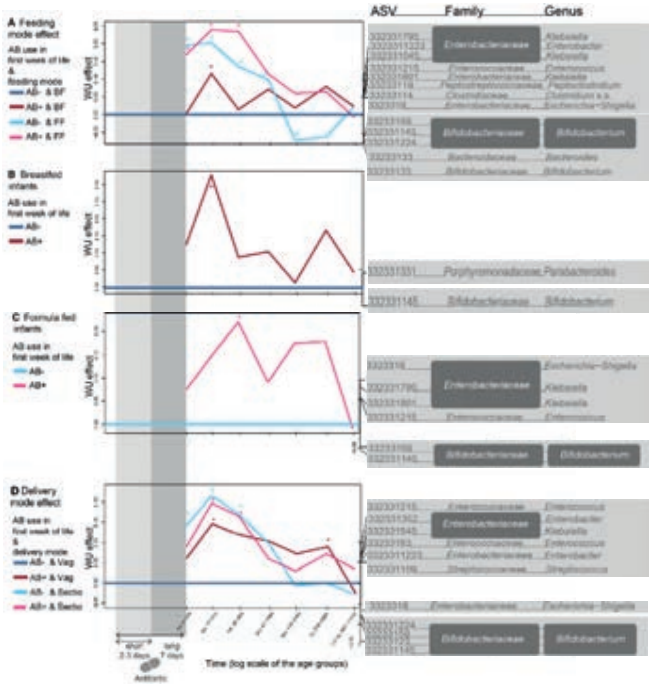

Figure 4. Weighted UniFrac (WU)-based Principal Response Curve (PRC) analyses in the different delivery and feeding mode groups. Bacterial genera shown are the main drivers for differences between the groups and the baseline: positive effect on the curve is linked to increased ASVs in the positive spectrum and decrease of those in the negative spectrum. (a) Breastfed controls (AB-BF) were compared as a baseline to antibiotic exposed (AB+) and formula fed (FF) infants. (b) Specifically featuring the AB effect in breastfed children, with breastfed control children as a baseline (AB-BF), and (c) formula fed children, with formula fed control children as a baseline (AB-). (d) Vaginally delivered control infants (AB-Vag) were compared as a baseline to antibiotic exposed (AB+) and C-section delivered (Sectio) infants. Significance was tested at the different time points using an ANOVA like permutation test (* = p-value < 0.05). Delivery mode analyses were controlled for feeding mode and vice versa. AB-: unexposed infants AB+: infants who received AB during their first week of life also indicated within grey shading, ASV: Amplicon sequence variants, BF: infants exclusively breast-fed in the first three months of life, FF: infants exclusively formula-fed in the first three months of life, Sectio: infants delivered through C-section, Vag: vaginally delivered infants, WU: weighted UniFrac.

DISCUSSION:

In this prospective, observational INCA study, we examined the microbiota development after AB exposure during the first week of life and found perturbations in the faecal microbiota development from one week until one year of age. These perturbations included decreased relative abundance of Bifidobacteriaceae, while potentially pathogenic Enterobacteriaceae increased. This study adds new insights about long-term compositional shifts after neonatal AB exposure (16,41). Our results corroborate findings in older infants with increased Enterobacteriaceae and decreased Bifidobacteriaceae after AB administration (16,20,42–44). Importantly, the severity and duration of AB-mediated microbiota perturbations increased with longer AB administration (5-7 versus 2-3 days). The results also align with a small study in preterm infants, where >5 days AB exposure intensified perturbations compared to 2-3 days (45).

Bifidobacteriaceae form a cornerstone in the early development of the immune system. They were shown to promote B-cell maturation and associations with decreased inflammatory responses and T-regulatory cell acquisition (46). Enterobacteriaceae on the other hand produce toxins and have lipopolysaccharides on their outer membranes, which causes inflammation (47–49). Therefore, it is not surprising that reduced Bifidobacteriaceae, often combined with an increase in potentially pathogenic Enterobacteriaceae, like Shigella, Klebsiella and Enterobacter, have been associated with immune-mediated disorders like asthma (50). Similar deviations were also found in functional disorders like infantile colic (51) and irritable bowel syndrome (52).

The long-term microbiota effect of ABs and its associated negative health outcomes reinforce the need for implementing AB stewardship programs (53–55) to avoid AB overuse (21,56,57). The microbiota perturbations were only significant after 5-7 days compared to 2-3 days AB which could explain previous findings from the INCA cohort: namely higher incidence of infantile colic, wheezing and food allergies in infants exposed for 5-7 days, but not for 2-3 days (10,58). If this observed difference between 2-3 days and 5-7 days exposure is the result of longer antibiotic exposure or the result of a concomitant infection or inflammatory response is yet unclear. The AB7 infants were treated because of suspected early onset sepsis (EOS). EOS is rare in term infants (59–61), but it is difficult to distinguish from normal neonatal physiology after birth, and laboratory tests cannot always reliably detect or rule out EOS (62). Because the consequences of delaying treatment are significant, on average 82 newborns without EOS are treated for each case (15,63–65). In our study population, only two of the 36 AB7 infants had a positive blood culture. The others were also treated for five to seven days because of elevated inflammatory markers or clinical symptoms. Uzan-Yulzari et al. showed that the association between neonatal AB exposure and growth was independent of the neonatal infection state (6).

This suggests that the differences in microbiota development after AB treatment in our study are more likely the result of the AB treatment duration itself than caused by a possible EOS. Our new findings emphasize the need for microbiota restoration to minimize aberrant immune development. Suggested strategies include prebiotics, probiotics and synbiotics (66) but also faecal transfers, which partially restored the microbiota of mice exposed to AB for seven days (12).

In vaginally delivered infants, the AB effect was most pronounced with microbiota deviations in the second week of life. C-section born infants, however, showed similar perturbations regardless of AB exposure. After C-section, microbiota perturbations occurred due to reduced vertical mother-infant transmission of important intestinal microbes such as Bacteroides and Bifidobacterium, while transmission of other microbes like skin and mouth bacteria increased (67), as well as due to maternal AB administration prior to cord clamping (3). C-section delivery already showed decreases in Bifidobacterium spp. and increases in opportunistic pathogens from hospital environments like Enterobacter, Enterococcus and Klebsiella (68). This resembled the AB effect, which might explain the lack of an additional AB effect in C-section infants as these infants already lack the affected microbial groups from birth.

Feeding also has a major impact on early life microbiota development (69). In our study, ABs in the first week of life perturbed the microbiota of both BF and FF infants, but potentially pathogenic Enterobacteriaceae only increased in FF infants. Moreover, AB perturbations were still notable at one month in FF infants but only until two weeks in BF infants. Breastmilk probably aids restoration through components like human milk oligosaccharides and live bacteria, which stimulate the growth of bifidobacteria and reduce (potential) pathogens (70).

The strengths of this study are the quantity of samples and long-term follow-up. This enabled the investigation of the interplay of AB with delivery and feeding modes. Moreover, the quantity of sampling points allowed for detailed and long-term detection of AB-induced perturbations within individuals. This was relevant as AB impact was not uniform over time, suggesting that limited sampling points could lead to misinterpretation. Finally, the number of infants receiving additional courses of AB in the first year was low in this cohort, thereby reducing an important confounding factor.

The methodology for profiling the intestinal microbiota, which targeted the V3 hypervariable regions of the bacterial 16S rRNA gene, provides a cost-effective overview of bacterial community composition, however, resolution at species level is limited, and amplification bias cannot be unequivocally ruled out (71,72). The applied Probio_Uni and Probio_Rev primers were validated and, compared to other primers, they seemed to represent relevant members intestinal microbiota such as bifidobacteria more accurately, which makes

them especially fit for analyzing the intestinal microbiota of infants (23). For future research, whole genome shotgun sequencing could be used to increase the accuracy of species and strain detection (73,74). Another limitation may be that environmental factors and maternal-infant interactions could have been confounders, because AB+ infants were admitted to neonatal wards, whereas AB- infants stayed with their mothers on the maternity ward and were discharged earlier. Lastly, we did not have sufficient data on perinatal antibiotic exposure and were therefore unable to correct for it, although it is questionable to what extent this confounder is important to take into account (75,76). Additionally, the study was not primarily designed (and thus underpowered) to conclude on AB types. Nevertheless, our results suggest that AB^{AMX} induced less perturbations as it did not result in any differences from AB- (Supplementary Figure 3b). The addition of the β-lactamase inhibitor clavulanic acid (AB^{AMC}) was associated with higher levels of bifidobacteria compared to other AB types, which supports an earlier finding in a single subject (20). Dedicated studies are, however, needed to further elucidate the optimal regime with the least microbial perturbations.

In conclusion, AB exposure in the first week of life in term born infants disturbed the microbiota up to one year, with more significant deviations after longer AB exposure (5-7 days). Both C-section delivery and AB administration in the first week of life are associated with deviant intestinal microbiota, but the two combined are not associated with further deviation. Breastfeeding was associated with reduced severity and duration of perturbations compared to formula feeding. Our observations may help to elucidate why AB-exposed infants have more health problems. It may also support the development of preventive and curative strategies after AB exposure to stimulate the growth of beneficial microbiota in order to prevent future health problems.

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APPENDIX

Study design summary

In short, 436 infants born at term were recruited from maternity and neonatal wards of four teaching hospitals in the Netherlands. Infants with suspicion of infection, who received a combination of broad-spectrum ABs in their first week of life (AB+), and healthy, unexposed controls (AB-) were included. Blood cultures were taken before AB exposure was started. In case of a negative blood culture, combined with a low clinical suspicion of infection and low C-reactive protein, ABs were discontinued after two to thre days (AB2). Otherwise ABs were continued for five to usually seven days (AB7). Infants without sufficient follow up, less than seven samples over the first three years of age, were not included for this study.

Supplementary Table S2. Baseline characteristics of the subset included in this study

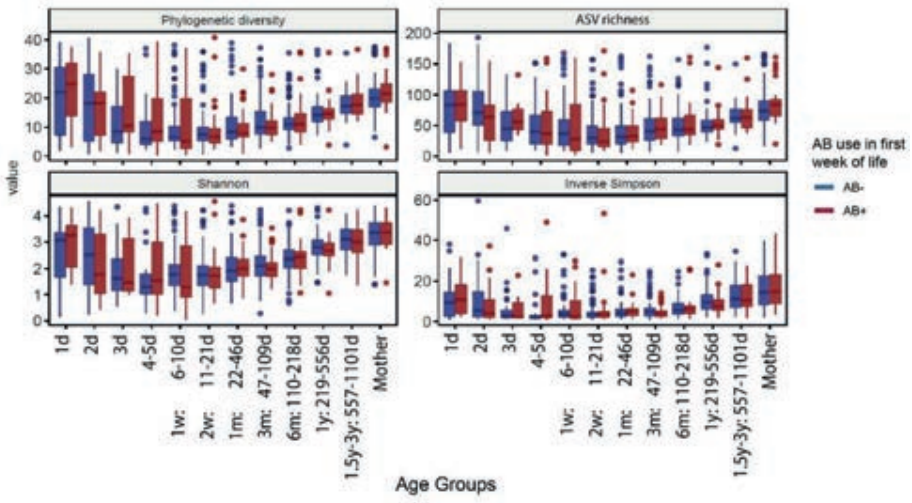
	AB-	**AB^{PEN}**	**AB^{AMX}**	**AB^{AMC}**
n	126	33	10	13
Gestational age (GA), weeks [IQR]	39.4 [38.5,40.4]	40.5 [39.3,41.1]	40.1 [39.4,40.5]	40.5 [40.2,41.3]
Birth weight, mean grams (SD)	3478 (515)	3733 (540)	3528 (326)	3797 (422)
Birth weight for GA z-score (SD)	0.15 (1.2%)	0.50 (1%)	0.05 (1%)	0.39 (1%)
Sex (Female %)	58 (46%)	16 (49%)	7 (70%)	5 (39%)
Delivery mode (Vaginal %)	83 (66%)	24 (73%)	7 (70%)	10 (77%)
Maternal intra-partum AB (Yes %)	15 (15%)	8 (31%)	5 (50%)	6 (50%)
Exclusive breastfeeding at 3m (Yes %)	47 (37%)	12 (36%)	7 (70%)	4 (31%)
Additional AB 1-6m (Yes %)	10 (8.2%)	9 (28%)	1 (10%)	3 (23%)
Additional AB 7-12m (Yes %)	30 (27%)	5 (17%)	0 (0%)	4 (36%)

There were no statistical differences between these groups. Birth weight for GA z-score is calculated according to the z-score formula (59). AB: antibiotics, AB-: control infants, AB^{Pen}: antibiotic exposure in first week of life with gentamicin and penicillin, AB^{AMX}: gentamicin and amoxicillin, AB^{AMC}: gentamicin with amoxicillin and clavulanic acid, GA: gestational age, IQR: not normally distributed variables are indicated by their median and inter quartile range [IQR], m: months, SD: standard deviation

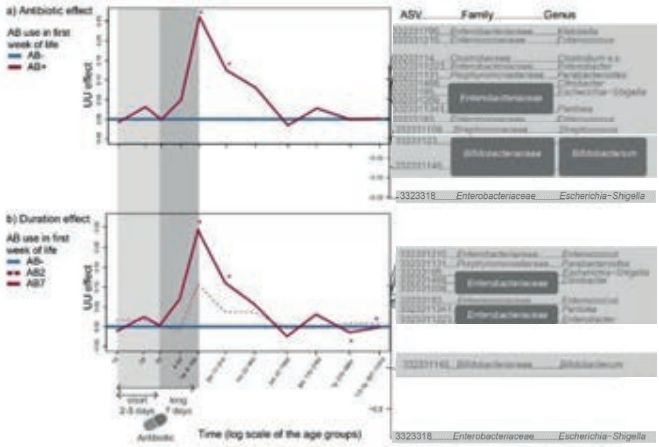
Detailed material and methods

Partial 16S rRNA gene amplicons were generated by using two consecutive PCR reactions. First, the V3 region of the 16S rRNA gene was amplified using primers (Probio_Uni/Probio_Rev) with adapters overhanging at the 5' end 23. Thermal cycling conditions were as follows: 5 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 55°C and 90 s at 72°C; followed

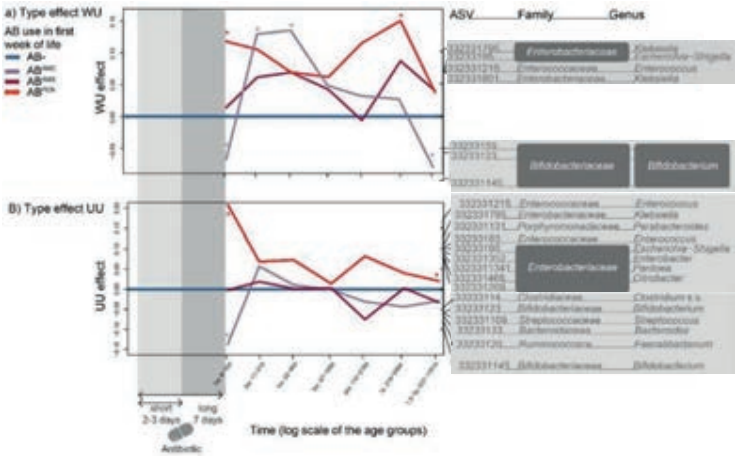
by 10 min at 72°C. The PCR amplicons, which were validated and checked on size by electrophoresis on a 2200 Tape Station instrument (Agilent Technologies, USA), were cleaned by magnetic Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH). To enable multiplexing, unique combinations of Tag barcodes (8 base pairs) were attached using a second PCR with the following cycling conditions: 3 min at 95 °C; 8 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C; followed by 5 min at 72°C. Each PCR was performed with a Verity Thermocycler (Applied Biosystems, USA). DNA obtained was purified by means of a magnetic purification step involving Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany). The DNA concentration of the amplified sequence library was determined by a fluorometric Qubit quantification system (Life Technologies, USA). Amplicons were diluted to a concentration of 4 nM in 10-µl quantities and combined to prepare the pooled final library. 16S rRNA gene amplicon sequencing was performed using an Illumina MiSeq sequencer and MiSeq Reagent Kit v3 chemicals (Illumina Inc., USA) at GenProbio srl (Parma, Italy) according to manufacturer's instructions.



Supplementary Figure 1: Alpha diversity in age categories from birth to 2.5 years of age. Alpha diversity at different age categories of the antibiotic (AB+) exposed and unexposed infants (AB-) did not differ at any of the time points, using one-way Analysis of Covariance (ANCOVA) corrected for additional AB exposure between one and six months of age, ASV: Amplicon sequence variants.



Supplementary Figure 2: Unweighted UniFrac (UU) -based Principal Response Curves (PRC). Complementary to the PRC in Figure 3. (a) unexposed infants (AB-), were compared as a baseline to the antibiotic exposed infants (AB+). Bacterial genera shown are the main drivers of the differences between AB+ and AB-: taxa on the same side of baseline as the curve are linked to an increased relative abundance at that time point, opposite sides indicate a decrease (b) AB- was also compared as a baseline with the different antibiotic durations of 2-3 (AB2) or 7 days (AB7). Significance was tested at the different time points using an ANOVA like permutation test (* = p-value < 0.05 compared to baseline AB-). Controlled for additional AB exposure between one and six months. AB-: unexposed infants, AB+: infants who received AB during their first week of life also indicated within grey shading, AB2: AB exposure for 2-3 days, AB7: AB exposure for seven days in the first week of life, ASV: Amplicon sequence variants, UU: unweighted UniFrac.



Supplementary Figure 3: Weighted (WU) (a) and Unweighted UniFrac (UU). (b) -based Principal Response Curves (PRC) comparing the impact of different antibiotic types during the first week of life. Unexposed infants (AB-), were compared as a baseline to the different types of antibiotics AB^{PEN} (antibiotic exposure in first week of life with gentamicin and penicillin), AB^{AMX} (gentamicin and amoxicillin), and AB^{AMC} (gentamicin with amoxicillin and clavulanic acid). Significance was tested at the different time points using an ANOVA like permutation test (* = p-value < 0.05 compared to baseline AB-). ASV: Amplicon sequence variants, UU: unweighted UniFrac, WU: weighted UniFrac

Chapter 8

Microbial effects of prebiotics, probiotics, and synbiotics after Caesarean section or exposure to antibiotics in the first week of life: a systematic review

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ABSTRACT

Background and aims: Disruption of the developing microbiota by Caesarean birth or early exposure to antibiotics may impact long-term health outcomes, which can potentially be prevented by nutritional supplements. This systematic review aimed to summarise the evidence regarding the effects of prebiotics, probiotics and synbiotics on the intestinal microbiota composition of term infants born by Caesarean section or exposed to antibiotics in the first week of life.

Methods: A systematic search was performed from inception to August 2022 in Medline and Embase. Two researchers independently performed title and abstract screening (n = 12,230), full-text screening (n = 46) and critical appraisal. We included randomised controlled trials which included term-born infants who were born following Caesarean section or who were exposed to postpartum antibiotics in the first week of life, pre-, pro- or synbiotics were administered <6 weeks after birth and outcome(s) consisted of microbiota analyses.

Results: Twelve randomised controlled trials investigating Caesarean born infants and one randomised controlled trial including infants exposed to antibiotics were included. Group sizes varied from 11 to 230 with 1193 infants in total. Probiotic (n=7) or synbiotic (n=3) supplementation significantly increased the abundance of the supplemented bacterial species (of the Bifidobacterium and Lactobacillus genus), and there was a decrease in Enterobacteriaceae, especially <4 weeks of age. At phylum level, Actinobacteria (two studies), Proteobacteria (one study) and Firmicutes (one study) increased after probiotic supplementation. In three studies on prebiotics, two studies reported a significant increase in Bifidobacteria and one study found a significant increase in Enterobacteriaceae.

Discussion: Prebiotic, probiotic and synbiotic supplements seem to restore dysbiosis after Caesarean section towards a microbial signature of vaginally born infants by increasing the abundance of beneficial bacteria. However, given the variety in study products and study procedures, it is yet too early to advocate specific products in clinical settings.

INTRODUCTION

The human gastrointestinal microbiota is a collection of all microorganisms (bacteria, viruses, fungi, protozoa) residing in the gastrointestinal tract. Together, these microorganisms affect processes such as metabolism [1, 2] and inflammatory and immunological responses [2], and also influence the integrity and structure of the gastrointestinal tract [2]. The normal gastrointestinal microbiota develops rapidly after birth and is highly dynamic until it shifts towards an adult-like composition around the age of three years [3]. This development is driven by exposure to microbes from maternal, environmental, and dietary sources [4] and can be disrupted by many factors, especially when they occur early in this developmental process.

Caesarean section is one of the most important causes of disrupted microbiota development due to reduced vertical mother-infant transmission of beneficial intestinal bacteria (specifically of the Lactobacillus and Bifidobacterium genus). It has been suggested that the prenatal antibiotic exposure during a Caesarean section also affects the infant's microbiota development, but a recent randomised controlled trial (RCT) reported that prenatal exposure to antibiotics during caesarean section does not further disrupt the microbiota colonization [5]. During and after a Caesarean section, the infant becomes predominantly colonized with bacteria from the hospital environment (e.g. Staphylococcus, Corynebacterium and Propionibacterium species) [1,2,6,7]. Dysbiosis after Caesarean section can persist for as long as seven years and is associated with a higher risk of obesity, atopy, and type 1 diabetes mellitus [6].

In addition to Caesarean deliveries, exposure to antibiotics in early life has been associated with dysbiosis [8]. Early life antibiotics have been shown to decrease the abundance of Bifidobacteria [9] and Bacteroidetes [10] and increase the amount of Clostridia [8] and Enterobacteriaceae [9]. Antibiotics are the most frequently prescribed drugs in neonates with 8% of all European infants exposed to antibiotics in the first week of life [11]. The effect of antibiotic exposure, specifically in the first week of life, has been associated with an altered gut microbiota [9, 12], a higher risk of wheezing [13], infantile colic [13], gastrointestinal disorders [14], impaired growth [12, 15], allergies [16], and asthma [17].

These short- and long-term health effects linked to early dysbiosis through Caesarean delivery and neonatal antibiotic exposure illustrate the need for interventions aimed at restoration of this dysbiosis, and consequently prevention of related health consequences. Supplementation with prebiotics, probiotics or synbiotics has been described as a promising intervention to reduce some of the risks associated with early microbiota disruption. Probiotics are live microorganisms such as Bifidobacteria and Lactobacilli [7], while prebiotics are nutrients that promote growth and activity of bacteria that already exist in the gut [18]. Synbiotics are a combination of pre- and probiotics [18].

The aim of this systematic review is to identify all studies investigating the effects of a pre-, pro- or synbiotic supplement on the gut microbiota of term-born infants born by Caesarean section or exposed to antibiotics in the first week of life.

METHODS

Literature search

OVID Medline and Embase were systematically searched from inception to August 10, 2022. The search strategy was constructed in collaboration with a medical librarian (JD) and was composed of the following components:

([c section] OR ([antibiotic treatment] AND [first week of life] OR [first week antibiotics])) AND

- [pre- pro- synbiotics]

OR

- [dietary supplements] AND [microbiome]

OR

- [dietary supplements brands]

In order to reduce recall bias and enhance search results precision VOS-viewer was used to identify terms for NOTing out irrelevant records from databases searched [19]. No other filters or limits were used. The full search term including the specific keywords and combinations of search components can be found in the S1 Table.

Eligibility criteria

The following inclusion criteria were applied, all criteria had to be met for inclusion: (1) study participants were term-born infants who were born following Caesarean section or exposed to antibiotics in the first week of life (born vaginally or following Caesarean section), (2) administration of pre-, pro- or synbiotic dietary supplements was started within six weeks after birth, (3) reported outcome(s) consisted of microbiota analyses, and (4) study design was a randomised controlled trial.

Exclusion criteria were:

(1) studies including infants with major congenital malformations, (2) studies written in a language other than English, (3) animal studies, (4) for the Caesarean-analyses: studies which included both vaginally and Caesarean-delivered infants but performed no subgroup analyses for only the Caesarean-delivered infants.

Data collection

All records found in the search were exported into Rayyan after deduplication [20]. Two researchers (NC and KK) independently performed title and abstract screening, as well as full-text screening. Titles and abstracts were screened by determining whether the article could meet the in- and exclusion criteria stated above. After consensus on the included articles, relevant data was extracted by NC in consultation with the other co-authors. Reference lists of the included articles were hand-searched to look for additional relevant studies.

All significant outcomes provided in the main text or supplemental information were summarised in a table, and non-significant results from studies investigating the same outcomes were reported in separate bar charts. If both “per protocol” and “(modified) intention to treat” analyses were available, only the results from the “(modified) intention to treat” analysis were included in the table.

Critical appraisal

To assess the risk of bias in the included articles, the Cochrane risk-of-bias tool for randomised controlled trials (RoB 2)[21] was used. The RoB 2 assesses the risk of bias of studies in five domains: bias arising from the randomisation process, bias due to deviations from the intended intervention, bias due to missing outcome data, bias in measurement of the outcome, and bias in selection of the reported results. Risk of bias was independently assessed by two researchers (NC and KK) and any discrepancies were discussed until a consensus was reached. The guidance document of the RoB 2 was used to determine whether articles had a high, some or a low risk of bias. If a study included both vaginally and Caesarean-delivered infants and performed a subgroup analysis on the Caesarean-delivered infants, only the methods used for the relevant subgroup analyses were assessed.

The review and protocol were not registered. This systematic review was conducted according to the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses [22].

RESULTS

Of the 15,756 records, 12,230 remained after deduplication. After title and abstract screening, 56 articles were deemed suitable for full-text screening. Finally, 13 articles were included for analysis (Fig 1). Hand-searching the reference lists of these articles did not result in any more inclusions.

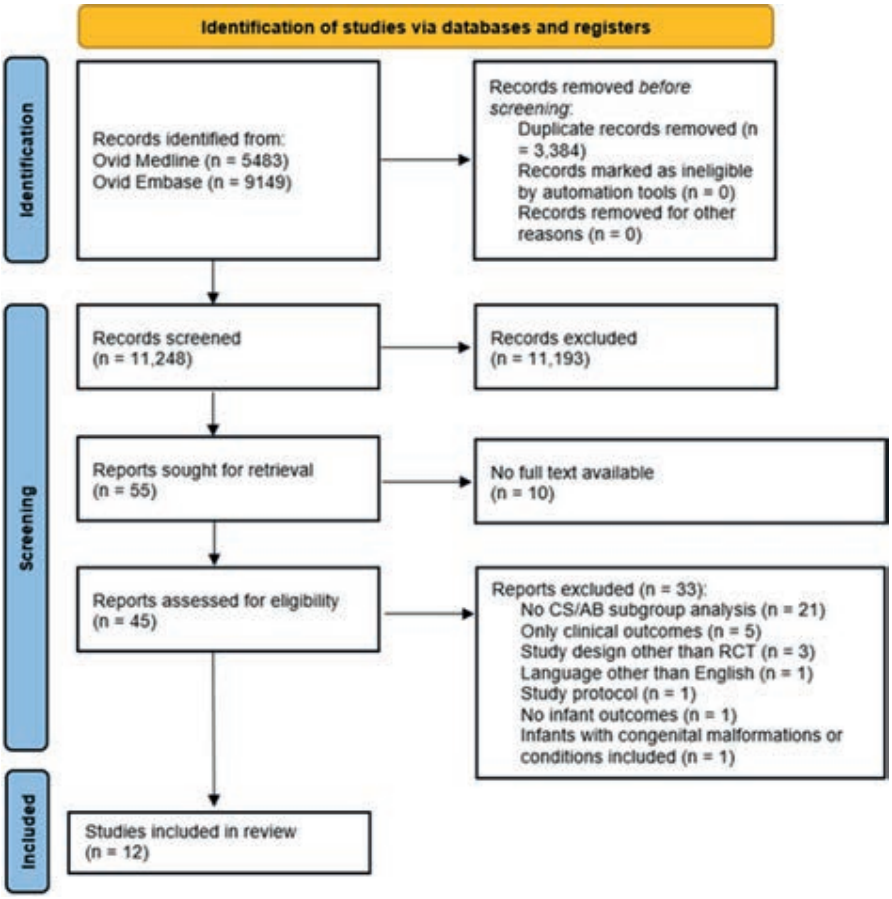

Fig 1. Flowchart showing article selection. Adapted from the PRISMA 2020 flow diagram [22].

Study characteristics

In total, 13 articles were included, based on 12 randomised controlled trials (Fig 1); Lay et al. [23] published results of a subgroup analysis based on the RCT by Chua et al [24].

The 12 microbiota studies investigated the effect of prebiotics [23-26] (n = 3), synbiotics [23, 24, 27, 28] (n = 3) and probiotics [29-35] (n = 7) (Table 1). The interventions were started between birth and the first three weeks of life, and treatment duration varied between five days after birth and six months of age. All studies investigated the effect of these interventions on infants born via Caesarean section, except for one study which included infants who received antibiotic treatment within three days after birth [35].

Table 1. General characteristics of the included randomised controlled trials

First author	Country	Study period (year published)	# Participants[1] I	C	T	AB or CS SG?	Feeding method	Intervention	Control	Start of intervention	Duration intervention	Outcomes (relevant subgroup)	Follow-up	Comments
Chua[24]	Singapore & Thailand	2011-2013 (2017, 2021)	52 + 51	50	153	CS	Mixed (FF + BF)	Prebiotic (scGOS/lcFOS) or synbiotic (scGOS/lCFOS and *Bifidobacterium breve* M-16V)	Control formula	1-3 D	16 W	Total faecal *Bifidobacterium*, *Bifidobacterium* species abundance, other members of the gut microbiota, pH, sc fatty acids, lactate	3, 5 D 2, 4, 8, 12, 16, 22 W	Infants born via CS were also exposed to intrapartum AB prophylaxis. Lay et al.: some results were based on a SG
Lay[23]			39 + 44	44	127									
Berger[25]	Italy & Belgium	2012-2015 (2020, 2017)	19	24	43	CS SG	Exclusive FF	Prebiotics: 2 HMOs (2'-fucosyllactose and lacto-N-neotetraose)	Control formula	0-14 D	6 M	Stool microbiota diversity	3, 12 M	-
Korpela[29]	Finland	2000-2003 (2018, 2018, 2009, 2017)	35	44	79	CS SG	Exclusive BF, mixed feeding or FF	Probiotic: *Lactobacillus rhamnosus* LC705, *Bifidobacterium breve* Bb99, *Propionibacterium freudenreichii* spp., *shermanii* JS	Placebo (micro-crystalline cellulose)	36 W gestation + from birth	6 M	Microbiota composition	3 M	Infants at risk for atopic disease (at least one parent with asthma, allergic rhinitis or eczema) and this intervention was initiated prenatally (36 W gestation)
Baglatzi [30]	Greece	2009-2011 (2016)	84	80	164	CS	Exclusive or mixed FF	Probiotic: regular dose of *Bifidobacterium lactis*	Low dose of *B. lactis*	Birth	6 M	Detection of *B. lactis*	12 M	No control group that was fed formula without pre-, pro-, or synbiotics
Cooper [27]	South Africa	2008-2013 (2016)	92	101	193	CS SG	Exclusive FF	Synbiotic: BMOs (containing GOS and MOS such as 3'- and 6' sialyllactose) + *Bifidobacterium lactis* CNCM-I-3446	Control formula	Birth (≤3 D)	6 M	Faecal (bifido)bacteria, anthropometrics, faecal pH, lean mass, fat mass and bone mineral content, digestive tolerance, immune parameters, HIV infection status, frequency of morbidity episodes	1 Y	All included infants had HIV+ mothers and all mothers and infants received antiretroviral medication. Infants who tested positive for HIV were excluded
Estorninos [26]	Philippines	2016-2018 (2022)	115	115	230	CS SG	Exclusive FF	Prebiotic: bovine MOS (GOS and sialylated-oligosaccharides)	Control formula	3 W	6 M	Phylogenetic distance/microbiota composition, *Bifidobacteria* abundance	4 M	At the 2.5 month time point, only a subgroup of 75 infants for each group provided a faecal sample
Frese[31]	USA	2015-2016 (2017)	11	9	20	CS SG	Any	Probiotic: *Bifidobacterium infantis* EVC001	None	7 D	27 D	Microbiota composition, relative abundances of the most abundant taxonomic groups	60 D	Significantly more mothers in the control group were primiparous
Garcia Rodenas[32]	Greece	2010-2011 (2016)	11	10	21	CS SG	Exclusive FF	Probiotic: *Lactobacillus reuteri* DSM 17938	Control formula	<72 H	6 M	Relative abundance of OTUs, weighted UniFrac distances, relative abundance of *Bifidobacterium*	4 M	-
Hurkala [33]	Poland	2014-2017 (2020)	71	77	148	CS	Exclusive FF	Probiotic: *Bifidobacterium breve* PB04 and *Lactobacillus rhamnosus* KL53A	None	<1 H	Until discharge (5 or 6 D)	Abundance of lactobacilli in faeces, populations of Bifidobacterium in faeces, populations of potentially pathogenic bacteria	1 M	Significantly more missing stool samples in the control group (29 versus 13 in intervention group)
Roggero [34]	Italy	2015-2016 (2020)	16	16	32	CS SG	Exclusive FF	Probiotic: *Lactobacillus paracasei* CBA L74	Control formula	<7 D	3 M	sIgA production, antimicrobial peptides, microbiota diversity, metabolome, abundance of bacterial genera	90 D	Infants may have been breastfed for a few days before enrolment

8

Table 1 continued

First author	Country	Study period (year published)	# Participants[1]			AB or CS SG?	Feeding method	Intervention	Control	Start of intervention	Duration intervention	Outcomes (relevant subgroup)	Follow-up	Comments
			I	C	T									
Yang[28]	China	2018 (2021)	7 + 7	9	23	CS	BF	Synbiotic: high and low dose of *Bifidobacterium lactis* Bi-07 and *Lactobacillus rhamnosus* HN001 + GOS	No probiotic	Birth	28 D	Diversity of gut microbiota, gut microbiota composition, COGs	28 D	-
Zhong[35]	China	2017-2018 (2021)	25 + 13	17	55	IW AB	Any	Probiotic: *Bifidobacterium longum*, *Lactobacillus acidophilus* and *Enterococcus faecalis*	None	Beginning or end of AB treatment (AB treatment started <3 D after birth)	42 D	Gut microbiota, relative abundance of OTUs	42 D	Children were not necessarily born via CS, but received AB in the first week of life

[1] # participants in a subgroup, if applicable. I Intervention, C Control, T Total, CS Caesarean section, SG subgroup, BF breastfeeding, FF formula feeding, HMOs human milk oligosaccharides, (sc) GOS: (short chain), galactooligosaccharides, (lc) FOS: (long chain) fructooligosaccharides, Spp. Several species, BMOs bovine milk oligosaccharides, MOS: milk oligosaccharides, D days, M months, W weeks, H hours, Y year, AB antibiotic, LRTI lower respiratory tract infection, URTI upper respiratory tract infection, OTU operational taxonomic unit, sIgA seScretory Immunoglobulin A, COG: clusters of orthologous groups of proteins

Critical appraisal

The assessment of the risk of bias of the included studies is provided in Table 2. Of the 12 studies, 10 were determined to have a high risk of bias, mainly due to issues in adhering to the intervention. Most studies did not address the extent to which participants adhered to the intervention, and if they did, the appropriate analyses necessary to estimate the effect of the non-adherence to the intervention were not applied.

Table 2. Risk of bias of the included studies

First author	Domains of the Cochrane risk-of-bias tool for randomised controlled trials (RoB-2)					
	Domain 1	Domain 2	Domain 3	Domain 4	Domain 5	Total
Chua[24]						
Lay[23]						
Berger[25]						
Estorninos[26]						
Korpela[29]						
Baglatzi[30]						
Cooper[27]						
Frese[31]						
Garcia Rodenas[32]						
Hurkala[33]						
Roggero[34]						
Yang[28]						
Zhong[35]						

Green: low risk of bias, yellow: some risk of bias, red: high risk of bias

Domain 1: Risk of bias arising from the randomisation process

Domain 2: Risk of bias due to deviations from the intended interventions (*adhering to intervention*)

Domain 3: Missing outcome data

Domain 4: Risk of bias in measurement of the outcome

Domain 5: Risk of bias in selection of the reported result

The effect of pre-, pro- and synbiotics after antibiotics in the first week of life.

The effects of the interventions were divided in three time clusters: 0-1 weeks, 1-4 weeks, or >4 weeks. The only study during or after antibiotic treatment in the first week of life investigated the effect of a probiotic supplement containing Bifidobacterium longum, Lactobacillus acidophilus and Enterococcus faecalis [35]. At the phylum level they found a significant increase in abundance of Actinobacteria and Proteobacteria at 0-1 weeks and >4 weeks and an increase in Actinobacteria at 1-4 weeks. At the genus level, they reported a significant increase in the relative abundance of Bifidobacterium at 0-1 weeks and >4 weeks (Table 3).

Table 3. Effects of pre-, pro- or synbiotic interventions on microbiota composition

First author!	Intervention: Pre-/pro-/synbiotic; Start; Duration	# Participants[1]	TP	Outcomes: microbiota composition: Diversity + differences in composition	Phylum level	Family level	Genus level	Species level	Other
					0-1 Week				
Cooper [27]	Synbiotic: BMOs (containing GOS and MOS such as 3'- and 6' sialyllactose) + *B. lactis* CNCM-I-3446 Birth (≤3 D) 6 M	I 92 C 101 T 193	3 D			n.s.			
Chua [24]	Prebiotic (scGOS/lcFOS) 1-3 D 16 W	I 39 C 45 T 84	3/ 5 D				% *Bifido bacteria*: ↑		
	Synbiotic (scGOS/lCFOS and *B. breve* M-16V) 1-3 D 16 W	I 45 C 45 T 90	3/ 5 D			% *Entero bacteriaceae*: ↓	Estimated mean of total *Bifidobacterium* gene count: ↑ % and count *Bifidobacteria*: ↑	*B. breve* M-16V [interven tion]: ↑	Acetate: ↑ pH: ↓
Lay[23]	Prebiotic (scGOS/lcFOS) 1-3 D 16 W	I 39 C 44 T 83	3/ 5 D			n.s.			
Lay[23]	Synbiotic (scGOS/lCFOS and *B. breve* M-16V) 1-3 D 16 W	I 44 C 44 T 88	3 & 5 D	Compo sitional difference		Relative abundance of : -strict anaerobes**: ↑ -facultative (an)aerobes***: ↓ *Bifidobacteriaceae*: ↑	*Bifido bacterium*: ↑ *Haemophilus*: ↓ (day 5 only)	Abundance of *B. breve* [intervention]: ↑	
Yang [28]	High dose of synbiotic: *B. lactis* Bi-07 and *L. rhamnosus* HN001 + GOS Birth 28 D	Day 3 I 7 C 9 T 16 Day 7 I 6 C 8 T 14	3 & 7 D				Relative abundance of *Bifido bacterium*: ↑ (day 3) *Lactobacillus*: ↑ (day 3 & 7)		
	Low dose of synbiotic: *B. lactis* Bi-07 and *L. rhamnosus* HN001 + GOS Birth 28 D	Day 3 I 7 C 9 T 16 Day 7 I 5 C 8 T 13	3 & 7 D				Relative abundance of: *Bifido bacterium*: ↑ (day 3) *Lactobacillus*: ↑ (day 3 & 7)		
Hurkala [33]	Probiotic: *B. breve* PB04 and *L. rhamnosus* KL53A <1 H 5 or 6 D	I 71 C 77 T 148	5/ 6 D				Abundance of *Lactobacilli*: ↑ and of *Bifidobacterium*: ↑	*L.rhamnosus*[x]: ↑ *B.breve*[x]: ↑ [[x]interven tion]	
Zhong [35]	Probiotic: *B. longum*, *L. acidophilus* and *E. faecalis* during AB treatment <3 D 42 D	I 25 C 17 T 42	1 W		Relative abundance: Actinobacteria: ↑ Proteobacteria: ↑		Relative abundance of *Bifido bacterium*: ↑		
	Probiotic: *B. longum*, *L. acidophilus* and *E. faecalis* after AB treatment 7 D 42 D	I 13 C 17 T 30	1 W			n.s.			

*: *g. bifidobacterium, o. pseudomonadales, f. actinomycetaceae, k. bacteria, g. staphylococcus, g. streptococcus, f. streptococcaceae, f. bifidobacteriaceae, o. enterobacteriales, f. enterobacteriaceae* **: *f. Prevotellaceae, f. peptostreptococcaceae, f. ruminococcaceae, o. clostridiales, f. porphyromonadaceae, f. clostridiaceae, f. lachnospiraceae, f. veillonellaceae, f. bacteroidaceae, f. bifidobacteriaceae* ***: *o. lactobacillales, o. bacillales, f. pasteurellaceae, f. staphylococcaceae, f. lactobacillaceae, f. enterococcaceae, o. enterobacteriales, f. streptococcaceae, f. Enterobacteriaceae*

Table 3 continued

First author!	Pre-/pro-/synbiotic Start Duration	# Participants[1]	TP	Diversity + differences in composition	Phylum level	Family level	Genus level	Species level	Other
					1-4 Weeks				
Cooper [27]	Synbiotic: BMOs (containing GOS and MOS such as 3'- and 6' sialyllactose) + *B. lactis* CNCM-I-3446 Birth (≤3 D) 6 M	I 92 C 101 T 193	10 D & 1 M				*Bifidobacterium* counts: ↑ detection rate of: -*Bifido bacteria*: ↑ -*Bacteroides*: ↑ (10 days) -*Lactobacillus*: ↑ (1M)	detection of *B. lactis* CNCM I-3446 [intervention]: ↑	pH: ↓
Chua [24]	Prebiotic (scGOS/lcFOS) 1-3 D 16 W	I 39 C 45 T 84	2 W			%*Enterobacteriaceae*: ↑			
			4 W						pH: ↓
Chua [24]	Synbiotic (scGOS/lCFOS and *B. breve* M-16V) 1-3 D 16 W	I 45 C 45 T 90	2 W & 4 W			% *Enterobacteriaceae*: ↓	Estimated mean of total *Bifidobacterium* gene count: ↑ % and count *Bifidobacteria*: ↑	*B. breve* M-16V [intervention] ↑	pH: ↓
Lay [23]	Prebiotic (scGOS/lcFOS) 1-3 D 16 W	I 39 C 44 T 83	2 + 4 W			n.s.			
	Synbiotic (scGOS/lCFOS and *B. breve* M-16V) 1-3 D 16 W	I 44 C 44 T 88	2 W	Compositional difference		Relative abundance of: strict anaerobes**: ↑ and facultative (an)aerobes***: ↓ *Bifidobacteriaceae*: ↑	*Bifidobacterium*: ↑	Abundance of *B. breve* [intervention]: ↑	
			4 W			*Clostridiaceae*: ↓		Abundance of *B. breve* [intervention]: ↑	
Garcia Rodenas [32]	Probiotic: *L. reuteri* DSM 17938 ≤72 H 6 M	I 11 C 10 T 21	2 W	Compositional difference	Relative abundance of: Actinobacteria: ↑ Firmicutes: ↑	Relative abundance of: *Enterobacteriaceae*: ↓ *Bifidobacteriaceae*: ↑ *Lactobacilliaceae*: ↑	Detectable *Bifidobacterium*: ↑ Relative abundance of *Lactobacillus*: ↑	Abundance of *L. reuteri* [intervention]: ↑	
Zhong [35]	Probiotic: *B. longum*, *L. acidophilus* and *E. faecalis* during AB ≤3 D 42 D	I 25 C17 T 42	2 W			n.s.			
	Probiotic: *B. longum*, *L. acidophilus* and *E. faecalis* after AB treatment 7 D 42 D	I 13 C 17 T 30	2 W		Relative abundance of Actinobacteria: ↑				
Hurkala [33]	Probiotic: *B. breve* PB04 and *L. rhamnosus* KLS3A ≤1 H 5 or 6 D	I 58 C 48 T 106	1 M				Abundance of *Lactobacilli*: ↑		
Yang [28]	High dose of synbiotic: *B. lactis* Bi-07 and *L. rhamnosus* HN001 + GOS Birth 28 D	I 6 C 5 T 11	1 M			n.s.			
	Low dose of synbiotic: *B. lactis* Bi-07 and *L. rhamnosus* HN001 + GOS Birth 28 D	I 7 C 5 T 12							

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Table 3 continued

First author!	Pre-/pro-/synbiotic Start Duration	# Participants[1]	TP	Diversity + differences in composition	Phylum level	Family level	Genus level	Species level	Other
					>4 weeks				
Zhong [35]	Probiotic: *B. longum*, *L. acidophilus* and *E. faecalis* during AB treatment <3 D 42 D	I 25 C17 T 42	42 D		Relative abundance of Actinobacteria: ↑ and of Proteobacteria: ↑		Relative abundance of *Bifidobacterium*: ↑		
	Probiotic: *B. longum*, *L. acidophilus* and *E. faecalis* after AB 7 D 42 D	I 13 C 17 T 30	42 D			n.s.			
Chua [24]	Prebiotic (scGOS/lcFOS) 1-3 D 16 W	I 39 C 45 T 84	12 W			n.s.			
			16 W			%*Enterobacteriaceae*: ↑			
	Synbiotic (scGOS/lCFOS and *B. breve* M-16V) 1-3 D 16 W	I 45 C 45 T 90	12 W			% *Enterobacteriaceae*: ↓	*Bifidobacteria* count: ↑	*B. breve* M-16V: ↑	
			16 W					*B. breve* M-16V: ↑	
Lay[23]	Prebiotic (scGOS/lcFOS) 1-3 D 16 W	I 39 44 C 44 44 T 83 88	8 W			*Staphylococcaceae*: ↓			
			12, 16, 22 W			n.s.			
Lay[23]	Synbiotic (scGOS/lCFOS and *B. breve* M-16V) 1-3 D 16 W	I 10 C 10 T 20	8 W	Shannon diversity: ↑				*B. longum*: ↓	
			12, 16 W			n.s.			
			22 W					*V. dispar*: ↑	
Estorninos [26]	Prebiotic: bovine MOS (GOS and sialylated-oligo saccharides) 3 W 6 M	I 75 C75 T 150	2.5 M	Compositional difference					
		I 114 C 112 T 226	4 M	Compositional difference			Abundance of *Bifidobacterium*: ↑		
Berger [25]	Prebiotics: 2 HMOs (2'-fucosyllactose and lacto-N-neotetraose) 0-14 D 6 M	I 19 C 24 T 43	3 M			n.s.			
Korpela [29]	Probiotic: *L. rhamnosus* LC705, *B. breve* Bb99, *P. freudenreichii* spp., *shermanii* JS and GOS 36 W gest. + from birth 6 M	I 35 C 44 T 79	3 M			*Bifidobacteriaceae*: ↑ *Coriobacteriaceae*: ↑ *Porphyromonadaceae*: ↑ *Bacteroidaceae*: ↑			
Cooper [27]	Synbiotic: BMOs (containing GOS and MOS such as 3'- and 6' sialyllactose) + *B. lactis* CNCM-I-3446 Birth (≤3 D) 6 M	I 92 C 101 T 193	3 M				*Bifidobacteria* counts: ↑ detection rate of *Clostridium*/ *Eubacterieum*: ↓	detection of *B. lactis* CNCM I-3446 [intervention]: ↑	Mean faecal pH: ↓
Roggero [34]	Probiotic: *L. paracasei* CBA L74 <7 D 3 M	I 16 C 16 T 32	3 M						sIgA production: ↑
Baglatzi [30]	Probiotic: regular dose of *B. lactis* Birth 6 M	I 84 C 80 T 164	4 M					Positive detection of *B. lactis* [intervention]: ↑	

Table 3 continued

First author!	Pre-/pro-/synbiotic Start Duration	# Partici pants[1]	TP	Diversity + differences in composition	Phylum level	Family level	Genus level	Species level	Other
Garcia Rodenas [32]	Probiotic: *L. reuteri* DSM 17938 <72 H 6 M	I 11 C 10 T 21	4 M					Abundance of *L. reuteri* [intervention]: ↑	

[1] # participants in a subgroup, if applicable. TP time points, I Intervention, C Control, T Total, CS Caesarean section, SG subgroup, HMOs human milk oligosaccharides, (sc) GOS: (short chain) galactooligosaccharides, (lc) FOS: (long chain) fructooligosaccharides, BMOs bovine milk oligosaccharides, MOS: milk oligosaccharides, D days, M months, W weeks, H hours, Y year, AB antibiotics

!Analysis techniques: Cooper [27] PCR, FISH; Chua [24] 16S rRNA sequencing + FISH + qPCR; Lay[23] Shotgun 16S rRNA sequencing of the V3-V6 region, shotgun metagenomics, metatranscriptomics and metabolomics; Yang [28] and Zhong[35] 16S rRNA gene sequencing of the V3-V4 region + PCR; Hurkala [33] and Baglatzi[30] PCR; Garcia Rodenas[32] 16S rRNA gene sequencing of the V1- V3 regions + PCR; Estorninos[26], Berger[25] and Korpela[29] 16S rRNA gene sequencing of the V3-V4 region; Roggero[34] 16s RNA gene sequencing of the V3 region

The effect of pre-, pro- and synbiotics after Caesarean section

Fig 2 summarises the number of statistically significant and non-significant differences that were found in the three time clusters. The significant microbiota changes in the experimental groups compared to the control groups (described in Table 1) are discussed in further detail below.

Diversity and compositional differences

One study [23] found compositional differences (using a distance-based redundancy analysis) at 0-1 weeks, 1-4 weeks, and >4 weeks in the infants who received a synbiotic compared to those who received a prebiotic or placebo, and an increased diversity at 8 weeks using the Shannon diversity index. Researchers of two other studies [26, 32] also measured compositional differences (phylogenetic distance) at 1-4 weeks [32] and >4 weeks [26] and reported a significantly different microbiota composition in infants who received a probiotic [32] or prebiotic [26] compared to a placebo.

Phylum level

Only one study [32] investigated the effect of a probiotic after Caesarean delivery on the phylum level. At 1-4 weeks, they found an increase in both Actinobacteria and Firmicutes in the probiotic group.

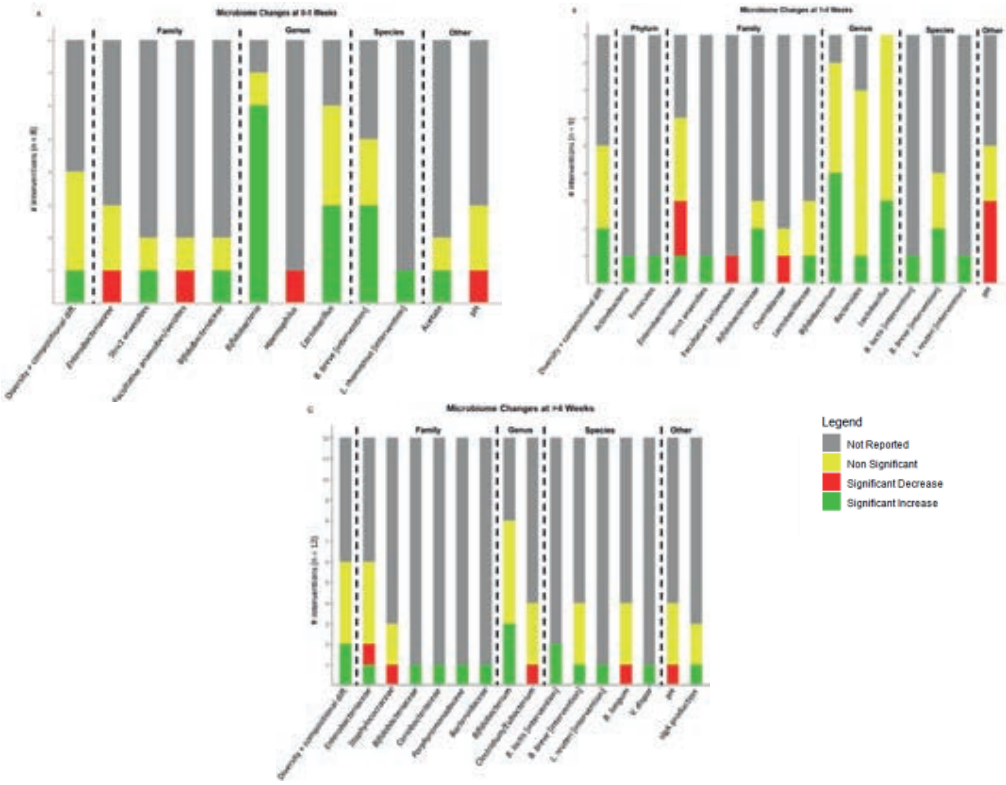

Fig 2. Bar charts showing the number of interventions (several studies used more than one intervention) with a significant effect on the microbiota composition at at least one time point in the clusters of 0-1 weeks (a), 1-4 weeks (b) or >4 weeks (c).

Family level

At 0-1 weeks, Chua et al. [24] found a significant decrease in the percentage of Enterobacteriaceae present in the stool of infants who received the synbiotic, but not those who received the prebiotic. Lay et al. [23], who analysed a subgroup of infants from the same study, reported an increase in relative abundance of strict anaerobes, a decrease in relative abundance of facultative anaerobes/aerobes and an increase in Bifidobacteriaceae in the synbiotic group.

At 1-4 weeks, Chua et al. again report a significant decrease in the percentage of Enterobacteriaceae in the synbiotic group, but a significant increase in the prebiotic group [24]. In a subgroup analysis by Lay et al., no significant differences were found in the prebiotic group, but an increase in strict anaerobes, decrease in facultative anaerobes/aerobes and Clostridiaceae and an increase in Bifidobacteriaceae was observed after a synbiotic supplement

[23]. In line with their findings, another study [32] also found a significant increase in Bifidobacteriaceae. Additionally, they found a significant increase in Lactobacillaceae. Furthermore, they reported a significant decrease in the percentage of Enterobacteriaceae in their probiotic group, which is similar to Chua et al.'s findings in their synbiotic intervention group.

At >4 weeks, Chua et al. found the same results as at 1-4 weeks: a significant decrease in the percentage of Enterobacteriaceae in the synbiotic group, and a significant increase in the prebiotic group [24]. Moreover, in a subgroup a decrease in Staphylococcaceae was reported [23]. Another study found an increase in Bifidobacteriaceae, Coriobacteriaceae, Porphyromonadaceae and Bacteroidaceae [29].

Genus level

At 0-1 weeks, four articles [23,24,28,33] based on three RCTs reported a significant increase in abundance of the Bifidobacterium genus after administration of a probiotic [33], prebiotic [24], and synbiotic [23,24,28]. Two studies [28,33] also found an increase abundance of the Lactobacillus genus [28,33], and one study [23] reported a decrease of the Haemophilus genus.

At 1-4 weeks, four articles [23,24,27,32] from three RCTs found an increase in the Bifidobacterium genus after administration of a probiotic [27,32] or symbiotic [23,24]. Three [27,32,33] reported an increased (relative) abundance of Lactobacillus and one of these [27] also observed an increased abundance of Bacteroides.

At >4 weeks, three studies [23,26,27] reported an increased Bifidobacterium genus abundance, and one of the two [27] also found a decreased faecal detection rate of Clostridium/Eubacterium.

Species level

At 0-1 weeks, Chua et al. [24] and Lay et al. [23] (based on the same RCT) reported a significant increase of Bifidobacterium breve M-16V detected in the infants who received a synbiotic, which included this Bifidobacterium species.

At 1-4 weeks, three studies [24,27,32] found an increase in the faecal detection of the bacterial species they included in their intervention: Bifidobacterium lactis CNCM I-3446 [27], Bifidobacterium breve [23] and Lactobacillus reuteri [32].

At >4 weeks, three studies again reported an increase in faecal detection of their intervention: Bifidobacterium lactis [27,30] and Lactobacillus reuteri [32]. Another study also

found a decreased abundance of Bifidobacterium longum and an increase in Veillonella dispar [23].

Intestinal microenvironment

At 0-1 weeks, one article [24] found a decreased faecal pH and increased acetate after administration of a synbiotic. At 1-4 weeks, the same study [24] and another [27] both reported a decreased faecal pH after administration of a synbiotic [24,27] or prebiotic [24]. At >4 weeks, one of the studies [27] still found a decreased faecal pH in the synbiotic-group [27], and another article [34] observed an increased secretory IgA (sIgA) production in infants who received a probiotic.

DISCUSSION

The aim of this systematic review was to describe the effects of a pre-, pro- or synbiotic supplement on the gut microbiota following Caesarean section or exposure to antibiotics in the first week of life. Only one article investigated the effect of a probiotic on antibiotic-exposed infants; a mixture of three probiotics resulted in an increase in Actinobacteria, Proteobacteria and Bifidobacterium. For the Caesarean-born infants, the key finding was an increase in the supplemented bacterial species (of the Bifidobacterium and Lactobacillus genus) after probiotic or synbiotic supplements, and a decrease in Enterobacteriaceae after synbiotic but an increase after prebiotic supplementation. Furthermore, there were significant increases in Actinobacteria, Proteobacteria and Firmicutes in the probiotic groups compared to the control groups. Moreover, the microbiota composition of the probiotic or synbiotic group was significantly different from the control group in two studies, and bacterial species diversity was increased in one study after administration of a synbiotic.

Prebiotics are less extensively studied, and only few outcome parameters reached statistical significance. However, according to one included study, prebiotics increased the abundance of Enterobacteriaceae, which has been associated with potentially negative health effects such as an increased risk of atopic eczema [36], food allergy [37] and delayed colonisation of beneficial bacterial species [36]. Three articles based on two prebiotic studies reported an increase in Bifidobacteria [23,24,26] and two of the three also found a significantly different microbiota composition [23,26].

Because of the heterogeneity in the interventions in terms of study design and composition of the supplement, it is difficult to compare their efficacy. Generally, probiotics and synbiotics seem more effective in increasing the abundance of beneficial bacteria. Bifidobacteria and Lactobacilli, which were increased in the intervention groups of eight and five studies respectively, are associated with various health effects: both Bifidobacteria and Lactobacilli

seem to protect from allergies [38,39] and infantile colic [38] and they are associated with healthy microbiota development [38]. Several species of the Bifidobacterium genus are commonly present in the infant gut, and their function is to digest sugars in human milk, reduce intestinal pH and improve the integrity of the intestinal wall [40]. Delivery via Caesarean section, which was the case in five [23,24,26,28,33] of the six studies investigating the microbiota at the genus level, results in a disrupted vertical transmission of Bifidobacterium [40]. The results in Table 3 indicate that a pro- or synbiotic intervention can alleviate this disruption and shift the neonatal microbiota composition towards that of vaginally born infants. Human milk oligosaccharides present in breast milk can also stimulate colonisation by Bifidobacteria [40]. Interestingly, all five articles that reported a significant increase of Bifidobacterium levels at 0-1 weeks included infants that were (also) breastfed. However, at the time points after this first week, other studies that included infants who were exclusively formula fed also show significant increases in Bifidobacterium levels. Moreover, three studies on pro- and synbiotics also found a significantly different microbiota composition or a more diverse microbiota in the intervention groups. Birth following Caesarean section or exposure to antibiotics in the first week of life reduces bacterial diversity, which makes these infants susceptible to colonisation by bacteria usually found on the mother's skin such as Staphylococcus, Corynebacterium and Propionibacterium spp., which is associated with an increased risk of gastrointestinal and systemic disorders, including eczema allergies, later in life [41]. The results in table 3 show that this diversity may (partially) be restored by supplementation with pro- or synbiotics and possibly prebiotics.

To our knowledge, this is the first systematic review evaluating the effects of pre-, pro- and synbiotics specifically in both Caesarean born and antibiotic-exposed infants. While another systematic review about the effects of pre-, pro- and synbiotics on the microbiota of children born via Caesarean section was published recently [42], we identified four additional relevant articles that were not included by Martin-Pelaez et al. Furthermore, some of their included articles did not perform a separate subgroup-analysis for children born via Caesarean section.

It is crucial to analyse these Caesarean born infants separately from vaginally born infants who were not exposed to antibiotics, because the effect of pre-, pro- and synbiotics may differ in infants with a disrupted microbiota from those who were born vaginally. Illustratively, in one of the trials included in our review, the effects of pre-, pro- and synbiotics on the microbiota was only significant in the Caesarean born subgroup [32]. Additionally, Frese et al. [31] did not perform a statistical subgroup analysis for the Caesarean born infants but the differences in the microbiota composition of their cohort seems to be largely driven by Caesarean born infants. Specifically, in their intervention group, they found a significant increase

in faecal Bifidobacteriaceae and Bifidobacterium infantis, and a clear decrease in the relative abundances of Enterobacteriaceae, Clostridiaceae, Erysipelotrichaceae, Pasteurellaceae, Micrococcaceae and Lachnospiraceae. These findings suggest that especially infants with a disrupted microbiota might benefit most from an intervention with pre-, pro or synbiotics.

Important strengths of this review are the elaborate search strategy developed in collaboration with a medical librarian to include all relevant articles. We also looked for any subgroup analyses of Caesarean-born infants in the full texts, even when the title or abstract did not explicitly state that these were performed. We only included articles that performed analyses on Caesarean-born infants, and not articles that only analysed the total group of participants with vaginally born infants included.

Limitations of this study are that many articles that included a subgroup analysis of Caesarean-born infants reported only a selection of the outcomes for this subgroup. It is unclear whether more analyses were performed and only the significant results were published, which would result in publication bias. Similarly, many articles did not adjust for multiple testing. This is also reflected in the critical appraisal of the articles, which showed that 11 of the 13 included studies had a high risk of bias. Lastly, while some trials mentioned in this review included a reference group with vaginally born and/or exclusively breastfed infants, we chose to focus on analyses between intervention and placebo-controlled groups instead of also comparing the intervention groups to the reference group. For further research, it would be interesting to evaluate whether pre-, pro- and synbiotic interventions could restore the microbiota of infants born through Caesarean section or infants exposed to antibiotics to that of a healthy reference group.

Other recommendations for future research are firstly that, in order to be able to compare the results of different studies, studies should standardise their methods of faecal sample collection, storage, isolation, and analyses. Furthermore, microbiota studies should increase their follow-up time to see whether any differences that were found between intervention and control groups persist beyond the duration of the intervention and to search for associations with long-term health outcomes. In addition, since the effect of a pre-, pro- or synbiotic on infants after antibiotic treatment in the first week of life was only investigated by one study [35], more RCTs are necessary in this group of infants. Moreover, to assess the clinical potential of pre-, pro- or synbiotic supplementation, it is crucial that high quality RCTs with predetermined clinical outcomes are conducted. Lastly, only one study [34] explored the effects of their intervention on the metabolome. The metabolome gives an indication of the function of the microbiota, and how the microbiota affects metabolites in urine, faeces and blood serum [43]. While most included studies focused their microbiota analysis on the microbiota

composition, the metabolome might reveal important information on the mechanics by which the microbiota influences its host.

CONCLUSIONS

Supplementation of pre-, pro- or synbiotics in Caesarean-born infants and infants who received antibiotics early in life mostly increased the phyla, families, genera, and species that corresponded to the pro- or synbiotic intervention that was administered, while the effects of a prebiotic generally did not reach statistical significance. Supplementation of these at-risk children to restore the microbiota to a composition more similar to vaginally born infants (i.e. predominant colonisation by Bifidobacteria and Lactobacilli) may alleviate some of the negative consequences of a disrupted microbiota. However, more high-quality research is needed to explicate the clinical effects of such microbiota changes and to determine which pre-, pro- or synbiotic products are most effective.

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APPENDIX

Table S1 Full search strategy

	Ovid MEDLINE(R) ALL <1946 to August 02, 2021> **Search date: 3 August 2021**	
#	**Searches**	**Results**
1	exp Cesarean Section/	48041
2	(C-section* or C?esar?an or Abdominal deliver* or abdominal birth? or (Surg* adj2 deliver*) or (surg* adj2 birth) or Sectio or cesarien or caesarien or postc?esar*).ab,kf,ti.	69874
3	1 or 2 [cesarean section]	81595
4	exp Anti-Bacterial Agents/	758161
5	(antibiotic* or anti biotic* or anti bacterial or antibacterial or biocidal or antimicrobial or anti microbial).ab,kf,ti.	554317
6	4 or 5 [antibiotic treatment]	1033437
7	infant, newborn/	627066
8	(newborn? or neonat*).ab,kf,ti.	417342
9	(("1" adj1 week?) or (("0" or "1" or "2" or "3" or "4" or "5" or "6" or "7") adj1 day?) or (("12" or "24" or "48" or "72") adj1 hour?)).ab.	963246
10	or/7-9 [first week of life]	1723901
11	6 and 10 [antibiotic treatment AND first week of life]	84493
12	((amoxicillin or penicillin G or augmentin or benzylpenicillin or ampicillin) and (gentamicin or amikacin or cefotaxime or ceftazidime)).mp. [1st week antibiotics]	9134
13	or/3,11-12 [children antibiotic treatment c section]	172617
14	prebiotics/ or probiotics/ or synbiotics/	21966
15	(pre biotic* or pro biotic* or syn biotic* or prebiotic* or probiotic* or synbiotic*).ab,kf,ti.	35911
16	(Arachidonic acid or Bacteroides uniformis or Bifidobacteri* or Bovine-milk derived oligosaccharides or Beta-palmitate or Clostridium butyricum or "E coli" or Escherichia coli or "E faecium L3" or Fructo-oligosaccharides or fructooligosaccharides or Galacto-oligosaccharides or galactooligosaccharides or Inulin-galactooligosaccharide or Oligofructose-inulin or Pectin-derived acidic oligosaccharides or Human milk oligosaccharides or fucosyllactose or fukosyllactose or Lacto-N-neotetraose or Pectin-derived acidic oligosaccharides or Lactobacill* or Lactococcus lactis or Long-chain polyunsaturated fatty acids or Oligofructose-enriched inulin or Polidextrose or Propionibacterium or Propionibacterium freudenreichii or Prebiotic oligosaccharides or Polydextrose or Streptococcus thermophilus).ab,kf,ti.	421784
17	or/14-16 [pre pro synbiotics]	442980
18	exp dietary supplements/	85531
19	(food? or feed* or diet* or supplement* or nutr*ceutical?).ab,kf,ti.	1606621
20	nutri*.jw.	245096
21	or/18-20 [dietary supplements]	1734967
22	(Aptamil or Biogaia or Probi or Lallemand or Valio or Danone or Nutricia or Nestle or Winclove).ab,in. [dietary supplements manufacturers]	5610

23	21 or 22	1737653
24	(Vivomixx or Visbiome or DeSimone Formulation or Danisco-DuPont or Orafti Synergy 1 or oligofructose-enriched inulin or Colinfant or Yakult or Nutrilon or Ecologic Panda).ab,in,kf,ti. [dietary supplements brands]	894
25	(gut or microbi* or gastrointest* or intestin* or flora).mp. [microbiome]	1983259
26	13 and 17	9305
27	and/13,23,25	4400
28	13 and 24	17
29	or/26-28	12166
30	exp animals/ not humans/	4868512
31	(mouse or mice or rodent? or rat? or pig* or swine? or hog? or cattle or chicken?).ab,kf,ti.	5199761
32	30 or 31	7652474
33	29 not 32	7550
34	(drug resistance or ((Beta-lactam or Penicillin) adj1 resistan*) or needle biopsy or Burns or Carrier state or Colistin or Emergency service or Endocarditis or Gastroenteritis or Methicillin-resistant staphylococcus aureus or Minocycline or Neoplasms or Peritonitis or Premedication or Prostate or Prosthesis or Surgical wound or Appendicitis or Debridement or Device removal or Drug utilization or Half-life or Hydrocephalus or Middle aged or Preoperative care or Sentinal surveillance or Community-acquired infections or Thienamycins or Naphthyridines or Oxazinesor Oxazolidinones or Tigecycline or Abscess or Drug synergism or Drug tolerance or Sisomicin or Kanamycin or Levofloxacin or Endophthalmitis or Equipment contamination or Microbial sensitivity test*).ab,ti. [VOS cluster 1]	872358
35	(Premature birth or Cholera or Poliovirus vaccine or Pulmonary surfactants or attenuated vaccines or Nitric oxide or Progesterone or Cholera toxin or Anti-hiv agents or "Caco-2 cells" or Cattle or Coculture techniques or animal Disease models or Fetal blood or Germ-free life or Healthy volunteers or ht29 cells).ab,ti. [VOS cluster 2]	445905
36	(Fetal blood or Oryza or Aging or Fetal development or Oxygen consumption or Swine).ab,ti. [VOS cluster 3]	304932
37	(Cardiac surgical procedures or Chewing gum or Gastric bypass or Gastrostomy or Ileostomy or Helicobacter infection? or Helicobactor pylori or Octreotide or Reconstructive surgical procedures or Short bowel syndrome or Anti-ulcer agent? or chewing gum or surgical Anastomosis or Diabetes complication? or Familial duodenal atresia or Gastroschisis or Mesenteric artery or Short bowel syndrome).ab,ti. [VOS cluster 4]	62632
38	(Acne vulgaris or Dental or Drinking water or Fungi or H?ematopoietic stem cell transplantation or Metal nanoparticle? or Precursor cell lymphoblastic leuk?emia-lymphoma or Prosthesis-related infection? or Silver or Typhoid fever or Vegetable? or Waste water or Bone cements or mouthwash* or ophthalmic solutions or root canal or tea or Computer simulation or Ecosystem or Poultry or Poultry diseases or Tissue donor? or X-ray diffraction or Zoonoses).ab,ti. [VOS cluster 5]	744799
39	(molecular cloning or Rabbit?).ab,ti. [VOS cluster 6]	269631
40	(H?emolytic-uremic syndrome or Ambulatory care or Vibrio cholerae).ab,ti. [VOS cluster 7]	25880
41	or/34-40	2622024
42	33 not 41 [VOS NOTing out]	5483

	Ovid Embase Classic+Embase <1947 to 2021 July 30> **Search date: 3 August 2021**	
#	**Searches**	**Results**
1	exp *Cesarean Section/	34080
2	(C-section* or C?esar?an or Abdominal deliver* or abdominal birth? or (Surg* adj2 deliver*) or (surg* adj2 birth) or Sectio or cesarien or caesarien or postc?esar*).ab,kw,ti.	107002
3	1 or 2 [cesarean section]	109703
4	exp *antiinfective agent/	1631474
5	(antibiotic* or anti biotic* or anti bacterial or antibacterial or biocidal or antimicrobial or anti microbial).ab,kw,ti.	768767
6	4 or 5 [antibiotic treatment]	2089849
7	newborn/	642275
8	(newborn? or neonat*).ab,kw,ti.	559799
9	(("1" adj1 week?) or (("0" or "1" or "2" or "3" or "4" or "5" or "6" or "7") adj1 day?) or (("12" or "24" or "48" or "72") adj1 hour?)).ab.	1532622
10	or/7-9 [first week of life]	2326933
11	6 and 10 [antibiotic treatment AND first week of life]	188981
12	((amoxicillin or penicillin G or augmentin or benzylpenicillin or ampicillin) and (gentamicin or amikacin or cefotaxime or ceftazidime)).mp. [1st week antibiotics]	75009
13	or/3,11-12 [children antibiotic treatment c section]	364462
14	*prebiotic agent/ or *probiotic agent/ or *synbiotic agent/	24152
15	(pre biotic* or pro biotic* or syn biotic* or prebiotic* or probiotic* or synbiotic*).ab,kw,ti.	45775
16	(Arachidonic acid or Bacteroides uniformis or Bifidobacteri* or Bovine-milk derived oligosaccharides or Beta-palmitate or Clostridium butyricum or "E coli" or Escherichia coli or "E faecium L3" or Fructo-oligosaccharides or fructooligosaccharides or Galacto-oligosaccharides or galactooligosaccharides or Inulin-galactooligosaccharide or Oligofructose-inulin or Pectin-derived acidic oligosaccharides or Human milk oligosaccharides or fucosyllactose or fukosyllactose or Lacto-N-neotetraose or Pectin-derived acidic oligosaccharides or Lactobacill* or Lactococcus lactis or Long-chain polyunsaturated fatty acids or Oligofructose-enriched inulin or Polidextrose or Propionibacterium or Propionibacterium freudenreichii or Prebiotic oligosaccharides or Polydextrose or Streptococcus thermophilus).ab,kw,ti.	485192
17	or/14-16 [pre pro synbiotics]	510503
18	exp dietary supplements/	16362
19	(food? or feed* or diet* or supplement* or nutr*ceutical?).ab,kw,ti.	2096249
20	nutri*.jx.	302884
21	or/18-20 [dietary supplements]	2235811
22	(Aptamil or Biogaia or Probi or Lallemand or Valio or Danone or Nutricia or Nestle or Winclove).ab,in. [dietary supplements manufacturers]	11817
23	21 or 22	2242040

24	(Vivomixx or Visbiome or DeSimone Formulation or Danisco-DuPont or Orafti Synergy 1 or oligofructose-enriched inulin or Colinfant or Yakult or Nutrilon or Ecologic Panda).ab,in,kw,ti. [dietary supplements brands]	1624
25	(gut or microbi* or gastrointest* or intestin* or flora).mp. [microbiome]	2166306
26	13 and 17	21095
27	and/13,23,25	6238
28	13 and 24	40
29	or/26-28	25508
30	(animal/ or animal experiment/ or animal model/ or nonhuman/ or rat/ or mouse/) not human/	7159102
31	(mouse or mice or rodent? or rat? or pig* or swine? or hog? or cattle or chicken?).ab,kw,ti.	6979804
32	30 or 31	10747161
33	29 not 32	12843
34	(drug resistance or ((Beta-lactam or Penicillin) adj1 resistan*) or needle biopsy or Burns or Carrier state or Colistin or Emergency service or Endocarditis or Gastroenteritis or Methicillin-resistant staphylococcus aureus or Minocycline or Neoplasms or Peritonitis or Premedication or Prostate or Prosthesis or Surgical wound or Appendicitis or Debridement or Device removal or Drug utilization or Half-life or Hydrocephalus or Middle aged or Preoperative care or Sentinal surveillance or Community-acquired infections or Thienamycins or Naphthyridines or Oxazinesor Oxazolidinones or Tigecycline or Abscess or Drug synergism or Drug tolerance or Sisomicin or Kanamycin or Levofloxacin or Endophthalmitis or Equipment contamination or Microbial sensitivity test*).ab,ti. [VOS cluster 1]	1217809
35	(Premature birth or Cholera or Poliovirus vaccine or Pulmonary surfactants or attenuated vaccines or Nitric oxide or Progesterone or Cholera toxin or Anti-hiv agents or "Caco-2 cells" or Cattle or Coculture techniques or animal Disease models or Fetal blood or Germ-free life or Healthy volunteers or ht29 cells).ab,ti. [VOS cluster 2]	563387
36	(Fetal blood or Oryza or Aging or Fetal development or Oxygen consumption or Swine).ab,ti. [VOS cluster 3]	390731
37	(Cardiac surgical procedures or Chewing gum or Gastric bypass or Gastrostomy or Ileostomy or Helicobacter infection? or Helicobactor pylori or Octreotide or Reconstructive surgical procedures or Short bowel syndrome or Anti-ulcer agent? or chewing gum or surgical Anastomosis or Diabetes complication? or Familial duodenal atresia or Gastroschisis or Mesenteric artery or Short bowel syndrome).ab,ti. [VOS cluster 4]	99739
38	(Acne vulgaris or Dental or Drinking water or Fungi or H?ematopoietic stem cell transplantation or Metal nanoparticle? or Precursor cell lymphoblastic leuk?emia-lymphoma or Prosthesis-related infection? or Silver or Typhoid fever or Vegetable? or Waste water or Bone cements or mouthwash* or ophthalmic solutions or root canal or tea or Computer simulation or Ecosystem or Poultry or Poultry diseases or Tissue donor? or X-ray diffraction or Zoonoses).ab,ti. [VOS cluster 5]	862062
39	(molecular cloning or Rabbit?).ab,ti. [VOS cluster 6]	362539
40	(H?emolytic-uremic syndrome or Ambulatory care or Vibrio cholerae).ab,ti. [VOS cluster 7]	32757
41	or/34-40	3390676
42	33 not 41 [VOS NOTing out]	9149

Chapter 9

Clinical outcomes following pre-, pro-, and synbiotics supplementation after caesarean birth or antibiotic exposure in the first week of life in term born infants: a systematic review of the literature

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ABSTRACT

Background: Caesarean section and early exposure to antibiotics disrupt the developing gastrointestinal microbiome, which is associated with long-term health effects.

Objective: The aim of this systematic review was to summarise the impact of prebiotics, probiotics, or synbiotics supplementation on clinical health outcomes of term infants born by caesarean section or exposed to antibiotics in the first week of life.

Design: A systematic search was performed in Medline and Embase from inception to August 2021. Title and abstract screening (n=11,248), full text screening (n=48), and quality assessment were performed independently by two researchers.

Results: Six RCTs studying caesarean born infants were included, group sizes varied between 32-193 with in total 752 children. No studies regarding supplementation after neonatal antibiotic exposure were found. Three studies administered a probiotic, one a prebiotic, one a synbiotic, and one study investigated a prebiotic and synbiotic. Several significant effects were reported at follow-up varying between 10 days and 13 years: a decrease in atopic diseases (n=2 studies), higher immune response to tetanus and polio vaccinations (n=2), lower response to influenza vaccination (n=1), fewer infectious diseases (n=2), and less infantile colic (n=1), although results were inconsistent.

Conclusions: Supplementation of caesarean-born infants with prebiotics, probiotics, or synbiotics resulted in significant improvements in some health outcomes as well as vaccination responses. Due to the variety of studied products and the paucity of studies, no recommendations can be given yet on the routine application of prebiotics, probiotics, or synbiotics to improve health outcomes after caesarean section or neonatal antibiotic exposure.

INTRODUCTION

Early life is an important period as the infant's immune system is still developing (1). The development of the immune system is influenced by the gut microbiome (1), which develops rapidly after birth (2). Disruption of the developing gut microbiome (dysbiosis) due to environmental factors have been associated with adverse long-term health effects (3, 4).

Caesarean section (CS) is one of the main causes of aberrant microbiome development because it affects the diversity and colonization pattern of the gut microbiome (5-7). Due to reduced vertical mother-infant transmission of beneficial gut bacteria, the infant is predominantly colonized with bacteria from the skin, mouth, and hospital environment (8-14). This is associated with an altered immune development, a higher risk of childhood obesity, atopy, allergy, asthma, and type 1 diabetes mellitus (10,15,16).

Another important cause of early-life dysbiosis is antibiotic exposure (17-19). Antibiotics are the most frequently prescribed drugs for neonates in their first week of life (20, 21), but their effects on later health outcomes have not yet been fully elucidated. So far, a few observational studies have shown that infants exposed to antibiotics in their first week of life had an altered gut microbiota (22-25) and it was associated with an increased risk of wheezing (26-28), infantile colic (26), gastrointestinal disorders (29) impaired growth (22,30), allergies (31), allergic rhinitis (27), functional abdominal pain (32), and asthma (33,34).

Potential interventions to reduce some of these long-term effects of early life dysbiosis include supplementation with prebiotics, probiotics, or synbiotics. Prebiotics are nutrients that promote growth and activity of beneficial bacteria that already exist in the gut (35), probiotics are live microorganisms such as Bifidobacteria and Lactobacilli (13), and synbiotics are a combination of pro- and prebiotics (36). The aim of this systematic review was to summarise the impact of prebiotics, probiotics, or synbiotics supplementation on clinical health outcomes of term infants born by caesarean section or exposed to antibiotics in the first week of life.

METHODS

Literature search

This systematic review was conducted according to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (37). OVID Medline and Embase were systematically searched from inception to August 3, 2021. A multi stranded search approach comprised the following concept combinations:

([c section] OR ([antibiotic treatment] AND [first week of life] OR [first week antibiotics])) AND

- [pre- pro- synbiotics]

OR

- [dietary supplements] AND [microbiome]

OR

- [dietary supplements brands]

To reduce recall bias and enhance search results precision VOS-viewer was used to identify terms for NOTing out irrelevant records from databases searched (38,39). No other filters or limits were used (Appendix chapter 8).

Inclusion criteria

(1) study participants were term-born infants (born between 37 and 42 weeks of gestation) and born via caesarean section or exposed to antibiotics in the first week of life; (2) exposure to pre-, pro- or synbiotic dietary supplements administered within six weeks after birth; (3) clinical outcomes were reported; (4) study design was a randomised controlled trial (RCT).

Exclusion criteria

(1) including infants with major congenital malformations; (2) written in a language other than English; (3) animal studies; (4) for the caesarean-analyses: if a study includes both vaginally and caesarean-delivered infants and there were no subgroup analyses for only the caesarean-delivered infants

Data collection

After the search, all records were imported into Rayyan after deduplication (40). Two researchers (NC and KK) independently performed title and abstract screening, as well as full-text screening. After consensus about the included articles, relevant data were extracted by NC in consultation with the other co-authors. Odds ratios (ORs), 95% confidence intervals (95% CI) and P-values were included in the table if these were provided in the original articles. If both “per protocol” and “(modified) intention to treat” analyses were available, only the results from the “(modified) intention to treat” analysis were included.

Critical appraisal

To assess the risk of bias in the included articles, the Cochrane risk-of-bias tool for randomised controlled trials (RoB 2)(41) was used. The RoB 2 assesses the risk of bias in the studies in five domains (Table 1). The risk of bias was independently assessed by two researchers (NC and KK) and any discrepancies were discussed until a consensus was reached.

Data analyses

Due to the heterogeneity in the interventions and outcomes evaluated in this systematic review, it is not possible to synthesize data from these studies in a meta-analysis. Therefore, a descriptive synthesis of the data was performed.

RESULTS

Of the 14,632 records, 11,248 remained after removing duplicates. After title and abstract screening, 55 articles were read in full-text, and eight articles were included for analysis (see Figure 1).

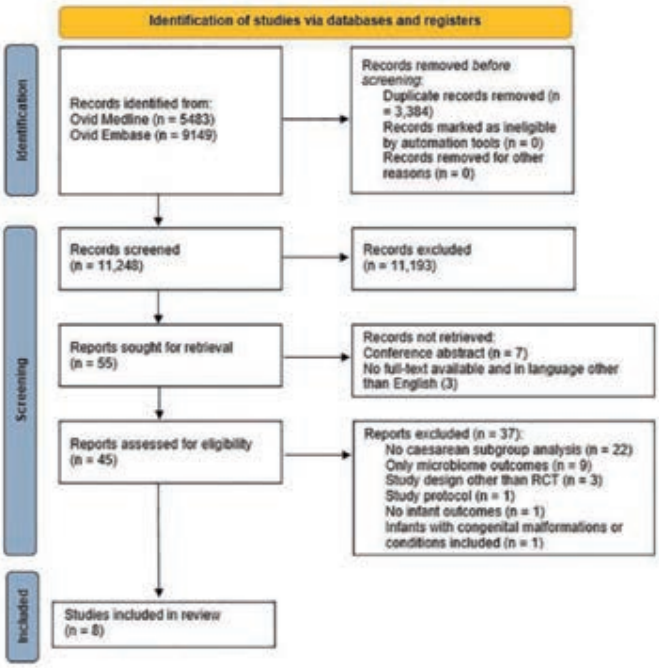

Figure 1. Flowchart showing article selection. Adapted from the PRISMA 2020 flow diagram.

Study characteristics

Eight articles were included, based on six RCTs (Figure 1), with a total of 752 children. Most studies scored a high risk of bias (Table 1). The characteristics of the included studies are summarised in Table 2. In all studies, supplementation was administrated to infants born by CS; no studies were found after antibiotics in the first week of life. The antibiotic policy for CS was not described in most studies, only Chua et al. (42) stated that infants born via CS were exposed

to intrapartum antibiotics prophylaxis. It is likely that in more studies caesarean-born infants were exposed to antibiotics in utero.

In three articles, based on the same study, the intervention was a probiotic mixture (43-45) (see Table 2)). In two other studies, the intervention group was also given a probiotic (46,47) and the interventions of the other three studies were prebiotics (48), synbiotics (49), and either pre- or synbiotics (42). All interventions were started within two weeks after birth, except for one study in which the intervention was started at six weeks of age (47). The intervention was administered for six months in most studies, except for two studies in which the intervention was continued until 12 weeks of age (47) or 16 weeks of age (42). In five RCT's, the intervention group was only compared with the placebo control group and not with the breastfed reference group for the clinical outcomes. Therefore, only the results between the intervention and the control groups are reported.

Table 1: Risk of bias of the included studies

First author	Domains of the Cochrane risk-of-bias tool for randomised controlled trials (RoB-2)					
	Domain 1	Domain 2	Domain 3	Domain 4	Domain 5	Total
Puccio[46]						
Chua[42]						
Kallio[43]						
Kuitunen[44]						
Peldan[45]						
Baglatzi[48]						
Cooper[49]						
Holscher[47]						

Domain 1: Risk of bias arising from the randomisation process; Domain 2: Risk of bias due to deviations from the intended interventions (effect of adhering to intervention); Domain 3: Missing outcome data; Domain 4: Risk of bias in measurement of the outcome; Domain 5: Risk of bias in selection of the reported result; Green: low risk of bias, yellow: some risk of bias, red: high risk of bias

If a study included both vaginally and caesarean-delivered infants and a subgroup analysis on the caesarean-delivered infants was performed, only the methods used for this relevant subgroup analyses were assessed

Table 2 general characteristics of the included studies

First author Country	Study period (year published)	# Participants[1]	AB or CS SG ?	Intervention	Control	Start duration intervention	Outcomes (relevant subgroup)	Follo w-up	Comments
Puccio[40] Italy & Belgium	2012-2015 (2017)	I 32 C 32 T 64	CS SG	Prebiotics: 2 HMOs (2'-fucosyllactose and lacto-N-neotetraose)	Control formula	0-14 D 6M	Colic, nighttime awakenings, bronchitis, LRTI	1, 2, 3, 4, 6, 12 M	Safety study, CS SG results are only reported if they are significant
Chua[42] Singapore & Thailand	2011-2013 (2017)	I 52 + 51 C 50 T 153	CS	Prebiotic (scGOS/lcFOS) or synbiotic (scGOS/lCFOS and *Bifidobacterium breve* M-16V)	Control formula	1-3 D 16W	Total faecal Bifidobacteria, *Bifidobacterium* species abundance, other members of the gut microbiota, pH, sc fatty acids, lactate, atopic dermatitis/eczema	3, 5 D 2, 4, 8, 12, 16, 22 W	Infants born via CS were also exposed to intrapartum AB prophylaxis*.
Kultunen[44] Finland	2000-2003 (2009,2017, 2019)	I 70 C 76 T 146	CS	Probiotic: *Lactobacillus rhamnosus* LC705, *Bifidobacterium breve* Bb99, *Propionibacterium freudenreichii* spp., *shermanii* JS	Placebo (micro-crystal line cellulose)	initiated prenatally (36 W gestation) + from birth 6M	(IgE-mediated) allergic disease, eczema, food allergy, asthma, allergic rhinitis, IgE sensitisation alone	5 Y	Infants were at risk for atopic diseases (at least one parent with asthma, allergic rhinitis or eczema)[2]. Peldan et al.: more use of probiotics after the study period in the intervention group
Peldan[45] Finland		I 69 C 75 T 144	SG				Allergic disease, eczema, allergic rhinitis, asthma, URTIs, LRTIs, AB use, food allergy	10 Y	
Kallio[43] Finland	2000-2003 (2009,2017, 2019	I 53 C 56 T 109		Probiotic: *Lactobacillus rhamnosus* LC705, *Bifidobacterium breve* Bb99, *Propionibacterium freudenreichii* spp., *shermanii* JS	Placebo (micro-crystalline cellulose)	initiated prenatally (36 W gestation) + from birth 6M	Allergic disease (doctor-diagnosed, ISAAC), sensitisation	13 Y	[2] and significant differences between sub- and main group: longer BF, older maternal age in CS SG, more use of prebiotics after 13 years in inter vention group
Baglatzi[46] Greece	2009-2011 (2016)	I 84 C 80 T 164	CS	Probiotic: regular dose of *Bifidobacterium lactis*	Low dose of *B. lactis*	Birth 6M	Diarrhoea, immune and gut maturation, microbiota, immune response to vaccines, anthropometry	12 M	No control group that was fed formula without pre/pro/ synbiotics
Cooper[49] South Africa	2008-2013 (2016)	I 92 C 101 T 193	CS SG	Synbiotic: BMOs (containing GOS and MOS such as 3'- and 6' sialyllactose) + *Bifidobacterium lactis* CNCM-I-3446	Control formula	Birth (≤3 D) 6M	Faecal (bifido)bacteria, anthropometrics, faecal pH, lean mass, fat mass and bone mineral content, digestive tolerance,immune parameters, HIV infection status, frequency of morbidity episodes	1 Y	The included infants all have HIV+ mothers and all mothers and infants received antiretroviral medication. Infants who tested positive for HIV were excluded
Holscher[47] USA	2007-2008 (2012)	I 16 C 16 T 32	CS SG	Probiotic: *Bifidobacterium animalis* subspecies lactis (Bb12)	Control formula	6 W 12W	Faecal sIgA, anti-rotavirus-specific IgA, faecal anti-poliovirus-specific IgA	12 W	

1 # participants in a subgroup, if applicable. CS Caesarean section, SG subgroup, I intervention, C controls, T total, BF breastfeeding, FF formula feeding, HMOs human milk oligosaccharides, (sc) GOS: (short chain) galactooligosaccharides, (lc) FOS: (long chain) fructooligosaccharides, Spp. several species, BMOs bovine milk oligosaccharides, MOS: milk oligosaccharides, D days, M months, W weeks, H hours, Y year, AB antibiotic, LRTI lower respiratory tract infection, URTI upper respiratory tract infection, ISAAC International study of asthma and allergies in childhood , HIV human immunodeficiency virus, sIgA secretory Immunoglobulin A. Outcome underlined was the primary outcome of the study. *The antibiotic policy for CS was not described in most studies, only Chua et al. (42) stated that infants born via CS were exposed to intrapartum antibiotics prophylaxis. It is likely that in more studies caesarean-born infants were exposed to antibiotics in utero

Atopic diseases

Four articles examined the effect of supplementation on atopy. Three articles (43-45) based on the same RCT evaluated the effect of a prenatally started probiotic supplement until six months of age on allergic disease in infants (n=146) at risk for atopic diseases at 5, 10 and 13 years of age. There was no significant difference between the intervention and control group for most outcomes regarding eczema, sensitisation, any allergic disease, and rhinitis until 13 years of follow-up (Table 3). The reported significant results were a decrease in IgE-associated eczema, and a positive (food) skin prick test (SPT) response and/or food-specific IgE >0.7 kU/L at 0-5 years of age in the intervention group (44). At 13 years of age, there was a significant decrease in eczema and any allergic disease experienced in the last 12 months, based on the ISAAC questionnaire (43,50). The study by Chua et al. (42) examined the effect of a prebiotic and a synbiotic supplementation administrated until 16 weeks of age (n=153). In post-hoc analyses, fewer skin disorders and atopic dermatitis/eczema were found in the synbiotic group, but not in the prebiotic group compared to the control group at 22 weeks.

Infectious diseases

Two studies (45,48) examined the effects of prebiotic (48) or synbiotic (45) supplementation in the first six months of life on infectious diseases. Puccio et al. found that infants (n=64) in the prebiotic intervention group had a lower risk of lower respiratory infection at six months OR 0.17 (95% CI 0.02-0.96), or 12 months OR 0.21 (95% CI 0.04-0.83) or bronchitis at 12 months OR 0.06 (95% CI 0.00-0.50) than those in the control group (48). Peldan et al. found after 5-10 years follow-up (n=144) that the probiotic intervention was associated with a reduced risk of receiving antibiotics over the past five years OR 3.19 (95% CI 1.02-9.97) and a lower risk of having four or more upper respiratory infections in one year 0.29 (95% CI 0.12-0.72) (45).

Gastrointestinal effects

Three articles assessed the effect of a prebiotic (48), probiotic (46), and a synbiotic (49) supplementation in the first six months of life on diarrhea (46), stool pattern (49) and colic (48) in the first year of life. Cooper et al. found up to six months of age, more liquid stools and fewer formed and hard stools were reported in the probiotic group compared to the control group (n=193) (49). Baglatzi et al. (n=164) found no differences in diarrhoea during the first year (46). Puccio et al. (n=64) found a significantly lower incidence of parent-reported infantile colic at four months of age in the intervention group, which was collected in a diary with the options ‘‘never,’’ ‘‘sometimes,’’ and ‘‘often’’.

Table 3: Clinical outcomes

First author	Intervention		Participants	Significant outcomes				Non-significant outcomes	Comments
	Type	Start Period		Outcome	Reply/specific outcome	Time point	P-value OR (95% CI)		
				Allergic disease					
Chua[42]	Prebiotic (scGOS/lcFOS)	1-3 D 16 W	I 52 C 50 T 102	-	-	22 W	- -	1. All skin disorders at 22 W 2. Atopic dermatitis/eczema at 22W	Post-hoc analysis
	Synbiotic (scGOS/lCFOS and *B. breve* M-16V)	1-3 D 16 W	I 51 C 50 T 101	Skin disorders	All skin disorders Atopic dermatitis/eczema	22 W 22 W	P=0.017 - P=0.037 -	-	
Kuitunen [44]	Probiotic: *L. rhamnosus* LC705, *B. breve* Bb99, *P. freudenreichii* spp., *shermanii* JS	36 W gestation + from birth 6 M	I 70 C 76 T 146	Allergic disease	Positive SPT response	0-5 Y	P<0.05 OR 0.47 (0.23 - 0.96)	1. Positive SPT response at 0-2Y, specific IgE >0.7 kU/L at 0-5Y 2. Positive SPT response and/or specific IgE >0.7 kU/L at 0-2 and 0-5 Y 3. all eczema at 0-2 and 0-5Y, IgE-associated eczema at 0-2Y, IgE-associated asthma and rhinitis at 0-2 and 0-5 Y	-
					Eczema: IgE-associated	0-5 Y	P<0.05 OR 0.43 (0.19 - 0.95)		
				Sensitisa-tion	Positive food SPT response and/or food-specific IgE >0.7 kU/L	0-5 Y	P <0.05 OR 0.33 (0.12 - 0.85)		
Peldan[45]	Probiotic: *L. rhamnosus* LC705, *B. breve* Bb99, *P. freudenreichii* spp., *shermanii* JS	36 W gestation + from birth 6 M	I 69 C 75 T 144	-	-	-	- -	ISAAC + doctor-diagnosed: 1. Any allergic disease 2. Eczema 3. Allergic rhinitis 4. Asthma 5. Doctor-diagnosed food allergy	Allergic rhinitis was significantly decreased in the intervention group in the unadjusted OR
Kallio[46]	Probiotic: *L. rhamnosus* LC705, *B. breve* Bb99, *P. freudenreichii* spp., *shermanii* JS	36 W gestation + from birth 6 M	I 53 C 56 T 109	Allergic disease (ISAAC, last 12 M)	Allergic disease	13 Y	P=0.006 0.336 (0.154 - 0.736)	1. Allergic disease: any/specific IgE >0.7 kU/L 2. Sensitization: IgE >0.7/>0.35 kU/L 3. Doctor-diagnosed allergy 4. ISAAC-diagnosed allergic disease in last 12 M: allergic disease, specific IgE >0.7 kU/L, IgE-associated eczema, asthma and rhinitis	Holm method was used to adjust for multiple comparisons
					Eczema		P=0.031 0.388 (0.162 - 0.930)		

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Table 3 continued

First author	Type	Start Period	Participants	Outcome	Reply/specific outcome	Time point	P-value OR (95% CI)	Non-significant outcomes	Comments
Infectious diseases									
Puccio[48]	Prebiotics: 2 HMOs (2'-fucosyllactose and lacto-N-neotetraose)	0-14 D 6 M	I 32 C 32 T 64	LRTI	Any	6 M	P=0.043 OR 0.17 (0.02 - 0.96)	-	Unclear if other analyses were performed
					Any	12 M	P=0.022 OR 0.21 (0.04 - 0.83)		
				Bronchitis	Any	12 M	P=0.003 OR 0.06 (0.00 - 0.50)		
Peldan[45]	Synbiotic: *L. rhamnosus* LC705, *B. breve* Bb99, *P. freudenreichii* spp., *shermanii* JS and GOS	36 W gestation + from birth 6 M	I 69 C 75 T 144	No AB	During last 5 years	5-10 Y	P=0.046 OR 3.19 (1.02 - 9.97	1. LRTI, ≥1/5 years	ORs are adjusted ORs
				URTI	≥4/year	5-10 Y	P=0.004 OR 0.29 (0.12-0.72)		
Gastrointestinal effects									
Puccio[48]	Prebiotics: 2 HMOs (2'-fucosyllactose and lacto-N-neotetraose)	0-14 D 6 M	I 32 C 32 T 64	Colic	↓ Colic reported	4 M	P=0.035 -	-	Unclear if other analyses were performed
Baglatzi[46]	Probiotic: regular dose of *B. lactis* (vs. low dose)	Birth 6 M	I 84 C 80 T 164	-	-	1 Y	- -	1. Diarrhoea: prevalence, incidence and days	high dose considered intervention, low dose considered control
Cooper[44]	Synbiotic: BMOs (containing GOS and MOS such as 3'- and 6' sialyllactose) + *B. lactis* CNCM-I-3446	Birth (≤3 D) 6 M	I 92 C 101 T 193	Stool consistency	↑ Liquid	10 D, 4 W, 6 W, 3 M, 4 M, 6 M	P<0.001	1. Frequency of daily stools at 3 D, 10 D, 28 D and 3 M 2. Frequencies of flatulence, spitting up, vomiting, crying, fussing, or colic at 10 D, 28 D, 6 W, 3 M and 4 M	-
					↓ days (mean %) in which hard stool was reported		P=0.001 -		
					↓ Proportion of days with formed stools		P=0.045 -		
Anthropometrics									
Baglatzi[46]	Probiotic: regular dose of *B. lactis* (vs. low dose)	Birth 6 M	I 84 C 80 T 164	-	-	1 M, 4 M, 12 M	- -	1. Weight-for-age, length-for-age, BMI-for-age and head-circumference-for-age at 1 M, 4 M and 12 M	high dose considered intervention, low dose considered control
Cooper[44]	Synbiotic: BMOs (containing GOS and MOS such as 3'- and 6' sialyllactose) + *B. lactis* CNCM-I-3446	Birth (≤3 D) 6 M	I 92 C 101 T 193	Daily weight gain	Between 10 D and 4 M (mean)	4 M	P=0.010 (Non- inferiority p-value) -	1. Weight-for-age, length-for-age, BMI-for-age and head-circumference-for-age at 10 D, 4 W, 6 W, 3 M, 4 M and 6 M 2. Fat mass and percentage mean fat mass at 4 M and 12 M	Body weight was adjusted for baseline value and sex Non-inferiority analysis

Table 3 continued

First author	Type	Start Period	Participants [1]	Outcome	Reply/specific outcome	Time point	P-value OR (95% CI)	Non-significant outcomes	Comments
					Behaviour				
Puccio[48]	Prebiotics: 2 HMOs (2'-fucosyllactose and lacto-N-neotetraose)	0-14 D 6 M	I 32 C 32 T 64	Nighttime awakenings	↓	2 M	P=0.036 -	-	Unclear if other analyses were performed
					Vaccination response				
Baglatzi[46]	Probiotic: regular dose of *B. lactis* (vs. low dose)	Birth 6 M	I 84 C 80 T 164	Immune responses to vaccinations	↑ Response to Tetanus IU/mL	12 M	P=0.0411 -	1. Immune responses to vaccination at 7 M and 1 Y: diphteria, B. pertussis, tetanus, polio, infants who reached anti-HIB protective antibody over the 12 M	high dose considered intervention, low dose considered control
					↓ Response to H. influenza B µg/mL		P=0.0186 -		
Cooper[49]	Synbiotic: BMOs (containing GOS and MOS such as 3'- and 6' sialyllactose) + *B. lactis* CNCM-I-3446	Birth (≤3 D) 6 M	I 92 C 101 T 193	-	-	6 W, 4 M, 12 M	- -	1. Immune measurements: positive anti-hepatitis B IgG antibody response	-
Holscher[47]	Probiotic: *B. animalis* subspecies lactis (Bb12)	6 W 12 W	I 16 C 16 T 32	Immune responses to vaccinations	↑ anti-polio-specific IgA (U/g) after vaccination	12 W compared to 8 W	P=0.026	1. Change in anti-rotavirus-specific IgA after vaccination between 8 W and 12 W	-

1 # participants in a subgroup, if applicable. I intervention, C controls, T total, CS Caesarean section, SG subgroup, OR odds ratio, HMOs human milk oligosaccharides, (sc) GOS: (short chain) galactooligosaccharides, (lc) FOS: (long chain) fructooligosaccharides, BMOs bovine milk oligosaccharides, MOS: milk oligosaccharides, SPT skin prick test, ISAAC International study of asthma and allergies in childhood, D days, M months, W weeks, H hours, Y year, AB antibiotic, LRTI lower respiratory tract infection, URTI upper respiratory tract infection. Outcome underlined was the primary outcome of the study.

Anthropometrics

Two studies (46,49) examined the effect of a probiotic (n=164) (46) and synbiotic (49) supplement (n=193) on anthropometric measurements during the first year of life. Both studies found no differences in anthropometric measurements including weight-for-age, length-for-age, BMI-for-age, head-circumference-for-age and fat mass between intervention and control group infants (46, 49).

Behaviour

Puccio et al. (48) found significantly fewer parent-reported night time awakenings at two months in the prebiotic group compared to the placebo group (n=64) (48). Parents reported these awakenings as ‘‘never,’’ ‘‘sometimes,’’ and ‘‘often’’. The difference did not persist after two months of age.

Immune response

Three studies (46,47,49) investigated the effect of a probiotic (46,47) or a synbiotic (49) supplement on the infants' immune system. Holscher et al. (47) found after probiotic supplementation between six and twelve weeks of age a significantly higher increase in anti-polio-specific IgA after vaccination at twelf weeks compared to eight weeks (n=32). Baglatzi et al. (46) found after six months of probiotic supplementation a significantly higher immune response to tetanus vaccinations (n=164), but a lower immune response to H. influenza B vaccinations at twelf months for the regular dose group compared to the low dose group. In contrast to Holscher at al. (47), no significant differences in immune response to polio vaccinations was found (46). Cooper et al. (49) found no significant differences after synbiotic supplementation (n=193).

Safety

All the included studied reported safety in terms of growth and gastrointestinal tolerance and none noted significant differences in these parameters or in the number of adverse events between the intervention and the control group.

DISCUSSION

This systematic review on the clinical effects of pre-, pro- or synbiotic supplementation after CS or antibiotic exposure in the first week of life shows several significant differences in clinical outcomes. The reported effects consisted of a decrease in atopic diseases, fewer infectious diseases, and difference in immune response to vaccinations. The results with regard to immune response to vaccinations were, however, inconsistent and only shown in CS born children. No studies were found regarding the effects of pre-, pro- or synbiotics supplementation on clinical outcomes after neonatal antibiotic treatment.

Only one RCT was included in this review in which allergy was the primary outcome (44). It showed some promising results of probiotics for CS born children in a post-hoc analysis, but not for vaginally born children (43,44). Both this RCT and the study of Chua et al. (42) showed that caesarean-born children in the intervention group had less eczema. The mechanisms behind the prevention of eczema following probiotics stem from the hygiene hypothesis, where early exposure to gut microbes directs the immune system away from a Th-2 skew (51) or upregulates Tk1-cytokine production (52). The protective effects of prebiotics may be by promoting bacterial growth of by immunomodulatory effects (52). Eczema in early life is an important risk factor itself for later allergy development (53), probably due to epicutaneous sensitization. We hypothesize that if pre-, pro- or synbiotic administration reduce the incidence of eczema, these

children may have less atopic diseases later in life. Adequately powered studies on the effect of probiotic supplementation in children born following CS are needed to confirm this hypothesis.

Two other included studies in this systematic review support the results that supplementation promotes the development of a healthier immune system in caesarean-born infants. Both studies found fewer infectious diseases in the caesarean-born intervention group (45,48). These studies also showed that the differences between the intervention and control groups persisted even after the intervention period. The potential immune modulation of the intervention can be long lasting; meaning that early supplementation can support the immune system to protect against later infectious diseases as found by Peldan et al. (45) after 5-10 years of follow-up. As the follow-up of one year in the study of Puccio et al. (48) was however relatively short, more studies with longer follow up are required to confirm these promising results

Two of the three studies on immune response to vaccinations after probiotic supplementation found significant effects (46, 47). The immune response to vaccination is a valuable marker reflecting the development of the responsiveness of the immune system to foreign antigens (54,55). These immunological benefits may be due to an enriched Bifidobacterium population in the gut microbiome. In the literature, an association has been found between reduced abundance of Bifidobacterial species and immune disorders such as pathogenic infections, and allergies (56,57). Furthermore, an aberrant gut microbiome development has been observed in preterm infants, infants born by CS and after antibiotic exposure in early life, which are all characterized by reduced abundance of Bifidobacterium species (58,59). Supplementation of a Bifidobacterium probiotic in caesarean-born infants may therefore contribute to a shift in the gut microbiome towards that of vaginally delivered infants, resulting in immunological benefits. However, more studies on the effect of probiotics are needed.

One of the strengths of this review is that, to our knowledge, this is the first review examining the clinical effects of pre-, pro- and synbiotics rather than microbiome differences whose clinical effect is still unclear in caesarean-born infants or infants exposed to antibiotics in the first week of life. One systematic review has recently been published about the effects of probiotics, prebiotics and synbiotics on the microbiome of children born via CS (60). However, no clinical outcome measures were reported in this review, which is the ultimate goal for optimizing health in children born following CS or after antibiotic exposure in the first week of life. Furthermore, all full-texts were studied to see if any subgroup analyses of caesarean-born infants were performed, even if this was not explicitly stated in the title or abstract. As a result, only articles that performed analyses on caesarean-born infants were included, and not articles that only analysed the total group of participants, including vaginally born infants.

The main limitation of this review is that nearly all studies were not powered for the clinical outcomes. In most studies, the outcomes for the caesarean-born infants resulted from a subgroup analysis. Moreover, many articles did not adjust for multiple testing, which may have resulted in false positive results. In addition, six of the eight studies scored a high risk of bias, and the included studies were very heterogeneous with regard to the type of supplement studied, the start and duration of the supplementation and the outcome measures. It was therefore not possible to perform a meta-analysis. Furthermore, in the included studies the intervention groups were compared with control groups who received a placebo and, except for one study, not with a "gold standard": the reference groups of vaginally born and/or breastfed infants that were included in some of the articles. Finally, the follow-up durations of most studies were only one year or less and are therefore too short to investigate the long-term effects.

For future research, several recommendations can be made. Studies need to be adequately powered on clinical outcome measures to investigate the effect of the supplementation. The clinical outcomes of interest, where changes could be expected based on the literature, are infections, type 1 diabetes, obesity, and atopic diseases such as eczema, allergy, and asthma. These outcome measures need adequate follow-up time. More studies with the same supplement are needed in order to advocate a specific supplement.

CONCLUSIONS

Supplementation of pre-, pro or synbiotics to infants delivered by caesarean section may result in significant improvements in various health outcomes. However, the results were sometimes contradictory or only found in a limited number of studies, and most studies were not adequately powered for the clinical outcome measures. Currently, no studies have been performed examining the effect of supplementation after antibiotic exposure in the first week of life. Due to the variety of study products and the lack of studies, to date no recommendations can be made on how to influence the gut microbiome to improve health outcomes in infants born by caesarean section or with antibiotic exposure in the first week of their life.

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Chapter 10

Predictive factors for allergy at 4-6 years of age based on machine learning: a pilot study

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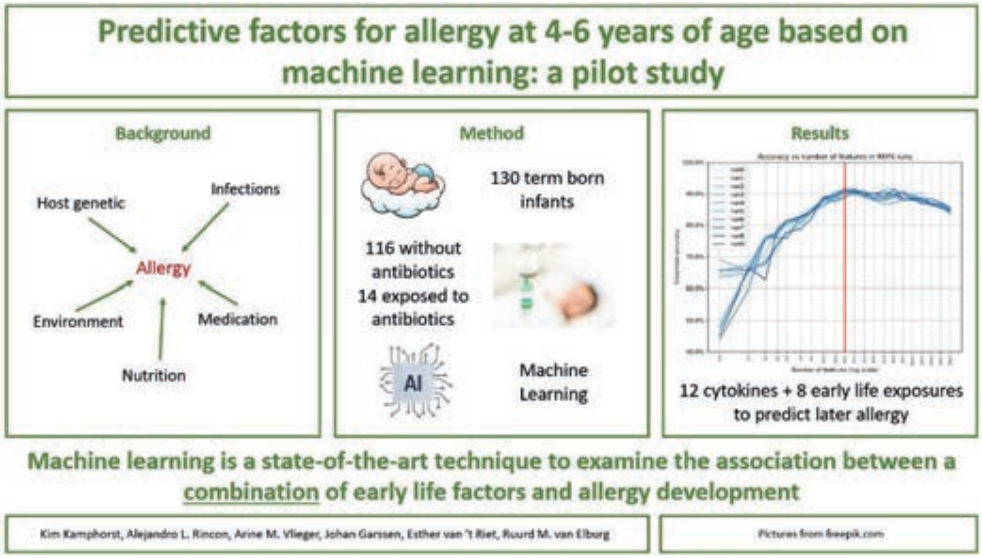
ABSTRACT

Background: In Europe, allergic diseases are the most common chronic childhood illnesses and the result of a complex interplay between genetics and environmental factors. A new approach for analyzing this complex data is to employ machine learning (ML) algorithms. Therefore, the aim of this pilot study was to find predictors for the presence of parental-reported allergy at 4-6 years of age by using feature selection in ML.

Methods: A recursive ensemble feature selection (REFS) was used, with a 20% step reduction and with eight different classifiers in the ensemble, and resampling given the class unbalance. Thereafter, the Receiver Operating Characteristic Curves for five different classifiers, not included in the original ensemble feature selection technique, were calculated.

Results: In total, 130 children (14 with and 116 without parental-reported allergy) and 248 features were included in the ML analyses. The REFS algorithm showed a result of 20 features and particularly, the Multi-layer Perceptron Classifier had an area under the curve (AUC) of 0.86 (SD 0.08). The features predictive for allergy were: tobacco exposure during pregnancy, atopic parents, gestational age, days of: diarrhea, cough, rash, and fever during first year of life, ever being exposed to antibiotics, Resistin, IL-27, MMP9, CXCL8, CCL13, Vimentin, IL-4, CCL22, GAL1, IL-6, LIGHT, and GMCSF.

Conclusions: This ML model shows that a combination of environmental exposures and cytokines can predict later allergy with an AUC of 0.86 despite the small sample size. In the future, our ML model still needs to be externally validated.



INTRODUCTION

In most European countries, allergic diseases are the most common chronic childhood illnesses [1]. Allergic diseases are the result of a complex interplay between genetics and environmental factors [2]. So far, allergic diseases have been associated with a variety of early life events such as: infections, nutrition, and exposure to medication [3]. However, most univariate/multivariate analysis techniques currently in use are not sufficient to study associations in a large dataset because of the complex interplay of all these factors [4]. A new approach for analyzing this complex data is to employ machine learning (ML) algorithms.

ML finds patterns in the data and applies these patterns to new data to make predictions [5]. Within the INCA study (INtestinal microbiota Composition after Antibiotic treatment in early life), a Dutch prospective birth cohort study, a large dataset has been built that incorporates various early life exposures/characteristics. Previously, we showed that antibiotic exposure in the first week of life was associated with allergy at 4-6 years of age [6]. The aim of this pilot study was to find predictors for the presence of parental-reported allergy at 4-6 years of age by using feature selection in ML.

MATERIALS AND METHODS

Study design and data collection

The study design and the data collection of the prospective birth-cohort INCA study has been previously described [7]. In short, between 2012 and 2015, 436 infants born at term were recruited from maternity and neonatal wards of four teaching hospitals in the Netherlands. The method of collection and the collected variables are presented in Table 1. Around one year of age, a blood sample was obtained if parents gave additional informed consent (n=149). The serum samples were aliquoted after centrifugation and stored at −80◦C until further use. The method of analyzing the immune markers with a multiplex immunoassay and the measurement of the IgE antibodies and the radioallergosorbent test (RAST) have been described elsewhere [8, 9]. At one year of age, doctors' diagnoses were requested using the general practitioner electronic medical database based on the International Classification of Primary Care (ICPC) [10]. Between May 2018 and May 2019, at 4-6 years of age depending on the year of inclusion, parents of the included children were approached to complete an online questionnaire. In this questionnaire, information about parental-reported allergies (food, drug, insect venom, inhalation or contact allergy) was collected.

Table 1 data collection of variables used in the machine learning model

Source	Time of collection	Variables
Hospital records	First week of life	Sex, birth mode, gestational age in days, birth weight, exposure to antibiotics, antibiotic type and duration, and hospital of birth
Parental questionnaire	At inclusion	Tobacco exposure during pregnancy, presence of siblings, birth order, presence of pets, atopy in the family, household education, parental country of birth, and living environment.
A daily checklist	During the first year of life	Start and duration of: wheezing, eczema, infantile colic, rash, fever, cough, runny nose, otalgia/otorrhea, diarrhea, daycare, and exposure to tobacco smoke
A monthly questionnaire	During the first year of life	Weight, length, type of feeding, duration of (exclusively) breastfeeding, presence of pets, number and type of additional antibiotic courses, general practioner visits, and probiotic use
doctors' diagnoses (general practitioner) based on the International Classification of Primary Care (ICPC)	At one year of age	A14 (infantile colic), D10 (vomiting), D70 (infectious diarrhea), D73 (susceptible gastrointestinal infection), R02 (dyspnea), R03 (wheezing), R05 (cough), R96 (asthma like symptoms), R74_R74.1_R74.01 (acute respiratory tract infection (RTI)), R78 (acute bronchiolitis), R81 (pneumonia), R21 (symptoms of the throat), R25 (abnormal sputum), R27 (concern about respiratory illness), R77 (acute laryngitis), R83 (other RTI), R88 (influenza), R99 (other RT diseases), S21 (other symptoms/ complaints of the skin), S21.01 (dry skin/flaking), S87 (eczema).
Blood sample in a subgroup	At one year of age	IL1RA, IL1b, IL4, IL5, IL6, IL10, IL12, IL13, IL17, IL17F, IL18, IL21, IL22, IL25, IL26, IL27, IL31, IL33, IL37, TNFa, IFNa, IFNg, TSLP, LIGHT, APRIL, MIF, TGFb, YKL40, CCL2, CCL7, CCL8, CCL11, CCL13, CCL17, CCL18, CCL22, CCL25, CCL26, CCL27, CCL28, CXCL4, CXCL8, CXCL9, CXCL10, CXCL11, CXCL13, GMCSF, VEGF, BDNF, sICAM1, sVCAM1, sCD14, sCD19, sCD25, sCD27, sCD40L, IL1R1, IL1R2, TNFR1, TNFR2, sIL6R, sIL7Ra, sVEGFR1, Gal1, Gal3, Gal7, Gal9, Eselectin, S100A8, HSP70, SAA1, MMP9, TIMP1, Apelin, Vimentin, TPO, Adipsin, Resistin, RBP4, Adiponectin, CRP, Leptin, and Chemerin, IgE, and the radioallergosorbent test (RAST) for: dust mite, cat, dog, hence egg, cow's milk, peanut, and grass pollen mix.
Parental questionnaire	4-6 years of age	Presence of allergies (food, drug, insect venom, inhalation or contact allergy), length, weight, exposure to antibiotics, and exposure to antibiotics and number of courses before two years of age. Information on conditions related to upper respiratory tract infection, pseudocroup, and ear nose and throat (ENT) disorders including treatments and the treating specialist were retrieved.
doctors' diagnoses (general practitioner) based on the ICPC	4-6 years of age	The same doctors' diagnoses as at one year of age were requested, supplemented with: D01 (generalized abdominal pain), D06 (other localized abdominal pain), D12 (obstipation), H71 (otitis media acute), H72 (otitis media with effusion), R08 (other nose symptoms/complaints), and R90 (hypertrophy/chronic infection tonsils/adenoid).

Ethics

Both parents of all participating children gave informed consent. At 4-6 years of age, doctors' diagnoses were only collected after additional informed consent. The study was approved by the ethical board of the St. Antonius Hospital in Nieuwegein, the Netherlands and was registered in the clinical trials register as NCT02536560.

Data analyses with machine learning

In order to predict allergy from early life exposures/characteristics, it is necessary to select the characteristics that can optimally distinguish between allergic children and healthy not allergic children. In this sense, popular approaches used for feature selection range from univariate statistical considerations, to iterated runs of the same classifier with an increasingly reduced number of features to assess the contribution of the features to the overall result [11, 12]. Because allergy is multifactorial and therefore very complex, it is not sufficient to rely on simple statistical analyses. In addition, features extracted using an iterative method on one classifier are likely to work well only for that specific classifier. Following the idea behind ensemble feature selection [11], multiple algorithms should be used to obtain a more robust and general predictive performance. An ensemble approach has the advantage of obtaining features that are effective across several classifiers, with a better likelihood of being more representative of the data, and not just of the inner workings of a single classifier. For this study, a set of classifiers was trained to extract a sorted list of the most relevant features from each. Features were only included if there was less than 10% missing data. Three features had more than 10% missing data and were therefore excluded: duration of rupture of membranes, GBS status of the mother, and exposure to antibiotics intrapartum. For the features with less than 10% missing data, the missing data was imputated with the mean or median for the continuous variables, depending on the distribution of the data, or with the mode for nominal and ordinal variables.

To find the most important features, a recursive ensemble feature selection (REFS) was used, with a 20% step reduction and with eight different classifiers in the ensemble [13], and resampling given the class unbalance. The classifiers are from the scikit-learntoolbox [14]: Gradient Boosting [15], Random Forest [16], Logistic Regression [17], Passive Aggressive [18], Stochastic Gradient Descent [19], Support Vector Machine [20], Ridge [21] and Bagging [22]. Each classifier ranks the features independently and then the features with the highest ranking were chosen with a reduction step of 20%. Next, the best set of features was selected with the highest average accuracy in ten independent runs in a nested cross-validation scheme [23]. In each run, the accuracy at each step is calculated in a 10-fold cross-validation. Finally, to test the results the Receiver Operating Characteristic (ROC) curve [24] was calculated in five classifiers

that were not part of the ensemble to assure no overfitting, namely: Adaboost [25], Extra Trees [26], K-Neighbours [27], LASSO [28] and Multi-layer Perceptron (MLP) [29] classifier.

RESULTS AND DISCUSSION

From 149 children with a blood sample at one year of age, 130 (87%) children had complete follow-up until the age 4-6 years. These 130 children (14 with and 116 without parental-reported allergy) and 248 features were included in the ML analyses.

The recursive ensemble feature selection (REFS) algorithm gets a result of 20 features as shown in Figure 1 in a stratified 10-fold cross validation scheme with oversampling.

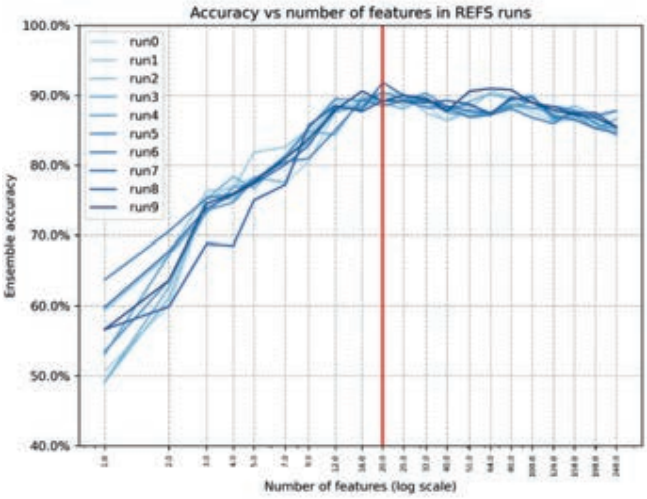
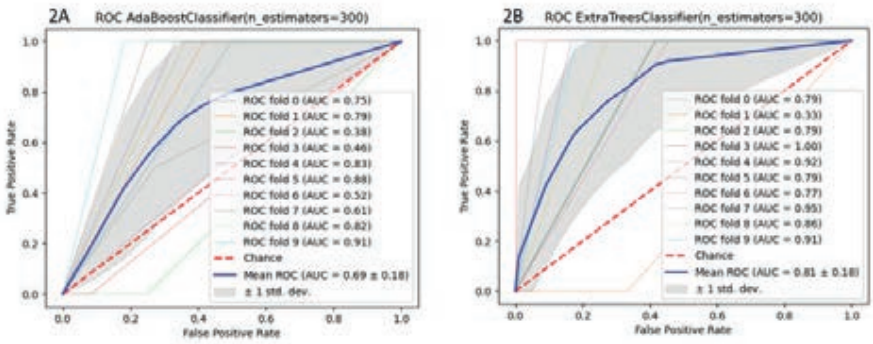


Figure 1 10 Runs of the recursive ensemble feature selection (REFS) algorithm. The best number of features is 20.

Next, the Receiver Operating Characteristic Curves (ROC) for five different classifiers, not included in the original ensemble feature selection technique, were calculated (Figure 2).



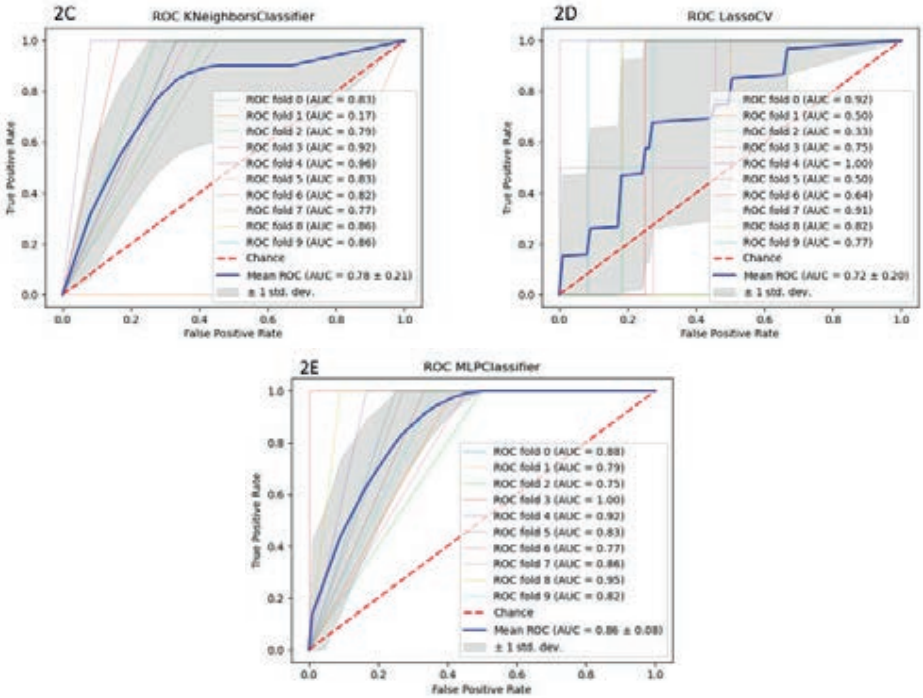


Figure 2A-E the Receiver Operating Characteristics (ROC) curves for five different classifiers, that were not included in the original ensemble feature selection technique: 2A=Adaboost, 2B=Extra Trees, 2C=K-Neighbours, 2D=LASSO and 2E=Multi-layer Perceptron classifier.

Particularly, the Multi-layer Perceptron Classifier showed an area under the curve (AUC) of 0.86 (standard deviation 0.08) which is considered as “very good” in diagnostic accuracy.

The resulting features that are predictive for allergy at 4-6 years of age, and presented in Figure 3, were: tobacco exposure during pregnancy, atopic parents, gestational age in days, days of: diarrhea, cough, rash, and fever during first year of life, and ever being exposed to antibiotics. The included immune mediators/cytokines determined at one year of age were: Resistin, Interleukin 27, Matrix metallopeptidase 9, C-X-C Motif Chemokine Ligand 8, C-C Motif Chemokine Ligand 13, Vimentin, Interleukine-4, C-C motif chemokine 22, Galactokinase 1, Interleukine 6, levels of tumor necrosis factor superfamily 14, and Granulocyte-macrophage colony-stimulating factor.

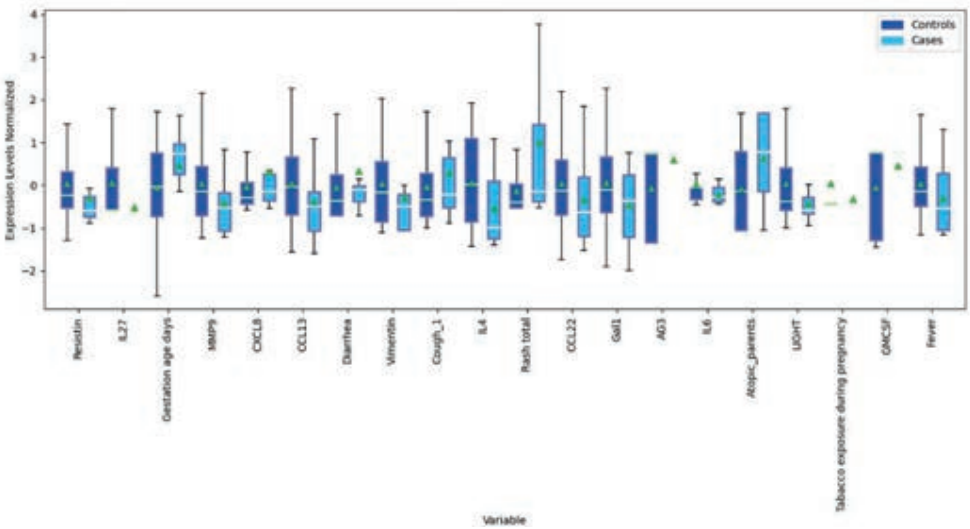


Figure 3 boxplot of the included features. IL-27=Interleukin 27*, MMP9=Matrix metallopeptidase 9*, CCL13=C-C Motif Chemokine Ligand 13*, Diarrhea=days of diarrhea during the first year of life, Cough_1=days of cough during the first year of life, IL-4=Interleukine 4*, Rash total=days of rash during the first year of life, CCL22=C-C motif chemokine 22*, Gal 1=Galactokinase 1*, AG3=ever being exposed to antibiotics, IL-6=Interleukin 6*, Atopic_Parents=the presence of atopic parents, LIGHT=levels of tumor necrosis factor superfamily 14*, GMCSF=Granulocyte-macrophage colony-stimulating factor*, Fever=days of fever during the first year of life. *determined at one year of age.

The use of ML to analyze the complex interplay between early life variables in this large prospectively collected dataset is an important strength of this study. Since ML-based models have a better predictive capacity than statistical-based models, they should have a more important role in future prediction studies, but this requires specific expertise from experts in bioinformatics. Limitations of the study were that we had only 14 children with a parental-reported allergy in our cohort. The results should therefore be interpreted as a pilot. We did not validate the model externally. Moreover, only parental-diagnosed allergy could be used as an outcome measure, because the doctor-diagnosed allergy group was even smaller.

CONCLUSIONS

We used a state-of-the-art technique to examine the combination of early life factors and later allergy development using ML. Our pilot study shows that an ML-model with 20 exposures/characteristics in early life can predict later allergy with an AUC of 0.86 despite the small sample size. This model emphasizes the importance of early life, since almost all included variables occur in the first year of life. In the future, our ML model still needs to be externally validated in a larger doctor diagnosed allergy dataset.

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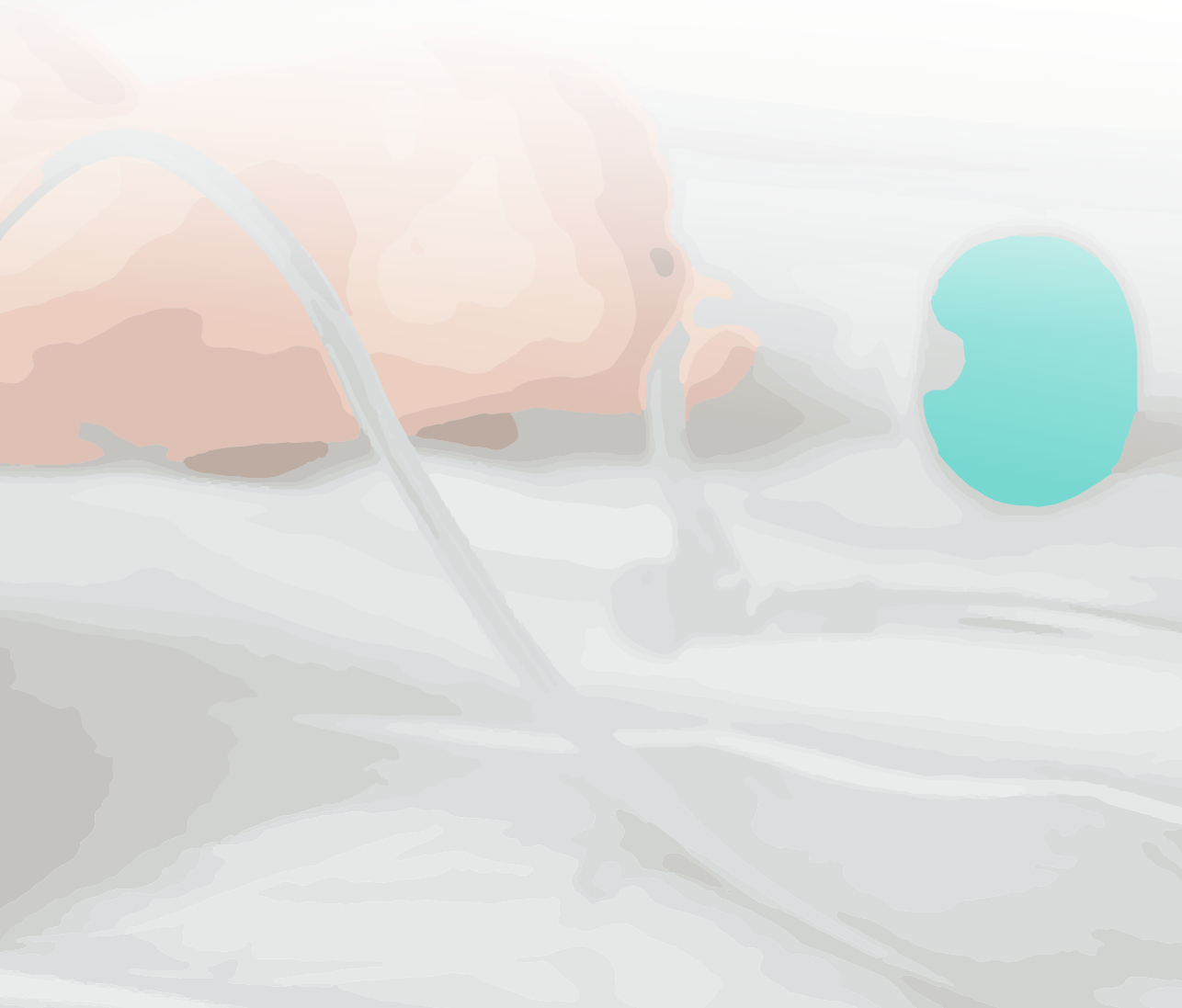
Chapter 11

General discussion and future perspectives



GENERAL DISCUSSION AND FUTURE PERSPECTIVES

This thesis aimed to provide insight into the association between neonatal antibiotics and their effects on the developing immune system and long-term health. In addition, its impact on the composition of the gut microbiome and the extent and duration of dysbiosis after antibiotic exposure were investigated. The INCA study showed associations between antibiotics in the first week of life and impaired growth, allergies, functional abdominal pain, and alterations in the gut microbiome. In conclusion, antibiotics in the first week of life can have long-term consequences for health in later life.

The outcomes of the INCA study emphasize the importance of the first week of life for later health and the need for well-considered decisions in the antibiotic policy of newborns. Although antibiotics are the most frequently prescribed drugs for neonates in the first week of life (1, 2), the effects of exposure in this specific timeframe (when the microbiome and the immune system are still developing) have been studied poorly. Only a few observational studies have shown that infants exposed to antibiotics in the first week of life had an altered gut microbiota (3, 4), and an increased risk of impaired growth (3), wheezing (5, 6), gastrointestinal disorders (7), allergic rhinitis (5), and asthma (8, 9). Some of these findings will be discussed in more detail.

Antibiotics and growth

In the INCA study, we found less weight and length gain in the first year of life after treatment with antibiotics for suspected early-onset sepsis. These results are partially in line with Uzan-Yulzari et al., who observed impaired growth in boys until six years of age after antibiotic exposure in the first week of life (3). It is known that minor impairments in early life growth patterns may impact later growth and health, such as shorter adult height, lower attained schooling, reduced adult income, and several chronic diseases such as cardiovascular disorders and diabetes (10). Interestingly, when antibiotics were administered after the first week of life, we found that they were associated with an increase in weight gain, which is in line with systematic reviews showing associations between antibiotic treatment in the first two years of life and childhood obesity (11, 12). Remarkably, none of the studies included in the reviews evaluated the association between neonatal antibiotics and growth. There are several possible explanations for the differences in growth after antibiotic exposure, as found in the INCA study.

Firstly, antibiotic-induced perturbations on the developing intestinal microbiome may not be uniform over time. It is known that the microbiome and immune system rapidly change in the first weeks of life (13, 14). The neonatal intestine is considered almost sterile at birth and becomes colonized with the first microbes in a highly dynamic process during the adaptation to

postnatal life (15). Delayed maturation of the gut microbiome in malnourished children, for example, appears to be causally related to their growth restriction (16). At later exposures, the microbiome composition is already more developed, and then reduced numbers of Bifidobacteria are particularly associated with the development of overweight and obesity (17, 18). Antibiotics given shortly after birth may have a different impact on the developing microbiome and thus with the association with growth than later exposures.

Secondly, the route of administration (parenteral vs. enteral) can play a role in the association with antibiotics. In the first week of life, antibiotics are administrated intravenously, while antibiotic treatment later in the first year of life is mainly prescribed orally. It has been shown previously that the combination of intravenous antibiotics followed by oral antibiotics has a larger detrimental effect on the gut microbiome than when only intravenously administered (19). Recently, a Dutch study showed that an early intravenous-to-oral antibiotic switch was similarly efficacious and safe in treating suspected EOS compared to a full course of intravenous antibiotics in term-born neonates (20). It would be interesting to know whether infants from this study, treated with the combination of intravenous and oral antibiotics, have a different growth pattern than infants who were treated only intravenously.

Thirdly, the type of antibiotics could influence the association with growth. In the INCA study, we found that infants exposed to gentamicin and amoxicillin in the first week showed lesser weight gain than infants exposed to gentamicin and amoxicillin with clavulanic acid and lesser length increase than infants exposed to gentamicin and penicillin in the first year of life. After the first week of life, children were treated with a single antibiotic (mostly amoxicillin). The metabolomic function of the gut is strongly affected by bacterial composition and thus its association with growth, which in turn is affected by the type and combination of antibiotics (21). Nevertheless, as there is no unequivocal explanation for the differences found in growth, it is strongly recommended to study health outcomes after exposure to antibiotics in the first week of life separately from later exposure.

Antibiotics and ear infections

Since the composition of the gut microbiome plays a role in the maturation of the immune system (22), we hypothesized that we would find a higher prevalence of acute otitis media (AOM) and otitis media with effusion (OME) in the antibiotic-exposed group. To date, no studies have examined the association between antibiotic exposure and otitis media (OM), but a recent review showed that children with rAOM/OME have aberrant immune status (23). Although the causes of OM are multifactorial and associated with complex interactions of various factors, aberrant development of the immune system after early-life antibiotic treatment

could be reflected by an increase in common childhood diseases such as OM. This is, however, in contrast with our findings. A possible explanation is that antibiotic exposure in the first week of life is just one of many factors that play a role in the susceptibility for OM. Until now, many studies have attempted to identify factors inducing OM, as well as investigating inflammatory responses and mediators, and innate and acquired immune responses, to elucidate the pathogenesis and pathophysiology of OM (23-27). Unfortunately, our study did not contribute to unravel this problem. For future research, we recommend studying multiple factors, like antibiotic exposure in combination with oral, nasopharyngeal, and gut microbiome data, rather than single factors.

Antibiotics and atopic disorders

Besides timing, type, and route of antibiotic administration, the duration of exposure may also be an essential factor for the association with health outcomes, as shown in **chapter 6**. Children exposed to seven days of antibiotics in the first week of life had more often a food allergy at 4-6 years of age, compared to unexposed children. In contrast, children exposed to 2-3 days of antibiotics in the first week of life had no increased risk of allergy. Previous studies have shown that a longer duration of antibiotic exposure is associated with a more disturbed gut microbiome (28, 29). Our data confirm these earlier results: the microbiota perturbations observed in the INCA cohort were only significant after 5-7 days of antibiotic exposure and not after 2-3 days of antibiotic treatment. Moreover, we showed that the gut microbiome composition restored more quickly after 2-3 days of antibiotic exposure, compared with 5-7 days, after which dysbiotic changes persisted for a more extended period. This is in line with the study of Uzan-Yulzari et al., showing a more pronounced growth impairment in neonates receiving a full course of antibiotics than those receiving a shorter empirical treatment (3). Early cessation of antibiotics in the first week of life in infants without proven or probable infection is, therefore, essential to reduce the risk for detrimental health outcomes.

To gain more insight into the contributing factors to allergy, we conducted a machine learning (ML) pilot study in **chapter 10**. This ML model showed that a set of twelf immune markers measured in blood samples at one year of age combined with clinical factors, predict later allergies. It is known that the ratio between the so-called Th1 and Th2 types of cytokines can influence allergy development earlier and later in life (30, 31). Th2 is more related to type 1 IgE-mediated allergies, such as food allergies, and Th1 is related to delayed-type hypersensitivity, such as allergy to nickel (32). Critical to developing IgE-mediated allergic diseases is an increased production of Th2 cytokines, which are not adequately counter-regulated by Th1 cytokines (33). A range of studies has linked single cytokines to allergic diseases (34-37). However, to unravel

the overall outcome and allergy risk, it is strongly advised to use a systems-immunology approach that takes into account all different cytokines. Further research in larger cohorts is needed to confirm whether the combination of cytokines found in our machine learning pilot study is indeed predictive for allergies later in life.

The INCA study demonstrated no association between antibiotic exposure in the first week of life and asthma/wheezing or allergic rhinitis at 4 to 6 years of age. This seems to be in contrast to a retrospective study where antibiotics in the first week of life were associated with atopic asthma at twelf years of age (38) and a prospective study where it was associated with allergic rhinitis at eight years of age (39). Our cohort, however, may have been too young to find this association since asthma is often diagnosed after six years of age, and the prevalence of allergic rhinitis increases with age (40, 41). Longer follow-up of the INCA cohort would gain more insight into the association between antibiotics in the first week of life and asthma or allergic rhinitis after six years of age.

Antibiotic exposure and gastrointestinal disorders

The gestational age at birth is another factor in the association between antibiotic exposure in the first week of life and health outcomes. In our childhood gastrointestinal disorders review (**chapter 4**), only one study specifically investigated the association between antibiotic exposure in the first week of life and functional gastrointestinal disorders (FGIDs) besides the INCA study (7). The study of Salvatore et al. demonstrated that neonatal antibiotic treatment was associated with an increased risk of FGIDs in the first year of life. However, the study population consisted of preterm and term-born infants (42). If only term-born infants were analyzed, this association was no longer significant (43). These results show that the effect of antibiotic exposure on health outcomes in term-born infants cannot be compared to preterm-born infants and vice versa. Therefore, it is recommended to analyze the outcomes in preterm and term-born infants separately in future studies.

We found strong evidence for an association between antibiotics in the first two years of life and inflammatory bowel disease (IBD), eosinophilic esophagitis (EoE), and celiac disease (CeD) (**chapter 4**). The question remains to what extent the association with IBD, EoE, and CeD can be attributed to antibiotic exposure or other factors such as infections and parental health-seeking behavior. Infections in early life have been proposed to contribute to the development of chronic GI disorders (44, 45). It is difficult to differentiate between the role of infections and antibiotics prescribed for (suspected) infections. Furthermore, several GI disorders like CeD can remain undiagnosed for a long time. Higher parental health-seeking behavior can lead to higher use of antibiotics and a higher chance of diagnosing a chronic GI disorder. Therefore, it remains

unknown whether antibiotics are the actual causative agent in the observed associations or whether they are intermediates in different mechanistic pathways through microbiome perturbations or changes in immune development after (suspected) infections.

The development of the immune system, as measured by cytokines at one year of age, was different in antibiotic exposed and unexposed children, as shown in **chapter 5** and **chapter 10**, and may play a role in the differences in health outcomes found in the INCA study. In **chapter 5**, we explored whether gut-associated immune markers that were significantly different at one year of age in children with and without a history of infantile colic were different for children with FGIDs at 4-6 years of age. We found differences in these gut-associated immune markers between children with and without FGIDs. We did, however, not find an association between infantile colic and the development of an FGID at 4-6 years of age. In contrast to our study, three studies showed an association between infantile colic and FGIDs later in life (46-48), but the FGIDs were diagnosed at an older age. The lack of an association in the present study could be explained by the younger age (4-6 years) at follow-up, as many FGIDs manifest later in childhood. Since we already found differences in the cytokine profile in children with colic and FGIDs, colic may be linked to FGIDs later in life with longer follow-up of the INCA cohort.

Antibiotics and gut microbiome

Differences in gut microbiome composition between antibiotic-exposed and unexposed infants may explain some of the differences in health outcomes. It is, for example, known that *Bifidobacteriaceae* are essential in the early development of the immune system (49). They promote B-cell maturation and are associated with decreased inflammatory responses and T-regulatory cell acquisition (50). *Enterobacteriaceae*, on the other hand, produce toxins and have lipopolysaccharides on their outer membranes, causing inflammation (51, 52). Reduced *Bifidobacteriaceae* are often found in combination with an increase in potentially pathogenic *Enterobacteriaceae*, like *Shigella*, *Klebsiella*, and *Enterobacter* species. This combination has been associated with immune-mediated disorders (53). This may also play a role in the association between food allergy and the presence of an FGID, as shown in the INCA study, since there are similarities in the pathophysiological mechanism of developing allergies and FGIDs (54-56). For example, both allergies and FGIDs, like IBS, have been associated with increased intestinal permeability and inflammatory signals (54-56). Microbiota composition can substantially influence epithelial barrier function (57). Commensals and probiotic bacteria can enhance barrier integrity (58). This suggests that early-life dysbiosis, caused by antibiotics, can play a role in developing both food allergies and FGIDs (59, 60). If these health outcomes are

indeed related to changes in the gut microbiome composition, it emphasizes the need for early interventions to restore dysbiosis after antibiotic exposure.

Although the INCA study was not designed nor powered to show differences between the different combinations of antibiotics, we observed some differences in the microbiome composition between the various combinations of antibiotics. The results from the microbiota analyses in a subset of 56 infants exposed to antibiotics suggest that the combination of gentamicin and amoxicillin is the least harmful combination, as the microbiota did not differ from the unexposed infants. In contrast, the combination of gentamicin and penicillin was associated with increased relative abundance of *Enterobacteriaceae* and the combination amoxicillin with clavulanic acid was associated with increased *Enterobacteriaceae* and *Enterococcaceae* but also with higher levels of bifidobacteria than other antibiotic types. In a recent RCT, 147 infants requiring broad-spectrum antibiotics for suspected EOS were randomized 1:1:1 to receive three commonly prescribed intravenous antibiotic combinations (61). In contrast to our findings, penicillin and gentamicin exhibited the least effects on both microbial community composition and antimicrobial resistance gene profile. However, the duration of antibiotic treatment varied substantially between this study (48 hours) and the INCA study (5-7 days in more than half of the infants). These conflicting results demonstrate that more research is needed to draw firmer conclusions on the detrimental effects of different antibiotic types.

Interventions potentially preventing adverse health effects

In the INCA study, we showed that infants born by cesarean section had similar microbiota perturbations as vaginally born infants after antibiotic exposure (**chapter 7**). Delivery by cesarean section decreases Bifidobacterium spp. and increases opportunistic pathogens from hospital environments, such as Enterobacter, Enterococcus, and Klebsiella (62). This is similar to the effect on the microbiome after antibiotic exposure and could explain the lack of an additional impact of antibiotics in cesarean-born infants.

Because of these similarities in the gut microbiome composition between cesarean-born infants and antibiotic-exposed infants, the results from the cesarean-born infants in the pre-, pro-, and synbiotics review might also apply to infants with antibiotic exposure after birth. This means that supplementing pre-, pro, or synbiotics to infants exposed to antibiotics in the first week of life may significantly improve the gut microbiome composition and various health outcomes similar to those shown in cesarean-born infants.

Another option is to administer probiotics prenatally to reduce antepartum Group B streptococcus colonization with less postpartum antibiotic exposure as a consequence (63). Nevertheless, further studies are necessary before recommendations can be given on the

routine application of prebiotics, probiotics, or synbiotics to improve health outcomes after neonatal antibiotic exposure.

Future perspectives: artificial intelligence

As shown in **chapter 10** of this thesis, a new approach for analyzing a combination of factors instead of individual factors in relation to allergies is artificial intelligence using machine learning. ML finds patterns in the data and applies these patterns to new data to make predictions (64). ML methods have several advantages over current statistical analyses used in epidemiological studies. Most statistical methods cannot hold categorical data, deal with missing values, and large data points (65, 66). In contrast, ML can assess large amounts of data and discover specific trends and patterns that do not require assumptions about the sample or population (67). ML algorithms are good at handling multi-dimensional and multivariable data in dynamic or uncertain environments (67). Since ML-based models have a better predictive capacity than statistical-based models, these models should have a more prominent role in future prediction research, but this requires specific expertise from bioinformaticians. ML could also be used, for example, to map the gut microbiome composition in cohorts of infants exposed to antibiotics in the first week of life. Based on these findings, supplementation with pre-, pro-, or synbiotics could be adjusted to the specific needs of infants exposed to antibiotics. The ultimate goal is to mimic the gut microbiome composition of vaginally born breastfed infants, which is the gold standard (68).

Future perspectives: prevention of antibiotic exposure in the first week of life

To prevent adverse health effects after antibiotic exposure in the first week of life, several strategies are conceivable. First, antibiotic stewardship to avoid unnecessary antibiotic use is pivotal. A promising development is, for example, the implementation of the neonatal early-onset sepsis calculator, which has been associated with a substantial reduction in empirical antibiotic therapy for suspected EOS (69). Second, the application of new biomarkers to predict EOS better is also a promising method to prevent unnecessary antibiotic treatment (70-72). The prediction of EOS by biomarkers may be even more effective if biomarker profiles are studied, for example, with machine learning, instead of individual biomarkers. Thirdly, more research is needed to determine which effective (combination of) antibiotics in the treatment of EOS has the least impact on the developing gut microbiome. The impact on the developing microbiome could be further reduced by limiting antibiotic exposure in neonates without positive blood cultures and increased inflammatory markers. Besides a positive effect on the developing microbiome, reducing the exposure time from 5-7 days to 2-3 days could also limit the adverse

health outcomes, as shown in our study. Finally, a promising strategy for preventing dysbiosis and its negative health effects is the modulation of the gut microbiome, for example, through administering pro-, pre, and synbiotics. However, further studies are necessary to determine which (combination of) pre-, pro-, or synbiotic supplements and which dosages should be used.

Future perspectives: future research

In the INCA study, we have shown differences in the gut microbiome composition between the antibiotics exposed and unexposed infants. Currently, we are investigating whether specific changes in the gut microbiome composition can be related to the differences in health outcomes. This may help to explain the associations found in the INCA study. For future studies, the effect of antibiotics in the first week of life must be studied more extensively. Moreover, longer follow-ups of the INCA cohort and other large cohorts of infants exposed to antibiotics in the first week of life will provide more insight into the duration of the adverse effects of antibiotic exposure. Furthermore, more insight is needed into the impact of different combinations of antibiotics and the routes of administration on the gut microbiome and health outcomes. Finally, these studies should include only term-born infants, as effects on the gut microbiome and health outcomes may differ from those in preterm infants.

In conclusion, this thesis showed that antibiotic exposure in the first week of life in term-born infants was associated with a different growth pattern during the first year of life, an increase in both parental-reported and doctor-diagnosed allergies, and functional abdominal pain at 4-6 years of age compared to infants without antibiotic exposure in the first week. All these health outcomes may have been the result of differences in microbiota development between exposed and unexposed infants. Finally, we reviewed the literature and showed that pre-, pro-, and synbiotics supplementation are promising strategies to restore dysbiosis after antibiotic exposure. Future research should focus on preventing unnecessary antibiotic administration and interventions restoring the dysbiotic microbiome in children needing antibiotics in the first week of life.

The results of this thesis illustrate the importance of early life factors for later health and emphasize the need for well-considered decisions in the antibiotic policy of term-born neonates. We are still far from safeguarding optimal gut microbiome and immune system development in neonates, especially those requiring antibiotics for suspected infection. The results described in this thesis contribute to the knowledge of the long-term consequences of antibiotic exposure in the first week of life and show the need for interventions to restore dysbiosis after antibiotic exposure.

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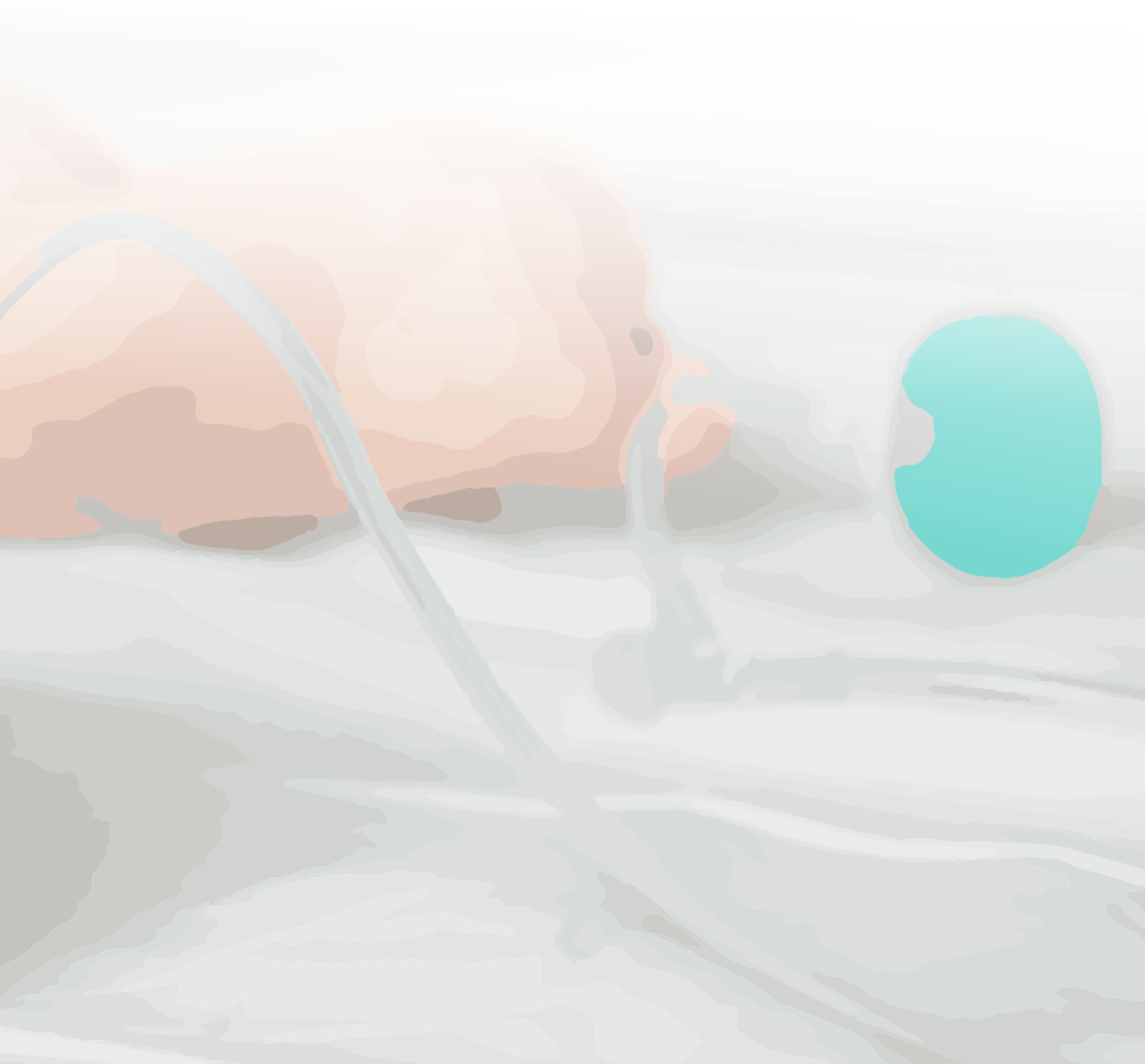
Chapter 12

English and Dutch summary



English summary: Antibiotic exposure in the first week of life, microbiota development and health outcomes

In Europe, between 2 and 16% of all newborns are exposed to antibiotics in their first week of life for suspected early-onset sepsis (EOS). Antibiotics are the most commonly prescribed drugs in children and are potentially lifesaving. However, its use has also been associated with negative long-term effects, such as atopic disorders, celiac disease, overweight and obesity, and behavioral problems. The effect of antibiotic exposure specifically in the first week of life on health outcomes in term-born infants has poorly been studied. Therefore, the INCA study "INtestinal microbiota Composition after Antibiotic treatment in early life" was designed, in which clinical and microbiome outcomes were studied in term-born infants after antibiotic treatment in the first week of life for presumed early onset sepsis compared to unexposed infants.

The first results of the INCA study showed that infants who were exposed to antibiotics in their first week of life had an altered gut microbiome composition in their first three months of life compared to unexposed infants. In addition, infants had a higher risk of wheezing and infantile colic in their first year of life after antibiotic exposure in the first week of life, and we found differences in immune markers measured in the blood at one year of age between the exposed and unexposed infants. This thesis aimed to provide further insight into the potential effects of neonatal antibiotics on the developing immune system and long-term health. In addition, the composition of the gut microbiome, and the extent and duration of dysbiosis after antibiotic exposure were investigated.

As it is known that the microbiome composition can play a role in the growth of infants and young children, the association between antibiotics administered in the first week of life and the growth trajectory in the first year of life was studied in **chapter 2**. A decreased weight gain of 200 grams and length gain of 1 cm was observed in the first year of life in the infants exposed to antibiotics in their first week of life compared to unexposed infants. After the first week of life, a different association between antibiotic exposure and growth was observed: for each additional course of antibiotics later in the first year of life, a significant additional weight gain of 76 grams was observed without change in length gain, irrespective of antibiotic exposure in the first week of life.

Growing evidence shows that the composition of the gut microbiome also plays an important role in the development and regulation of the immune system, especially in early life when the microbiome and immune system develop concurrently. Antibiotic exposure could compromise the developing immune system resulting in an increased risk of common childhood infections like upper respiratory tract infections and otitis media (OM). Therefore, in **chapter 3**

the association between antibiotic treatment in the first week of life and later OM was investigated. In contrast to our hypothesis, antibiotic use was not associated with either parent-reported or doctor-diagnosed acute otitis media (AOM) or otitis media with effusion (OME) in the first 4-6 years of life.

Chapter 4 is a systematic review with best evidence syntheses that studied the association between exposure to antibiotics in the first two years of life and the presence of chronic gastrointestinal disorders during childhood. Strong evidence was found for an association between antibiotic exposure in the first two years of life and inflammatory bowel disease and celiac disease and moderate evidence for this association with eosinophilic esophagitis. For the other studied GI disorders, insufficient evidence was found.

To further explore the association between early-life antibiotics and the development of functional gastrointestinal disorders (FGIDs) later in childhood, the exposure to antibiotics in the first week of life and the presence of FGIDs were studied in the INCA cohort (**chapter 5**). We found that significantly more children in the antibiotic group had functional abdominal pain at 4-6 years of age compared to the unexposed infants (4% vs. 0,4% respectively, p=0.045). Moreover, there was a significantly higher frequency of both irritable bowel syndrome (IBS) aOR 2.821 (95% CI 1.231-6.463, p=0.014) corrected for age, sex, and presence of siblings, and abdominal migraine (7% and 1%, p=0.043) in children with a parentally reported food allergy at 4-6 years of age. In contrast, no association was found between infantile colic and the presence of an FGID at 4-6 years of age, whereas differences in gut-associated immune markers were observed between children with and without FGIDs.

In **chapter 6**, we investigated the presence of atopic disorders at 4 to 6 years of age in the INCA cohort. The odds of having an allergy at 4-6 years of age was almost three times higher in children exposed to 5-7 days of antibiotics in the first week of life compared to unexposed children. This association was independent of additional antibiotic exposure later in the first two years of life. In contrast, no association was found between 2-3 days of antibiotic exposure in the first week of life and allergy. No associations were found between antibiotic exposure in the first week of life and other atopic disorders (eczema, wheezing/asthma, and allergic rhinitis).

To explore the pathophysiological factors involved in the association between antibiotic exposure in the first week of life and later health outcomes, the microbiota development after neonatal antibiotic exposure was studied in **chapter 7**. We observed perturbations in the faecal microbiota development from one week until one year of age. These perturbations included a decreased relative abundance of *Bifidobacteriaceae* and an increase in potentially pathogenic *Enterobacteriaceae*. The severity and duration of antibiotic-mediated

microbiota perturbations increased with longer antibiotic administration (2-3 versus 5-7 days). In addition, microbiota perturbations varied between the three studied antibiotic regimes.

To investigate whether clinicians can potentially diminish the adverse effects of antibiotic exposure in the future, a systematic review was performed on studies investigating the microbial and health effects of pre-, pro-, or synbiotic supplementation after antibiotic exposure in the first week of life or cesarean section (**chapters 8** and **9** respectively). Only one study investigated the effect of probiotic supplementation after antibiotic exposure on the microbiome; the supplementation of a mixture of three probiotics increased the Actinobacteria, Proteobacteria, and *Bifidobacterium*. For the Caesarean-born infants, the key finding was an increase in the supplemented bacterial species (of the *Bifidobacterium* and *Lactobacillus genus*) after probiotic or synbiotic supplements, and a decrease in *Enterobacteriaceae* after synbiotic but an increase after prebiotic supplementation. Furthermore, there were significant increases in Actinobacteria, Proteobacteria, and Firmicutes in the probiotic groups compared to the control groups.

To date, no studies were found regarding the clinical effects of pre-, pro-, or synbiotics supplementation after neonatal antibiotic treatment (**chapter 9**). The reported clinical effects after supplementation of pro-, pre, or synbiotics in cesarean-born children were a decrease in atopic diseases, fewer infectious diseases, and differences in the immune response to vaccinations. The results concerning immune response to vaccinations were however inconsistent.

Finally, in **chapter 10**, a pilot study was performed to study the value of machine learning by using feature selection to find predictive values for the presence of allergy at 4-6 years of age. In our small dataset, we showed that a combination of 20 early-life features, both clinical parameters, and cytokines, predict the presence of later allergy with an area under the curve (AUC) of 0.84. The resulting 20 features that are predictive for allergy at 4-6 years of age in our pilot study were tobacco exposure during pregnancy, the presence of atopic parents, gestational age in days, days of diarrhea, cough, rash, and fever during the first year of life, and ever being exposed to antibiotics. The included immune mediators/cytokines determined at one year of age were: Resistin, Interleukin 27 (IL-27), Matrix metallopeptidase 9 (MMP9), C-X-C Motif Chemokine Ligand 8 (CXCL8), C-C Motif Chemokine Ligand 13 (CCL13), Vimentin, Interleukine-4 (IL-4), C-C motif chemokine 22 (CCL22), Galactokinase 1 (Gal1), Interleukin 6 (IL-6), levels of tumor necrosis factor superfamily 14 (LIGHT), and Granulocyte-macrophage colony-stimulating factor (GMCSF).

In conclusion, this thesis showed that antibiotic exposure in the first week of life in term-born infants was associated with a different growth pattern during the first year of life, an increase in both parental-reported and doctor-diagnosed allergies, and functional abdominal pain at 4-6 years of age compared to infants without antibiotic exposure in the first week. All these health outcomes may have been the result of differences in microbiota and immune development between exposed and unexposed infants. Finally, we reviewed the literature and showed that pre-, pro-, and synbiotics supplementation are promising strategies to restore dysbiosis after antibiotic exposure. Future research should focus on preventing unnecessary antibiotic administration and interventions to restore the dysbiotic microbiome in infants needing antibiotics in the first week of life.

The results of this thesis illustrate the importance of early life factors for later health and emphasize the need for well-considered decisions in the antibiotic policy of term-born neonates. We are still far from safeguarding optimal gut microbiome and immune system development in neonates, especially those requiring antibiotics for suspected infection. The results described in this thesis contribute to the knowledge of the long-term consequences of antibiotic exposure in the first week of life and show the need for interventions to restore dysbiosis after antibiotic exposure.

Samenvatting in het Nederlands voor niet medici: blootstelling aan antibiotica in de eerste levensweek, ontwikkeling van de darmflora en gezondheidsuitkomsten

In Europa krijgen tussen de 2 en 16% van alle pasgeborenen antibiotica in hun eerste levensweek vanwege een verdenking op een infectie. Antibiotica zijn de meest voorgeschreven medicijnen bij kinderen en kunnen levensreddend zijn, maar worden daarentegen ook in verband gebracht met negatieve lange termijneffecten, zoals atopische aandoeningen, coeliakie, overgewicht en obesitas en gedragsproblemen. Het effect van antibiotica blootstelling specifiek in de eerste levensweek op gezondheidsuitkomsten bij voldragen baby's is echter nog weinig onderzocht. Daarom werd de INCA-studie "INtestinal microbiota Composition after Antibiotic treatment in early life" opgezet. In de INCA-studie werden klinische uitkomsten en de darmflora bestudeerd bij voldragen baby's na behandeling met antibiotica in de eerste levensweek vanwege een mogelijke infectie en deze groep werd vergeleken met een groep baby's die niet aan antibiotica zijn blootgesteld in de eerste levensweek.

De eerste resultaten van de INCA-studie lieten zien dat baby's die antibiotica gekregen hadden in hun eerste levensweek een veranderde darmflora hadden in de eerste drie levensmaanden ten opzichte van kinderen die geen antibiotica hadden gekregen. Daarnaast hadden baby's na antibiotica in de eerste levensweek een hoger risico op een piepende ademhaling en waren er significant meer huilbaby's in de antibioticagroep. Verder vonden we verschillen in immuunmarkers gemeten in het bloed op 1-jarige leeftijd tussen de aan antibiotica blootgestelde en niet blootgestelde baby's. Het doel van dit proefschrift was om meer inzicht te krijgen in de mogelijke negatieve lange termijneffecten van antibiotica blootstelling specifiek in de eerste levensweek. Hiervoor hebben we het verband onderzocht tussen antibiotica blootstelling in de eerste levensweek en de effecten op het zich ontwikkelende immuunsysteem en de gezondheid op de lange termijn. Daarnaast werd de samenstelling van de darmflora bestudeerd en de mate en duur van afwijkingen in de darmflora na deze antibiotica blootstelling.

Het is bekend dat de samenstelling van de darmflora een rol speelt bij groei van baby's en jonge kinderen. Daarom werd het verband tussen antibiotica blootstelling in de eerste levensweek en het groeipatroon in het eerste levensjaar bestudeerd in **hoofdstuk 2**. We zagen een verminderde groei van 200 gram en 1 centimeter in de groep baby's die in hun eerste week antibiotica toegediend hadden gekregen in vergelijking met de baby's die geen antibiotica kregen. Na de eerste levensweek werd een ander verband tussen blootstelling aan antibiotica en groei waargenomen: bij elke antibioticakuur later in het eerste levensjaar werd een

significante extra gewichtstoename van 76 gram per kuur waargenomen zonder verandering in de lengtetoename, ongeacht blootstelling aan antibiotica in de eerste levensweek.

Er is steeds meer bewijs dat de samenstelling van de darmflora ook een belangrijke rol speelt bij de ontwikkeling en regulering van het immuunsysteem, vooral in het vroege leven wanneer de darmflora en het immuunsysteem zich gelijktijdig ontwikkelen. Blootstelling aan antibiotica kan het zich ontwikkelende immuunsysteem aantasten, wat kan leiden tot een verhoogd risico op veel voorkomende kinderinfecties, zoals infecties van de bovenste luchtwegen en middenoorontsteking. Daarom werd in **hoofdstuk 3** het verband tussen antibioticabehandeling in de eerste levensweek en latere middenoorontstekingen onderzocht. In tegenstelling tot onze hypothese was er geen verband tussen zowel door ouders gerapporteerde als door artsen gediagnosticeerde acute middenoorontsteking of slijmoor in de eerste 4-6 levensjaren.

Hoofdstuk 4 is een systematische review (literatuuroverzicht) met "best evidence syntheses" om het verband te bestuderen tussen blootstelling aan antibiotica in de eerste twee levensjaren en de aanwezigheid van chronische functionele maagdarmaandoeningen tijdens de kindertijd. Er werd sterk bewijs gevonden voor een verband tussen blootstelling aan antibiotica in de eerste twee levensjaren en inflammatoire darmaandoeningen (de ziekte van Crohn en colitis ulcerosa) en coeliakie. Matig bewijs voor dit verband met eosinofiele oesofagitis (een chronische ontsteking van de slokdarm). Voor de overige onderzochte maagdarmaandoeningen werd onvoldoende bewijs gevonden.

Om het verband tussen antibiotica blootstelling op jonge leeftijd en de ontwikkeling van functionele maagdarmaandoeningen later op de kinderleeftijd verder te onderzoeken, werd in **hoofdstuk 5** blootstelling aan antibiotica in de eerste levensweek en de aanwezigheid van functionele maagdarmaandoeningen bestudeerd in het INCA-cohort. We vonden in de groep kinderen die antibiotica kregen in de eerste levensweek significant meer kinderen met functionele buikpijn op 4-6 jarige leeftijd in vergelijking met de niet blootgestelde kinderen (4% versus 0.4%). Daarnaast hadden kinderen met een door ouders gerapporteerde voedselallergie significant vaker het prikkelbare darm syndroom en buikmigraine dan kinderen zonder een voedselallergie. Er werd daarentegen geen verband gevonden tussen huilbaby's en de aanwezigheid van functionele maagdarmaandoeningen op 4-6 jarige leeftijd. Ten slotte vonden we verschillen in darm geassocieerde immuun markers, gemeten in het bloed op 1-jarige leeftijd, tussen kinderen met en zonder functionele maagdarmaandoeningen.

In **hoofdstuk 6** onderzochten we de aanwezigheid van atopische aandoeningen op 4-6 jarige leeftijd in de INCA-studie. De kans op het hebben van een allergie op 4-6 jarige leeftijd was bijna drie keer hoger bij kinderen die in de eerste levensweek 5-7 dagen antibiotica kregen dan

bij niet blootgestelde kinderen. Dit verband was onafhankelijk van blootstelling aan extra kuren antibiotica later in de eerste twee levensjaren. Bij kinderen die 2-3 dagen antibiotica kregen werd daarentegen geen verband gevonden tussen antibiotica blootstelling in de eerste levensweek en allergie. Er werd ook geen verband gevonden tussen antibiotica blootstelling in de eerste levensweek en andere atopische aandoeningen (eczeem, piepende ademhaling/astma en hooikoorts).

Om te onderzoeken welke factoren een rol spelen bij de gevonden associaties tussen antibiotica blootstelling in de eerste levensweek en latere gezondheidsuitkomsten, werd in **hoofdstuk 7** de ontwikkeling van de darmflora na blootstelling aan antibiotica in de eerste levensweek onderzocht. We vonden hierin verstoringen vanaf de leeftijd van een week tot een jaar. Deze verstoringen omvatten een verminderde relatieve aanwezigheid van bifidobacteriën en een toename van potentieel ziekteverwekkende enterobacteriën. De ernst en duur van de met antibiotica in verband gebrachte darmflora verstoringen namen toe met langere toediening van antibiotica (2-3 versus 5-7 dagen). Daarnaast varieerden de verstoringen van de darmflora tussen de drie verschillende types antibiotica die bestudeerd zijn in de INCA-studie.

Om te onderzoeken of de nadelige effecten van antibiotica blootstelling in de toekomst mogelijk verminderd kunnen worden, hebben we in **hoofdstukken 8** en **9** naar studies gezocht die de darmflora en klinische effecten onderzochten na toediening van pre-, pro- of synbiotica na blootstelling aan antibiotica in de eerste levensweek of geboorte doormiddel van een keizersnede. Slechts één studie onderzocht het effect van probiotica (levende micro-organismen zoals bifidobacteriën en lactobacillen) toediening na blootstelling aan antibiotica op de darmflora; een mengsel van drie probiotica verhoogde de aanwezigheid van Actinobacteria, Proteobacteria en Bifidobacterium. Voor de baby's geboren met een keizersnede was de belangrijkste bevinding dat er een toename van de toegediende bacteriesoorten (bifidobacteriën en lactobacillen) na probiotica of synbiotica suppletie werd gevonden en een afname van enterobacteriën na synbiotica (combinatie van pre- en probiotica) maar een toename na prebiotica suppletie (voedingsstoffen die de groei en activiteit bevorderen van bacteriën die al in de darm voorkomen). Bovendien waren er significante toenames in Actinobacteriën, Proteobacteriën en Firmicutes in de probiotica groepen in vergelijking met de controlegroepen. Tot op heden zijn er geen studies gevonden over de klinische effecten van pre-, pro- of synbiotica toediening na antibioticabehandeling in de eerste levensweek (**hoofdstuk 9**). De gerapporteerde klinische effecten na suppletie van pro-, pre- of synbiotica bij kinderen geboren doormiddel van keizersnede waren een afname van atopische ziekten, minder infectieziekten en verschillen in de immuunrespons op vaccinaties. De resultaten met betrekking tot de immuunrespons op vaccinaties waren echter inconsistent.

Ten slotte werd in **hoofdstuk 10** een pilotstudie uitgevoerd om de waarde van machine learning te bestuderen door deze te gebruiken om voorspellende waarden te vinden voor de aanwezigheid van allergie op de leeftijd van 4-6 jaar. In onze kleine dataset hebben we aangetoond dat een combinatie van 20 vroege levenskenmerken, zowel klinische parameters als cytokines, de aanwezigheid van latere allergie voorspelt met een “area under the curve” van 0,84. De 20 kenmerken die voorspellend zijn voor allergie op de leeftijd van 4-6 jaar in onze pilotstudie waren: een moeder die gerookt heeft tijdens de zwangerschap, de aanwezigheid van atopische ouders, de zwangerschapsduur, dagen van diarree, hoesten, huiduitslag en koorts in het eerste levensjaar en ooit blootgesteld zijn aan antibiotica. De opgenomen immuunmediatoren/cytokines bepaald op de leeftijd van één jaar waren: Resistin, Interleukin 27 (IL-27), Matrix metallopeptidase 9 (MMP9), C-X-C Motif Chemokine Ligand 8 (CXCL8), C-C Motif Chemokine Ligand 13 (CCL13), Vimentin, Interleukine-4 (IL-4), C-C-motief chemokine 22 (CCL22), Galactokinase 1 (Gal1), Interleukine 6 (IL-6), niveaus van tumornecrosefactor-superfamilie 14 (LIGHT) en granulocyt-macrofaag-koloniestimulerend factor (GMCSF).

Concluderend hebben we in dit proefschrift verbanden aangetoond tussen blootstelling aan antibiotica in de eerste levensweek en een ander groeipatroon tijdens het eerste levensjaar, een toename van zowel door de ouders gerapporteerde als door de arts gediagnosticeerde allergieën en functionele buikpijn op de leeftijd van 4-6 jaar in vergelijking met kinderen die niet blootgesteld zijn aan antibiotica in de eerste levensweek. Al deze gezondheidsuitkomsten kunnen het gevolg zijn van verschillen in de ontwikkeling van de darmflora tussen blootgestelde en niet-blootgestelde baby's. Ten slotte hebben we de literatuur bestudeerd en aangetoond dat pre-, pro- en synbiotica toediening veelbelovende strategieën zijn om de afwijkingen in de darmflora te herstellen na blootstelling aan antibiotica. Toekomstig onderzoek moet zich richten op het voorkomen van onnodige toediening van antibiotica en interventies om verstoringen in de darmflora te herstellen bij baby’s die in de eerste levensweek antibiotica nodig hebben.

De uitkomsten van dit proefschrift laten nog eens het belang van het vroege leven zien voor de latere gezondheid. Dit benadrukt de noodzaak voor weloverwogen beslissingen rondom het antibioticabeleid van baby’s. We zijn nog ver verwijderd van het waarborgen van een optimale ontwikkeling van de darmflora en het immuunsysteem bij pasgeborenen, vooral bij degenen die antibiotica krijgen in verband met een vermoedelijke infectie. De resultaten beschreven in dit proefschrift dragen bij aan de kennis over de langetermijngevolgen van antibioticablootstelling in de eerste levensweek en tonen de noodzaak voor interventies om de verstoringen in de darmflora te herstellen na blootstelling aan antibiotica.

Appendices

Curriculum Vitae
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Curriculum vitae

Kim Kamphorst werd geboren op 2 augustus 1987 in Twello. Na het behalen van haar vmbo-diploma aan het Veluws College Twello, heeft ze mbo-verpleegkunde gestudeerd aan het ROC Aventus in Deventer, waar zij in 2007 haar diploma heeft behaald.

Na haar diplomering is zij gaan werken als verpleegkundige op de neonatologie en kinderafdeling in het Gelre Ziekenhuis Apeldoorn. In 2010 heeft zij de opleiding tot kinderverpleegkundige succesvol afgerond en in 2011 de opleiding tot neonatologieverpleegkundige. Vervolgens wilde ze graag klinische gezondheidswetenschappen richting verplegingswetenschappen studeren aan de universiteit van Utrecht. Hiervoor heeft ze in 2013 haar vwo-wiskunde C diploma gehaald en is ze met twee aanbevelingsbrieven toegelaten tot de pre-master klinische gezondheidswetenschappen. In 2016 heeft ze deze master succesvol afgerond.

Vanaf 2015 combineerde ze het werk als kinder- en neonatologieverpleegkundige met een functie als researchcoördinator in het Deventer Ziekenhuis, voor de vakgroep kindergeneeskunde en gynaecologie, verloskunde en voortplantingsgeneeskunde. In deze functie is ze betrokken bij al het wetenschappelijke onderzoek waar deze vakgroepen aan deelnemen, maar worden ook zelf studies opgezet en begeleidt ze studenten bij hun wetenschappelijke onderzoek. Vanaf 2018 is ze gestopt met het werk als verpleegkundige en gestart met haar promotieonderzoek, waar ze in totaal 5 jaar parttime aan heeft gewerkt, twee keer onderbroken voor een zwangerschapsverlof. Daarnaast is haar opleidingsplan voor een registratie als epidemioloog B goedgekeurd, welke naar verwachting in september 2023 afgerond zal worden.

Per januari 2023 blijft Kim als inmiddels senior researchcoördinator/senior onderzoeker voor de twee vakgroepen werken en zal dit combineren met een functie bij het wetenschapsbureau in het Deventer ziekenhuis. Hierbij zal ze zich voornamelijk richten op het verpleegkundig wetenschappelijk onderzoek, de epidemiologische vraagstukken en het onderwijs.

Kim heeft een geregistreerd partnerschap met Erwin Kempink en samen hebben zij 3 kinderen: Joost (2017), Leah (2019) en Morris (2021).

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Antibiotic treatment in the first week of life impacts the growth trajectory in the first year of life in term infants

The author's responsibilities were as follows: Data collection (AMV, NBR, CMB); Data Analysis (KK, BCO, ECW, AMW, RMvE); Writing manuscript (KK, BCO), Data interpretation and critical review of manuscript (KK, BCO, AMV, CMB, ECW, RMvE)

The association between exposure to antibiotics in the first week of life and later otitis media: the INCA Study

The author's responsibilities were as follows: Data collection (KK, BCO, AMV, RMvE); Data Analysis (KK, LCR, EvtR); Writing manuscript (KK), Data interpretation and critical review of manuscript (KK, BCO, LCR, EvtR, AMV, RMvE)

Neonatal Antibiotics and Food Allergy are Associated with FGIDs at 4–6 years of age

The author's responsibilities were as follows: Data collection (KK, AMV, RMvE, BCO); Data Analysis (KK); Writing manuscript (KK), Data interpretation and critical review of manuscript (KK, AMV, RMvE, BCO, JG)

Higher risk of allergies at 4-6 years of age after systemic antibiotics in the first week of life

The author's responsibilities were as follows: Data collection (KK, AMV, RMvE, BCO); Data Analysis (KK, SW); Writing manuscript (KK), Data interpretation and critical review of manuscript (KK, AMV, RMvE, BCO, SW)

Early life antibiotics and childhood gastrointestinal disorders: a systematic review

The author's responsibilities were as follows: Data collection (KK, EVD, JGD); Data Analysis (KK, EVD); Writing manuscript (KK, EVD), Data interpretation and critical review of manuscript (KK, EVD, AMV, RMvE, JGD, JK)

Effect of antibiotics in the first week of life on faecal microbiota development

The author's responsibilities were as follows: Data collection (EvD, AMV); Data Analysis (EvD, CM, GDAH); Writing manuscript (EvD, CM, KK, AMV, RMvE), Data interpretation and critical review of manuscript (EvD, KK, AMV, GDAH, CM, MV, CV, CB, HS, RMvE, JK)

Microbial effects of prebiotics, probiotics and synbiotics after Caesarean section or exposure to antibiotics in the first week of life: a systematic review
The author's responsibilities were as follows: Data collection (KK, NCC, JGD); Data Analysis (KK, NCC, TGJdM, RMvE); Writing manuscript (KK, NCC), Data interpretation and critical review of manuscript (KK, NCC, AMV, RMvE, JGD, TGJdM)

Clinical outcomes following pre-, pro-and synbiotic supplementation after caesarean birth or antibiotic exposure in the first week of life in term born infants: A systematic review of the literature
The author's responsibilities were as follows: Data collection (KK, NCC, JGD); Data Analysis (KK, NCC, TGJdM, RMvE); Writing manuscript (KK, NCC), Data interpretation and critical review of manuscript (KK, NCC, AMV, RMvE, JGD, TGJdM)

Predictive factors for allergy at 4-6 years of age based on machine learning: a pilot study
The author's responsibilities were as follows: Data collection (KK, AMV, RMvE); Data Analysis (ALR); Writing manuscript (KK, ALR), Data interpretation and critical review of manuscript (KK, ALR, AMV, JG, EvtR, RMvE)

PhD portfolio

Name PhD student: K. Kamphorst PhD period: 17-01-2018 – 31-12-2022

Promotor: prof. dr. R.M. van Elburg Co-promotor: dr. A.M. Vlieger

Training activities

Courses	Year	ECTS
Scientific English writing	2018	0.5
Practical Biostatistics	2018	1.4
Presentation skills training	2018	-
Good Clinical Practice (GCP) re-registration	2018	0.5
Advanced topics in biostatistics	2020	2.1
Scientific writing in English	2022	1.5
Clinical epidemiology: evaluation of medical tests	2022	0.9
Multilevel and longitudinal data analyses	2022	4.0

Symposia and congresses

Amsterdam Kindersymposium	2018	0.3
EAPS in Paris	2018	0.5
EAPS online	2020	0.5
EAPS in Barcelona	2022	0.5

Oral presentations

NVK Congress Tulips late breakers	2018	0.5
Antibiotic treatment in the first week of life impacts the growth trajectory in the first year of life		
EAPS in Paris	2018	0.5
Antibiotic treatment in the first week of life impacts the growth trajectory in the first year of life		
EAPS online	2020	1.0
1. *Early life antibiotic exposure and later childhood chronic gastrointestinal disorders: a systematic review*		
2. *Antibiotics administrated in the first week of life are associated with allergies at 4-6 years of age*		

EAPS in Barcelona	2022	0.5
The association between antibiotic exposure in the first week of life and later otitis media: the INCA study		

Teaching activities

Co-supervision of 6 students

4 during their Scientific Internship for BSc thesis	2018-2022	4.0
2 master students during their Scientific Internship	2020-2021	2.0

Awarded

TULIPS late breakers	2018	-
Young investigator award ESPGHAN	2020	-

Publications included in this thesis

Kamphorst, K.*, Oosterloo, B. C.*, Vlieger, A. M., Rutten, N. B., Bunkers, C. M., Wit, E. C., & Van Elburg, R. M. Antibiotic treatment in the first week of life impacts the growth trajectory in the first year of life in term infants. *J Pediatr Gastroenterol Nutr.* 2019;69(1):131-136.

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Carpay, N. C.*, **Kamphorst, K**.*, de Meij, T. G., Daams, J. G., Vlieger, A. M.*, & van Elburg, R. M.* Microbial effects of prebiotics, probiotics and synbiotics after Caesarean section or exposure to antibiotics in the first week of life: A systematic review. *PLoS One*. 2022;17(11):e0277405.

Kamphorst, K.*, Carpay, N. C.*, De Meij, T. G., Daams, J. G., Van Elburg, R. M.*, & Vlieger, A. M.* Clinical outcomes following pre-, pro-and synbiotic supplementation after caesarean birth or antibiotic exposure in the first week of life in term born infants: A systematic review of the literature. *Front Pediatr*. 2022;1757.

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*Both authors contributed equally

Not included in this thesis

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Kamphorst, K., Faber, J., & van der Linden, P. J. Q. (2021). The Presence of Antisperm Antibodies in Semen of Subfertile Men—Positive ASA in Subfertile Men. *Open J Obstet Gynecol*. 2021;11(02):88.

Bouwknegt, A., Jongen, S., **Kamphorst, K**., van Pampus, M. G., van der Linden, P. J., & Zwart, J. J. Foley Balloon Catheter versus Oral Misoprostol for Induction of Labour after Prelabour Rupture of Membranes: A Retrospective Data Analysis. *Open J Obstet Gynecol.* 2022;12(7):579-589.

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