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Study protocol for the ABERRANT study: Antibiotic-induced disruption of the maternal and infant microbiome and adverse health outcomes

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Study protocol for the ABERRANT study: Antibiotic-induced disruption of the maternal and infant microbiome and adverse health outcomes

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ABSTRACT

Introduction: There is compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome. Antibiotics cause profound changes in the microbiome. However, the effect of intrapartum and early-life antibiotics on the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome, and whether effects are only short-term or persist long-term remain uncertain.

Methods and analyses: In this prospective cohort study, we will use metagenomic sequencing to determine: (i) the effect of *intrapartum antibiotics* on the composition of the breast milk, and the infant oral and intestinal microbiome, including the development and persistence of antibiotic resistance; (ii) the effect of *antibiotic exposure in the first year of life* on the composition of the infant oral and intestinal microbiome, including the development and persistence of antibiotic resistance; (iii) the effect of disruption of the infant oral and intestinal microbiome on health outcomes; and (iv) the compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.

Ethics and dissemination: The ABERRANT study has been approved by the Commission cantonale d'éthique de la recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-01567). Outcomes will be disseminated through publication and will be presented at scientific conferences.

Trial registration number: The U.S. National Institutes of Health NCT04091282.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- This study will use metagenomic sequencing to comprehensively determine the effect of intrapartum and early-life antibiotics on the composition of the maternal breast milk and the infant oral and intestinal microbiome.
- High quality clinical data combined with cutting-edge microbiome analyses will enable the identification of bacterial species, together with resistance genes and other important components of the microbiome such as archaea, eukaryotes (fungi) and viruses.
- The study includes investigation of the association between the early-life intestinal microbiome and clinical health outcomes.
- The knowledge gained by this study will form the basis for the development of evidence-based interventions to prevent adverse outcomes in situations where antibiotics cannot be avoided, including modifying the intestinal microbiome with directed pre- and probiotics, or bacteriophages.
- Meticulous precautions will be used to avoid contamination of potentially low microbial biomass breast milk samples, such as working in a laminar flow cabinet and including negative controls to identify microbial DNA signals from the environment or extraction and sequencing kits.

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1
2
3 63 **INTRODUCTION**

64 Antibiotics are amongst the most commonly used drugs, especially in infants and children.
65 Even before birth, more than 40% of infants are exposed to antibiotics.[1] Additionally, more
66 than two-thirds of children receive antibiotics before reaching the age of two years.[2 3] The
67 human intestine is the habitat for a large community of microbes, the intestinal microbiome.
68 Colonisation of the intestinal tract increases rapidly after birth and the microbiome of the
69 maternal intestine and breast milk are an important source for the infant intestinal
70 microbiome.[4] Conversely, as retrograde flow of breast milk into mammary ducts has been
71 documented,[5] the infant oral microbiome might be responsible for colonising the mammary
72 ducts and therefore could contribute to the breast milk microbiome. Consequently, there is
73 compositional overlap between the maternal intestinal microbiome, the breast milk
74 microbiome and the infant oral and intestinal microbiome.[6 7]

75 Growing evidence shows that the composition of the intestinal microbiome in infants plays an
76 important role in the development and regulation of the immune system, especially in the
77 early-life 'critical window' during which the microbiome and the immune response develop
78 concurrently.

79 Antibiotics cause profound changes in the microbiome.[8 9] However, the magnitude of the
80 effect of intrapartum and early-life antibiotics on the breast milk, and the infant oral and
81 intestinal microbiome, and whether effects are only short-term or persist long-term remain
82 uncertain. Preliminary studies suggest that disruption of intestinal microbiome in the early-life
83 period is associated with the development of a number of immune- and non-immune-
84 mediated diseases, including allergies,[10] eczema,[10] asthma,[10] chronic inflammatory bowel
85 disease,[11] obesity[12] and diabetes mellitus.[13] Antibiotic exposure *in utero* and during infancy
86 has been associated with an increased risk for the same diseases[14-16] and it is likely that the
87 association between antibiotic exposure and the subsequent development of these diseases is
88 mediated through changes in the infant microbiome. However, the features and composition

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characteristics of the intestinal microbiome associated with the development of these conditions are unclear.

Importantly, antibiotic exposure can also lead to the development of antibiotic resistance,[8] resulting in infections that are more difficult and costly to treat, often requiring longer duration of hospital stay, and the use of antibiotics with more adverse effects. Currently, in children, there is sparse data available on the effect of antibiotic exposure on the development and persistence of antibiotic resistance in their intestinal microbiome.

In this prospective cohort study, we will use metagenomic sequencing to determine (i) the effect of *intrapartum antibiotics* on the composition of the breast milk, and the infant oral and intestinal microbiome (including the development and persistence of antibiotic resistance); (ii) the effect of *antibiotic exposure in the first year of life* on the composition of the infant oral and intestinal microbiome (including the development and persistence of antibiotic resistance); (iii) the effect of disruption of the infant oral and intestinal microbiome on health outcomes; and (iv) determine the compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.

Determining the relationship between antibiotic exposure and changes in the breast milk, and the infant oral and intestinal microbiome, and their potential association with adverse health outcomes, will provide stronger evidence for strict antibiotic stewardship. Additionally, it will form the basis for designing studies to investigate interventions to prevent adverse outcomes in situations where antibiotics cannot be avoided, including modifying the intestinal microbiome with directed pre- and probiotics, or bacteriophages.

OBJECTIVES

METHODS AND ANALYSIS

Study design

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A prospective single-centre cohort study of 400 mother-infant pairs.

Aims

Aim 1: To determine the extent to which, and for how long, intrapartum antibiotics affect the composition of the breast milk microbiome and the infant oral and intestinal microbiome, as well as the prevalence of antibiotic resistance genes.

Aim 2: To determine the extent to which, and for how long, antibiotics in the first year of life affect the composition of the oral and intestinal microbiome in infants, as well as the prevalence of antibiotic resistance genes.

Aim 3: To determine health outcomes (Table 1) in children up to the age of 2 years who have or have not been exposed to intrapartum antibiotics or antibiotics in the first year of life and determine whether there is an association with the composition of the oral and intestinal microbiome.

Aim 4: To determine the degree to which the maternal intestinal and the breast milk microbiome affect the composition of the infant oral intestinal microbiome and the prevalence of antibiotic resistance genes.

Aim 5: To determine the degree to which the infant oral microbiome affects the composition of the breast milk microbiome.

Outcomes

Primary endpoints:

- Composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome and the prevalence of antibiotic resistance genes within the infant microbiome at birth and when infants are 7 days, 1, 2, 4, 6, 12 and 24 months of age.

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- Number of episodes of lower respiratory tract illnesses and acute otitis media in the first two years of life.
- Prevalence of allergic sensitisation and eczema at 1 and 2 years of age.
- Weight at 1 and 2 years of age.

Setting and participants

Sampling frame and study sample**:** Women who give birth at the Hôpital fribourgeois (HFR) in Fribourg, Switzerland and their infants will be followed over a two-year period. If recruitment is slow a second study site will be added.

Recruitment: Pregnant women attending the antenatal clinic will be given information about the study by a research study nurse or doctor and asked to consider enrolling themselves and their infant in the study.

Blinding of outcome assessment: Doctors and study nurses will be blinded to the group of infants (control or antibiotic-exposed) when outcomes are measured.

Eligibility criteria

Inclusion criteria*:* Healthy babies born at 37 weeks or more gestation who are breastfed.

Exclusion criteria*:* Women with the following criteria: HIV, hepatitis B or C infection or unscreened for these infections, antibiotics or probiotics in pregnancy or postpartum period other than during delivery. Infants with the following criteria: low birth weight (<2500 g) or severe congenital abnormality.

Study outcome measures

We will use internationally accepted validated measures for clinical outcomes. The study protocol is depicted in **Table 2**.

Diary: Parents will be given a structured diary where they can record information about their

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166 infant's diet (introduction of formula and new foods), illnesses, medical visits, hospital
167 admissions, use of antibiotics and other medications. This will help them when filling in the
168 questionnaires at the required time points.

169 ***Questionnaire:*** We will do computer-assisted interviews at birth, and when infants are 1, 6,
170 12 and 24 months of age using best practice international protocols. The following data will
171 be recorded: demographic variables including parental ancestry and education, family history
172 of atopy, eczema, asthma and other immune disorders, antenatal variables such as maternal
173 age, weight, smoking habits, underlying diseases, medication and supplementation use (e.g.
174 probiotics and vitamins). In addition, we will collect data on delivery history, perinatal course
175 (e.g. hospitalisation, infections, antibiotics or oxygen administration), breast-feeding
176 (including episodes of mastitis and maternal antibiotic and probiotic use), age of introduction
177 of formula and new foods, administration of probiotics and vitamins, use of antibiotics,
178 antacids and other medications, GP and other medical visits, illnesses including infections and
179 hospital admissions, number of siblings, child care attendance, parental smoking habits, pet
180 ownership, suspected food allergy and eczema (presence, medications). Data will be stored
181 using the Research Electronic Data Capture (REDCap Consortium) database.[17]

182 ***Clinical examination:*** Participants will be reviewed at 12 and 24 months of age in a specially
183 designated clinic at the HFR by a study nurse or doctor using a structured interview and
184 clinical eczema assessment.

185 ***Weight:*** Weight will be assessed during the clinical examination at 12 and 24 months of age.
186 The WHO Child Growth Standards will be used as a reference for percentiles.[18]

187 ***Lower respiratory tract illness (LRTI) and acute otitis media (AOM):*** Symptoms of acute
188 lower respiratory illness (such cough and wheeze) and the number of episodes of LRTI and
189 AOM will be recorded by parents, and specific questions will be asked in the questionnaires.
190 We will use the definitions for LRTI developed by Oddy *et al* and Kusel *et al.*[19 20]

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Eczema: Prevalence of eczema at 12 and 24 months of life will be assessed by the Williams' UK diagnostic criteria of the International Study of Asthma and Allergy in Childhood (ISAAC).[21] This will be assessed by using parent-reported eczema from diary information and from the clinical examinations at 12 and 24 months of age. We will assess the severity of eczema using the SCORAD.[22] We will also collect data on age of onset of eczema, distribution of eczema, use of eczema medications, and medical consultations and hospital admissions.

Skin prick tests: Sensitisation to the following panel of allergens will be assessed at 24 months of age: cow's milk, egg, peanut, sesame, house dust mite (*Dermatophagoides pteronyssinus 1*), cat, dog and grass pollen. Skin prick allergy testing will be performed according to standard guidelines.[23] A positive skin prick test will be defined as an average wheal diameter at least 3 mm greater than that produced by a negative control solution at 15 minutes.[24]

Blood sampling: We will collect maternal blood at time of delivery. We also will obtain cord blood at birth. During the clinical examination at 12 and 24 months of age, blood will be collected by personnel trained in infant venipuncture, whose parents consent to this component of the study. The 5-10 ml volume required is safe and within limits for weight recommended by the US-based Office of Human Research Protections guidelines for blood collection from healthy infants.

***Breast milk samples*:** Study nurses will collect one colostrum sample as soon as possible after birth. Mothers will be asked to collect breast milk (with date and time recorded) from their first feed of the day (a minimum of 2 hours required to the previous feed) when infants are 7 days, 1, 2, 4, and 6 months old. If breastfeeding is ceased earlier than 6 months, breast milk will be collected before breastfeeding is discontinued. Mothers will be instructed to wash their hands and breasts meticulously and to then extract breast milk manually without touching the

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areola. The first few drops will be discarded. Reminders will be sent by SMS. Sterile containers will be provided.

Oral swabs: Oral swabs will be taken from infants as soon as possible after birth by a study nurse. Additionally, parents will then be asked to collect buccal swabs (with date and time recorded) before the first feed of the day when infants are 7 days, 1, 2, 4, and 6 months old. Sterile containers will be provided. Reminders will be sent by SMS.

Stool samples: Mothers will be asked to collect stool from their first bowel movement of the day at 38 weeks of pregnancy and on the day after delivery (with date and time recorded). A meconium sample will be collected from infants as soon as possible after birth by a study nurse. Parents will then be asked to collect stool samples from their infants when they are 7 days, 1, 2, 4, 6, 12 and 24 months old. Reminders will be sent by SMS. To minimise variation, parents are asked to collect stool from the first bowel movement of the day (with date and time recorded). Sterile containers will be provided.

Storage of samples**:** Parents will be instructed to freeze breast milk, oral swabs and stool samples in sealed bags in their domestic freezer at -20°C until collection by the research team. Samples will be kept frozen during transportation to the laboratory where they will be aliquoted and stored at -80°C.

DNA extraction and sequencing**:** DNA from breast milk, oral swabs and stool samples (approximately 200 mg) will be extracted using the FastDNA Spin Kit for soil (MP Biomedicals, Santa Ana, California, USA). DNA concentrations will be quantified using a Qubit 4.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and high sensitivity DNA reagents. Bacterial DNA will be quantified by broad-range bacterial qPCR. The library preparation will be done using Nextera DNA Flex Library Preparations Kits. Extracted DNA will be indexed with IDT Illumina Nextera DNA Unique Dual Indexes to allow analysis of pooled samples. 150-bp pair-end sequencing will be done using the Illumina NextSeq. The required sequencing depth to provide adequate coverage of microbial

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communities for taxonomic profiling will be determined by rarefaction curves. We will aim for a minimum yield of $2x10^6$ read-pairs per sample. Appropriate negative controls (including controls from sterile containers, extraction kits etc.) and positive controls of mock communities will be included. These controls will be sequenced together with the samples to identify potential environmental and laboratory contaminants.

Researchers carrying out the microbial analyses will be blinded to the group identity of infants (control or antibiotic exposed group).

Storage of blood samples: Peripheral blood mononuclear cells will be separated from whole blood and stored in liquid nitrogen. Plasma will be stored frozen at -80°C. These will be retained for future analysis in projects to evaluate the effect of microbiome on the immune system, including immunophenotyping and analysis of markers of immune function.

Bioinformatics and statics plan

Bioinformatics: Sequences will be trimmed according to quality scores and sequencing adaptors will be removed using Trimmomatic.[25] Host sequences will be removed by mapping against the Human genome with Bowtie2.[26] High-quality sequences will be used to create taxonomic and functional profiles using MetaphlAn2[27] and HUMAnN2[28], respectively. Antibiotic resistance genes will be identified using ResFinder.[29] The outputs will be tables with taxonomically classified sequence counts and gene abundances.

Statistical and association analysis: Metrics describing and summarising the different dimensions of microbiome composition will be considered for statistical analyses. Relative abundances of bacterial and non-bacterial (archaea, eukaryotes and viruses) taxa, as well as metabolic functional and antibiotic resistance genes profiles, will be directly integrated for some analyses. Microbial abundances will also be summarised in alpha-diversity indexes to describe the number of different taxa (Chao richness) and their distribution (Simpson diversity) within each sample. Inter-samples distances will be described in standard beta-

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diversity indexes matrices (Jaccard, Bray-Curtis, UniFrac and weighted UniFrac). All these different metrics will be studied in appropriate statistical analyses to investigate the relationships between sample groups of interest and correlation between clinical metadata and microbiome composition.

Statistical analyses will compare these metrics in different sample-groups of interest. Antibiotics exposed samples will be compared to non-exposed samples for significant changes in relative abundance of bacterial taxa, antibiotic resistance genes content and alpha-diversity by Pearson x^2 test and logistic regression.

Infant age, demographics, delivery mode and feeding method will be modelized in permutational multivariate analysis of variance (PERMANOVA, adonis2 in VEGAN package, R Foundation) using beta-diversity distance matrices to identify significant contributors to the infant stool microbiome composition. To compare paired maternal stool, breast milk, and infant stool samples, we will do clustering analysis and perform a Wilcoxon rank sum test on beta-diversity distances between true mother-infant pairs and randomly paired mothers and infants matched by infant age. The same will be done for comparison of the breast milk and the infant oral microbiome. These different analyses should allow to describe the relationships between samples of different origins and identify determinants of microbiome composition.

To further characterise the mother-to-infant microbiome transmission, the StrainPhlAn software will be used.[30] This software enables same-species sequence comparisons at the single nucleotide polymorphism (SNPs) level to define sample-specific strains. Combined results for shared species allow precise description of the phylogenetic distance between samples. Added to the beta-diversity analyses, this will allow to better disentangle the hypothesised seeding from maternal stool and breast milk to the infant oral and stool microbiome.

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For integrated analysis of the microbiome data and clinical outcomes, allergic sensitisation, eczema, and overweight cases will be defined using the prospectively collected outcome measures. The relationship between bacterial taxa and these clinical outcomes will be determined by hierarchical clustering of communities using heatmaps and principal component analysis (PCA). Significance of grouping of clinical categories using permutational multivariate analysis of variance (PERMANOVA). Microbes that have significantly different abundance between the clinical outcome groups will be identified using the multiple testing ("mt") function in phyloseq.[31] The potential influence of antenatal and postnatal factors on the microbiome or clinical outcomes will be accounted for in all analyses by PERMANOVA and unsupervised hierarchical clustering.

ETHICS AND DISSEMINATION

Ethics approval

The ABERRANT study has been approved by the Commission cantonale d'éthique de la recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-01567).

Recruitment and consent

Written informed consent will be obtained from all participants included in the trial. Participants will be informed that they are not obliged to take part in the study and are free to withdraw at any time without any impact on their future care.

Data collection and storage

Data will be sourced from medical maternal and infant records, as well as by questionnaire from parents. Data will be de-identified and entered in to a secure, web-based electronic database.

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Safety

No serious adverse reactions are anticipated but these will be checked for by the Data Safety and Monitoring Committee.

Dissemination of results

Outcomes will be disseminated through publication according to the SPIRIT statement and will be presented at scientific conferences.

Study duration

We aim to recruit participants over a two-year period.

DISCUSSION

The intestinal microbiome is crucial in the development of the immune system and regulation of immune responses, especially during infancy, when the intestinal microbiome and the immune response develop concurrently.[32] The development of intestinal microbiome is easily disrupted by external factors and perturbation during this vulnerable period may have a large influence on immune development. A number of factors influence the development of the infant intestinal microbiome, including gestational age, delivery mode, feeding, and maternal and infant antibiotic exposure **(Figure 1)**.[33] While the effect of delivery mode and feeding methods on the establishment of microbial communities has been well studied, much less is known about the effects of intrapartum and early-life antibiotic exposure on the establishment of microbial communities in the intestinal microbiome.[9]

A number of commonly used antibiotics have profound effects on specific bacteria within the intestinal microbiome, as detailed in a recent systematic review.[8] This 'collateral damage' includes changes in abundance of microbial taxa, a decrease in 'colonisation resistance' (protection against colonisation with potentially pathogenic organisms) and the development

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of antibiotic resistance. To date, most studies on the effect of antibiotic exposure on the intestinal microbiome have been done in adults.[8] The main findings of these studies are that antibiotics often lead to a decreased bacterial diversity, a decrease in the abundance of anaerobic bacteria, an increase in abundance of *Enterobacteriaceae* other than *E. coli* and an increase in the abundance of yeast.[8] These studies show that changes in the intestinal microbiome after just one course of antibiotics can persist up to four years.[8] However, the clinical consequences of changes in the composition of the intestinal microbiome with antibiotic treatment are unknown. An increase in Enterobacteriaceae, which are often resistant to beta-lactam and other antibiotics, might render the host more susceptible to infections with antibiotic-resistant bacteria. This phenomenon has been observed in infants in neonatal intensive care units, who become more often colonised with *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp., when treated with antibiotics.[34]

Intrapartum antibiotic prophylaxis (IAP) has become common practice in obstetric medicine and is used in up to 40% of deliveries, which makes it the most common source of antibiotic exposure in neonates.[1] IAP is routinely used in both elective and emergency Caesarean section (CS). It is also routinely used in women who are colonised with group B streptococcus (GBS). Despite the benefits, the risks associated with exposing a large number of infants to antibiotics, especially the long-term effects on health through changes in the microbiome, remain unclear. Infants who were exposed to IAP have been reported to have a lower alpha-diversity, a lower relative abundance of Actinobacteria, especially *Bifidobacteriaceae,* and a larger relative abundance of Proteobacteria in their intestinal microbiome compared to non-exposed infants.[9]

Breastfeeding has been shown to be beneficial in preventing many communicable and non-communicable diseases.[35] Despite intensive research into the positive health effects of breastfeeding, the underlying mechanisms are still not understood. However, a large part of the beneficial effects of breast milk is likely mediated through the microbiome and its

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associated immunomodulatory, anti-inflammatory and antimicrobial components. The breast milk microbiome also plays a large part in shaping the infant's intestinal microbiome.[4]

There is relatively little data about the effects of IAP on the composition of the breast milk microbiome.[36 37] Mothers who receive IAP have been reported to have a lower abundance or even an absence of the beneficial bacteria *Bifidobacterium* spp. in their breast milk.[36 37] There is also some evidence suggesting that mothers who receive IAP have a higher bacterial richness and diversity in their breast milk microbiome compared with mothers who do not receive antibiotics.[36] However, these findings have to be interpreted with caution: it could be that antibiotics lead to lower bacterial numbers and therefore signals from contamination, e.g. bacteria found in DNA extraction or sequencing kits might be amplified more leading to a the detection of a higher diversity. Use of broad-range qPCR to quantity bacterial load in milk samples will allow to assess this potential bias.

Interestingly, recent preliminary studies have also shown that delivery mode affects the composition of the breast milk microbiome.[36 38 39] However, during suckling, a high degree of retrograde flow of milk into the mammary ducts can occur,[5] transferring bacteria from the infant to the mother, as postulated for GBS.[40] It is therefore possible that the differences in the breast milk microbiome observed with different delivery modes are mediated through differences in the oral microbiome of infants.

This study will determine the effect of intrapartum and early-life antibiotics on the composition of the breast milk, and the infant oral and intestinal microbiome and antibiotic resistance. The knowledge gained by this study will make an important contribution to the growing field of research investigating the importance of the immunological role of the breast milk microbiome and the infant intestinal microbiome on infant health. It will form the basis for investigating the interplay between the microbiome and the regulation of the human

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397 immune system and possible adverse health outcomes, such as the development of immune
398 and non-immune mediated diseases, including allergic diseases.
399 The results of this study will also build a stronger evidence base for strict antibiotic
400 stewardship and form the basis for development of evidence-based interventions to prevent
401 adverse outcomes in situations where antibiotics cannot be avoided, including modifying the
402 intestinal microbiome with directed pre- and probiotics or bacteriophages.

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Contributors VS, LF, NC and PZ were responsible for study conception and design. JW, AL and PZ were responsible for funding acquisition and implementation. MV drafted the manuscript and coordinated the manuscript preparation and revision. PZ has developed the statistical analysis plan. MV and PZ have developed the online questionnaires and database set-up in REDCap, based on the database setup by the Melbourne Infant Study: BCG for Allergy and Infection Reduction (MIS BAIR).[41] All authors provided critical evaluation and revision of manuscript and have given final approval of the manuscript accepting responsibility for all aspects.

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Table 1 Clinical outcomes for aim 3

Outcome	Main measure	Timing
Lower respiratory tract illness	Number of episodes & hospitalisations[19 20]	first 2 years of life
Acute otitis media	Number of episodes[19 20]	first 2 years of life
Allergic (atopic) sensitisation	Prevalence (positive skin prick test)[23]	at 2 years of age
Eczema	Prevalence (Williams criteria)[21]	at 1 and 2 years of age
Weight	Centile (WHO Child Growth Standards)[18]	at 1 and 2 years of age

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Table 2 Study protocol

Time	Ant	Birth	7d	1m	2m	4m	6m	12m	24m
Diary			✓	✓	✓	✓	✓	✓	✓
Questionnaire			✓		✓	✓	✓	✓	✓
Maternal blood sample		✓							
Maternal stool sample	✓	✓							
Breast milk sample		✓col	✓	✓	✓	✓	✓*		
Infant oral swab		✓	✓	✓	✓	✓	✓		
Infant stool sample		✓mec	✓	✓	✓	✓	✓	✓	✓
Clinical examination								✓	✓
Skin prick test									✓
Blood sampling (optional)		✓cb						✓	✓

Ant = antenatal; col = colostrum; mec = meconium; [3] cb = cord blood;
*or before breastfeeding is discontinued if earlier than 6 m

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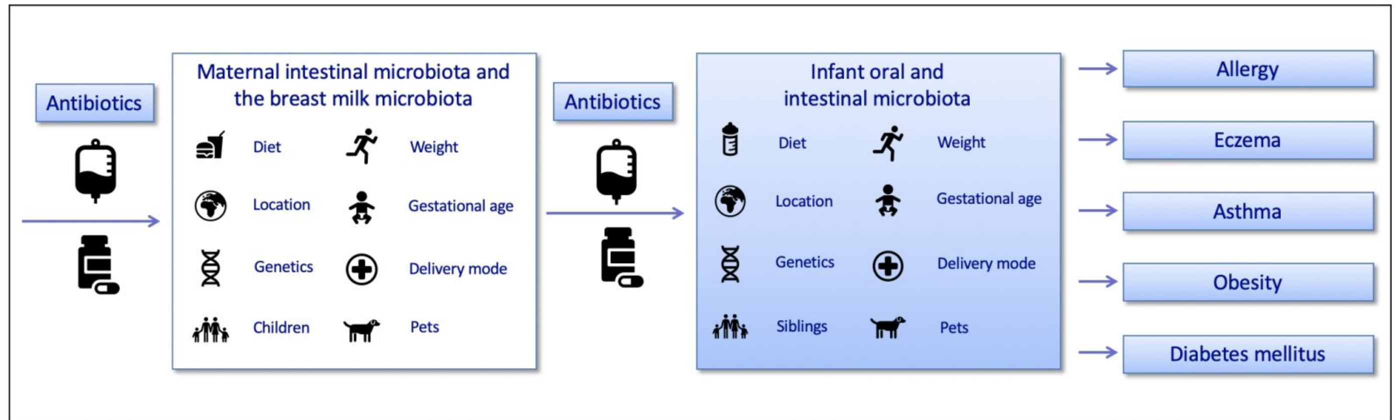
Figure 1 Summary of factors that might influence the composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome together with possible associated adverse health outcomes.



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Study protocol for the ABERRANT study: Antibiotic-induced disruption of the maternal and infant microbiome and adverse health outcomes - A prospective cohort study among children born at term

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ABSTRACT

Introduction: There is compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome. Antibiotics cause profound changes in the microbiome. However, the effect of intrapartum and early-life antibiotics on the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome, and whether effects are only short-term or persist long-term remain uncertain.

Methods and analyses: In this prospective cohort study, we will use metagenomic sequencing to determine: (i) the effect of *intrapartum antibiotics* on the composition of the breast milk, and the infant oral and intestinal microbiome, including the development and persistence of antibiotic resistance; (ii) the effect of *antibiotic exposure in the first year of life* on the composition of the infant oral and intestinal microbiome, including the development and persistence of antibiotic resistance; (iii) the effect of disruption of the infant oral and intestinal microbiome on health outcomes; and (iv) the compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.

Ethics and dissemination: The ABERRANT study has been approved by the Commission cantonale d'éthique de la recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-01567). Outcomes will be disseminated through publication and will be presented at scientific conferences.

Trial registration number: The U.S. National Institutes of Health NCT04091282.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- The use of metagenomic sequencing to comprehensively determine the effect of intrapartum and early-life antibiotics on the composition of the maternal breast milk and the infant oral and intestinal microbiome.
- The possibility to identify bacterial species, together with resistance genes and other important components of the microbiome such as archaea, eukaryotes (fungi) and viruses.
- The investigation of the association between the early-life intestinal microbiome and clinical health outcomes.
- The potential for contamination of low microbial biomass such as breast milk or meconium samples from the environment or extraction and sequencing kits.

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INTRODUCTION

Antibiotics are amongst the most commonly used drugs, especially in infants and children. Even before birth, more than 40% of infants are exposed to antibiotics.[1] Additionally, more than two-thirds of children receive antibiotics before reaching the age of two years.[2 3] The human intestine is the habitat for a large community of microbes, the intestinal microbiome. Colonisation of the intestinal tract increases rapidly after birth and the microbiome of the maternal intestine and breast milk are likely important source for the infant intestinal microbiome.[4] Conversely, as retrograde flow of breast milk into mammary ducts has been documented,[5] the infant oral microbiome might be responsible for colonising the mammary ducts and therefore could contribute to the breast milk microbiome. Consequently, there is compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.[6 7]

Growing evidence shows that the composition of the intestinal microbiome in infants plays an important role in the development and regulation of the immune system, especially in the early-life 'critical window' during which the microbiome and the immune response develop concurrently.

Antibiotics cause profound changes in the microbiome.[8 9] However, the magnitude of the effect of intrapartum and early-life antibiotics on the breast milk, and the infant oral and intestinal microbiome, and whether effects are only short-term or persist long-term remain uncertain. Preliminary studies suggest that disruption of intestinal microbiome in the early-life period is associated with the development of a number of immune- and non-immune-mediated diseases, including allergies,[10] eczema,[10] asthma,[10] chronic inflammatory bowel disease,[11] obesity[12] and diabetes mellitus.[13] Antibiotic exposure *in utero* and during infancy has been associated with an increased risk for the same diseases[14-16] and it is likely that the

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association between antibiotic exposure and the subsequent development of these diseases is mediated through changes in the infant microbiome. However, the features and composition characteristics of the intestinal microbiome associated with the development of these conditions are unclear.

Importantly, antibiotic exposure can also lead to the development of antibiotic resistance,[8] resulting in infections that are more difficult and costly to treat, often requiring longer duration of hospital stay, and the use of antibiotics with more adverse effects. Currently, in children, there is sparse data available on the effect of antibiotic exposure on the development and persistence of antibiotic resistance in their intestinal microbiome.

In this prospective cohort study, we will use metagenomic sequencing to determine (i) the effect of *intrapartum antibiotics* on the composition of the breast milk, and the infant oral and intestinal microbiome (including the development and persistence of antibiotic resistance); (ii) the effect of *antibiotic exposure in the first year of life* on the composition of the infant oral and intestinal microbiome (including the development and persistence of antibiotic resistance); (iii) the effect of disruption of the infant oral and intestinal microbiome on health outcomes; and (iv) determine the compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.

Determining the relationship between antibiotic exposure and changes in the breast milk, and the infant oral and intestinal microbiome, and their potential association with adverse health outcomes will provide stronger evidence for strict antibiotic stewardship. Additionally, it will form the basis for designing studies to investigate interventions to prevent adverse outcomes in situations where antibiotics cannot be avoided, including modifying the intestinal microbiome with directed pre- and probiotics, or bacteriophages.

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OBJECTIVES

METHODS AND ANALYSIS

Study design

A prospective single-centre cohort study of 400 mother-infant pairs.

Aims

Aim 1: To determine the extent to which, and for how long, intrapartum antibiotics affect the composition of the breast milk microbiome and the infant oral and intestinal microbiome, as well as the prevalence of antibiotic resistance genes.

Aim 2: To determine the extent to which, and for how long, antibiotics in the first year of life affect the composition of the oral and intestinal microbiome in infants, as well as the prevalence of antibiotic resistance genes.

Aim 3: To determine health outcomes (**Table 1**) in children up to the age of 2 years who have or have not been exposed to intrapartum antibiotics or antibiotics in the first year of life and determine whether there is an association with the composition of the oral and intestinal microbiome.

Aim 4: To determine the degree to which the maternal intestinal and the breast milk microbiome affect the composition of the infant oral intestinal microbiome and the prevalence of antibiotic resistance genes.

Aim 5: To determine the degree to which the infant oral microbiome affects the composition of the breast milk microbiome.

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Outcomes

Primary endpoints:

- Composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome and the prevalence of antibiotic resistance genes within the infant microbiome at birth and when infants are 7 days, 1, 2, 4, 6, 12 and 24 months of age.
- Number of episodes of lower respiratory tract illnesses and acute otitis media in the first two years of life.
- Prevalence of allergic sensitisation and eczema at 1 and 2 years of age.
- Weight at 1 and 2 years of age.

Setting and participants

Sampling frame and study sample: Women who give birth at the Hôpital fribourgeois (HFR) in Fribourg, Switzerland and their infants will be followed over a two-year period. If recruitment is slow a second study site will be added.

Recruitment: Pregnant women attending the antenatal clinic will be given information about the study by a research study nurse or doctor and asked to consider enrolling themselves and their infant in the study. Either both parents or only mothers are present during the antenatal consent interview. We explicitly encourage caregivers to discuss participation with their partners, other family members, doctors and midwives (this is clearly stated in the consent form). We will re-evaluate the willingness to participate when mothers are admitted to the hospital for delivery. Paediatricians will be informed about the children's participation in the study.

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Blinding of outcome assessment: Doctors and study nurses will be blinded to the group of infants (control or antibiotic-exposed) when outcomes are measured.

Patient and Public Involvement

Patients and public were not involved in the design of this study. The results of this study will be disseminated to parents of the study participants via a participant newsletter distributed by email.

Eligibility criteria

Inclusion criteria: Healthy infants born at 37 weeks or more gestation who are breastfed. Mothers will be asked at an antenatal consent interview if they intend to breastfeed. This will be reassessed at delivery. Mothers will only be included if they breastfeed their infants. However, if breastfeeding is stopped before the infant reaches six months of age, this will not be a reason for exclusion. All the breast milk samples up to that point, as well as stool samples and oral swabs collected afterwards will be analysed.

Exclusion criteria: Women with the following criteria: HIV, hepatitis B or C infection or unscreened for these infections, antibiotics or probiotics in pregnancy or postpartum period other than during delivery. Infants with the following criteria: low birth weight (<2500 g) or severe congenital abnormality.

Study outcome measures

We will use internationally accepted validated measures for clinical outcomes. The study protocol is depicted in **Table 2**.

Diary: Parents will be given a structured diary where they can record information about their

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infant's diet (introduction of formula and new foods), illnesses, medical visits, hospital admissions, use of antibiotics and other medications. This will help them when filling in the questionnaires at the required time points.

Questionnaire: We will do computer-assisted interviews at birth, and when infants are 1, 6, 12 and 24 months of age using best practice international protocols. The following data will be recorded: demographic variables including parental ancestry and education, family history of atopy, eczema, asthma and other immune disorders, antenatal variables such as maternal age, weight, smoking habits, underlying diseases, medication and supplementation use (e.g. probiotics and vitamins). In addition, we will collect data on delivery history, perinatal course (e.g. hospitalisation, infections, antibiotics or oxygen administration), breast-feeding (including episodes of mastitis and maternal antibiotic and probiotic use), age of introduction of formula and new foods, administration of probiotics and vitamins, use of antibiotics, antacids and other medications, GP and other medical visits, illnesses including infections and hospital admissions, number of siblings, child care attendance, parental smoking habits, pet ownership, suspected food allergy and eczema (presence, medications). Data will be stored using the Research Electronic Data Capture (REDCap Consortium) database.[17]

Clinical examination: Participants will be reviewed at 12 and 24 months of age in a specially designated clinic at the HFR by a study nurse or doctor using a structured interview and clinical eczema assessment.

Weight: Weight will be assessed during the clinical examination at 12 and 24 months of age. The WHO Child Growth Standards will be used as a reference for percentiles.[18]

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Lower respiratory tract illness (LRTI) and acute otitis media (AOM): Symptoms of acute lower respiratory illness (such cough and wheeze) and the number of episodes of LRTI and AOM will be recorded by parents, and specific questions will be asked in the questionnaires. We will use the definitions for LRTI developed by Oddy *et al* and Kusel *et al.*[19 20]

Eczema: Prevalence of eczema at 12 and 24 months of life will be assessed by the Williams' UK diagnostic criteria of the International Study of Asthma and Allergy in Childhood (ISAAC).[21] This will be assessed by using parent-reported eczema from diary information and from the clinical examinations at 12 and 24 months of age. We will assess the severity of eczema using the SCORAD.[22] We will also collect data on age of onset of eczema, distribution of eczema, use of eczema medications, and medical consultations and hospital admissions.

Skin prick tests: Sensitisation to the following panel of allergens will be assessed at 24 months of age: cow's milk, egg, peanut, sesame, house dust mite (*Dermatophagoides pteronyssinus 1*), cat, dog and grass pollen. Skin prick allergy testing will be performed according to standard guidelines.[23] A positive skin prick test will be defined as an average wheal diameter at least 3 mm greater than that produced by a negative control solution at 15 minutes.[24]

Blood sampling: We will collect maternal blood at time of delivery. We also will obtain cord blood at birth. During the clinical examination at 12 and 24 months of age, blood will be collected by personnel trained in infant venipuncture, whose parents consent to this component of the study. The 5-10 ml volume required is safe and within limits for weight recommended by the US-based Office of Human Research Protections guidelines for blood collection from healthy infants.

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***Breast milk samples*:** Study nurses will collect one colostrum sample as soon as possible after birth. Mothers will be asked to collect breast milk (with date and time recorded) from their first feed of the day (a minimum of 2 hours required to the previous feed) when infants are 7 days, 1, 2, 4, and 6 months old. If breastfeeding is ceased earlier than 6 months, breast milk will be collected before breastfeeding is discontinued. Mothers will be instructed to wash their hands and breasts meticulously and to then extract breast milk manually without touching the areola. The first few drops will be discarded. Reminders will be sent by SMS. Sterile containers will be provided.

Oral swabs: Oral swabs will be taken from infants as soon as possible after birth by a study nurse. Additionally, parents will then be asked to collect buccal swabs (with date and time recorded) before the first feed of the day when infants are 7 days, 1, 2, 4, and 6 months old. Sterile containers will be provided. Reminders will be sent by SMS.

Stool samples: Mothers will be asked to collect stool on or after the day of the delivery (with date and time recorded). A meconium sample will be collected from infants as soon as possible after birth by a study nurse. Parents will then be asked to collect stool samples from their infants when they are 7 days, 1, 2, 4, 6, 12 and 24 months old. Reminders will be sent by SMS. To minimise variation, parents are asked to collect stool from the first bowel movement of the day (with date and time recorded). Sterile containers will be provided.

***Storage of samples*:** Parents will be instructed to freeze breast milk, oral swabs and stool samples in sealed bags in their domestic freezer at -20°C until collection by the research team. Samples will be kept frozen during transportation to the laboratory where they will be aliquoted and stored at -80°C.

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267 ***DNA extraction and sequencing*:** DNA from breast milk, oral swabs and stool samples
268 (approximately 200 mg) will be extracted using the FastDNA Spin Kit for soil (MP
269 Biomedicals, Santa Ana, California, USA). DNA concentrations will be quantified using a
270 Qubit 4.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and high
271 sensitivity DNA reagents. Bacterial DNA will be quantified by broad-range bacterial
272 quantitative polymerase chain reaction (qPCR). The library preparation will be done using
273 Nextera DNA Flex Library Preparations Kits. Extracted DNA will be indexed with IDT
274 Illumina Nextera DNA Unique Dual Indexes to allow analysis of pooled samples. 150-bp
275 pair-end sequencing will be done using the Illumina NextSeq. The required sequencing depth
276 to provide adequate coverage of microbial communities for taxonomic profiling will be
277 determined by rarefaction curves. We will aim for a minimum yield of $2x10^6$ read-pairs per
278 sample. Appropriate negative controls (including controls from sterile containers, extraction
279 kits etc.) and positive controls of mock communities will be included. These controls will be
280 sequenced together with the samples to identify potential environmental and laboratory
281 contaminants.
282 Researchers carrying out the microbial analyses will be blinded to the group identity of
283 infants (control or antibiotic exposed group).
284
285 ***Storage of blood samples:*** Peripheral blood mononuclear cells will be separated from whole
286 blood and stored in liquid nitrogen. Plasma will be stored frozen at -80°C. These will be
287 retained for future analysis in projects to evaluate the effect of microbiome on the immune
288 system, including immunophenotyping and analysis of markers of immune function.
289
290 **Statistical power calculation**
291 The analysis for the a priori computation of the required sample size was conducted using the
292 G*Power 3.1 software. For the power analysis, the repeated measurements MANOVAs were

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considered. The calculation was based on F-tests for the interaction effect of the between-subject factor antibiotics vs no antibiotics and the within-subject factor time (8 time points). We chose a small to medium effect size of 0.22 for the calculation. With a significance level of 5% per test and a power of 80% the power analysis yields a sample size of 304. While we will attempt to retain and follow up all participants, we are powering our study on 'a worst-case scenario' assumption that complete 24-month data will be available for 76% of participants (this corresponds to a drop-out rate of 12% per year). We will therefore recruit approximately 400 infants in total (plus their mothers, therefore 800 participants). Approximately 40-50% of infants are exposed to prophylactic intrapartum antibiotics at our institution for colonisation with Group B *streptococcus* or premature rupture of membranes. Additional 50-60% of children are expected to be exposed to antibiotics during the first two years of life.

Bioinformatics

Sequences will be trimmed according to quality scores and sequencing adaptors will be removed using Trimmomatic.[25] Host sequences will be removed by mapping against the Human genome with Bowtie2.[26] High-quality sequences will be used to create taxonomic and functional profiles using MetaphlAn2[27] and HUMAnN2[28], respectively. Antibiotic resistance genes will be identified using ResFinder.[29] We will share our metagenomic data through the European Nucleotide Archive (ENA).

Statistical analysis

Considered metrics:

Alpha-diversity: Alpha-diversity indexes are descriptive of the intra-sample richness (number of taxonomic functional features), evenness (features distribution) or diversity (richness weighted by evenness).[30] We will use Chao richness (number of different taxa) and Simpson

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1
2
3 319 diversity (distribution of taxa) to summarise the alpha-diversity for each sample.
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7
8 321 *Beta-diversity:* For beta-diversity, samples are compared for their composition in features of
9
10 322 interest (taxonomic or functional units).[31] All-versus-all distances between samples are
11
12 323 computed in pairwise comparisons and summarised in distance-matrices. We will use indices,
13
14 324 with distance equal to 1-index value, to capture different dimensions of microbial structures,[31]
15
16 325 restricting ourselves to non-Euclidean indices and excluding the "joint absences" as sharing of
17
18 326 unseen features would have doubtful significance in this context. Feature presence/absence will
19
20 327 be described by Jaccard indices and quantitative overlap by Bray-Curtis indices. In taxonomic
21
22 328 comparisons, Unifrac (presence/absence) and Weighted Unifrac (quantitative) will be used to
23
24 329 account for features phylogenetic distances.[32] Statistics on distances matrices require adapted
25
26 330 methods to assess for significant differences in average location (centroid) of the samples of
27
28 331 groups of interest. For this, we will use the PERMANOVA approach implemented in the
29
30 332 Adonis2 function of the vegan R package. PERMANOVA can be sensitive to variance
31
32 333 heterogeneity in unbalances groups. Thus, variance homogeneity will be tested by ANOVA to
33
34 334 centroids.
35
36 335
37
38 336 *Differential abundance testing:* Metagenomics data are compositional due to technical
39
40 337 limitations.[33,34] Relative abundances of bacterial and non-bacterial (archaeal, eukaryotic and
41
42 338 virusal) taxa and antibiotic resistance genes, will be directly integrated for analyses. For
43
44 339 bacteria, we will transform observed proportions into absolute quantities by multiplying
45
46 340 proportions (%) by measured microbial loads, quantified by broad-range qPCR. Thus, we will
47
48 341 explore correlation abundance testing of transformed counts for taxa and bacterial resistance
49
50 342 genes. Statistical challenges of metagenomics data are the high number of features (and
51
52 343 related multiple-testing false-discovery) and features sparsity (a given features – species or
53
54 344 genes – will be observed only in a few samples, leading to a high proportion of zeros in count
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345 tables). These limitations in metagenomics statistics were only recently recognised and the
346 developments of methods accounting for these is a field of active research and publication.
347 Thus, we will follow on future developments, recommendation and consensuses in the field
348 regarding these challenges. Currently identified solutions accounting for these limitations are
349 MetagenomSeq, Aldex2 and Maaslin2, which all integrate normalisation and correlation
350 testing to account for the pre-mentioned limitations while trying to identify differentially
351 abundant features (genes or speices) between tested groups.
352
353 *Statistical plan for aim 1 and 2:* Antibiotics exposed samples will be compared to non-
354 exposed samples (grouped per received drug) for differences in alpha-diversity metrics and
355 abundances of bacterial and non-bacterial taxa and antibiotic resistance genes with significant
356 changes defined as a > 0.5% change in abundance between groups. Alpha-diversity metrics
357 are continuous numeric values. Normality of the data will be checked by the Kolmogorov-
358 Smirnov test and QQ-plots. For normal variables, a one-way ANOVA will be used. If
359 normality cannot be reached by transformation, differences between groups will be analysed
360 using a nonparametric Kruskal-Wallis test, or in case of pairwise comparisons, a Mann-
361 Whitney U-test. For comparison of abundance Pearson x^2 tests will be used. Infant age,
362 demographics, delivery mode, feeding method and antibiotic type and dose will be modelized
363 in permutational multivariate analysis of variance (PERMANOVA, adonis2 in VEGAN
364 package, R Foundation) using beta-diversity distance matrices to identify significant
365 contributors to the microbiome composition.
366
367 *Statistical plan for aim 3:* For integrated analysis of the microbiome data and clinical
368 outcomes, allergic sensitisation, eczema, and overweight cases will be defined using the
369 prospectively collected outcome measures. The relationship between alpha-diversity and
370 clinical outcomes will be investigated using logistic regression. The relationship between taxa

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and these clinical outcomes will be determined by hierarchical clustering of communities using heatmaps and principal component analysis (PCA). Significance of grouping of clinical categories using permutational multivariate analysis of variance (PERMANOVA). Microbes that have significantly different abundance between the clinical outcome groups will be identified using Aldex2.[35] The potential influence of antenatal and postnatal factors on the microbiome or clinical outcomes will be accounted for in all analyses by PERMANOVA and unsupervised hierarchical clustering.

Statistical plan for aim 4: To compare paired maternal stool, breast milk, and infant stool samples, we will do clustering analysis and perform a Wilcoxon rank sum test on beta-diversity distances between true mother-infant pairs and randomly paired mothers and infants matched by infant age. These different analyses should allow to describe the relationships between samples of different origins and identify determinants of microbiome composition.

To further characterise the mother-to-infant microbiome transmission, the StrainPhlAn software will be used.[36] This software enables same-species sequence comparisons at the single nucleotide polymorphism (SNPs) level to define sample-specific strains. Combined results for shared species allow precise description of the phylogenetic distance between samples. Added to the beta-diversity analyses, this will allow to better disentangle the hypothesised seeding from maternal stool and breast milk to the infant oral and stool microbiome.

Statistical plan for aim 5: The same as for aim 4 will be done for comparison of the breast milk and the infant oral microbiome.

Missing data

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If the fraction of missing data is less than 5%, the primary analysis will be a complete case analysis. If not, the rate and patterns of missing data will be examined and, if appropriate, multiple imputation models will be applied for the outcome variables.

ETHICS AND DISSEMINATION

Ethics approval

The ABERRANT study has been approved by the Commission cantonale d'éthique de la recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-01567), Switzerland.

Recruitment and consent

Written informed consent will be obtained from all participants included in the trial. Participants will be informed that they are not obliged to take part in the study and are free to withdraw at any time without any impact on their future care.

Data collection and storage

Data will be sourced from medical maternal and infant records, as well as by questionnaire from parents. Data will be de-identified and entered in to a secure, web-based electronic database.

Safety

No serious adverse reactions are anticipated but these will be checked for by the Data Safety and Monitoring Committee.

Dissemination of results

Outcomes will be disseminated through publication according to the SPIRIT statement and will be presented at scientific conferences.

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Study duration

We aim to recruit participants over a two-year period.

DISCUSSION

The intestinal microbiome is crucial in the development of the immune system and regulation of immune responses, especially during infancy, when the intestinal microbiome and the immune response develop concurrently.[37] The development of intestinal microbiome is easily disrupted by external factors and perturbation during this vulnerable period may have a large influence on immune development. A number of factors influence the development of the infant intestinal microbiome, including gestational age, delivery mode, feeding, and maternal and infant antibiotic exposure **(Figure 1)**.[38] While the effect of delivery mode and feeding methods on the establishment of microbial communities has been well studied, much less is known about the effects of intrapartum and early-life antibiotic exposure on the establishment of microbial communities in the intestinal microbiome.[9]

A number of commonly used antibiotics have profound effects on specific bacteria within the intestinal microbiome, as detailed in a recent systematic review.[8] This 'collateral damage' includes changes in abundance of microbial taxa, a decrease in 'colonisation resistance' (protection against colonisation with potentially pathogenic organisms) and the development of antibiotic resistance. To date, most studies on the effect of antibiotic exposure on the intestinal microbiome have been done in adults.[8] The main findings of these studies are that antibiotics often lead to a decreased bacterial diversity, a decrease in the abundance of anaerobic bacteria, an increase in abundance of *Enterobacteriaceae* other than *E. coli* and an increase in the abundance of yeast.[8] These studies show that changes in the intestinal microbiome after just one course of antibiotics can persist up to four years.[8] However, the

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clinical consequences of changes in the composition of the intestinal microbiome with antibiotic treatment are unknown. An increase in Enterobacteriaceae, which are often resistant to beta-lactam and other antibiotics, might render the host more susceptible to infections with antibiotic-resistant bacteria. This phenomenon has been observed in infants in neonatal intensive care units, who become more often colonised with *Klebsiella* spp*., Enterobacter* spp. and *Citrobacter* spp., when treated with antibiotics.[39]

Intrapartum antibiotic prophylaxis (IAP) has become common practice in obstetric medicine and is used in up to 40% of deliveries, which makes it the most common source of antibiotic exposure in neonates.[1] IAP is routinely used in both elective and emergency Caesarean section (CS). It is also routinely used in women who are colonised with group B streptococcus (GBS). Despite the benefits, the risks associated with exposing a large number of infants to antibiotics, especially the long-term effects on health through changes in the microbiome, remain unclear. Infants who were exposed to IAP have been reported to have a lower alpha-diversity, a lower relative abundance of Actinobacteria, especially *Bifidobacteriaceae,* and a larger relative abundance of Proteobacteria in their intestinal microbiome compared to non-exposed infants.[9] Furthermore, they have been reported to have a higher number of beta-lactamase encoding genes.[40]

Breastfeeding has been shown to be beneficial in preventing many communicable and non-communicable diseases.[41] Despite intensive research into the positive health effects of breastfeeding, the underlying mechanisms are still not understood. However, a large part of the beneficial effects of breast milk is likely mediated through the microbiome and its associated immunomodulatory, anti-inflammatory and antimicrobial components. The breast milk microbiome also likely plays a part in shaping the infant's intestinal microbiome,[4] to which extent we will be investigated in this study.

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3 475 There is relatively little data about the effects of IAP on the composition of the breast milk
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5 476 microbiome.[42-44] Mothers who receive IAP have been reported to have a lower abundance or
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7 477 even an absence of the beneficial bacteria *Bifidobacterium* spp. in their breast milk.[42 43]
8
9 478 Furthermore, in a small study IAP has been shown to increase antibiotic resistance genes in
10
11 479 the breast milk microbiota.[45] There is also some evidence suggesting that mothers who
12
13 480 receive IAP have a higher bacterial richness and diversity in their breast milk microbiome
14
15 481 compared with mothers who do not receive antibiotics.[42] However, these findings have to be
16
17 482 interpreted with caution: it could be that antibiotics lead to lower bacterial numbers and
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19 483 therefore signals from contamination, e.g. bacteria found in DNA extraction or sequencing
20
21 484 kits might be amplified more leading to a the detection of a higher diversity. Use of broad-
22
23 485 range qPCR to quantity bacterial load in milk samples will allow to assess this potential bias.
24
25 486 Interestingly, recent preliminary studies have also shown that delivery mode affects the
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27 487 composition of the breast milk microbiome.[42 44 46 47] However, during suckling, a high degree
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29 488 of retrograde flow of milk into the mammary ducts can occur,[5] transferring bacteria from the
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31 489 infant to the mother, as postulated for GBS.[48] It is therefore possible that the differences in the
32
33 490 breast milk microbiome observed with different delivery modes are mediated through
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35 491 differences in the oral microbiome of infants.
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37 492 This study will determine the effect of intrapartum and early-life antibiotics on the
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39 493 composition of the breast milk, and the infant oral and intestinal microbiome and antibiotic
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41 494 resistance. The knowledge gained by this study will make an important contribution to the
42
43 495 growing field of research investigating the importance of the immunological role of the breast
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45 496 milk microbiome and the infant intestinal microbiome on infant health. It will form the basis
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47 497 for investigating the interplay between the microbiome and the regulation of the human
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49 498 immune system and possible adverse health outcomes, such as the development of immune
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51 499 and non-immune mediated diseases, including allergic diseases.
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3 501 The results of this study will also build a stronger evidence base for strict antibiotic
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6 502 stewardship and form the basis for development of evidence-based interventions to prevent
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8 503 adverse outcomes in situations where antibiotics cannot be avoided, including modifying the
9
10 504 intestinal microbiome with directed pre- and probiotics or bacteriophages.
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13 505
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15 506 **Contributors** PZ is the lead investigator. VS, LF, NC and PZ were responsible for study
16
17 507 conception and design. JW, AL and PZ were responsible for funding acquisition and
18
19 508 implementation. MV drafted the manuscript and coordinated the manuscript preparation and
20
21
22 509 revision. PZ and VS has developed the statistical analysis plan. MV and PZ have developed
23
24 510 the online questionnaires and database set-up in REDCap. WJ will be responsible for sample
25
26 511 analysis. MV, VS, WJ, DB, VDM, ALB, JW, LF and NC provided critical evaluation and
27
28
29 512 revision of manuscript and have given final approval of the manuscript accepting
30
31 513 responsibility for all aspects.
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35
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39
40 517 F. Taroni). None of the funders had a role in designing the study or in the study conduct and
41
42
43 518 they will not be involved in the publication of the results from the study.
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47 520 **Competing interests** None.
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52 522 **Provenance and peer review** Not commissioned; externally peer reviewed.
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Table 1 Clinical outcomes for aim 3

Outcome	Main measure	Timing
Lower respiratory tract illness	Number of episodes & hospitalisations[19 20]	first 2 years of life
Acute otitis media	Number of episodes[19 20]	first 2 years of life
Allergic (atopic) sensitisation	Prevalence (positive skin prick test)[23]	at 2 years of age
Eczema	Prevalence (Williams criteria)[21]	at 1 and 2 years of age
Weight	Centile (WHO Child Growth Standards)[18]	at 1 and 2 years of age

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Table 2 Study protocol

Time	Birth	7d	1m	2m	4m	6m	12m	24m
Diary		✓	✓	✓	✓	✓	✓	✓
Questionnaire		✓		✓	✓	✓	✓	✓
Maternal blood sample	✓							
Maternal stool sample	✓							
Breast milk sample	✓col	✓	✓	✓	✓	✓*		
Infant oral swab	✓	✓	✓	✓	✓	✓		
Infant stool sample	✓mec	✓	✓	✓	✓	✓	✓	✓
Clinical examination							✓	✓
Skin prick test								✓
Blood sampling (optional)	✓cb						✓	✓

col = colostrum; mec = meconium; [3] cb = cord blood;
*or before breastfeeding is discontinued if earlier than 6 m

Figure 1 Summary of factors that might influence the composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome together with possible associated adverse health outcomes

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

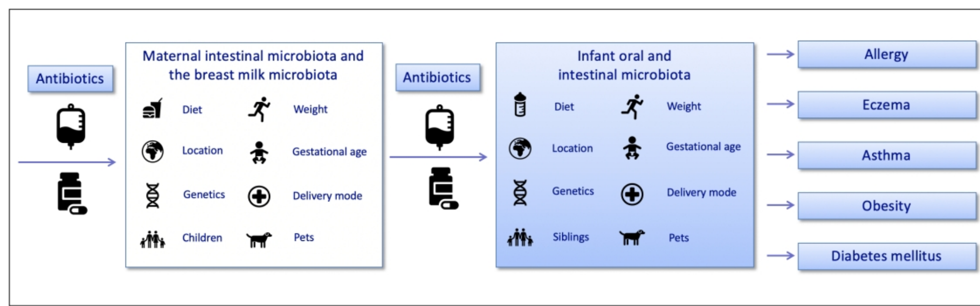

Figure 1

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Page Line
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1 1-2
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2 46
	2b	All items from the World Health Organization Trial Registration Data Set	Available at Clinical trials.gov
Protocol version	3	Date and version identifier	Approved by the CER-VD
Funding	4	Sources and types of financial, material, and other support	18 450
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1 5-20 18 441-4448
	5b	Name and contact information for the trial sponsor	1 22-24
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	18 452-453
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	18 441-448
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4-5

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	6b	Explanation for choice of comparators	4-5
Objectives	7	Specific objectives or hypotheses	6-7
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	7
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	7
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	7-8
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	no intervention
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	no intervention
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	NA
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	8-9 Table 1
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Table 2
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	11-12
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	7
Methods: Assignment of interventions (for controlled trials)			

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Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	NA
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	7
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	8-9
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	8-9
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	14
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	11-13
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	NA
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	14
Methods: Monitoring			

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Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	15
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	NA
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	14
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	NA
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	14
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	14
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Approved by the CER-VD
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	19
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Approved by the CER-VD
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Approved by the CER-VD
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	7
	31b	Authorship eligibility guidelines and any intended use of professional writers	NA

	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	14
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Attachment
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	9-11

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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Study protocol for the ABERRANT study: Antibiotic-induced disruption of the maternal and infant microbiome and adverse health outcomes - A prospective cohort study among children born at term

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ABSTRACT

Introduction: There is compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome. Antibiotics cause profound changes in the microbiome. However, the effect of intrapartum and early-life antibiotics on the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome, and whether effects are only short-term or persist long-term remain uncertain.

Methods and analyses: In this prospective cohort study, we will use metagenomic sequencing to determine: (i) the effect of *intrapartum antibiotics* on the composition of the breast milk, and the infant oral and intestinal microbiome, including the development and persistence of antibiotic resistance; (ii) the effect of *antibiotic exposure in the first year of life* on the composition of the infant oral and intestinal microbiome, including the development and persistence of antibiotic resistance; (iii) the effect of disruption of the infant oral and intestinal microbiome on health outcomes; and (iv) the compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.

Ethics and dissemination: The ABERRANT study has been approved by the commission cantonale d'éthique de la recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-01567). Outcomes will be disseminated through publication and will be presented at scientific conferences.

Trial registration number: The U.S. National Institutes of Health NCT04091282.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- The use of metagenomic sequencing to determine the effect of intrapartum and early-life antibiotics on the composition of the maternal breast milk and the infant oral and intestinal microbiome.
- The possibility to identify bacterial species, together with resistance genes and other important components of the microbiome such as archaea, eukaryotes (fungi) and viruses.
- The investigation of the association between the early-life intestinal microbiome and clinical health outcomes.
- The potential for contamination of low microbial biomass such as breast milk or meconium samples from the environment or extraction and sequencing kits.

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INTRODUCTION

Antibiotics are amongst the most commonly used drugs, especially in infants and children. Even before birth, more than 40% of infants are exposed to antibiotics.[1 2] Additionally, more than two-thirds of children receive antibiotics before reaching the age of two years.[3 4] The human intestine is the habitat for a large community of microbes, the intestinal microbiome. Colonisation of the intestinal tract increases rapidly after birth and the microbiome of the maternal intestine and breast milk are likely important source for the infant intestinal microbiome.[5] Conversely, as retrograde flow of breast milk into mammary ducts has been documented,[6] the infant oral microbiome might be responsible for colonising the mammary ducts and therefore could contribute to the breast milk microbiome. Consequently, there is compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.[7 8]

Growing evidence shows that the composition of the intestinal microbiome in infants plays an important role in the development and regulation of the immune system, especially in the early-life 'critical window' during which the microbiome and the immune response develop concurrently.[9-11]

Antibiotics cause profound changes in the microbiome.[12 13] However, the magnitude of the effect of intrapartum and early-life antibiotics on the breast milk, and the infant oral and intestinal microbiome, and whether effects are only short-term or persist long-term remain uncertain. Preliminary studies suggest that disruption of intestinal microbiome in the early-life period is associated with the development of a number of immune- and non-immune-mediated diseases, including allergies,[14] eczema,[14] asthma,[14] chronic inflammatory bowel disease,[15] obesity[16] and diabetes mellitus.[17] Antibiotic exposure *in utero* and during infancy has been associated with an increased risk for the same diseases[18-20] and it is likely that the

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association between antibiotic exposure and the subsequent development of these diseases is mediated through changes in the infant microbiome. However, the features and composition characteristics of the intestinal microbiome associated with the development of these conditions are unclear.

Importantly, antibiotic exposure can also lead to the development of antibiotic resistance,[12] resulting in infections that are more difficult and costly to treat, often requiring longer duration of hospital stay, and the use of antibiotics with more adverse effects. Currently, in children, there is sparse data available on the effect of antibiotic exposure on the development and persistence of antibiotic resistance in their intestinal microbiome.

In this prospective cohort study, we will use metagenomic sequencing to determine (i) the effect of *intrapartum antibiotics* on the composition of the breast milk, and the infant oral and intestinal microbiome (including the development and persistence of antibiotic resistance); (ii) the effect of *antibiotic exposure in the first year of life* on the composition of the infant oral and intestinal microbiome (including the development and persistence of antibiotic resistance); (iii) the effect of disruption of the infant oral and intestinal microbiome on health outcomes; and (iv) determine the compositional overlap between the maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.

Determining the relationship between antibiotic exposure and changes in the breast milk, and the infant oral and intestinal microbiome, and their potential association with adverse health outcomes will provide stronger evidence for strict antibiotic stewardship. Additionally, it will form the basis for designing studies to investigate interventions to prevent adverse outcomes in situations where antibiotics cannot be avoided, including modifying the intestinal microbiome with directed pre- and probiotics, or bacteriophages.

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OBJECTIVES

METHODS AND ANALYSIS

Study design

A prospective single-centre cohort study of 400 mother-infant pairs.

Aims

Aim 1: To determine the extent to which, and for how long, intrapartum antibiotics affect the composition of the breast milk microbiome and the infant oral and intestinal microbiome, as well as the prevalence of antibiotic resistance genes.

Aim 2: To determine the extent to which, and for how long, antibiotics in the first year of life affect the composition of the oral and intestinal microbiome in infants, as well as the prevalence of antibiotic resistance genes.

Aim 3: To determine health outcomes (**Table 1**) in children up to the age of 2 years who have or have not been exposed to intrapartum antibiotics or antibiotics in the first year of life and determine whether there is an association with the composition of the oral and intestinal microbiome.

Aim 4: To determine the degree to which the maternal intestinal and the breast milk microbiome affect the composition of the infant oral intestinal microbiome and the prevalence of antibiotic resistance genes.

Aim 5: To determine the degree to which the infant oral microbiome affects the composition of the breast milk microbiome.

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Outcomes

Primary endpoints:

- Composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome and the prevalence of antibiotic resistance genes within the infant microbiome at birth and when infants are 7 days, 1, 2, 4, 6, 12 and 24 months of age.
- Number of episodes of lower respiratory tract illnesses and acute otitis media in the first two years of life.
- Prevalence of allergic sensitisation and eczema at 1 and 2 years of age.
- Weight at 1 and 2 years of age.

Setting and participants

***Sampling frame and study sample*:** Women who give birth at the Hôpital fribourgeois (HFR) in Fribourg, Switzerland and their infants will be followed over a two-year period. If recruitment is slow a second study site will be added.

Recruitment: Pregnant women attending the antenatal clinic will be given information about the study by a research study nurse or doctor and asked to consider enrolling themselves and their infant in the study. Either both parents or only mothers are present during the antenatal consent interview. We explicitly encourage caregivers to discuss participation with their partners, other family members, doctors and midwives (this is clearly stated in the consent form). We will re-evaluate the willingness to participate when mothers are admitted to the hospital for delivery. Paediatricians will be informed about the children's participation in the study.

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Blinding of outcome assessment: Doctors and study nurses will be blinded to the group of infants (control or antibiotic-exposed) when outcomes are measured.

Patient and Public Involvement

Patients and public were not involved in the design of this study. The results of this study will be disseminated to parents of the study participants via a participant newsletter distributed by email.

Eligibility criteria

Inclusion criteria*:* Healthy infants born at 37 weeks or more gestation who are breastfed. Mothers will be asked at an antenatal consent interview if they intend to breastfeed. This will be reassessed at delivery. Mothers will only be included if they breastfeed their infants. However, if breastfeeding is stopped before the infant reaches six months of age, this will not be a reason for exclusion. All the breast milk samples up to that point, as well as stool samples and oral swabs collected afterwards will be analysed.

Exclusion criteria*:* Women with the following criteria: HIV, hepatitis B or C infection or unscreened for these infections, antibiotics or probiotics in pregnancy or postpartum period other than during delivery. Infants with the following criteria: low birth weight (<2500 g) or severe congenital abnormality.

Study outcome measures

We will use internationally accepted validated measures for clinical outcomes. The study protocol is depicted in **Table 2**.

Diary: Parents will be given a structured diary where they can record information about their

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infant's diet (introduction of formula and new foods), illnesses, medical visits, hospital admissions, use of antibiotics and other medications. This will help them when filling in the questionnaires at the required time points.

Questionnaire: We will do computer-assisted interviews at birth, and when infants are 7 days, 2, 4, 6, 12 and 24 months of age using best practice international protocols. The following data will be recorded: demographic variables including parental ancestry and education, family history of atopy, eczema, asthma and other immune disorders, antenatal variables such as maternal age, weight, smoking habits, underlying diseases, medication and supplementation use (e.g. probiotics and vitamins). In addition, we will collect data on delivery history, perinatal course (e.g. hospitalisation, infections, antibiotics or oxygen administration), breast-feeding (including episodes of mastitis and maternal antibiotic and probiotic use), age of introduction of formula and new foods, administration of probiotics and vitamins, use of antibiotics, antacids and other medications, GP and other medical visits, illnesses including infections and hospital admissions, number of siblings, child care attendance, parental smoking habits, pet ownership, suspected food allergy and eczema (presence, medications). Data will be stored using the Research Electronic Data Capture (REDCap Consortium) database.[21]

Clinical examination: Participants will be reviewed at 12 and 24 months of age in a specially designated clinic at the HFR by a study nurse or doctor using a structured interview and clinical eczema assessment.

Weight: Weight will be assessed during the clinical examination at 12 and 24 months of age. The WHO Child Growth Standards will be used as a reference for percentiles.[22]

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Lower respiratory tract illness (LRTI) and acute otitis media (AOM): Symptoms of acute lower respiratory illness (such cough and wheeze) and the number of episodes of LRTI and AOM will be recorded by parents, and specific questions will be asked in the questionnaires. We will use the definitions for LRTI developed by Oddy *et al* and Kusel *et al.*[23 24]

Eczema: Prevalence of eczema at 12 and 24 months of life will be assessed by the Williams' UK diagnostic criteria of the International Study of Asthma and Allergy in Childhood (ISAAC).[25] This will be assessed by using parent-reported eczema from diary information and from the clinical examinations at 12 and 24 months of age. We will assess the severity of eczema using the SCORAD.[26] We will also collect data on age of onset of eczema, distribution of eczema, use of eczema medications, and medical consultations and hospital admissions.

Skin prick tests: Sensitisation to the following panel of allergens will be assessed at 24 months of age in children whose parents consent to this component of the study: cow's milk, egg, peanut, sesame, house dust mite (*Dermatophagoides pteronyssinus 1*), cat, dog and grass pollen. Skin prick allergy testing will be performed according to standard guidelines.[27] A positive skin prick test will be defined as an average wheal diameter at least 3 mm greater than that produced by a negative control solution at 15 minutes.[27]

Blood sampling: We will collect maternal blood at time of delivery. We also will obtain cord blood at birth. During the clinical examination at 12 and 24 months of age, blood will be collected by personnel trained in infant venipuncture, whose parents consent to this component of the study. The 5-10 ml volume required is safe and within limits for weight recommended by the US-based Office of Human Research Protections guidelines for blood collection from healthy infants.

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Breast milk samples**:** Study nurses will collect one colostrum sample as soon as possible after birth. Mothers will be asked to collect breast milk (with date and time recorded) from their first feed of the day (a minimum of 2 hours required to the previous feed) when infants are 7 days, 1, 2, 4, and 6 months old. If breastfeeding is ceased earlier than 6 months, breast milk will be collected before breastfeeding is discontinued. Mothers will be instructed to wash their hands and breasts meticulously and to then extract breast milk manually without touching the areola. The first few drops will be discarded. Reminders will be sent by SMS. Sterile containers will be provided.

Oral swabs: Oral swabs will be taken from infants as soon as possible after birth by a study nurse. Additionally, parents will then be asked to collect buccal swabs (with date and time recorded) before the first feed of the day when infants are 7 days, 1, 2, 4, and 6 months old. Sterile containers will be provided. Reminders will be sent by SMS.

Stool samples: Mothers will be asked to collect stool on or after the day of the delivery (with date and time recorded). A meconium sample will be collected from infants as soon as possible after birth by a study nurse. Parents will then be asked to collect stool samples from their infants when they are 7 days, 1, 2, 4, 6, 12 and 24 months old. Reminders will be sent by SMS. To minimise variation, parents are asked to collect stool from the first bowel movement of the day (with date and time recorded). Sterile containers will be provided.

Storage of samples**:** Parents will be instructed to freeze breast milk, oral swabs and stool samples in sealed bags in their domestic freezer at -20°C until collection by the research team. Samples will be kept frozen during transportation to the laboratory where they will be aliquoted and stored at -80°C.

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***DNA extraction and sequencing*:** DNA from breast milk, oral swabs and stool samples (approximately 200 mg) will be extracted using the FastDNA Spin Kit for soil (MP Biomedicals, Santa Ana, California, USA). DNA concentrations will be quantified using a Qubit 4.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and high sensitivity DNA reagents. Bacterial DNA will be quantified by broad-range bacterial quantitative polymerase chain reaction (qPCR). The library preparation will be done using Nextera DNA Flex Library Preparations Kits. Extracted DNA will be indexed with IDT Illumina Nextera DNA Unique Dual Indexes to allow analysis of pooled samples. 150-bp pair-end sequencing will be done using the Illumina NextSeq. The required sequencing depth to provide adequate coverage of microbial communities for taxonomic profiling will be determined by rarefaction curves. We will aim for a minimum yield of 2-5x10^6 read-pairs per sample. Appropriate negative controls (including controls from sterile containers, extraction kits etc.) and positive controls of mock communities will be included. These controls will be sequenced together with the samples to identify potential environmental and laboratory contaminants.

Researchers carrying out the microbial analyses will be blinded to the group identity of infants (control or antibiotic exposed group).

Storage of blood samples: Peripheral blood mononuclear cells will be separated from whole blood and stored in liquid nitrogen. Plasma will be stored frozen at -80°C. These will be retained for future analysis in projects to evaluate the effect of microbiome on the immune system, including immunophenotyping and analysis of markers of immune function.

Statistical power calculation

The analysis for the a priori computation of the required sample size was conducted using the

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G*Power 3.1 software. For the power analysis, the repeated measurements MANOVAs were considered. The calculation was based on F-tests for the interaction effect of the between-subject factor antibiotics vs no antibiotics and the within-subject factor time (8 time points). We chose a small to medium effect size of 0.22 for the calculation. With a significance level of 5% per test and a power of 80% the power analysis yields a sample size of 304. While we will attempt to retain and follow up all participants, we are powering our study on 'a worst-case scenario' assumption that complete 24-month data will be available for 76% of participants (this corresponds to a drop-out rate of 12% per year). We will therefore recruit approximately 400 infants in total (plus their mothers, therefore 800 participants). Approximately 40-50% of infants are exposed to prophylactic intrapartum antibiotics at our institution for colonisation with Group B *streptococcus* or premature rupture of membranes. Additional 50 to 60% of children are expected to be exposed to antibiotics during the first two years of life.

Bioinformatics

Sequences will be trimmed according to quality scores and sequencing adaptors will be removed using Trimmomatic.[28] Host sequences will be removed by mapping against the Human genome with Bowtie2.[29] High-quality sequences will be used to create taxonomic and functional profiles using MetaphlAn2[30] and HUMAnN2[31], respectively. Antibiotic resistance genes will be identified using ResFinder.[32] We will share our metagenomic data through the European Nucleotide Archive (ENA).

Statistical analysis

Considered metrics:

Alpha-diversity: Alpha-diversity indexes are descriptive of the intra-sample richness (number of taxonomic functional features), evenness (features distribution) or diversity (richness

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320 weighted by evenness).[33] We will use Chao richness (number of different taxa) and Simpson
321 diversity (distribution of taxa) to summarise the alpha-diversity for each sample.
322
323 *Beta-diversity:* For beta-diversity, samples are compared for their composition in features of
324 interest (taxonomic or functional units).[34] All-versus-all distances between samples are
325 computed in pairwise comparisons and summarised in distance-matrices. We will use indices,
326 with distance equal to 1-index value, to capture different dimensions of microbial structures,[34]
327 restricting ourselves to non-Euclidean indices and excluding the "joint absences" as sharing of
328 unseen features would have doubtful significance in this context. Feature presence/absence will
329 be described by Jaccard indices and quantitative overlap by Bray-Curtis indices. In taxonomic
330 comparisons, Unifrac (presence/absence) and Weighted Unifrac (quantitative) will be used to
331 account for features phylogenetic distances.[35] Statistics on distances matrices require adapted
332 methods to assess for significant differences in average location (centroid) of the samples of
333 groups of interest. For this, we will use the PERMANOVA approach implemented in the
334 Adonis2 function of the vegan R package. PERMANOVA can be sensitive to variance
335 heterogeneity in unbalances groups. Thus, variance homogeneity will be tested by ANOVA to
336 centroids.
337
338 *Differential abundance testing:* Metagenomics data are compositional due to technical
339 limitations.[36 37] Relative abundances of bacterial and non-bacterial (archaeal, eukaryotic and
340 viral) taxa and antibiotic resistance genes, will be directly integrated for analyses. For
341 bacteria, we will transform observed proportions into absolute quantities by multiplying
342 proportions (%) by measured microbial loads, quantified by broad-range qPCR. Thus, we will
343 explore correlation abundance testing of transformed counts for taxa and bacterial resistance
344 genes. Statistical challenges of metagenomics data are the high number of features (and
345 related multiple-testing false-discovery) and features sparsity (a given features – species or

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346 genes – will be observed only in a few samples, leading to a high proportion of zeros in count
347 tables). These limitations in metagenomics statistics were only recently recognised and the
348 developments of methods accounting for these is a field of active research and publication.
349 Thus, we will follow on future developments, recommendation and consensuses in the field
350 regarding these challenges. Currently identified solutions accounting for these limitations are
351 MetagenomSeq, Aldex2 and Maaslin2, which all integrate normalisation and correlation
352 testing to account for the pre-mentioned limitations while trying to identify differentially
353 abundant features (genes or species) between tested groups.
354

355 *Statistical plan for aim 1 and 2:* Antibiotics exposed samples will be compared to non-
356 exposed samples (grouped per received drug) for differences in alpha-diversity metrics and
357 abundances of bacterial and non-bacterial taxa and antibiotic resistance genes with significant
358 changes defined as a > 0.5% change in abundance between groups. Alpha-diversity metrics
359 are continuous numeric values. Normality of the data will be checked by the Kolmogorov-
360 Smirnov test and QQ-plots. For normal variables, a one-way ANOVA will be used. If
361 normality cannot be reached by transformation, differences between groups will be analysed
362 using a nonparametric Kruskal-Wallis test, or in case of pairwise comparisons, a Mann-
363 Whitney U-test. For comparison of abundance Pearson x^2 tests will be used. Infant age,
364 demographics, delivery mode, feeding method and antibiotic type and dose will be modelized
365 in permutational multivariate analysis of variance (PERMANOVA, adonis2 in VEGAN
366 package, R Foundation) using beta-diversity distance matrices to identify significant
367 contributors to the microbiome composition.
368

369 *Statistical plan for aim 3:* For integrated analysis of the microbiome data and clinical
370 outcomes, allergic sensitisation, eczema, and overweight cases will be defined using the
371 prospectively collected outcome measures. The relationship between alpha-diversity and

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clinical outcomes will be investigated using logistic regression. The relationship between taxa and these clinical outcomes will be determined by hierarchical clustering of communities using heatmaps and principal component analysis (PCA). Significance of grouping of clinical categories using permutational multivariate analysis of variance (PERMANOVA). Microbes that have significantly different abundance between the clinical outcome groups will be identified using Aldex2.[38] The potential influence of antenatal and postnatal factors on the microbiome or clinical outcomes will be accounted for in all analyses by PERMANOVA and unsupervised hierarchical clustering.

Statistical plan for aim 4: To compare paired maternal stool, breast milk, and infant stool samples, we will do clustering analysis and perform a Wilcoxon rank sum test on beta-diversity distances between true mother-infant pairs and randomly paired mothers and infants matched by infant age. These different analyses should allow to describe the relationships between samples of different origins and identify determinants of microbiome composition.

To further characterise the mother-to-infant microbiome transmission, the StrainPhlAn software will be used.[39] This software enables same-species sequence comparisons at the single nucleotide polymorphism (SNPs) level to define sample-specific strains. Combined results for shared species allow precise description of the phylogenetic distance between samples. Added to the beta-diversity analyses, this will allow to better disentangle the hypothesised seeding from maternal stool and breast milk to the infant oral and stool microbiome.

Statistical plan for aim 5: The same as for aim 4 will be done for comparison of the breast milk and the infant oral microbiome.

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Missing data

If the fraction of missing data is less than 5%, the primary analysis will be a complete case analysis. If not, the rate and patterns of missing data will be examined and, if appropriate, multiple imputation models will be applied for the outcome variables.

ETHICS AND DISSEMINATION

Ethics approval

The ABERRANT study has been approved by the commission cantonale d'éthique de la recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-01567), Switzerland.

Recruitment and consent

Written informed consent will be obtained from all participants included in the trial. Participants will be informed that they are not obliged to take part in the study and are free to withdraw at any time without any impact on their future care.

Data collection and storage

Data will be sourced from medical maternal and infant records, as well as by questionnaire from parents. Data will be de-identified and entered in to a secure, web-based electronic database.

Safety

No serious adverse reactions are anticipated but these will be checked for by the Data Safety and Monitoring Committee.

Dissemination of results

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Outcomes will be disseminated through publication according to the SPIRIT statement and will be presented at scientific conferences.

Study duration

We aim to recruit participants over a two-year period.

DISCUSSION

The intestinal microbiome is crucial in the development of the immune system and regulation of immune responses, especially during infancy, when the intestinal microbiome and the immune response develop concurrently.[40] The development of intestinal microbiome is easily disrupted by external factors and perturbation during this vulnerable period may have a large influence on immune development. A number of factors influence the development of the infant intestinal microbiome, including gestational age, delivery mode, feeding, and maternal and infant antibiotic exposure **(Figure 1)**.[41] While the effect of delivery mode and feeding methods on the establishment of microbial communities has been well studied, much less is known about the effects of intrapartum and early-life antibiotic exposure on the establishment of microbial communities in the intestinal microbiome.[13]

A number of commonly used antibiotics have profound effects on specific bacteria within the intestinal microbiome, as detailed in a recent systematic review.[12] This ‘collateral damage’ includes changes in diversity and abundance of microbial taxa, a decrease in ‘colonisation resistance’ (protection against colonisation with potentially pathogenic organisms) and the development of antibiotic resistance. To date, most studies on the effect of antibiotic exposure on the intestinal microbiome have been done in adults.[12] The main findings of these studies are that antibiotics often lead to a decreased bacterial diversity, a decrease in the abundance of anaerobic bacteria, an increase in abundance of *Enterobacteriaceae* other than *E. coli* and an

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increase in the abundance of yeast.[12] These studies show that changes in the intestinal microbiome after just one course of antibiotics can persist up to four years.[12] However, the clinical consequences of changes in the composition of the intestinal microbiome with antibiotic treatment are unknown. An increase in Enterobacteriaceae, which are often resistant to beta-lactam and other antibiotics, might render the host more susceptible to infections with antibiotic-resistant bacteria. This phenomenon has been observed in infants in neonatal intensive care units, who become more often colonised with *Klebsiella* spp*., Enterobacter* spp. and *Citrobacter* spp., when treated with antibiotics.[42]

Intrapartum antibiotic prophylaxis (IAP) has become common practice in obstetric medicine and is used in up to 40% of deliveries, which makes it the most common source of antibiotic exposure in neonates.[1] IAP is routinely used in both elective and emergency Caesarean section (CS). It is also routinely used in women who are colonised with group B streptococcus (GBS). Despite the benefits, the risks associated with exposing a large number of infants to antibiotics, especially the long-term effects on health through changes in the microbiome, remain unclear. Infants who were exposed to IAP have been reported to have a lower alpha-diversity, a lower relative abundance of Actinobacteria, especially *Bifidobacteriaceae,* and a larger relative abundance of Proteobacteria in their intestinal microbiome compared to non-exposed infants.[13] Furthermore, they have been reported to have a higher number of beta-lactamase encoding genes.[43]

Breastfeeding has been shown to be beneficial in preventing many communicable and non-communicable diseases.[44] Despite intensive research into the positive health effects of breastfeeding, the underlying mechanisms are still not understood. However, a large part of the beneficial effects of breast milk is likely mediated through the microbiome and its associated immunomodulatory, anti-inflammatory and antimicrobial components. The breast

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milk microbiome also likely plays a part in shaping the infant's intestinal microbiome,[5] to which extent we will be investigated in this study.

There is relatively little data about the effects of IAP on the composition of the breast milk microbiome.[45-47] Mothers who receive IAP have been reported to have a lower abundance or even an absence of the beneficial bacteria *Bifidobacterium* spp. in their breast milk.[45 46] Furthermore, in a small study IAP has been shown to increase antibiotic resistance genes in the breast milk microbiome.[48] There is also some evidence suggesting that mothers who receive IAP have a higher bacterial richness and diversity in their breast milk microbiome compared with mothers who do not receive antibiotics.[45] However, these findings have to be interpreted with caution: it could be that antibiotics lead to lower bacterial numbers and therefore signals from contamination, e.g. bacteria found in DNA extraction or sequencing kits might be amplified more leading to a the detection of a higher diversity. Use of broad-range qPCR to quantity bacterial load in milk samples will allow to assess this potential bias. Interestingly, recent preliminary studies have also shown that delivery mode affects the composition of the breast milk microbiome.[45 47 49 50] However, during suckling, a high degree of retrograde flow of milk into the mammary ducts can occur,[6] transferring bacteria from the infant to the mother, as postulated for GBS.[51] It is therefore possible that the differences in the breast milk microbiome observed with different delivery modes are mediated through differences in the oral microbiome of infants.

This study will determine the effect of intrapartum and early-life antibiotics on the composition of the breast milk, and the infant oral and intestinal microbiome and antibiotic resistance. The knowledge gained by this study will make an important contribution to the growing field of research investigating the importance of the immunological role of the breast milk microbiome and the infant intestinal microbiome on infant health. It will form the basis for investigating the interplay between the microbiome and the regulation of the human

immune system and possible adverse health outcomes, such as the development of immune and non-immune mediated diseases, including allergic diseases.

The results of this study will also build a stronger evidence base for strict antibiotic stewardship and form the basis for development of evidence-based interventions to prevent adverse outcomes in situations where antibiotics cannot be avoided, including modifying the intestinal microbiome with directed pre- and probiotics or bacteriophages.

Contributors PZ is the lead investigator. VS, LF, NC and PZ were responsible for study conception and design. PZ, JW and ALB were responsible for funding acquisition and implementation. MV drafted the manuscript and coordinated the manuscript preparation and revision. PZ and VS has developed the statistical analysis plan. MV and PZ developed the online questionnaires and database set-up in REDCap. WJ will be responsible for sample analysis. MV, VS, WJ, DB, VDM, ALB, JW, LF and NC provided critical evaluation and revision of manuscript and have given final approval of the manuscript accepting responsibility for all aspects.

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Competing interests None.

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Table 1 Clinical outcomes for aim 3

Outcome	Main measure	Timing
Lower respiratory tract illness	Number of episodes & hospitalisations[23 24]	first 2 years of life
Acute otitis media	Number of episodes[23 24]	first 2 years of life
Allergic (atopic) sensitisation	Prevalence (positive skin prick test)[27]	at 2 years of age
Eczema	Prevalence (Williams criteria)[25]	at 1 and 2 years of age
Weight	Centile (WHO Child Growth Standards)[22]	at 1 and 2 years of age

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3 684 **Table 2** Study protocol
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Time	Birth	7d	1m	2m	4m	6m	12m	24m
Diary		✓	✓	✓	✓	✓	✓	✓
Questionnaire		✓		✓	✓	✓	✓	✓
Maternal blood sample	✓							
Maternal stool sample	✓							
Breast milk sample	✓col	✓	✓	✓	✓	✓*		
Infant oral swab	✓	✓	✓	✓	✓	✓		
Infant stool sample	✓mec	✓	✓	✓	✓	✓	✓	✓
Clinical examination							✓	✓
Skin prick test (optional)								✓
Blood sampling (optional)	✓cb						✓	✓

686
687 col = colostrum; mec = meconium; [3] cb = cord blood;
688 *or before breastfeeding is discontinued if earlier than 6 m

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Figure 1 Summary of factors that might influence the composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome together with possible associated adverse health outcomes

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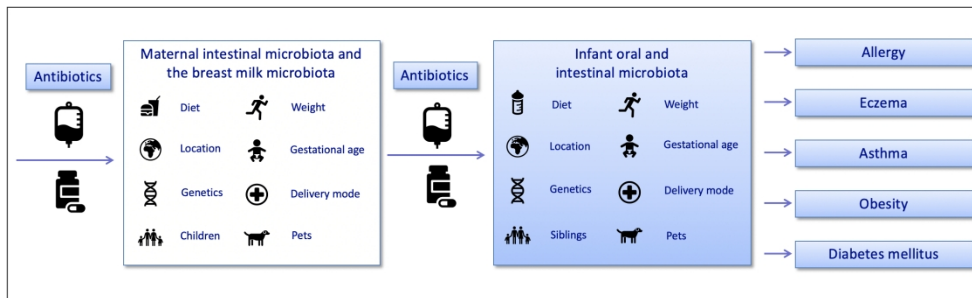

Figure 1

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Page Line
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1 1-2
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2 46
	2b	All items from the World Health Organization Trial Registration Data Set	Available at Clinical trials.gov
Protocol version	3	Date and version identifier	Approved by the CER-VD
Funding	4	Sources and types of financial, material, and other support	18 450
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1 5-20 18 441-4448
	5b	Name and contact information for the trial sponsor	1 22-24
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	18 452-453
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	18 441-448
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4-5

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	6b	Explanation for choice of comparators	4-5
Objectives	7	Specific objectives or hypotheses	6-7
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	7

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	7
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	7-8
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	no intervention
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	no intervention
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	NA
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	8-9 Table 1
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Table 2
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	11-12
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	7

Methods: Assignment of interventions (for controlled trials)

Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	NA
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	7
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	8-9
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	8-9
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	14
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	11-13
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	NA
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	14
Methods: Monitoring			

Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	15
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	NA
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	14
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	NA
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	14
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	14
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Approved by the CER-VD
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	19
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Approved by the CER-VD
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Approved by the CER-VD
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	7
	31b	Authorship eligibility guidelines and any intended use of professional writers	NA

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	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	14
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Attachment
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	9-11

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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