UiT Norges arktiske universitet

Faculty of Health Sciences

Determination of Short Chain Fatty Acids in Meconium from Healthy Term Infants

Sigrid Sandli

Master theses medicine MED-3950, June 2022

Supervisor: Veronika Kuchařová Pettersen, Associate professor at the Centre for New Antibacterial Strategies, Host-Microbe Interactions and Paediatric Research groups, Department for Medical Biology, UiT

Co-supervisor: Gaute Hovde Bø, PhD Fellow, Department for Medical Biology, UiT

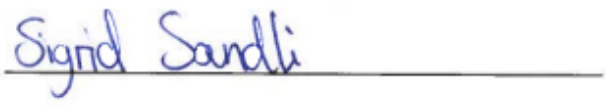
1 Preface

I had my first experience with academic writing in the second year of medical school when I wrote a literature review. This time, for the master thesis, I wanted to learn something new, a more practical type of research. When the opportunity came to join this project, I didn't hesitate. To work with chemicals and biological samples in a laboratory was always the most fun part of natural science in my opinion, and to combine this with a topic that is so relevant at the time, sounded just perfect.

I started writing the project description in the autumn of 2020, and together with my supervisor Veronika, we had a pretty clear vision of what the project should investigate. However, as so often with research project on human subjects, it ends up being a lot more complicated and time consuming than foreseen. That is one thing I have learned through this process. After changing and narrowing the project a little bit, we were back on track in 2022.

It has been some intense and all-consuming months of work and I have for sure had my moments of frustration. But what I'm left with is a whole new perspective on research, and I have experienced the importance of staying patient throughout the process. I also have learnt a lot about the topic of gut microbiota, a pretty unknown field for me to begin with.

I want to express my gratitude to the people who made it possible for me to carry out this project all the way to the finish line. First of all, my supervisor Veronika who has guided me steadily all the way, followed me closely throughout the writing, and included me in every part of the process. I also have to give a big thank you to my co-supervisor Gaute, for helping me in the lab with the metabolomics and all of the analyses. He was also the one to prepare the method for metabolomics for me to follow, and he performed the mass spectrometry. Last, but not least, medical students Synnøve Holmebukt and Oda Marie Aspenes Gundersen deserve credit for their work with collecting all of the faecal samples used in this study.



Sigrid Sandli, 23.05.2022

Table of content

2 Summary

Background: Short-chain fatty acids (SCFA) are metabolites produced by bacteria that can be found in the human gut. They have been shown to have several beneficial effects on the human health, one of them is to enhance the immune system by colonization resistance. The purpose of this study was to investigate the abundance of short chain fatty acids in meconium from healthy term born infants, as well as to develop sample preparation methods for metabolomics and DNA extraction from stool samples. The results will be used as a reference in a comparison with age-matched group of infants treated with antibiotics, to characterise how antibiotics use affects SCFA profile of the neonatal gut.

Method: Stool samples were collected from 39 healthy infants born week 35-42, within 48 hours after birth. The samples were mixed with 95% ethanol, homogenised, aliquoted, and stored in a -80°C freezer until preparation. When preparing, the samples were divided in three parts: for metabolomics, dry weight measurement and DNA-extraction. We detected SCFA concentrations by targeted mass spectrometry-based technique and used the dry weight determination to normalise for different solvent content between stool samples. We also measured the DNA-concentration in 29 of the 39 samples. For statistical analysis available metadata to correlate with SCFA-profiles were: age at the time of sample collection (hours), birth weight (grams), time from sample collection to the sample was put in the freezer (hours), and DNA-concentration.

Results: The most abundant SCFA detected were acetate, propionate and butyrate. Also the organic acids (OA) lactate and succinate were analysed and came out with relatively high values. The remaining 10 SCFA had very low concentrations. The 15 compounds showed slightly positive correlation with age the infants, and the number of hours passed from sample collection until it was put in the freezer. They also showed slightly negative correlation with birth weight of infants, and DNA concentration in the faecal samples.

Conclusion: Main conclusion is that acetate, propionate and butyrate are the three most abundant SCFA of the infant gut metabolome. No conclusions are made based on correlation analyses, due to a small population and some inaccuracies in the method.

3 Abbreviations

SCFA – Short-chain fatty acids

OA – Organic acids

IMPALE – Impact of Antibiotics on the Neonatal Metabolome

UiT – Universitetet i Tromsø

UNN – Universitetssykehuset Nord-Norge

DW – Dry weight

MET – metabolite extraction, metabolomics

3NPH – 3-nitrophenylhydrazine

$^{13}C_6$-3NPH – 3-nitrophenylhydrazine, carbon 13-isotop

EDC – N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide

ACN – Acetonitrile

HPLC-MS/MS – High performance liquid chromatography tandem mass spectrometry

PCA – Principal Component Analysis

4 The project

This project is a part of the Impact of Antibiotics on the Neonatal Metabolome (IMPALE) study performed by the Paediatric Research group at Universitetet i Tromsø (UiT)/Universitetssykehuset Nord-Norge (UNN). The objective of IMPALE is to investigate how antimicrobial therapy affects the gut microbiota and metabolome of term infants.

A part of the IMPALE study has been to determine a baseline dataset for metabolome of healthy term infants who have not received antimicrobial treatment. The purpose of this Master thesis project was to test the method for preparing and analysing stool samples, as well as investigate the faecal metabolome of healthy infants, specifically the content of SCFA. We measured the abundance of different SCFA in meconium through mass spectrometry and statistically compared the results to parameters like birth weight, timing of sample collection and DNA-concentration in the meconium. The results will establish a reference for the later part of the IMPALE study, investigating infants treated with antibiotics.

5 Introduction

The term gut microbiota describes the collection of different microbial species within the intestines, while the gut microbiome refers to the microbes, their genetic composition and their surrounding environmental conditions (1). The human gut microbiota consists of a vast number of different microbes (bacteria, archaea, fungi, protozoa) as well as viruses. It is mostly bacteria for which there is an evidence of functions supporting the host health (2). One of them is colonization resistance, in which the resident microbiota prevents colonization of invading microbes and the blooming of opportunistic pathogens residing in the host intestines.

There are differences in the adult and infant microbiome in the effectiveness of colonization resistance function. Adults usually have a diverse microbiota that varies between individuals. Despite this personalised character, adults gut microbiota performs overlapping metabolic functions (3), and are therefore more resistant to gut microbiome perturbations, for example, by antibiotic treatment. Essentially, adults have many different bacteria to cover the necessary functions. Infants have a much lower microbiota diversity (4), making them more vulnerable to external perturbations. Since infant's gut microbes do not have the same functional overlap

as adults, they show a reduced capacity to maintain a balanced gut microbiome in response to disturbances (4).

The gut microbiota is thus an integral part of the body's defence against infectious diseases in any age. When giving an infant antibiotics with the intention to prevent or cure infection, the result may paradoxically be that the child is more susceptible to infections during infancy and possibly also later in life. In addition, research studies have found that the use of antibiotics early in life increases the risk of autoimmune and metabolic diseases, like asthma, celiac disease, obesity and inflammatory bowel disease (5).

5.1 Colonization resistance

Colonization resistance can be explained as a process where the intestinal microbiota prevents pathogenic microbes from colonising the gastrointestinal tract and causing infection. The mechanisms of colonisation resistance are often categorised into direct and indirect (2). In the indirect mechanisms, the intestinal microbiota influences the host's own protection against exogenous pathogens. This happens, for example, by stimulating antimicrobial peptide production, epithelial barrier maintenance, and influencing bile acid metabolism. With the direct mechanisms, the commensal microbiota inhibits the invading pathogens directly, for example, by outcompeting them for nutrients, producing bacteriocins and metabolites such as SCFA, or killing them through the type VI secretion system. The different colonisation resistance mechanisms work in concert to prevent the colonisation of exogenous microbes and restrict the overgrowth of indigenous microbes that may cause infection when becoming too dominant (opportunistic pathogens).

Production of short-chain fatty acids and their inhibitory actions

The project's focus has been on SCFA, and details of their involvement in colonization resistance are therefore discussed here. The commensal microbiota ferments carbohydrates and proteins that have either escaped absorption in the small intestines, or are derived from dietary fibers indigestible by human enzymes, producing a variety of metabolites, including SCFA. SCFA are 1-6 carbon volatile fatty acids, existing in both straight and branched chain formation (6). Acetic, propionic and butyric acids being the most abundant (90-95%) of the total intestinal SCFA. Studies have revealed several beneficial health effects attributed to the

production of SCFA, summarized in the review by Ríos-Covían *et al* (6), including the ones relevant to colonization resistance.

In infancy, obligate anaerobes such as bifidobacteria play a major role in the production of SCFA. *Bifidobacterium* is one of the first genera of bacteria that colonise the gut of humans (7), and they cooperate with the human host to confer colonisation resistance; a large portion of their genome is adapted to use human milk oligosaccharides as a carbon source, metabolising them into SCFA inhibitory to other bacteria (8). The resulting acidic pH is predicted to limit potentially pathogenic bacteria, such as Gram-negative *Enterobacterales* (7).

Colonization resistance is one important part of the immune system, but SCFA also play a part in the general immunological homeostasis of the gut. Because of the vast number of bacteria, there has to be a balance between tolerating the commensal microbiota and attacking pathogens. In other words, there must be a certain degree of both immune suppression and immune activation (9). SCFA function as signalling molecules in cells, and they can bind to receptors on the cell surface to activate different pathways, and the results are suppression of proinflammatory effectors, regulation of immune cell differentiation - both effector cells and regulatory immune cells, and generation of interleukin production (9, 10). Especially butyrate and propionate have immune suppressive and anti-inflammatory functions, contributing to immune homeostasis (9). Further, butyrate also fuels epithelial cells in the intestines and increases mucin production, resulting in improved tight junctions integrity and changes in bacterial adhesion (6). All of the above shows that SCFA are useful both in protection against pathogens and in suppression of the immune system, preventing it to be hypersensitive to the gut microbiota, and also providing protection against inflammatory bowel disease and colorectal cancer (9).

In general, most SCFA are produced through fermentation of carbohydrates, but they may also be a product of amino acid metabolism (less than 5% of the total) (6). A study on meconium from African American infants showed a correlation between the detection of certain bacteria in the meconium and the amount of certain metabolites, as well as the abundance of amino acids. The more bacteria detected in the meconium, they found higher levels of metabolites such as SCFA, and lower levels of amino acids. Concluding that the

bacteria used amino acids as their energy source, producing SCFA as a metabolic result (11). The study investigators detected 45 different metabolites, two of them were the SCFA acetate and propionate. In contrast, in this project we decided to investigate 13 SCFA.

5.2 The effect of perinatal and early life antibiotics on the infant's gut microbiome

Ever since the discovery of antibiotics at the beginning of the 20th century, the most famous being the discovery of penicillin in 1928 (12), these medicines have saved an enormous number of lives throughout the years. Infectious diseases that used to be fatal, can now easily be cured with antibiotic treatment. But there are downsides to this story as well, and one of them is how antibiotics affect beneficial commensal bacteria that live in our gastrointestinal system. Because most antibiotic drugs cannot separate between the "good bacteria" and the "bad bacteria". They wipe out all bacteria sensitive to the drug, including those beneficial to the health, causing trouble for the human host.

Current scientific evidence suggests that perinatal antibiotic use strongly impacts the neonatal gut microbiome composition, which might directly affect microbiome-mediated functions such as colonization resistance. A study performed by Tapiainen *et al.* (13) has investigated how perinatal antibiotic treatment affects the gut microbiome of infants. The study authors included infants borne at term, vaginally delivered, and recruited within 24 hours after birth. Compared to the control group, the infants receiving postnatal antibiotic treatment had a significantly lower relative abundance of the Bacteroidetes phylum and a significantly higher abundance of Firmicutes (13). After analysing follow-up samples at the age of six months, the authors also concluded that the differences in the gut microbiome after antibiotic treatment were long-term.

Early life antibiotic treatment given to infants after birth is one of several external factors that may affect the vulnerable process of the early life microbiota development (14). Other factors are birth mode (vaginally or C-section) and feeding mode (breastfeeding or formula). Especially bifidobacteria are found to decrease in abundance when the infant receives antibiotics, while *Clostridia* (14), *Enterobacterales*, and *Enterococcus* spp. often increase (15). Bifidobacteria are usually the most common member of the gut microbiota the first weeks of life, and as mentioned earlier, an important producer of SCFA. Antibiotic treatment in this period of time may take away this window for the bifidobacteria's dominance(14).

Even though the microbiome gradually recovers, it takes time. One study observed a small, but significant difference in the variety and stability of the microbial composition compared to a control group not treated with antibiotics, even after 12 months (15).

5.3 The effect of antibiotic use on the infant's gut metabolome

The commensal gut bacteria perform important colonization resistance functions in which microbial metabolites are crucial, as described in section 5.1. Studies of the gut microbiome using DNA sequencing give us the answer to what species are present - what specific microorganisms we can actually find - but they do not describe the metabolic functioning of the microbes under specific conditions. By analysing the faecal metabolome before and after antibiotic treatment, one can determine how the antibiotics alter amounts of different metabolites. In connection with DNA sequencing methods, it is also possible to predict which metabolites might be derived from host cells and which come from the microbes within the gut.

Findings from animal studies have shown that antibiotics affect several metabolic pathways, including sugar, fatty acid, bile acid, amino acid, steroid and eicosanoid metabolism (16). Also, levels of SCFA seem to be altered due to exposure to antibiotics, as observed in rats, where the concentrations of acetate, propionate, and butyrate were significantly decreased after ceftriaxone treatment (17). Changes in the gut microbiota also lead to changes in the metabolome, including SCFA profiles, because SCFA are microbiota generated products, and a change in microbial composition leads to a change in the products (18). Research on humans show similar results, for example in a study on adult humans with the antibiotic amoxicillin/clavulanate (19). This study also describes that for people eating yoghurt containing *Bifidobacterium animalis* together with the antibiotic, the levels of SCFA returned quicker to baseline and they had less antibiotic associated diarrhea. The main outcome in this study was acetate-levels, but also propionate and butyrate were analysed, and all three significantly decreased after 7 days of antibiotics.

Other studies as well support the hypothesis that antibiotics alters the gut content of SCFA. Children who went through allogeneic hematopoetic stem cell transplantation participated in a study (20) which looked at how concentrations of SCFA changed in children who received antibiotics due to infection following the stem cell transplant regime. This would typically be

broad spectrum antibiotics like first line piperacillin or tazobactam, or second line meropenem. The results showed an association between high exposure to these antibiotics with special activity against anaerobic bacteria, and lower concentrations of butyrate and propionate.

To summarize these studies, many findings point in the direction that antibiotic treatment is harmful to the gut microbiome and may alter the metabolome in a way that decreases the colonization resistance of the gut microbiota. This might, in turn, increase the risk of infectious diseases. More research on the topic is necessary, and especially research in infants born at term, as they are at the time quite an unexplored subject. One need to understand the consequences of treating new-borns with antibiotics to prevent long-term effects on the intestinal microbiome and metabolome.

6 Material and method

Study population: one group of 39 infants who met the inclusion/exclusion criteria (n=39).

Inclusion criteria: term and near-term infants born between 35-42 weeks, ≤ 48 hours of age, vaginal birth.

Exclusion criteria: born before week 35, congenital gastrointestinal anomalies or other severe gastrointestinal conditions, antibiotic treatment.

6.1 Ethical aspect and privacy considerations

This project is a part of the clinical observation study "IMPALE" led by Veronika K. Pettersen and Claus Klingenberg from the Paediatric Research group at UNN/UIT. A part of the IMPALE study is a reference study with the purpose of investigating the neonatal metabolome. The study coordinators have explained the nature of the research study to parents of infants who meet the study's inclusion criteria. Informed consent was obtained prior to study entry. The studies are conducted in accordance with "good clinical practice" and all applicable regulations, including the Declaration of Helsinki June 1964, as modified by the 48th World Medical Association, Republic of South Africa October 1996. Parents are advised that they are free to withdraw consent and discontinue their participation in the study at any time. There are no safety concerns associated with this study, as the only biological sample required will be faeces, which can be collected from a soiled diaper.

Subjects have been anonymized through the provision of a study ID number, which was used on all study-related documents and sample vials. The master list, which matches the subject to their numbers, is kept in a separate location and will be maintained only as a safety measure in case of a subject emergency. Databases will be stored on the UiT server/drive, which requires a username and password, and authorized access will be granted to staff members by Drs Pettersen/Klingenberg. Hard copies of subject files are kept in locked offices that are not accessible without a pre-authorized access card.

6.2 Sample collection

The collection of faecal samples was performed by medical students Synnøve Holmebukt and Oda Marie Aspenes Gundersen. They collected all samples at UNN, during the months of July, August and September 2021. There were made an agreement with the midwives at the hospital that they would notify the two students whenever there was a newborn who met the inclusion criteria. Then the students went to the hospital and explained the study to the parents and asked for permission to collect a stool sample from the newborn. The parents would give written consent to participate in the study, and then they were instructed on how to collect the sample themselves, by using a plastic spoon to fill a tube containing ethanol 95% and a metal bead (for homogenizing). Some of the samples were gathered from the diaper by the parents and some of them were gathered by the two medical students. The two medical students also processed the samples into about 1ml aliquots, which were then stored in a freezer at -80°C until further preparation.

6.3 Laboratory work

6.3.1 Preparing samples for metabolomics, dry weight and DNA-extraction

A stool sample (≈ 1ml) mixed with 95% ethanol was divided in three parts. One part for DNA extraction, one part for dry weight (DW) determination, and one part for metabolite extraction (MET). We had to measure the dry weight of the stool samples in order to normalize for the differences in water content and achieve accurate concentration of metabolites compared to biomass. The purpose of the DNA extraction was to establish a method for later use in the IMPALE study, where the faecal DNA will be used for taxonomic classification of the gut microbiota. In addition, the DNA-extracts were used to see if there was any correlation between the concentrations of DNA to the concentrations of SCFA.

Faecal samples were collected from 41 infants. The first 30 samples were divided in three parts (DNA, metabolomics and dry weight), while for the last 11 samples that were collected later, only metabolomics and dry weight were analysed. One of the samples was excluded because there was not enough volume for metabolomics, and another was excluded because the sample was not collected within 48 hours after birth. That left a total of 39 samples for the final metabolomics results and 29 samples for the DNA-concentrations.

Dividing the samples:

Using a pipette, each sample was divided in two or three parts, either for DNA, MET and DW (figure 1) or just for MET and DW. For DNA and MET 300 µl were used, and 100-200 µl for DW. When necessary, a volume of 95% ethanol (200-300 µl) was added to the samples with a thick texture that was difficult to work with, to provide enough volume for all parts. For the 30 samples that were divided in three parts, the ethanol was added after taking out 300 µl for DNA isolation. This was to make sure the concentration of DNA would be as high as possible. Independent of how much ethanol was added to each sample, the important thing was to have the same concentration of faecal matter in the MET and DW part.

6.3.2 Dry weight

The DW tubes did not need any further preparation and went straight into the freezer while preparing the others for MET and DNA. The next step here was to remove all the liquid from the sample by drying them for 5-6 hours in a vacuum centrifuge (Savant Speed Vac Concentrator). The tubes were weighted when empty, and then again when containing dried stool. Based on this, the dry weight was calculated.

6.3.3 Metabolomics

The tubes, containing 300µl of faeces in 95% ethanol were run through a 10-minute bead beating step. After bead beating, the MET tubes were centrifuged for 15 minutes (Eppendorf Centrifuge 5430 R) at a temperature of 4°C at a speed of 7830 rpm, and then the supernatant was transferred to new Eppendorf tubes, using a pipette. The tubes were always put on ice or stored in the freezer when not working with them.

Chemicals used:

See tables 1 and 2 in section 10.

External standard: An external standard with known concentrations of each SCFA and organic acid was used to obtain a standard curve by using linear regression. The regression line on the external standard curve can be utilized to calculate concentrations in samples with unknown content. First, 20 mM of each SCFA and OA standard were prepared in 50:50 (v/v) acetonitrile (ACN):H_2O. Then a mixture of these standards was made by transferring 5 ml of 20mM acetate, and 1 ml 20 mM propionate to the same tube. The rest of the 20mM stocks were mixed together and 1ml of this mixture was transferred to the same tube as acetate and propionate. This new mixture was then further diluted with 50:50 (v/v) ACN:H_2O to final concentrations of 10mM acetate and 2mM for all of the other compounds. Acetate had a range from 0,12 to 500 mM, the rest had a range from 0,02 to 100 mM.

Derivatization: Samples were derivatized with 3-nitrophenylhydrazine (3NPH) by mixing 40 µl of each supernatant, or 50 µl standard solution, with 20µl 200mM 3NPH (25 µl for the standard), and 20 µl of 120mM N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide (EDC) 6% pyridine (25 µl for the standard). They were incubated at 40°C for 30 minute, with slight shaking, and then they were diluted 1:3 in 10:90 (v/v) ACN:H2O.

200mM 3NPH were prepared by mixing 37,92 mg of 3NPH with 1ml of 50:50 (v/v) ACN:H_2O. 120mM EDC 6% pyridine were prepared by mixing 23,00 mg EDC with 940 µl 50:50 (v/v) ACN:H_2O and 60 µl of pyridine.

Internal standards: Internal standards were prepared by making stocks of 4 mM acetate, 2 mM propionate and 1 mM for each of the SCFA in 50:50 (v/v) ACN:H_2O. All these SCFA stocks were mixed together and then 50 µl of the mix was added to a tube containing 1 mg carbon 13 3-nitrophenylhydrazine ($^{13}C_6$-3NPH). 25 µl each of 120mM EDC in 50:50 (v/v) ACN:H_2O and 6% pyridine in 50:50 (v/v) ACN:H_2O were then added to 50 µl of SCFA mix. The tube was incubated at 40°C for 30 minutes with slight shaking, and at last the mixture was diluted to 100 ml in 10:90 (v/v) ACN:H_2O. Before mass spectrometry, the same volume of the internal standard mix as the supernatant volume was added to each of the derivatized samples, and also to the external standard.

Mass spectrometry: When all samples were derivatized and standards prepared, the final step was analysis through high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), and this was performed by PhD Fellow Gaute Hovde Bø. The method for targeted mass spectrometry was adapted from the research article by Han *et al.*

from 2015 (21). The instrument was QTRAP triple quadrupole mass spectrometer. The samples were injected to Waters BEH_{18} UPLC columns, with dimension 2.1 x 100mm, 1.7 μm, and electrospray ionized in negative mode. Solution A was 0,1% formic acid in water and solution B was ACN with 0,1% formic acid. The flow rate was 0,350ml/min with a column temperature at 40°C and autosampler at 5°C. Gradient elution as follows: 15% solution B for 2min, 15%-55% solution B in 9 minutes, 100% solution B for 1 min, and 1,5 minutes equilibration at 15% solution B between injections.

The software TargetLynx was used for processing of the samples, and metabolites were identified by known standards.

6.3.4 DNA-extraction

For DNA-extraction the DNeasy Power Soil Pro Kit (Qiagen) was used (22). The method that was followed came with the kit, as a step-by-step guide (23). It included chemical and mechanical cell lysis by bead beating of the faecal samples in PowerBead Pro Tubes that contained a buffer to enhance cell lysis and protect nucleic acids from degradation. Then the samples were centrifuged for 1 minute at 15,000 x g to separate the supernatant from the solid matter, moving on with just the supernatant that now contained the DNA. Further followed a process of carefully adding chemical solutions that came along with the DNA-kit, in a specific order. The full procedure is described in the instructions for the kit. For each solution added, homogenization was done by a vortexer. One solution would bind and remove contaminating matter, both organic and inorganic to ensure purity of the DNA. Then a high-concentrated salt solution improved the binding of DNA to the silica filter membrane in a spin column where all the supernatants were centrifuged through. Next there was a wash buffer and an ethanol-based wash solution used to clean the DNA sitting in the membrane, removing proteins and other contaminants from the membrane. The finish of the process was to wash through the filter membrane with PCR grade water, to release the DNA from the membrane, ending up with about 60 μl of water containing the DNA, ready for analysis. This was the product used to measure DNA-concentrations. After the DNA extraction, the concentration of DNA (ng/μl) was measured in each sample with a Nanodrop 2000 spectrophotometer (Thermo Scientific).

6.4 Statistical analysis

For statistical pathway analyses, web-based tool MetaboAnalyst 5.0 and Microsoft Excel were used. Calculations of concentrations and all descriptive statistics were performed in Excel. All samples with values outside of the external standard curve range from the mass spectrometry (16 samples) were excluded from the data set, and 23 samples were left for one factor statistical analyses. Even more samples were excluded for correlation analyses with metadata, due to missing values. For the age dataset a total of 19 samples were excluded, leaving 20 for statistical analyses. For the birth weight dataset a total of 23 samples were excluded, leaving 16 for analyses. For the pre-freezing time dataset a total of 22 samples were excluded, leaving 17 for analyses. And for the DNA concentration dataset a total of 11 samples were excluded, but since there were only 29 samples to begin with, 18 samples were left for analyses.

Correlation analyses was performed in MetaboAnalyst, using Spearman rank correlation. How to determine the strength of the correlation differs between research areas and specialities, but for the calculation in this study the correlation strength determination was based on commonly used table by *Chan* et al. for biostatistics and medicine (24, 25). A correlation coefficient less than 0,3 is a poor correlation, from 0,3 – 0,5 is a fair correlation, from 0,6 – 0,8 is a moderately strong correlation and above 0,8 is a very strong correlation.

7 Results

7.1 SCFA profiles

In the present study there was a total of 15 metabolites detected, 13 SCFA and 2 organic acids (OA) – lactic and succinic acid. Descriptive statistics of both compounds and metadata is shown in table 3. Of the SCFA, acetate was the most abundant with a mean concentration of 5,186 μmol/g (43,99% of the total metabolites analysed), followed by propionate 0,753 μmol/g (6,39%) and butyrate 0,433 μmol/g (3,67%). In addition, relatively large amounts of lactate 4,441 μmol/g (37,67%) and succinate 0,215 μmol/g (1,82%) were found. See figures 2 and 3 for concentrations and percentages. Principal Component Analyses (PCA) plot (figure 4) shows one cluster covering the majority of the samples, meaning the samples within this cluster have similar SCFA profiles. The closer they are in the plot, the more similar profiles. The PCA biplot (figure 5) shows how the SCFA concentrations correlates with each other.

Arrows pointing in the same direction indicates positive correlation, and the longer arrow the stronger correlation. Arrows pointing in opposite directions indicates negative correlation (none in this case), and perpendicular arrows indicates no correlation. The plot shows that several of the SCFA and OA correlates positively with each other, for example acetate and isobutyrate with almost identical arrows. There is no negative correlation.

7.2 Correlation to metadata

There were four types of variables included as a metadata in this study. The age of the infants when the sample was collected (in hours), birth weight (grams), the time it took from the sample being collected until it was put in the freezer (in hours), and the DNA concentration in some of the samples.

Correlation to age (figure 6): The correlation analysis shows a positive correlation between the detected SCFA and OA, and the age of the infants in hours. With a Spearman correlation coefficient of about +0,5, there is not a strong correlation, but still a tendency that the compound concentrations increase with increasing age. The line diagrams in figure 7 is also a visualization of the correlation between the 6 most abundant SCFA and OA, and the age. In these diagrams it is clear that the correlation is not strong, but looking at for example the graph for acetate, one can see the slightly increasing tendency.

Correlation to birth weight (figure 8): The figure shows that all compounds have a negative correlation with birth weight that ranges from poor to fair, they are all less than between 0 and -0,4. The negative coefficient is suggesting a pattern of lower SCFA concentrations with increasing birth weight.

Correlation to time from sampling until freezing the sample (figure 9): Here, all the compounds, except propionate, have a positive correlation coefficient between 0 and 0,5. Propionate is just below 0. This means a poor to fair positive correlation between increasing compound concentrations and increasing pre-freezing time.

Correlation to DNA concentration (figure 10): All compounds, except succinate, correlate negatively with DNA-concentration. The compound concentrations decrease with increasing DNA concentration. The coefficients ranges from about -0,2 to -0,6, meaning a range from poor to moderately strong correlation. Pivalate has the strongest negative correlation, and

succinate is the only one that is just above 0. The negative correlation means the compounds have lower values with increasing DNA concentration.

8 Discussion

The aim of this project was to investigate SCFA profiles in healthy term born infants as well as to test and evaluate the method.

The three most abundant SCFA in the gut are acetate, butyrate and propionate (6), but also others can be found in smaller amounts. The results from this reference study showed acetate to be superior in abundance compared to other SCFA, followed by propionate and butyrate. In addition, 10 other SCFA and the two OA lactate and succinate were detected. Of the two OA, lactate was by far most abundant. Out of all compounds acetate and lactate alone made up 82%.

Several studies have, with different purposes, investigated the intestinal microbiota and metabolome of infants and small children and measured concentrations of the different SCFA. The master thesis by Nilsen, M. (26) investigated the SCFA profile of 100 infants from newborn to one year of age. In the meconium samples they found acetate to be superior in abundance at about 83% of the total SCFA, while propionate made up about 5%. The amount of butyrate was close to zero, while there were approximately 5% isovalerate, 2% valerate and 1% isobutyrate. Compared to our results, both have found acetate and propionate to have the highest concentrations, but in our reference study butyrate had a higher concentration than isovalerate, valerate and isobutyrate. Both studies found isovalerate to be more abundant than valerate and isobutyrate. Differences in results, could possibly be explained by the differences to the method. Their study population was larger than our's, with 100 infants from the Scandinavian collaboration study PreventADALL, were inclusion criteria were wider and therefore the study population was more heterogeneous (27). They included infants born in week 35 and later, both vaginal births and C-sections. We only included infants born to through vaginal delivery. Also, for the detection of SCFA, they used gas chromatography instead of HPLC-MS/MS. One should also consider the accuracy of only reporting the results in percentages, like Nilsen M. did in his thesis, which means there were no absolute values for comparison to our concentrations.

An Australian study (28) investigating SCFA content in meconium and amnionic fluid from neonates born through planned caesarean sections, found the average acetate concentration to be 29,35 mmol/g and propionate concentration was 4,37 mmol/g. Butyrate and the rest of the SCFA were not detectable. Just like the thesis by Nilsen, M. this suggests that butyrate-producing microbes in meconium is very low or not existing, and that butyrate production increases with a more mature microbiota. Studies performed with stool samples from 2 days old infants (29) 3, and 6 days old infants (30) also found results like this, with acetate being superior in abundance, then propionate and then very small concentrations of butyrate. As a comparison to this, in our study we were able to detect butyrate in all of the 39 original samples, and some of them had relatively high concentrations, which means there must have been a certain butyrate production happening in the gut of the infants.

All the above mentioned studies on meconium show variations in the results when it comes to SCFA concentrations. In general these studies found higher concentrations of acetate and propionate than this reference study, and the reasons for that could be several. Variations to the method and it's accuracy being one of them. In this reference study, 16 samples, many of them with very high concentrations of SCFA, were excluded from the results because the values were above the external standard curve range. Therefor it is reasonable to assume that the average concentrations would have been significantly higher if the range was wider an included all of the samples. When it comes to percentages, it is not really comparable, since the number of detected compounds varies a lot and affects the final calculations. Still, it is interesting to see that acetate, propionate and butyrate, ends up as the top three SCFA in all of the presented studies, including our own.

The main focus for most researchers within this field is usually on acetate, propionate and butyrate, but there are more SCFA to investigate. One study by *Chan* et al. managed to detect 9 SCFA in stool from infants between 3 weeks and 12 months (31). They found acetate, propionate, butyrate, isobutyrate, isovalerate, caproate, valerate, 2-methylbutyrate and 4-methylvalerate. Additionally, they tried to detect pivalate, 2,2-dimethylbutyrate, 2-ethylbutyrate, 3,3-dimethylbutyrate, 2-methylvalerate and 3-methylvalerate, but all of these were below detection limit. In our reference study we detected 13 SCFA, which is an improvement compared to this study from 2017, and we managed to detect 4 out of their 6 undetectable SCFA; pivalate, 2-ethylbutyrate, 3,3-dimethylbutyrate and 2-methylvalerate. In

addition, we also included hexanoate. We did not include caproate in our study. This could mean that meconium contain more of the SCFA only detected in our study, and that these specific SCFA decrease as the infant ages. It could also be due to method differences, where the main difference was that we used 3NPH for derivatization and *Chan* et al. used aniline.

8.1 SCFA profiles and correlation

There was one result of particular interest, and that was the correlation between metabolite concentrations and age of the infants. Even though the correlation was not very strong, there was a tendency that concentrations increased with increasing age. With this being just a small study with a small number of analysed samples, and the correlations were weak, we cannot conclude with certainty that there is a connection. However, the pattern points in that direction, and literature supports the hypothesis that older infants have more microbes and therefor more microbial activity in the gut (11, 18, 26). In this study only infants below the age of 48 hours were included, so one could perhaps assume that the correlation to age would have been stronger if also older infants were included.

There was a negative correlation of the metabolites both with birth weight and with DNA concentration, but the correlation was not strong, and again, by using a very small population it is impossible to say that there is actually a connection between increasing birth weight and DNA, and decreasing SCFA and OA. There is also possible that the DNA concentrations were not so accurate, as explained in section 8.3.

8.2 Pre-analytic stability

Microbial activity may not end at defecation, but microbes keep producing metabolites even after (32). Therefore it is crucial to stop this activity as soon as possible to get the most accurate read out of SCFA concentrations in the collected stool samples. Homogenizing the sample with organic solvents like ethanol, methanol or isopropanol stabilizes the SCFA content in the stool, even at room temperature, by stopping metabolic processes (32). In the present study 95% ethanol was used as stabilizing agent and in addition the samples were stored at -80°C. The results showed a weak positive correlation between increasing metabolite concentrations and the time that passed from the samples were collected and mixed with ethanol until it was put in the freezer. With the weak correlation, in combination with the very small number of samples, one cannot suggest that there is a connection. Thus, it seems that as

long as the samples are mixed with ethanol immediately after collection, there are not any significant changes in microbial activity (32).

8.3 DNA-isolates

Most of the samples had low concentrations of DNA, which might be explained by a high content of other compounds in meconium, like bile acids and salts, glycolipids and urea, which makes it harder to extract and isolate DNA from the sample (33). Also, because meconium is a low biomass material, it often has low levels of bacterial DNA to begin with. This study (33) addressed the issues of DNA-extraction from meconium, and the authors tried to make an improved protocol for the method, by freeze-drying meconium samples and then put the samples through cycles of freezing and thawing. The conclusion from the study, and perhaps something to consider for future trials, was that the DNA extraction efficiency improved with three cycles of freeze-thaw treatment of meconium at a temperature of -20°C (rather than-80°C) before the extraction process. Both freezing and thawing should happen immediately instead of gradually, as it gave better results. Before freezing, the samples where mixed with a chemical cell lysis buffer and vortexed for 30 minutes.

8.4 Evaluation of method and potential limitations

The method was performed more or less according to the plan. However, a few challenges showed up during the process that could be useful to note.

The stool samples varied a lot in texture, some of them were runny and very much diluted by the ethanol, while others where thick, clumpy, and hard to work with using a pipette. Therefore, the volumes pipetted when dividing the samples were probably not completely accurate for many of them. It could have been better to divide the samples using weight, rather than volume.

The tubes for MET should have been pre-weighted before adding the sample, and again after, to determine the sample weight. However, the tubes were weighted only before adding the sample, and not after.

In hindsight, that would have made the normalization to dry weight a whole lot easier. Because the weights of the MET samples were unknown, the total volumes of supernatant

from these samples were used instead. That required an extra process of carefully pipetting all the supernatants to figure out the volume.

8.5 Weaknesses to the study

A small population: The original population of 39 infants was not a large population to begin with, and when it got reduced with about 50% for the final statistics, datasets were too small that one can only see trends and make assumptions based on them, no actual conclusions.

Little information: Very little information about the infants was gathered. The only information we had for the statistical analysis was the four metadata. It would have been useful to have more information for statistics, like the genders of the infants, and feeding status (breast-feeding or formula) since this may impact the microbiota and metabolome (34).

8.6 Future aspects

The results of this project will serve as a pilot data indicative of SCFA concentrations in meconium of term-born infants. The results showed that the targeted metabolomics method for SCFA needs to be further optimised, that is, by extending the range of concentrations included in the standard curves. Similarly, further optimisation of DNA extraction protocol will need to be carried out.

Besides determining concentrations of SCFA and OA in the stool samples, the next steps in this project will be to evaluate the carriage of *Enterobacterales* (from Faecal swab samples stored in preservation media), including those carrying antibiotic resistance genes, and determining the microbiota taxonomic profiles by 16S rRNA sequencing of the extracted DNA. Ultimately, findings from this reference study will be directly compared to results derived from stool samples produced by antibiotic treated infants.

9 Conclusions

The main conclusion to be made from this study is that the SCFA profiles of the study population match the literature within the field, showing that acetate is the superior SCFA in abundance, followed by propionate and butyrate. Correlation analyses to metadata showed some trends, but none of the correlations were strong, and the number of samples in the datasets were small, therefore no conclusions can be made based on correlation analyses. Studies with larger populations, more extensive metadata, and improved accuracy of the method must be achieved to get a broader picture and understanding of the infant metabolome.

References

1. Marchesi JR, Ravel J. The vocabulary of microbiome research: a proposal. Microbiome. 2015;3:31.
2. Kim S, Covington A, Pamer EG. The intestinal microbiota: Antibiotics, colonization resistance, and enteric pathogens. Immunol Rev. 2017;279(1):90-105.
3. Heintz-Buschart A, Wilmes P. Human Gut Microbiome: Function Matters. Trends Microbiol. 2018;26(7):563-74.
4. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012;486(7402):222-7.
5. Arrieta MC, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B. The intestinal microbiome in early life: health and disease. Front Immunol. 2014;5:427.
6. Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de Los Reyes-Gavilán CG, Salazar N. Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. Front Microbiol. 2016;7:185.
7. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 2011;469(7331):543-7.
8. Sorbara MT, Dubin K, Littmann ER, Moody TU, Fontana E, Seok R, et al. Inhibiting antibiotic-resistant Enterobacteriaceae by microbiota-mediated intracellular acidification. J Exp Med. 2019;216(1):84-98.
9. Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell. 2016;165(6):1332-45.
10. Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. Nat Rev Immunol. 2016;16(6):341-52.
11. Bittinger K, Zhao C, Li Y, Ford E, Friedman ES, Ni J, et al. Bacterial colonization reprograms the neonatal gut metabolome. Nat Microbiol. 2020;5(6):838-47.
12. Aminov RI. A brief history of the antibiotic era: lessons learned and challenges for the future. Front Microbiol. 2010;1:134.
13. Tapiainen T, Koivusaari P, Brinkac L, Lorenzi HA, Salo J, Renko M, et al. Impact of intrapartum and postnatal antibiotics on the gut microbiome and emergence of antimicrobial resistance in infants. Sci Rep. 2019;9(1):10635.
14. Korpela K, de Vos WM. Early life colonization of the human gut: microbes matter everywhere. Curr Opin Microbiol. 2018;44:70-8.
15. Reyman M, van Houten MA, Watson RL, Chu M, Arp K, de Waal WJ, et al. Effects of early-life antibiotics on the developing infant gut microbiome and resistome: a randomized trial. Nat Commun. 2022;13(1):893.
16. Antunes LC, Han J, Ferreira RB, Lolić P, Borchers CH, Finlay BB. Effect of antibiotic treatment on the intestinal metabolome. Antimicrob Agents Chemother. 2011;55(4):1494-503.
17. Holota Y, Dovbynchuk T, Kaji I, Vareniuk I, Dzyubenko N, Chervinska T, et al. The long-term consequences of antibiotic therapy: Role of colonic short-chain fatty acids (SCFA) system and intestinal barrier integrity. PLoS One. 2019;14(8):e0220642.
18. Tsukuda N, Yahagi K, Hara T, Watanabe Y, Matsumoto H, Mori H, et al. Key bacterial taxa and metabolic pathways affecting gut short-chain fatty acid profiles in early life. Isme j. 2021;15(9):2574-90.

19. Merenstein D, Fraser CM, Roberts RF, Liu T, Grant-Beurmann S, Tan TP, et al. Bifidobacterium animalis subsp. lactis BB-12 Protects against Antibiotic-Induced Functional and Compositional Changes in Human Fecal Microbiome. Nutrients. 2021;13(8).
20. Romick-Rosendale LE, Haslam DB, Lane A, Denson L, Lake K, Wilkey A, et al. Antibiotic Exposure and Reduced Short Chain Fatty Acid Production after Hematopoietic Stem Cell Transplant. Biol Blood Marrow Transplant. 2018;24(12):2418-24.
21. Han J, Lin K, Sequeira C, Borchers CH. An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography-tandem mass spectrometry. Anal Chim Acta. 2015;854:86-94.
22. QIAGEN 2022 [cited 2022 01.05.2022]. Available from: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-powersoil-pro-kit/?cmpid=PC_QF_MB_microbiome-sales-brand_0520_SEA_GA&gclid=Cj0KCQjw37iTBhCWARIsACBt1IxJ44OrC_9z1Phj1aI66cXB1oiK0ZZy2im7488-iFEwPSRArZmT1ZUaArMyEALw_wcB#orderinginformation.
23. DNeasy PowerSoil Pro Kit Handbook: Qiagen; 2021 [cited 2022 18.05.2022]. Available from: https://www.qiagen.com/us/resources/resourcedetail?id=9bb59b74-e493-4aeb-b6c1-f660852e8d97&lang=en.
24. Akoglu H. User's guide to correlation coefficients. Turk J Emerg Med. 2018;18(3):91-3.
25. Chan YH. Biostatistics 104: correlational analysis. Singapore Med J. 2003;44(12):614-9.
26. Nilsen M. Gut Microbiota and Short Chain Fatty Acids (SCFA) Composition the First Year of Life [Masters Thesis]: Norwegian University of Life Sciences; 2018.
27. Lødrup Carlsen KC, Rehbinder EM, Skjerven HO, Carlsen MH, Fatnes TA, Fugelli P, et al. Preventing Atopic Dermatitis and ALLergies in Children-the PreventADALL study. Allergy. 2018;73(10):2063-70.
28. Stinson LF, Boyce MC, Payne MS, Keelan JA. The Not-so-Sterile Womb: Evidence That the Human Fetus Is Exposed to Bacteria Prior to Birth. Front Microbiol. 2019;10:1124.
29. Nogacka A, Salazar N, Suárez M, Milani C, Arboleya S, Solís G, et al. Impact of intrapartum antimicrobial prophylaxis upon the intestinal microbiota and the prevalence of antibiotic resistance genes in vaginally delivered full-term neonates. Microbiome. 2017;5(1):93.
30. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, et al. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A. 2011;108 Suppl 1(Suppl 1):4578-85.
31. Chan JC, Kioh DY, Yap GC, Lee BW, Chan EC. A novel LCMSMS method for quantitative measurement of short-chain fatty acids in human stool derivatized with (12)C- and (13)C-labelled aniline. J Pharm Biomed Anal. 2017;138:43-53.
32. Liebisch G, Ecker J, Roth S, Schweizer S, Öttl V, Schött HF, et al. Quantification of Fecal Short Chain Fatty Acids by Liquid Chromatography Tandem Mass Spectrometry-Investigation of Pre-Analytic Stability. Biomolecules. 2019;9(4).
33. Xin Y, Xie J, Nan B, Tang C, Xiao Y, Wu Q, et al. Freeze-Thaw Pretreatment Can Improve Efficiency of Bacterial DNA Extraction From Meconium. Front Microbiol. 2021;12:753688.
34. Bridgman SL, Azad MB, Field CJ, Haqq AM, Becker AB, Mandhane PJ, et al. Fecal Short-Chain Fatty Acid Variations by Breastfeeding Status in Infants at 4 Months: Differences in Relative versus Absolute Concentrations. Front Nutr. 2017;4:11.

10 Tables

10.1 Table 1 – SCFA and organic acids

Table 1: 15 SCFA standards and 2 organic acid standards were used for targeted metabolomics. All MS-grade.

SCFA standards	**CAS number**	**Source**
Acetic acid	64-19-7	https://uk.vwr.com/store/product/723269/acetic-acid-glacial-99-8-100-5-analar-normapur-acs-reag-ph-eur-analytical-reagent
Propionic acid	79-04-9	https://www.sigmaaldrich.com/NO/en/product/sigald/p1386
Butyric acid	107-92-6	https://www.sigmaaldrich.com/NO/en/product/aldrich/b103500
Isobutyric acid	79-31-2	https://www.sigmaaldrich.com/NO/en/product/sigma/i1754
Valeric acid	109-52-4	https://www.sigmaaldrich.com/NO/en/product/aldrich/240370
Isovaleric acid	503-74-2	https://www.sigmaaldrich.com/NO/en/product/aldrich/129542
Pivalic acid	75-98-9	https://www.sigmaaldrich.com/NO/en/product/aldrich/t71803
2-methylbutyric acid	116-53-0	https://www.sigmaaldrich.com/NO/en/product/aldrich/193070
Hexanoic acid	142-62-1	https://www.sigmaaldrich.com/NO/en/product/aldrich/-153745?gclid=EAIaIQobChMI16ifiqS-9gIVuAWiAx0JYA-CEAAYASAAEgIgvPD_BwE
3,3-dimethylbutyric acid	1070-83-3	https://www.sigmaaldrich.com/NO/en/product/aldrich/b88403)
2-ethylbutyric acid	88-09-5	https://www.sigmaaldrich.com/NO/en/product/aldrich/109959
2-methylvaleric acid	97-61-0	https://www.sigmaaldrich.com/NO/en/product/aldrich/109878
4-methylvaleric acid	646-07-1	https://www.sigmaaldrich.com/NO/en/product/aldrich/277827
Organic acid standards		
Lactic acid	50-21-5	https://www.sigmaaldrich.com/NO/en/product/sial/phr1215
Succinic acid	110-15-6	https://www.sigmaaldrich.com/NO/en/product/sial/14079

10.2 Table 2 – Chemicals for derivatization and HPLC-MS/MS

Table 2: Chemicals used for derivatization of supernatant and standards, and for HPLC-MS/MS.

Chemicals for derivatization and HPLC-MS/MS	**CAS number**	**Source**
3NPH (3-nitrophenylhydrazine)	636-95-3	https://www.sigmaaldrich.com/NO/en/product/aldrich/n21804
EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide)	25952-53-8	https://www.sigmaaldrich.com/NO/en/product/sial/e1769
Pyridine	110-86-1	https://www.sigmaaldrich.com/NO/en/product/sial/270970
$^{13}C_6$-3NPH (3-nitrophenylhydrazine)	1977535-33-3	https://www.caymanchem.com/product/20744/13c6-3-nitrophenylhydrazine-(hydrochloride)
Acetonitrile (ACN)	75-05-8	https://www.sigmaaldrich.com/NO/en/product/sigald/34851
Formic acid		
MQ-water		

10.3 Table 3 – Descriptive statistics of metadata and compounds

Table 3: Descriptive statistics of metadata and compounds. The table shows average values and standard deviation of all metadata and metabolites.

Metadata	**Average**	**Standard deviation**
Age (hours)	20,56	7,88
Birth weight (grams)	3587	398,6
Time from sampling to freezer (hours)	17,92	12,51
DNA concentration (ng/µl)	26,12	53,42
SCFA concentrations (µmol/g)		
Lactate	4,441	4,789
Acetate	5,186	7,880
Propionate	0,753	0,903
Isobutyrate	0,114	0,238
Butyrate	0,433	1,227
Succinate	0,215	0,243
2methylbutyrate	0,052	0,101
Pivalate	0,16	0,193
Isovalerate	0,141	0,355
Valerate	0,046	0,063
2ethylbutyrate	0,03	0,039
3.3dimethylbutyrate	0,047	0,058
2methylvalerate	0,033	0,043
4methylvalerate	0,039	0,052
Hexanoate	0,098	0,146

11 Figures

11.1 Figure 1 – Illustration of sample preparation

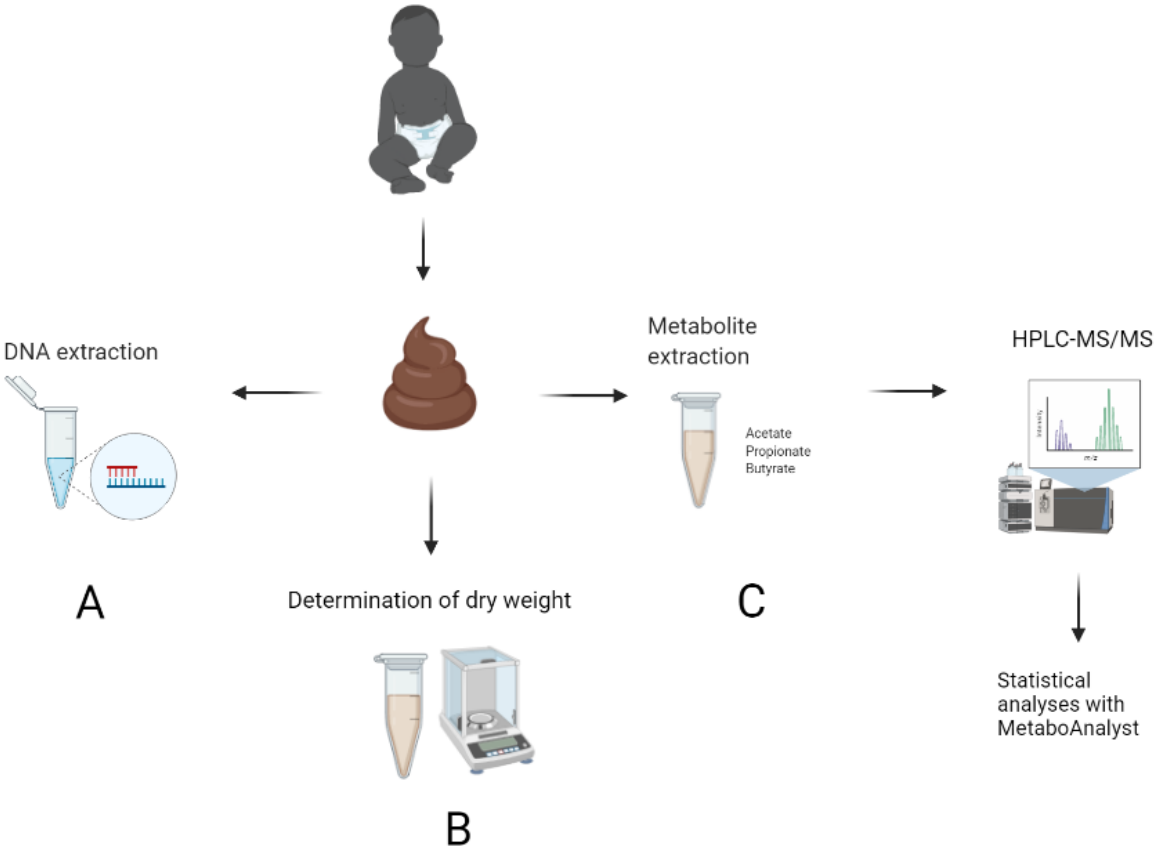


Figure 1: Illustration of sample preparation. Stool sample from infant is processed and divided into three tubes. A) DNA extraction B) Dry weight determination, necessary for normalizing concentration of metabolites. C) Metabolite extraction for metabolome analysis.

11.2 Figure 2 – Mean concentrations of 13 detected SCFA and 2 OA

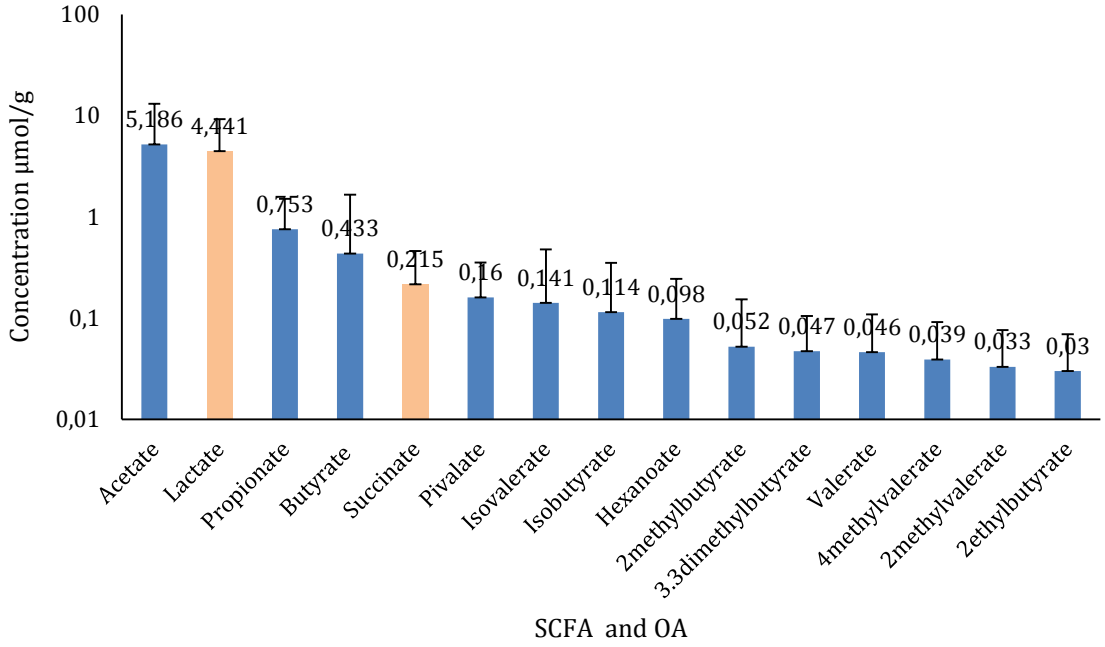


Figure 2: Mean concentrations of 13 detected SCFA (blue) and 2 OA (orange).

11.3 Figure 3 – Percentages of the total detected SCFA and OA

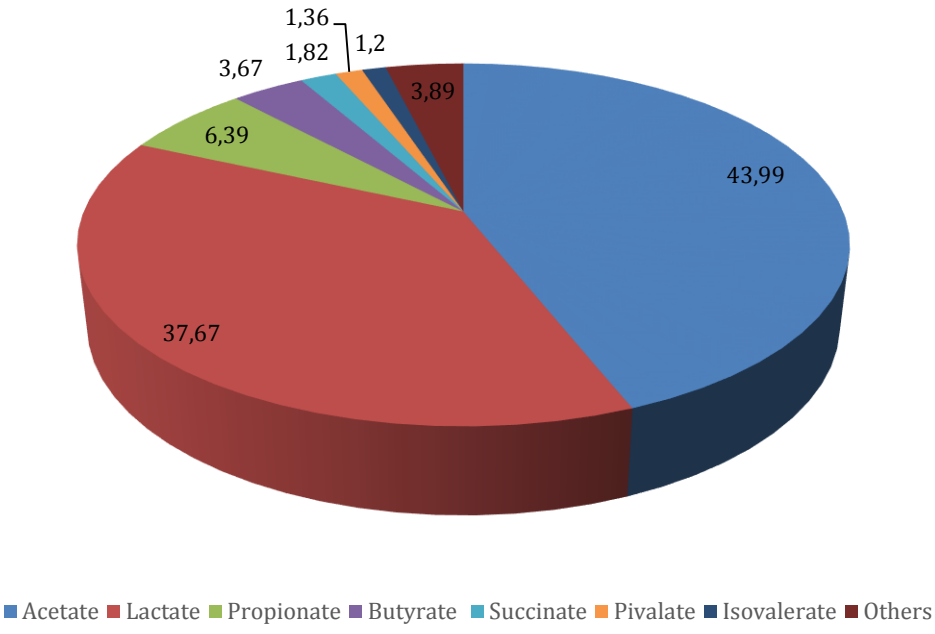


Figure 3: Percentages of the total detected SCFA and OA. The sector marked as "others" consists of isobutyrate, hexanoate, 2methylbutyrate, 3,3dimethylbutyrate, valerate, 4methylvalerate, 2methylvalerate and 2ethylbutyrate. These were all below 1% each.

11.4 Figure 4 – PCA plot based on concentrations of 15 compounds detected in 23 faecal samples of infants.

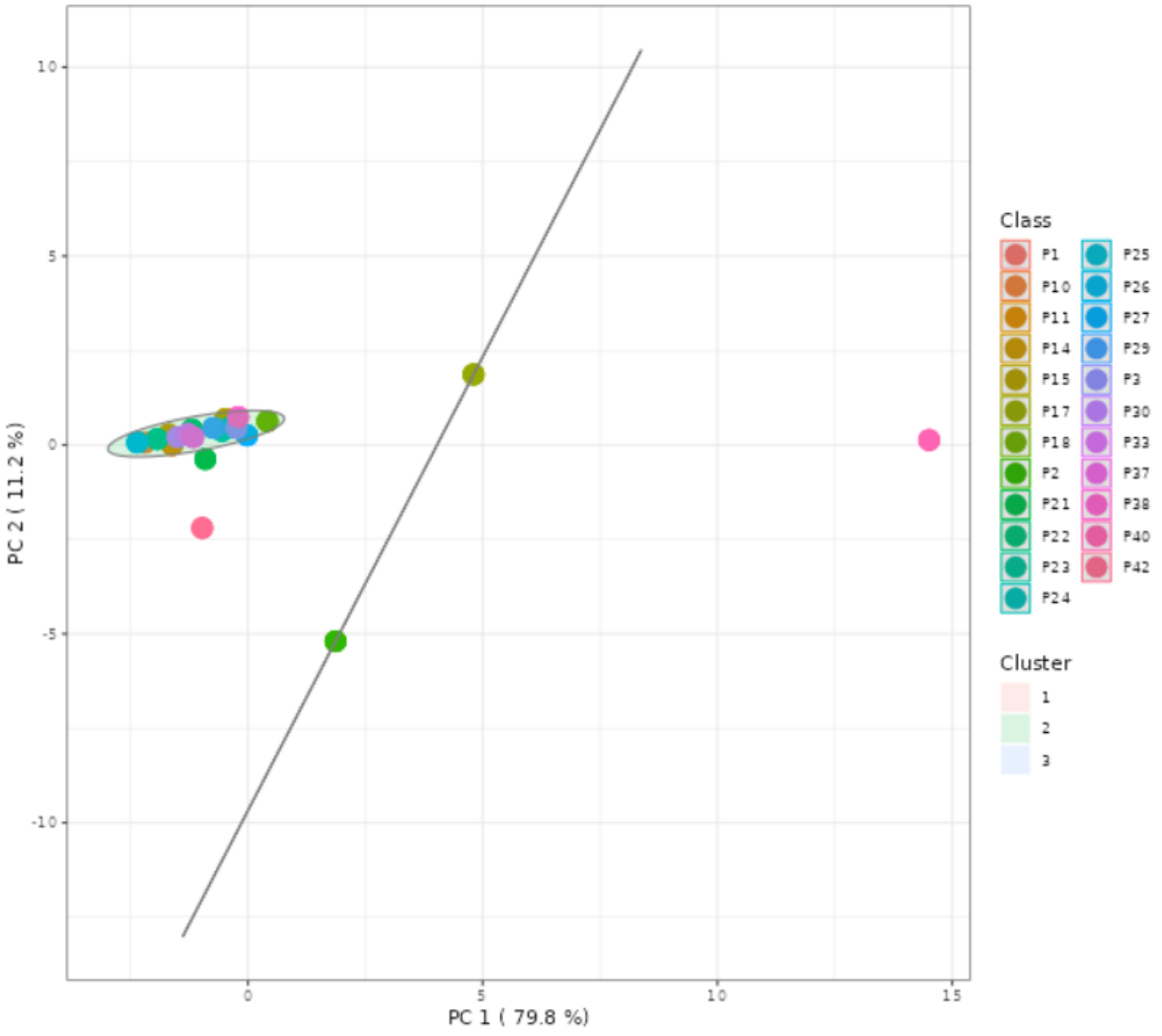


Figure 4: PCA plot based on concentrations of 15 compounds detected in 23 faecal samples of infants. The plot shows clustering of the samples with similar SCFA profiles. Most of the samples are gathered in one main cluster, and there are a few samples that are more spread out, having different profiles than the rest. PC1 explains 79,8% of the sample variance, and PC2 explains 11,2%.

11.5 Figure 5 – PCA biplot based on concentrations of 15 compounds detected in 23 faecal samples of infants.

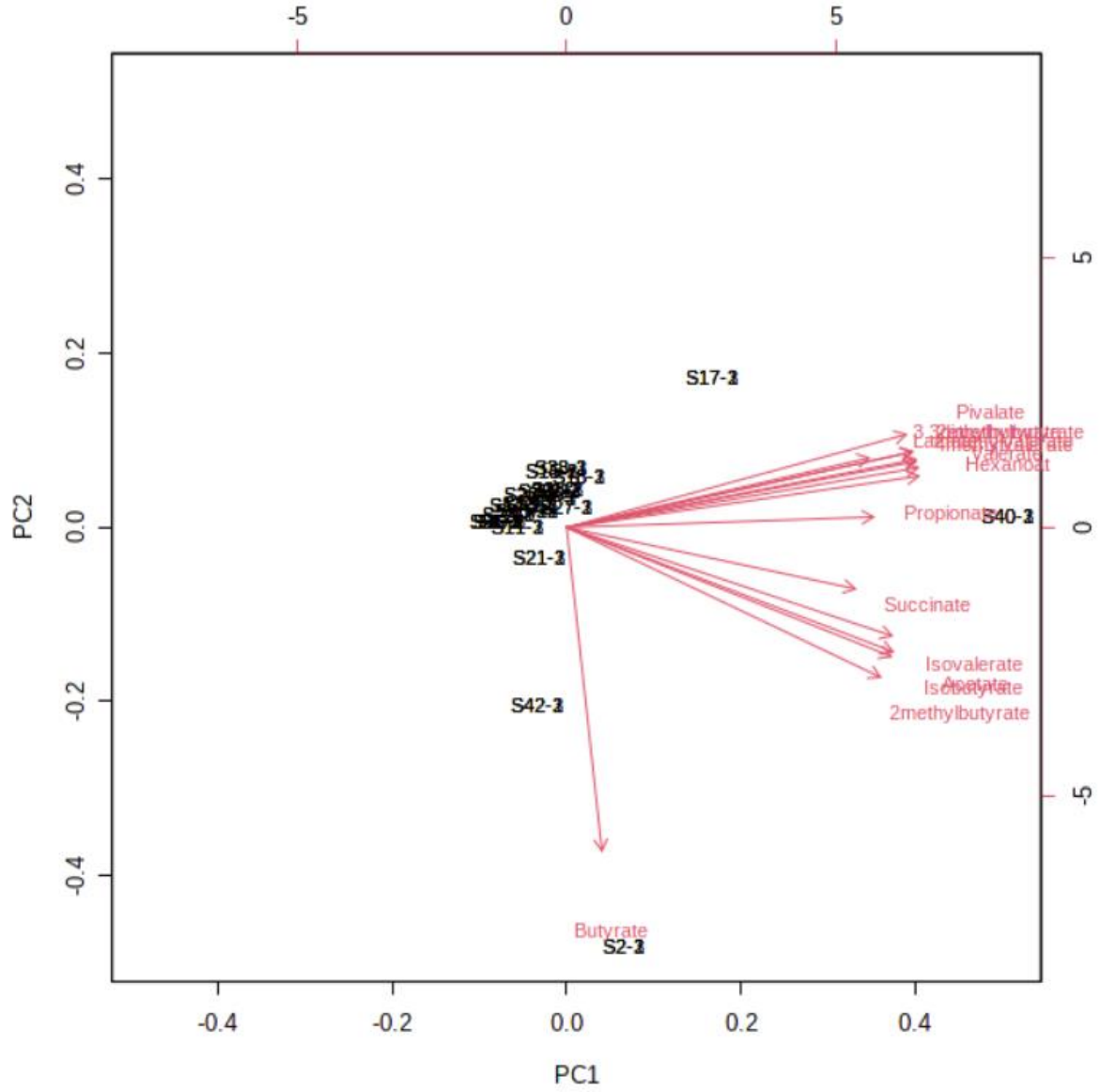


Figure 5: PCA biplot based on concentrations of 15 compounds detected in 23 faecal samples of infants. PCA biplot describes how much the individual SCFA correlate with each other, by the direction and length of arrows. Arrows pointing in the same direction means positive correlation, arrows pointing in opposite directions means negative correlations (not observed), and perpendicular arrows means no correlation. The longer arrow, the stronger correlation.

11.6 Figure 6 – Correlation of detected SCFA and OA with age

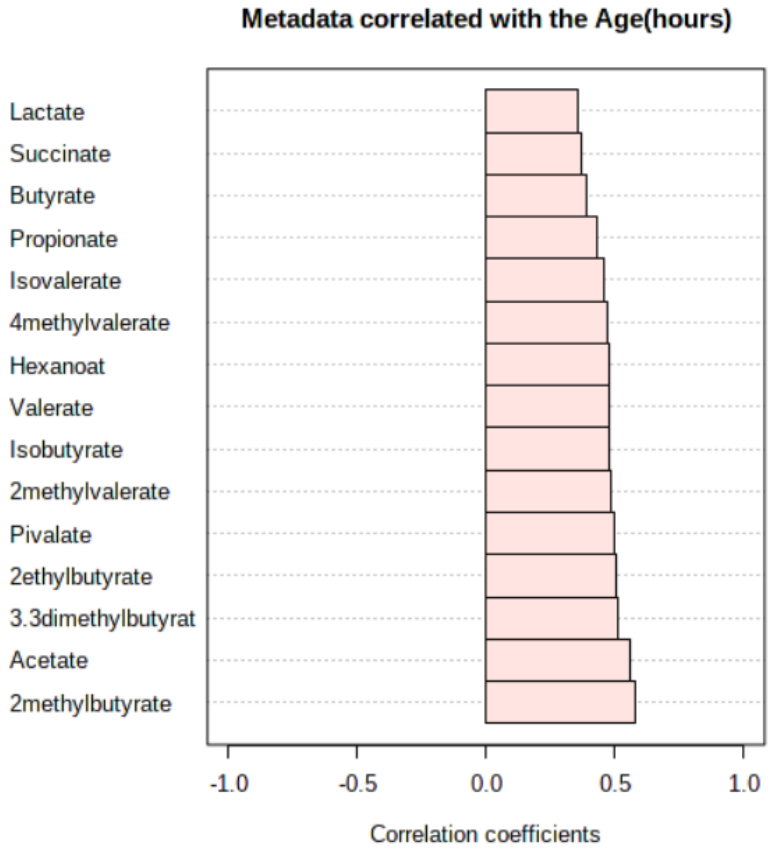


Figure 6: Correlation of detected SCFA and OA with age. All SCFA and OA correlates positively with age, with a correlation coefficient at approximately 0,5 for 2methylbutyrate and acetate, and slightly lower for the rest.

11.7 Figure 7 – Line diagrams of the 6 most abundant SCFA and OA in correlation with age of the infants

Figure 7: Line diagrams for the 6 most abundant SCFA and OA in correlation to age of the infants. The blue line indicates the age in hours, the orange line indicates SCFA or OA concentration and the orange stapled line shows the trend (linear regression) of the SCFA or OA concentration.

11.8 Figure 8 – SCFA correlation of detected SCFA and OA with birth weight of the infants

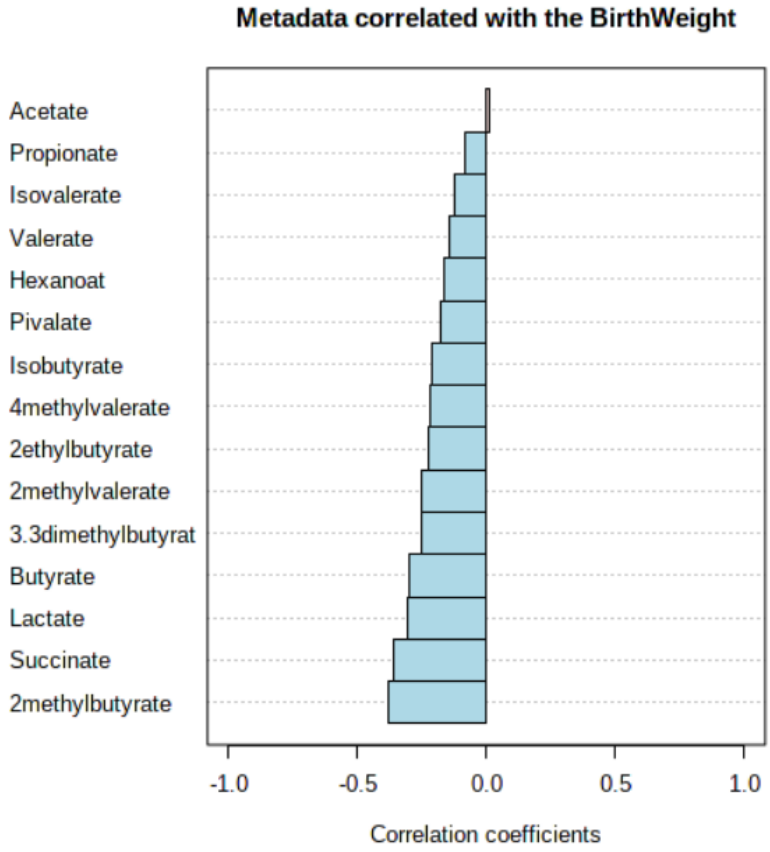


Figure 8: Correlation of detected SCFA and OA with birth weight of the infants. There is a pattern of slightly negative correlation with coefficients from about 0 to -0,4. Negative correlation means that the compounds have lower values with increasing birth weight.

11.9 Figure 9 – SCFA correlation of detected SCFA and OA with time from sample collection to freezing

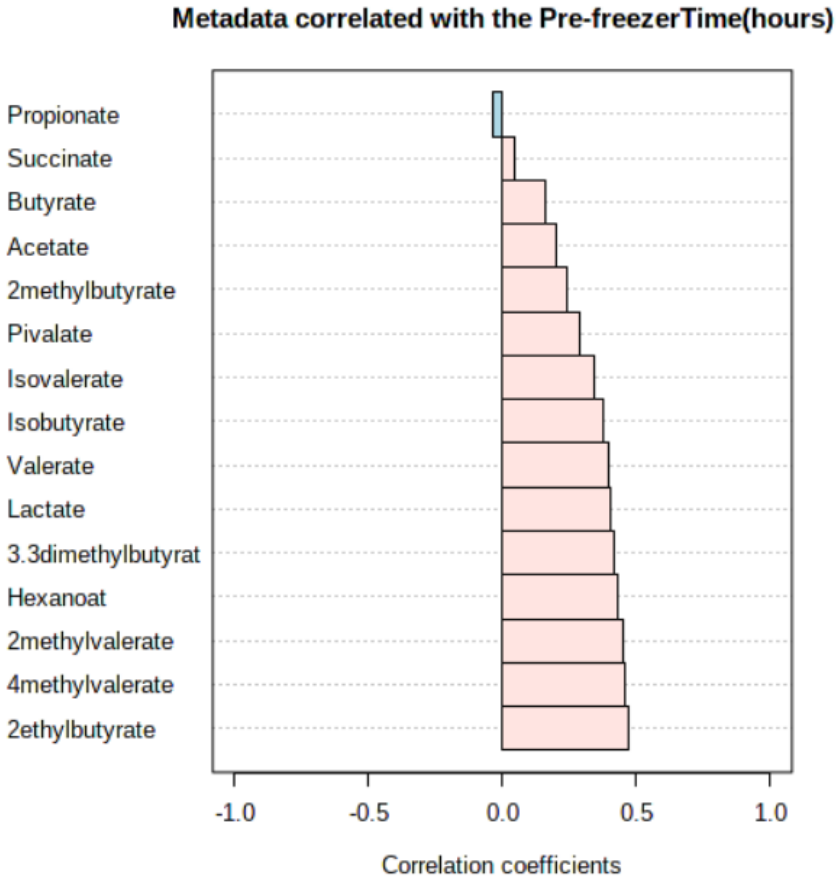


Figure 9: SCFA correlation of detected SCFA and OA with time from sample collection to freezing. All compounds have positive correlation coefficients, except propionate which is barely below 0. The rest ranges from about 0 to 0,5.

11.10 Figure 10 – SCFA correlation with DNA concentrations

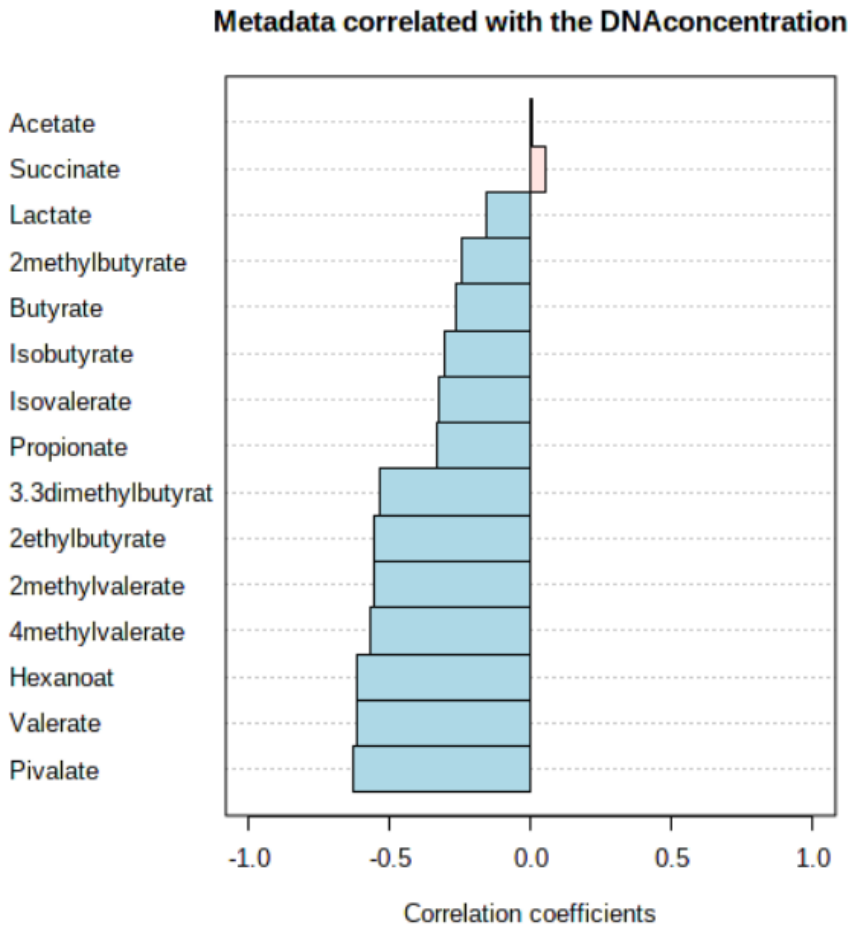


Figure 10: Correlation between metabolites and DNA concentration. Most of the compounds have a negative correlation to DNA concentration, with coefficients ranging from about -0,2 to -0,6, which means there is a pattern of lower concentrations with increasing DNA concentration. Succinate is the only one that is slightly above 0.