# Diet and gut microbiome in early life- a potential pathway for atopic disease development?

Carina Madelen Saunders

The University of Oslo, Institute of Clinical Medicine

Department of Paediatrics, Oslo University Hospital

ORAACLE

Oslo Research Group of Asthma and Allergy in Childhood; the Lung and Environment

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# 1 Preface

## 1.1 Acknowledgements

I remember the first time I heard about the vast world of microbes that live in our bodies. I had recently moved to Norway and started working as a resident doctor in Pediatrics in Haugesund. When I was given an opportunity to continue my pediatric training at the Oslo University Hospital in 2014, my ever-growing curiosity about the human microbiome and eagerness to be a part of this exciting new research field grew. It was Torbjørn Nag who encouraged me to approach Prof. Karin C. Lødrup Carlsen, an internationally acclaimed expert in the field of asthma and allergic disease, and head of ORAACLE (Oslo Research group of Asthma and Allergy in Childhood; the Lung and Environment)

Not long after, I found myself knocking on her office door, hoping to convince her of my potential value as a member of her team in the Preventing Atopic Dermatitis and ALLergies in children study (PreventADALL). Thankfully, she recognized my genuine enthusiasm for the field, and to my surprise, a few months later, I was given an opportunity to fill in on a one-year research and teaching fellowship at the University of Oslo, while Karin successfully secured funding for a continued PhD position.

Karin- Your enthusiasm for research is contagious! No one sees the big picture like you do. Thank you for always being incredibly positive, kind, and encouraging. You have taught me so much!

I would also like to express my deepest gratitude to my colleague and supervisor, Eva Rehbinder, for her invaluable support, and encouragement throughout my PhD journey. This would not have been the same experience without you. You are one of a kind! I hope we will continue to work together in research in the future.

Thank you to my supervisors, Monica Hauger Carlsen, for always having an open door and guiding me through nutritional research, and Håvard O. Skjerven, for your academic

knowledge and guidance. Thank you, Riyas Vettukattil, for your help- how you keep control over so much data baffles me. Thank you to Marissa Erin LeBlanc for your statistical help and guidance on Paper 3.

In the first three years, I performed over 2 000 individual study controls, including examinations of babies and toddlers and recruiting of pregnant women. Only after having kids myself, have I truly realized the extent of dedication, hard work, and commitment it takes to be a parent in the PreventADALL study. So, thank you to every one of you who took part in the study- without you, this study would not have been possible!

The numerous weeks of repeated study controls would have been a lot less fun without Mari Kjendsli, Ingvild Essén, Peder Annæus Granlund, Kim Advocaat Endre, and Malén Gudbrandsgard. Thank you for all the laughs and jokes. I am also grateful for all the other wonderful PhD students and study personnel I got to know. It was an absolute pleasure to work with you: Hrefna Katrín Gudmundsdóttir, Karen Eline Stensby Bains, Ina Kreyberg, Anine Lie, Hilde Aaneland, Oda Lødrup Carlsen, Thea Aspelund Fatvik, Live Nordhagen, Åshild Wiik Despriée and Johanne Uthus Hermansen.

Thank you also to Morten Nilsen and Knut Rudi. Your brilliant minds will never cease to amaze me, and I am grateful that I got to learn and take part in your microbiome world.

I am grateful to Lisa Bjarkø and Einar Stensvold for our time spent as colleagues and friends teaching medical students. Thank you to Marius Skram for your support and encouragement and for always having an open ear. You are a great department head for pediatric residents.

I would like to thank the University of Oslo, for providing me with a stimulating academic environment. I would like to acknowledge the financial support provided by HSØ, which has enabled me to pursue my research. I am grateful for the Unger-Vetlesen Medical fund, which made it possible for me and my husband to move to Melbourne in 2019 for a research stay. I want to thank Prof. Ben Marsland and his wife, Prof. Nicola Harris, for letting me

become part of their brilliant research team at the Department of Immunology and Pathology at Monash University, one of the leading academic immunology departments in Australia and worldwide. I not only learned laboratory skills but was able to take part in weekly meetings, lectures, and journal clubs and met lots of fantastic people. Thank you to Gillian Coakley, who spent hours teaching me how to grow intestinal organoids from stem cells, a technique, which had only been recently discovered.

As I'm handing in my thesis, I am about to begin a new job as a pediatric consultant at the Pediatric Department of Allergy- and Pulmonology at the Oslo University Hospital. I am grateful to Iren Matthews for believing in me and offering me a position. I look forward to spending more time with patients, increasing my clinical knowledge, and working closely with many skilled colleagues.

I would like to express my heartfelt gratitude to my family and friends, for their unwavering love, and belief in my abilities- You know who you are.

And lastly,
THANK YOU, Duane, for your constant love and support.
You and our two boys, Adam (3) and Macsen (2), are everything to me.

## 1.2 Summary of thesis

**Background**

The observed increases in immune-mediated illnesses have been associated with changes in the infant microbiome. Both maternal diet in pregnancy and infant diet are likely to impact atopic disease development, partly through influence on infant gut microbial composition and function. However, the underlying mechanisms are not clear. Species dominance or dysbiosis may affect later disease development. Microorganisms influence immune development by their ability to produce short-chain fatty acids (SCFA) through fermentation of indigestible carbohydrates in the infant's diet. Particularly butyrate has been recognized for its ability to exert anti-inflammatory functions.

**Aims**

Based on the hypothesis that atopic disease development in early childhood may be partly conveyed through gut modulation in early infancy, the overall objective of this thesis was to assess the impact of maternal and infant diet on the gut microbiome in early life and explore a possible impact of their metabolites on atopic dermatitis in infancy, through 4 specific aims; 1) to assess the dietary patterns in mid-pregnancy and their offspring in the first year of life, 2) to explore if early complementary food introduction affected infant diets including breastfeeding rates or diet diversity in the first year of life, 3) to explore the temporal development of gut microbiome and SCFA production, and the potential impact of breastfeeding and time of solid food introduction in the first year of life and 4) to explore if short-chain fatty acids are associated with atopic dermatitis.

**Methods**

The Preventing Atopic dermatitis and ALLergies in children (PreventADALL) study is a general population-based, randomized controlled interventional birth cohort study. In total, 2697 pregnant women were enrolled at the 18-week fetal ultrasound in Oslo, Østfold, and Stockholm. Overall,2397 infants were included at birth and randomized at birth to no intervention, skin intervention (oil baths and facial cream from 2 weeks- 9 months of age), early food introduction (small tastes of peanut, milk, wheat, and egg 4 days/week from 3-6 months of age), or both interventions. In this thesis focusing on mother and infant diets, infants were categorized by food intervention or no food intervention. Dietary intake in pregnancy was assessed by an electronically validated food frequency questionnaire (PrevFFQ) in the Norwegian part of the cohort. The FFQ consisted of 279 questions on the frequency and amount of intake of about 280 food items, grouped according to main food groups and meal patterns. Infant dietary data was assessed from electronic weekly questionnaires in weeks 2-26 and at 3-, 6-, 9-, and 12 months of infant age. Fecal samples were collected from mothers at 18 weeks of gestation, and their infants at birth, 3, 6, and 12 months of age. Fecal samples were analyzed for different marker genes (16S rDNA and RpOB) and metagenome sequencing on subsets of infant samples, in addition to metaproteomic and metabolomics analysis on infant sample subsets. The association between SCFAs and atopic dermatitis development was assessed in a smaller pilot study sample, including 100 mother-child pairs. Atopic dermatitis, in this thesis, was defined as the presence of eczematous lesions, excluding differential diagnoses to AD. Log-rank test and Cox regression was used to assess the impact of food intervention on age of breastfeeding

cessation. Mixed effects logistic regression was used to compare diet diversity, defined as the number of food categories consumed, between intervention groups.

**Results**

Food frequency questionnaires in mid-pregnancy were available for 1675 women. More than half of women reported consumption of excessive amounts of red meat, salt, and saturated fatty acids, surpassing the recommended limits. Alcohol intake of >1 g/day was reported by a 1/4 of pregnant women, and 1/5 exceeded recommended daily caffeine levels. Inadequate intake of essential micronutrients was observed in 54 % for folate, 50 % for iron, 41 % for selenium, 36 % for calcium, 29 % for vitamin D, and 24% for iodine.

Infant nutritional data was available for 2059/2394 infants. The rate of breastfeeding overall at 3, 6, 9 and 12 months was 95%, 88%, 67%, and 51%. In the No Food Intervention group mean age of complementary food introduction was 18.3 weeks (95% CI 18.1,18.5), while 95% of all infants in the food intervention group had their first taste of peanut at the 3-month follow-up visit (mean age 12 weeks). At 6 months 45% of infants received mostly commercially prepared foods, 47% at 9 months, and 32 % at 12 months. Early food introduction compared to no food intervention was not associated with reduced breastfeeding rates at 3 months (95 vs 95%), 6 months (87% vs 89%), 9 months (66% vs 68%) or 12 months (51% vs 50%), all p=0.96. In the Food Intervention group, diet diversity was 1.39 units (CI 1.16- 1.62) higher at 9 months ($p < 0.001$) and 0.7 units (CI 0.5-0.9) higher at 12 months ($p < 0.001$) compared to the No Food Intervention group.

The average number of unique species increased with age and reached the highest diversity at 12 months, with *Clostridiales* representing 67% of the microbiota composition. Within the *Clostridiales*, the most prominent genera were *Faecalibacterium, Ruminococcus gnavus (R. gnavus)* and *Eubacterium rectale (E. rectale)*, while the representation of *Bifidobacterium* declined from 32% at 6 months to 8% at 12 months. The delivery method significantly influenced the initial colonization of gut bacteria.

Breastfeeding at 3 months was positively associated with *Bifidobacterium* and negatively associated with *Bacteroides*, while no associations were observed with breastfeeding at 6 and 12 months and microbiota composition. Time of complementary food introduction did not explain the distinct bacterial networks observed in the infant gut at 12 months. Changes in microbiota composition influenced fecal SCFAs, particularly butyrate, which increased 4-fold from 6 to 12 months. We observed that *Eubacterium rectale* and *Faecalibacterium prasunitzii* are potential key bacterial members responsible for butyrate production in the infant gut, while *Ruminococcus gnavus* was associated with low butyrate levels.

We found significant associations between fecal butyrate levels at 12 months of age and atopic dermatitis development. Children with higher fecal butyrate levels (>75$^{th}$ percentile) were significantly less likely to develop atopic dermatitis (p=0.001).

**Conclusions**

Our data reveal nutritional inadequacy in many pregnancies and highlight the necessity for improved nutritional guidance to pregnant women across all educational levels. Introducing small amounts of complementary food early does not affect breastfeeding rates or duration but increases diet diversity. Our results indicate a shift towards an adult-like microbiota with butyrate-producing bacteria between 6 and 12 months of age. We observed two gut community states, represented by high and low fecal butyrate. Particularly butyrate seems to play an important role in preventing atopic dermatitis development in the first year of life. Given the strong associations between early-life alterations to the microbiota and immune-mediated diseases later in life, it is crucial to gain a deeper mechanistic understanding how microbes and microbiota-derived metabolites influence health and disease trajectories starting in infancy.

## 1.3 Sammendrag av avhandlingen

**Bakgrunn**

Økningen av immunmedierte sykdommer har blitt assosiert med endringer i spedbarnsmikrobiomet. Både mors kosthold i svangerskapet og spedbarns kosthold påvirker sannsynligvis utviklingen av atopisk sykdom, delvis gjennom påvirkning på spedbarnets tarmmikrobiota. De underliggende mekanismene er imidlertid ikke klare. Mikroorganismer påvirker immunutviklingen ved produksjon av kortkjedede fettsyrer (SCFA) gjennom fermentering av ufordøyelige karbohydrater i barnets kosthold. Spesielt butyrat har blitt anerkjent for sin evne til å utøve anti-inflammatoriske funksjoner.

**Forskningsspørsmål og målsetninger**

Basert på hypotesen om at atopisk sykdomsutvikling i tidlig barndom delvis kan formidles gjennom tarmmikrobiota, var det overordnede målet med oppgaven å vurdere effekten av mors og spedbarns kosthold på tarmmikrobiomet tidlig i livet og utforske en mulig påvirkning av dens metabolitter på atopisk dermatitt i spedbarnsalderen, gjennom 4 spesifikke målsetninger; 1) å vurdere kostholdet i midten av svangerskapet og barnets kosthold i første leveåret, 2) å undersøke om tidlig introduksjon av fast føde påvirket spedbarnets kosthold, inkludert amming eller kostholdets mangfold i første leveåret, 3) å undersøke utviklingen av tarmmikrobiomet og produksjonen av SCFA, og effekten av amming og tidspunktet for introduksjon av fast føde i det første leveåret og 4) å undersøke om SCFA er assosiert med atopisk dermatitt.

## Metode

Studien Preventing Atopisk Dermatitis and ALLergies in children (PreventADALL) er en generell populasjonsbasert, randomisert kontrollert intervensjonsstudie. Totalt ble 2697 gravide kvinner rekruttert ved 18-ukers rutine ultralyd i Oslo, Østfold og Stockholm.Totalt ble 2397 spedbarn inkludert ved fødselen. Ved fødselen ble spedbarnene tilfeldig fordekt i fire grupper: ingen intervensjon, hudintervensjon (oljebad og ansiktskrem fra 2 uker til 9 måneder), tidlig matintroduksjon (små smaker av peanøtter, melk, hvete og egg 4 dager/uke fra 3. -6 måneders alder), eller begge intervensjoner. I denne oppgaven ble barn kategorisert etter matintervensjon eller ingen matintervensjon. Kostholdet i svangerskapet ble undersøkt med hjelp av et elektronisk validert Food Frequency Questionnaire (PrevFFQ) i den norske delen av kohorten. FFQ besto av 279 spørsmål om frekvensen og mengden av inntak av omtrent 280 matvarer, gruppert etter matgrupper og måltidsmønstre. Barnets kostholdsdata ble analysert med hjelp av elektroniske spørreskjemaene ved 3-, 6-, 9- og 12 måneder. Avføringsprøver fra mødre i 18. uke svangerskap, og deres barn ved fødsel, 3, 6 og 12 måneder, ble analysert ved bruk av 16S rDNA og metagenomsekvensering, i tillegg til metaproteom og metabolomik analyse i 100 mor-barn par. Sammenhengen mellom SCFA og utvikling av atopisk dermatitt ble vurdert i en pilotstudie, inkludert 100 mor-barn-par med et komplett sett med biologiske prøver og kliniske undersøkelser. Atopisk dermatitt, i denne oppgaven, ble definert som tilstedeværelsen av eksematøse lesjoner, unntatt differensialdiagnoser til AD. Log-rank test og Cox-regresjon ble brukt for å vurdere effekten av matintervensjon på amming. Logistisk regresjon ble brukt for å sammenligne kostholdet mangfold.

**Resultater**

Analyse av kostholdsdata i svangerskapet blant 1675 kvinner viste at mer enn halvparten rapporterte inntak av rødt kjøtt, salt og mettede fettsyrer, som oversteg anbefalinger. Alkoholinntak på >1 g/dag ble rapportert av 1/4 av gravide kvinner, og 1/5 overskred anbefalt daglig koffeinnivå. Utilstrekkelig inntak av essensielle mikronæringsstoffer ble observert hos 54 % for folat, 50 % for jern, 41 % for selen, 36 % for kalsium, 29 % for vitamin D og 24 % for jod.

Kostholdsdata var tilgjengelig for 2059/2394 barn. Antall barn som ble ammet ved 3, 6, 9 og 12 måneder var 95 %, 88 %, 67 % og 51 %. I gruppen uten tidlig matintroduksjon var gjennomsnittlig alder for introduksjon 18,3 uker (95 % KI 18,1, 18,5), mens 95 % av alle spedbarn i matintervensjonsgruppen hadde sin første smak av peanøtt ved 3-måneders kontroll (gjennomsnittsalder 12 uker). Etter 6 måneder fikk 45 % av spedbarn for det meste ferdigmat, 47 % etter 9 måneder og 32 % etter 12 måneder. Tidlig introduksjon av fast føde sammenlignet med ingen matintervensjon var ikke assosiert med reduserte ammerate, etter 3 måneder (95 vs 95 %), 6 måneder (87 % vs 89 %), 9 måneder (66 % vs 68 %) eller 12 måneder (51 % vs 50%), alle p=0,96. I intervensjonsgruppen som introduserte fast føde tidlig var kostholdets mangfold 1,39 enheter (KI 1,16-1,62) høyere etter 9 måneder ($p < 0,001$) og 0,7 enheter (KI 0,5-0,9) høyere etter 12 måneder ($p < 0,001$).

Gjennomsnittlig antall unike arter i spedbarnets tarm økte med alderen og nådde det høyeste mangfoldet etter 12 måneder, med *Clostridiales* som representerte 67 % av mikrobiotasammensetningen etter 12 måneder. Innenfor *Clostridiales* var *Faecalibacterium,*

*Ruminococcus gnavus (R. gnavus*) og *Eubacterium rectale (E. rectale)* mest fremtredende, mens *Bifidobacterium* gikk ned fra 32 % etter 6 måneder til 8 % etter 12 måneder. Forløsningsmetoden påvirket den første koloniseringen av tarmbakterier betydelig.

Amming ved 3 måneder var positivt assosiert med *Bifidobacterium* og negativt assosiert med *Bacteroides*, mens ingen assosiasjoner ble observert med amming ved 6 og 12 måneder og mikrobiotasammensetning. Tidspunktet for introduksjon av fast føde forklarte ikke de distinkte bakterielle nettverkene som ble observert i spedbarnets tarm ved 12 måneder. Endringer i mikrobiotasammensetning påvirket fekale SCFAs, spesielt butyrat, som økte 4 ganger fra 6 til 12 måneder. Vi observerte at *Eubacterium rectale* og *Faecalibacterium prasunitzii* er viktige bakterier, som er ansvarlige for butyratproduksjon i spedbarnets tarm, mens *Ruminococcus gnavus* var assosiert med lave butyratnivåer.

Vi fant signifikante assosiasjoner mellom fekalt butyratnivå ved 12 måneders alder og eksemutvikling. Barn med høyere fekalt butyrat (>75. persentil) hadde betydelig mindre sannsynlighet for å utvikle atopisk dermatitt (p=0,001).

Konklusjon

Våre data avslører utilstrekkelig næringsinntak i mange svangerskap og fremhever nødvendigheten av forbedret ernæringsveiledning til gravide kvinner på tvers av alle utdanningsnivåer. Å introdusere små mengder fast føde tidlig påvirker ikke amming, men øker kostholdets mangfold. Resultatene våre indikerer et skifte mot en voksenlignende mikrobiota med butyratproduserende bakterier mellom 6 og 12 måneders alder. Vi

observerte to bakterienetverk, representert ved høy og lav fekalt butyrat. Spesielt butyrat ser ut til å spille en viktig rolle for å forhindre utvikling av atopisk dermatitt i det første leveåret. Gitt de sterke assosiasjonene mellom tidlige endringer i mikrobiotaen og immunmedierte sykdommer senere i livet, er det avgjørende å få en dypere mekanistisk forståelse av hvordan mikrober og mikrobiota-avledede metabolitter påvirker helse og sykdomsbaner som starter i spedbarnsalderen.

## 1.4 Abbreviations

| | |
|---|---|
| AD | Atopic dermatitis |
| CI | Confidence Interval |
| CS | Caesarean section |
| DD | Dietary diversity |
| FI group | Food Intervention group |
| GA | Gestational age |
| NCD | Non-communicable diseases |
| NFI group | No Food Intervention group |
| OR | Odds Ratio |
| PrevFFQ | PreventADALL Food Frequency Questionnaire |
| RCT | Randomised controlled trial |
| RI | Recommended Intake |
| SCFA | Short-chain fatty acids |
| SD | Standard Deviation |

# 2 Articles in the thesis

Paper I

Carina Madelen Saunders, Eva Maria Rehbinder, Karin C. Lødrup Carlsen, Malén Gudbrandsgard, Kai-Håkon Carlsen, Guttorm Haugen, Gunilla Hedlin, Christine Monceyron Jonassen, Katrine Dønvold Sjøborg, Linn Landrø, Björn Nordlund,Knut Rudi,Håvard O.Skjerven, Cilla Söderhäll, Anne Cathrine Staff, Riyas Vettukattil and Monica Hauger Carlsen study group. **Food and nutrient intake and adherence to dietary recommendations during pregnancy: a Nordic mother–child population-based cohort.** Food & Nutrition Research 2019; DOI: 10.29219/fnr.v63.3676

Paper II

Morten Nilsen, Carina Madelen Saunders, Inga Leena Angell, Magnus Ø. Arntzen, Karin C. Lødrup Carlsen, Kai-Håkon Carlsen, Guttorm Haugen, Live Heldal Hagen, Monica H. Carlsen, Gunilla Hedlin, Christine Monceyron Jonassen, Björn Nordlund, Eva Maria Rehbinder, Håvard O. Skjerven, Lars Snipen, Anne Cathrine Staff, Riyas Vettukattil and Knut Rudi. **Butyrate Levels in the Transition from an Infant-to an Adult-Like Gut Microbiota Correlate with Bacterial Networks Associated with *Eubacterium Rectale* and *Ruminococcus Gnavus.*** Genes 2020; DOI:10.3390/genes11111245

Paper III

Carina Madelen Saunders, Eva Maria Rehbinder, Karin Cecilie Lødrup Carlsen, Christine Monceyron Jonassen, Marissa LeBlanc, Björn Nordlund, Håvard Ove Skjerven, Cilla Söderhäll, Riyas Vettukattil and Monica Hauger Carlsen. **Feeding practices and dietary diversity in the first year of life; PreventADALL, a Scandinavian RCT and birth cohort study.** The Journal of Nutrition, 2023, DOI: 10.1016/j.tjnut.2023.06.015

IV Pilot study

Carina Madelen Saunders, Morten Nilsen, Eva Maria Rehbinder, Karin Cecilie Lødrup Carlsen, Kai-Håkon Carlsen, Monica Hauger Carlsen, Gunilla Hedlin, Benjamin Marsland, Christine Monceyron Jonassen, Bjørn Nordlund, Håvard Ove Skjerven, Cilla Söderhäll, Anne Cathrine Staff, Riyas Vettukattil, Knut Rudi, **The role of butyrate in atopic dermatitis development**, EAACI congress 2019, poster presentation

# 3 General Introduction

## 3.1 Environment, lifestyle, and Non-Communicable Diseases (NCDs)

Chronic inflammatory non-communicable diseases (NCDs), including atopic and autoimmune diseases, have increased over the last decades [1–3]. Although genes play a central role in the development of atopic disease, the environment, lifestyle, and, exposures throughout a lifetime could account for more than 80 % of an individual´s disease risk [4,5]. The initial discovery in 1989 that children from larger families were less likely to develop hay fever, possibly due to early exposure to infections, pointed to the importance of "hygiene-related" determinants of allergic disease [6]. Even before the advent of modern sequencing techniques, it was hypothesized that microbes are indispensable for normal tolerogenic immune development [7]. The concept that early-life exposure to microbes can facilitate healthy immune responses gained more interest as studies reported a decrease in allergy and asthma in children growing up in rural areas [8,9]. Over the past decade, more attention turned to the protective effects of extrinsic exposure to microbes in the environment and intrinsic exposures to commensal microbes in the gut on immune- and atopic disease development [10,11], a concept termed the "biodiversity hypothesis." [12] The underlying presumption being that a diverse gut microbiota and high turnover of microbes at the mucosal level stimulate the immune system in a balanced way and can thereby confer protective effects [13,14].

Key risk factors for NCDs appear to stem from the intrauterine environment and exposures early in life [15,16]. In line with the Developmental Origins of Health and Disease (DOHaD), the 'first 1000 days,' starting from conception until age 2 years, are recognized to be of

particular importance, as early life events, including diet in pregnancy, can exert profound effects on the offspring [15]. The concept that nutrition can affect a person´s health trajectory before conception through epigenetic fetal programming has increased the importance of a well-balanced diet during pregnancy [17–19].

A Western diet, characterized by a low intake of fiber and plant-based foods and excessive intake of foods high in saturated fat, salt, and refined carbohydrates, may be an additional contributor to the rise of atopic disease and other non-communicable illnesses [20–22]. Diet may modulate the composition and function of the gut microbiota, the community of microbes residing in the gut [23]. A mechanism in which beneficial microbes may exert immunomodulatory effects is through the release of metabolites, particularly SCFAs (short-chain fatty acids), derived from dietary fiber [24]. Moreover, breastfeeding versus formula-feeding as well as the types of complementary foods given and the time of introduction, may also impact immune modulation and tolerance induction, independent of the microbiome [25]. Early introduction of complementary allergenic foods into the infant diet has recently been found to be associated with a reduced risk of developing food allergies, as recently confirmed in large RCTs [26].

Figure 1 highlights diet-microbiome-related mechanisms for atopic disease development.

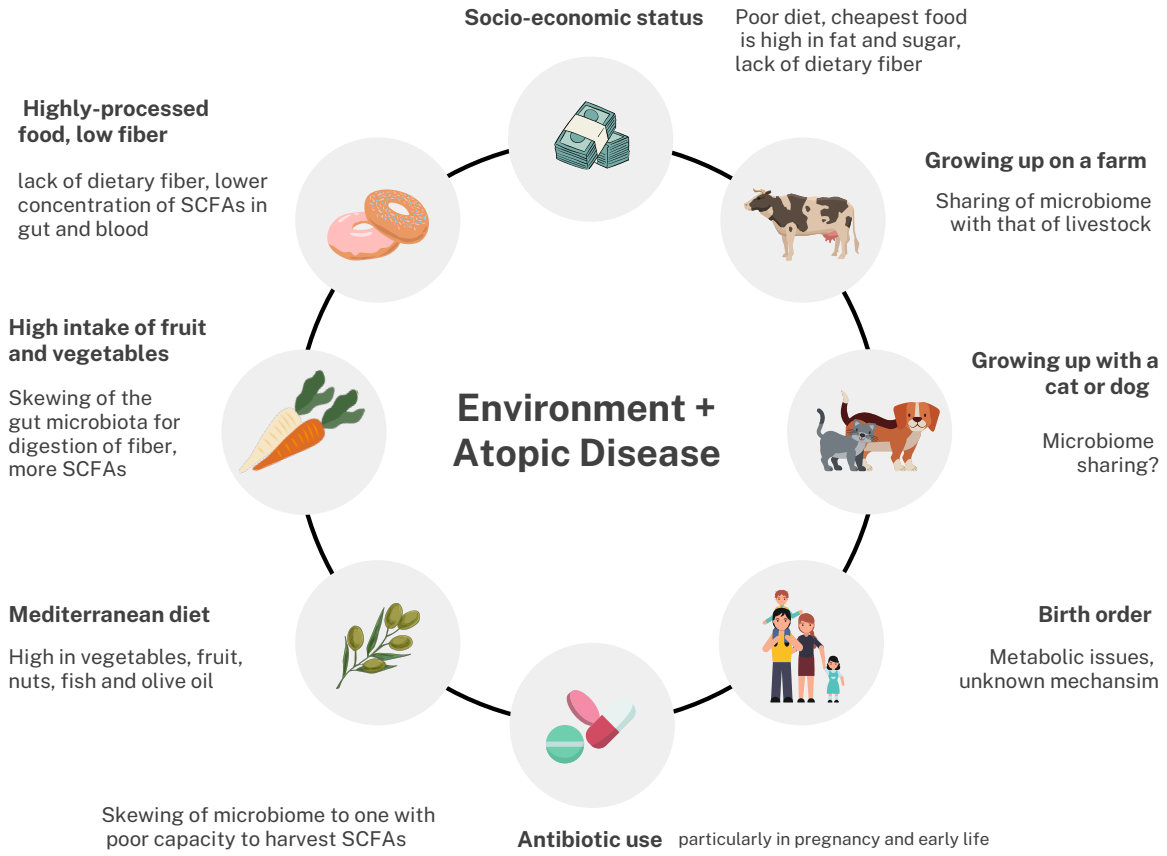


*Figure 1 Diet-microbiome related mechanisms and early-life environmental factors demonstrated to be associated with gut microbiota composition and the rise of atopic disease.*

## 3.2 Diet- General Aspects

The term “healthy diet” is generally used to describe an appropriate intake of macronutrients to support energetic and physiologic needs while also providing sufficient micronutrients to sustain the physiologic functions of the body [27,28]. A number of relevant dietary terms is depicted in Figure 2 List of nutritional definitions relevant to this thesis. The interrelation between diet and health can be assessed at the level of nutrients, foods, or dietary patterns. Analysis of dietary patterns may give a more comprehensive description of

dietary data and a better explanation of diet-health interactions compared with analyses of foods or single nutrients. Humans do not eat isolated nutrients but a diversity of foods with an intricate combination of nutrients [29]. Healthy dietary patterns normally consist of a variety of plant-based diets, including whole grains, fruits and vegetables, nuts, and sources of omega-3 fatty acids, while also having low amounts of saturated fats, trans-fats, and added sugars [30]. Such diets have been associated with a positive effect on health outcomes, with the strongest associations found for cardiovascular disease and metabolic syndrome [31]. Adherence to healthy diets and their negative associations with NCDs have been summarized in a meta-review examining the effect of these diets on health outcomes in 9 systematic reviews and 24 meta-analysis [32]. Western-type diets, on the other hand, including highly processed foods and "fast foods," contain high amounts of saturated fat and sugar but lack fiber, vitamins, and minerals. Such diets have been shown to trigger inflammatory responses with elevated serum markers of inflammation found in both studies of human and rodents [33,34], and have been associated with chronic diseases [35,36].

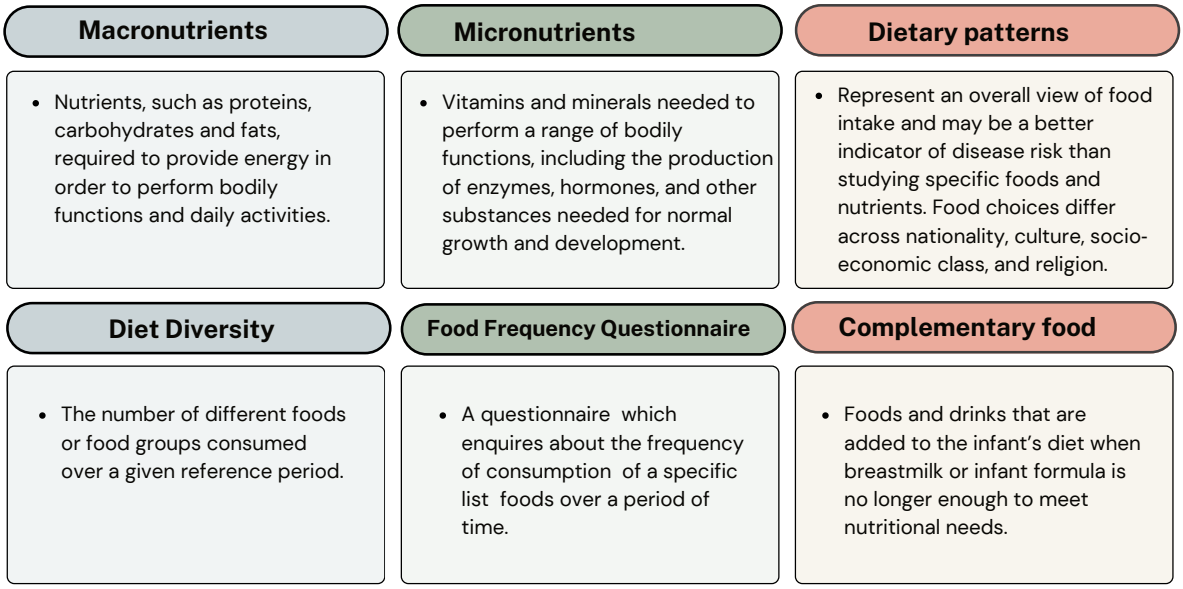


*Figure 2 List of nutritional definitions relevant to this thesis*

## 3.3 Diet during pregnancy

Optimal fetal development and growth depend on a healthy, nutrient-rich, and energy-appropriate diet in pregnancy [37]. Although energy requirements for the mother increase slightly towards the end of pregnancy, the need for nutrients considerably increases throughout pregnancy [37,38]. Consequently, pregnant women are at increased risk of micro- and macronutrient deficiency [39]. In high-income countries, an inadequate maternal diet is usually characterized as a combination of macronutrient overnutrition and micronutrient undernutrition [40]. Micronutrients particularly important in pregnancy, and commonly

provided as supplements, include vitamins A, D, E, folate, B12, B6, and C, iron, zinc, iodine, copper and selenium [41]. Low-quality diets, including inadequate intakes of folate, iron, and vitamin D during pregnancy, are prevalent in the United States, the United Kingdom, and other European countries [42,43]. Insufficient intakes of micronutrients in pregnancy have also been documented in Norway, with a recent study including 804 pregnant women finding iodine deficiency in a large proportion of women [44]. In another study including 40 108 pregnant women participating in the Norwegian Mother and Father-Child Cohort (MOBA) study, 63% and 80% did not reach the recommended intake of Vitamin D and iodine, respectively, and 34 % did not reach the recommended intakes of folate, despite supplementing [45]. Also, the intake of alcohol during pregnancy is prevalent in many European countries, despite recommendations to abstain from alcohol during pregnancy [46]. A study among over 7000 women in 11 European countries showed that the highest proportion of women who reported alcohol consumption during pregnancy were the UK (28.5 %) and Russia (26.5 %), with Norway (4.1 %) and Sweden (7.2) reporting the lowest alcohol consumption of the countries included [47].

Diet during pregnancy may influence health in later life, as illustrated in the Dutch Famine study [48], which examined the consequences of restricted maternal nutrition during different stages of pregnancy in a cohort of 2414 people born around the time of the Dutch famine. Exposure to famine was associated with glucose intolerance and a higher risk of coronary heart disease, disturbed blood coagulation, more obesity, and breast cancer among the offspring exposed to famine in early gestation. The study was among the first to exhibit that chronic diseases emanate in the womb through adjustments made by the fetus when

exposed to undernutrition. Notably, these changes were also passed to the next generation, highlighting the long-term consequences of an inadequate diet during pregnancy [49].

Guidelines for intake of nutrients are provided by the Norwegian Directory of Health, largely stemming from the Nordic Nutrition Recommendations (NNR) [50]. The NNR are regularly updated and are published by the Norwegian Directorate of Health [51]. The Norwegian Council for Nutrition also compiles the Norwegian food-based dietary guidelines (NFG) [51]. Pregnant women are advised to adhere to the same dietary guidelines as the rest of the population, with a special emphasis on a varied and mainly plant-based diet [52,53]. Micronutrient adequacy is usually assessed using a reference value AR (average requirement), and the percentage of individuals with an intake below the AR have an increased risk for inadequate intake. AR for vitamins and minerals, as well as the recommended intake range for macronutrients and food groups, is presented in Figure 3 Recommendations on intake of micro- and macronutrients, as well as food groups, based on the latest Nordic Nutrition Recommendations from 2023. Definitions: E% (percentage of total energy intake per day), AR (Average requirement), RIR (recommended intake range.

Studies exploring how well Nordic countries adhere to the NNR during pregnancy are few [45,54]. Knowledge about nutritional status and dietary habits during pregnancy is necessary when developing future health policies and intervention programs aimed at improving the health and well-being of both mothers and their offspring.

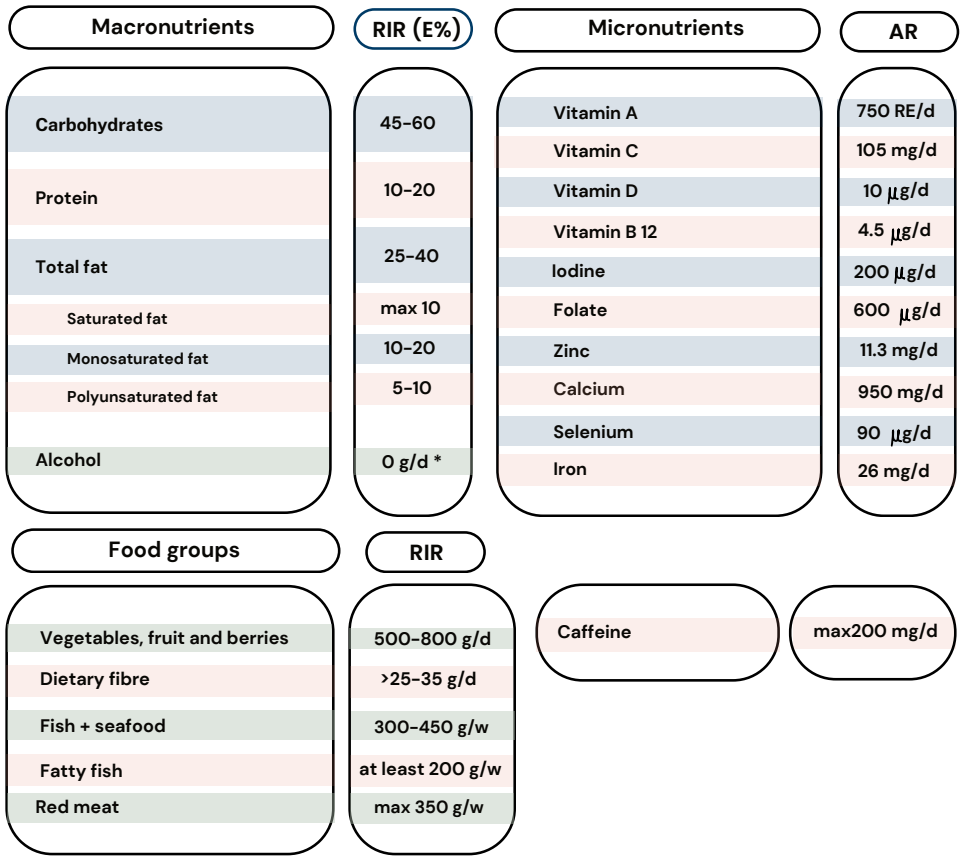


*Figure 3 Recommendations on intake of micro- and macronutrients, as well as food groups, based on the latest Nordic Nutrition Recommendations from 2023. Definitions: E% (percentage of total energy intake per day), AR (Average requirement), RIR (recommended intake range), *Alcohol is not an essential nutrient but confers energy*

## 3.4 Infant Diet

During the first year of life, infants progress from an all-milk diet (breastmilk and/or formula) to varied diets, including other food and beverages, referred to as complementary foods. The term “complementary feeding” or “weaning” refers to all solid and liquid foods other than breast milk or infant formula. This definition has been adopted by the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), commonly used in Europe for advice on weaning practices, and by other international societies [55–57].

The period of complementary feeding usually refers to the age range of 6–24 months [58] and is an important infant milestone intended to provide the growing infant with macro- and micronutrients needed for optimal growth once milk-based feeding alone becomes insufficient [59]. This transition is necessary to sustain the changing nutritional requirements in infancy, as well as adaptation to physiological changes in the infant related to feeding and processing foods. The time of weaning is generally recognized as being a time when infants are vulnerable to nutrient deficiencies and imbalances [60]. Additionally, introducing allergenic foods early on exposes infants to potential food allergens with the aim of developing tolerance [61].

To date, much of the focus in infant diet research has been directed toward milk feeding, the difference in long-term health outcomes between breastfed and formula-fed infants and assessing the timing and duration of milk feeding in infancy. Other perspectives on infant feeding, such as types of complementary foods given, is less clear [55]. Knowledge about what infants eat is crucial to correct an inadequate diet in early life and, to potentially prevent future health problems.

### 3.4.1 Timing of Complementary Food Introduction

Throughout the years, recommendations regarding the timing of complementary food introduction have changed and continue to be a topic of debate [62,63]. In the 1960s, for example, it was common to introduce complementary foods by 4 months of age, with the average age of introduction being 8 weeks [64]. In the late 1990s, a paradigm shift occurred as

expert bodies began advocating for the postponement of complementary food introduction until the age of 6 months [65]. This recommendation was partly made to control the rising incidence of food allergies, the notion being that exclusive breastfeeding for 6 months and avoidance of allergenic foods in infancy could prevent allergies [66]. However, it seems probable that the delay in introducing allergenic foods may have inadvertently contributed to the continued rise of food allergies, alongside other factors, as highlighted already by Prescott et al in 2008 [67]. A link between late introduction of complementary foods and food allergy has been documented in several observational studies, including in a large cohort study of 3097 German infants that were followed until 6 years of age. In this study Zutavern et al. found a higher risk of food sensitization in infants introduced to solid foods later [68,69]. The hypothesis that delaying solids confers no protection in terms of food allergy development, was later confirmed in the Learning Early about Peanut Allergy (LEAP) trial, being the first RCT to show a benefit of early introduction of allergenic foods [70]. Results from our own study show that the risk of food allergy can, in fact, be reduced through early introduction of common allergenic foods from 3 months of age [71], pointing in the same direction as the LEAP study and also the Enquiring About Tolerance (EAT) trial [70,72]. However, the topic of immunologic tolerance development through allergenic dietary modifications is beyond the scope of the present thesis.

Since the 1990s, weaning advice has remained controversial, and the lack of evidence supporting delayed complementary food introduction is reflected in differing recommendations across countries and governing authoritative bodies [55,59,73]. The World Health Organization (WHO) recommends exclusive breastfeeding for 6 months and

continued breastfeeding thereafter until 2 years of age or beyond [74]. These recommendations were adopted by the American Academy of Pediatrics (AAP) [75]. The latest Nordic Nutrition Recommendations from 2023, based on a systemic review of short- and long-term health benefits of breastfeeding, have concluded that the recommendation from 2004 about exclusive breastfeeding for 6 months and continued breastfeeding thereafter can remain unchanged [76]. Norwegian health authorities add that complementary foods can be gradually introduced from four months of age if the infant is developmentally ready and shows signs of needing more food, such as not gaining weight sufficiently, seeming hungry despite frequent breastfeeding, or displaying a strong interest in solid foods [77],[73]. Similarly, the ESPGHAN Committee guidelines state that complementary foods should not be introduced before four months but should not be delayed beyond six months of age [55].

The WHO's recommendation to exclusively breastfeed for 6 months was made in 2001 after the organization commissioned a meta-analysis [78] reviewing the optimal duration of exclusive breastfeeding based on studies from 11 low- and 12 high-income countries. The WHO's guidelines were particularly important in resource-constrained regions, where access to clean water and food may be scarce. The most relevant finding pertaining to high-income countries was an observational analysis from a trial of breastfeeding promotion in Belarus, which found a reduced risk of gastrointestinal infection in infants exclusively breastfed for 6 months children, compared to infants exclusively breastfed for 3 months [79].

Several high-income countries, including the US and UK [57,80], adopted the WHO's recommendation for the duration of exclusive breastfeeding, while other countries made

certain adaptions. For instance, Sweden and the Netherlands suggest that breastfed babies can be given "trial foods" or "small tastes" between 4 and 6 months, but these foods should not replace milk [81,82]. On the other hand, some countries continued to advise introducing CF between 4 and 6 months [59].

Despite widespread official support, the scientific basis of the WHO's recommendations has been debated based on the lack of research on the optimal timing of complementary foods [55]. European infants are less likely to experience deficiency of macro- and micronutrients in the complementary feeding period [55]. Therefore the WHO´s recommendations of exclusive breastfeeding for the first 6 months of life is particularly important for low-income countries dealing with high infant mortality rates, due to infections and malnutrition, among other things [83]. In contrast, high-income countries face different challenges, including an explosion of immune-mediated diseases, a loss of gut microbial diversity, and impaired immune development [84,85]. Recommendations on infant feeding, including the WHO's, stem from a time when most studies assessing the timing of complementary food introduction had been observational, mainly because of ethical challenges associated with conducting RCTs. Over the last decade, however, several trials have shown reduced food allergy by earlier introduction of allergenic foods, summarized in a recent systematic review and meta-analysis by Scarpone et al [86].

Studies assessing the timing of complementary food introduction in European countries show that very few mothers exclusively breastfeed for 6 months [87]. The most recent Norwegian Spedkost 3 survey from 2018/2019 including 2182 participants found that only

5% of infants are exclusively breastfed at 6 months, and 43% of infants are introduced to complementary food at 4 months [88]. If early complementary food introduction influences breastfeeding rates has not been well studied. Both the EAT study in a general population, and the LEAP study of children at high risk of peanut allergy, found that breastfeeding rates were not affected by early food introduction [72,89].

Overall, the decision on when to introduce solid foods seems to be mostly influenced by the readiness of the child, perceived need for additional nutrition, and parental factors rather than strict guidelines, reflected by the low adherence to current guidelines in both low- and high-income countries [55]. Many questions on the timing of complementary food introduction remain unanswered and more research is needed to address optimal and safe ways to introduce allergenic foods, which is outside the scope of this thesis.

### 3.4.2 Diet diversity in infancy

A growing interest in assessing food patterns rather than individual nutrients when studying disease outcomes has led to an increased focus on diet diversity (DD). Diet diversity is usually defined as the number of different foods or food groups consumed over a given time frame [90]. Dietary variety is a synonym often used in literature [90]. Diet diversity can also be defined as the diversity of foods consumed, the number of foods within a food group eaten, e.g., fruit and vegetable diversity, as the World Health Organization (WHO) definition of minimum diet diversity, or the number of allergens consumed, called food allergen diet diversity. An increased diet diversity has been linked to increased nutrient adequacy, both in low- and high-income countries [91,92].

A task force report from the European Academy of Asthma, Allergy, and Immunology (EAACI) suggested that increased diet diversity may affect allergic disease outcomes in several ways, such as via an effect on the microbiome, through increased nutrient intake, and/or through exposure to different food antigens [93]. One of the challenges highlighted by the task force was a lack of agreed definitions and inconsistencies across studies surrounding the terminology and use of diet diversity. The report summarized 14 papers assessing the link between diet diversity and allergy outcomes, with only one study reporting on the association between diet diversity and food allergy outcomes. This study by Roduit et al assessed the impact of diet diversity on allergy outcomes by using data from the prospective multi-center study Protection Against Allergy Study in Rural Environments study (PASTEUR/EFRAIM study) and specifically examined whether complementary food introduced in the first year of life was associated with asthma, food allergy, allergic rhinitis, and allergic sensitization up to 6 years of age. Infant feeding practices were reported monthly from 3-12 months of life and a diversity score was calculated based on major foods. Two definitions were used in this study, with one including 15 different foods (including any cow's milk, yogurt, other milk product, eggs, nuts, vegetables or fruits, cereals, bread, meat, fish, soy, margarine, butter, cake, and chocolate) frequently consumed by 80% of the population in the first ear of life and the second definition including 6 major foods (vegetables/fruits, cereals, bread, meat, cake, and yogurt) introduced in the first 6 or first 12 months of life. Children with a lower diet diversity score in the first year of life had an increased risk of developing food allergies up to six years of age compared to children with a more diverse diet, including both sensitization to food allergens and doctor-diagnosed food

allergy [94]. The same study found that also in the second year of life, increased diet diversity was inversely associated with the development of asthma [95]. Venter et al. recently also found an association between diet diversity in the first year of life in 900 children and reduced odds of developing food allergy by 10 years of age. Specifically, they showed that the odds of developing food allergy were reduced by 10.8% by each additional food item introduced by 6 months of life [96]. In a Finish birth cohort including 3142 infants reduced diet diversity by 12 months of age was associated with asthma and allergic rhinitis at 5 years [97]. A German prospective birth cohort study including 3097 infants, grouped 48 food items assessed through questionnaires into 8 food groups: vegetables, fruit, cereals, meat, egg, dairy products, fish, and others (nuts, soy products, chocolate). They found that children in the highest quartile who were introduced to all eight food groups during the first year of life have lower odds of developing atopic dermatitis up to 15 years of age compared to children in the lowest quartile consuming less than five food groups ( OR, 0.67; 95% CI, 0.48-0.94) [98].

Moreover, the effect of complementary food introduction and diet diversity on early microbiome composition has recently gained interest [99]. Although there are few studies on this topic, the hypothesis is that a higher diet diversity provides more fiber and other nutrients, which can lead to increased diversity of the gut microbiome [100]. In a Danish study of 227 children, the introduction of complementary food was found to be the major determinant for gut microbiota establishment at 9 and 18 months of age, and specifically, food with high fiber and protein content was associated with an increase in microbiome diversity [101]. Increased microbial diversity in the gut has been shown to reduce the risk of food allergies in different populations, including children [102–104]. In an observational study of

100 Korean children, for example, different diet diversity scores ≥1 at 3 and 4 months of life increased gut microbial diversity and reduced the development of egg allergy in a high-risk group of infants with a family history of allergic disease. However, they found no association between diet diversity scores at 6 to 12 months and the development of egg allergy [105]. A longitudinal study following 440 infants from birth until school age found an independent and inverse association between gut microbial diversity and atopic dermatitis [106].

However, a birth cohort study from New Zealand in the 1980s found that infants exposed to 4 or more different food types (including cereals, vegetables, dairy products, meat, fruits, and egg or related products) before 4 months of age had a 2,9 times increased risk of developing recurring or chronic eczema up to 10 years of age, than children not introduced to complementary food early [107].

Considering the impact of diet diversity in early childhood on disease outcomes, assessing if early complementary food introduction affects diet diversity in infancy seems important. However, this has not been the subject of any studies to the best of our knowledge.

## 3.5 Introduction to the gut microbiome

In the 17th century, Antonie van Leewuhoek discovered that microbes inhabit part of the human body, using a self-designed microscope to examine his own faeces [108]. His important observation was, hoqever disregarded for well over 200 years. In 1882, German physician Robert Koch discovered that tuberculosis was caused by a bacterium, marking the beginning of an era in which several infectious illnesses were recognized as caused by bacteria; the

"germ theory of disease". Consequently, several hygiene measures were implemented, vaccines were developed, and the concept of useful microorganisms was neglected. After Penicillin and other antibiotics were discovered in 1942, optimism arose that it was indeed possible to control infectious diseases. Over the next decades, the number of infectious illnesses decreased. In the same period, however, the incidence of immune-mediated diseases increased. Obesity, diabetes, inflammatory bowel disease, and atopic disease are but a few examples of conditions that have risen as part of collateral consequences [109]. The discovery of antibiotics and implementation of hygiene measures, coupled with changes in lifestyle and diet, had led to a loss of potential critical components of the human microbiome, the array of microorganisms, including bacteria, yeast and viruses, that live in and on humans [110].

The composition of microbes varies between body sites. Most of the human body microbes reside in the colon and fulfil several functions based on their genetic potential, including the fermentation of complex fibres and the production of vitamins and metabolites [112]. In 1972 the human body was estimated to harbour 10 times more bacteria than human cells, altogether approximately 2-3 kg [113]. Newer research from 2016 suggests that this ratio has been overestimated, and a ratio of 1:1.3 seems more probable (meaning 1.3 bacterial cells for every 1 human cell) [114,115]. Not accounting for fungi, viruses, and phages inhabiting the human body, which likely outnumber bacteria and whose function is still not entirely known [116]. It has been suggested that up to 400 bacterial species exist in the gut of each individual host [112], and that the gut may contain 100 times more genes than the estimated number of total human genes [117]. Four types of bacteria, Bacteroides, Firmicutes, Proteobacteria, and

Actinobacteria, have been found to dominate the gastrointestinal microbiota in humans, including a number of different species [118,119].

Key definitions used in microbiome research are summarized in Figure 4.

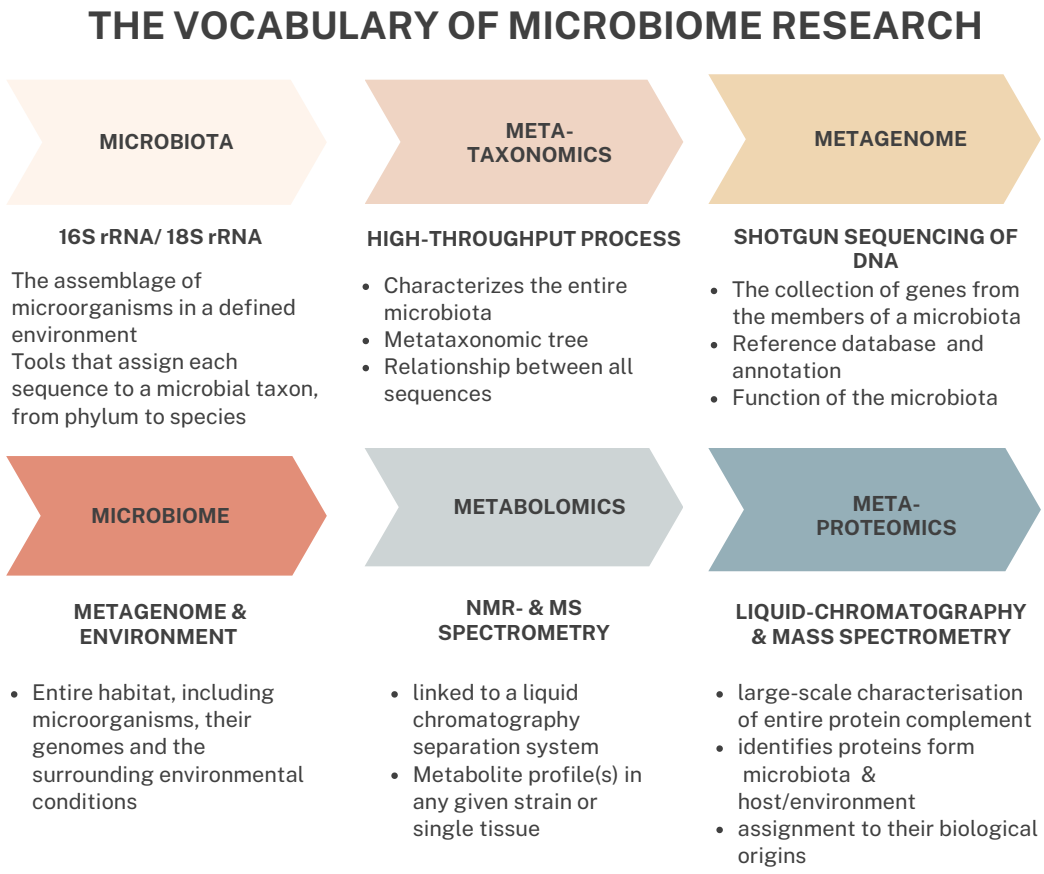


*Figure 4 The discovery of the human microbiome led to an explosion of scientific literature describing the composition and function of microbial communities, leading to an oftentimes inconsistent use of vocabulary. Marchesi and Ravel sought to address this issue by proposing clear definitions, which will be used in this thesis and are described in this figure. [111] The analyzes used are usually various 'omics' techniques that tell which microbes are found in the gut (metagenomics), which genes are active (meta transcriptomics), and what kind of activity the microbes have at any given time (metabolomics).*

To explore mechanisms by which microbes influence health and disease, approaches include combining microbiota identification and clinical data, coupled with gene expression and metabolome profiles of both the microbiota and its host, as well as animal research to understand how these factors interact. Ideally, these interactions are synergetic and mutualistic, meaning that the human host provides a living space and dietary substrate, and the microbes, in turn, produce essential nutrients, influence metabolism, and train the immune system [120–122]. Ways in which the microbiota may benefit the host are illustrated in Figure 5 Main functions and possible mechanisms of the human gut microbiota and Figure 6 lists common terms in microbiome research.

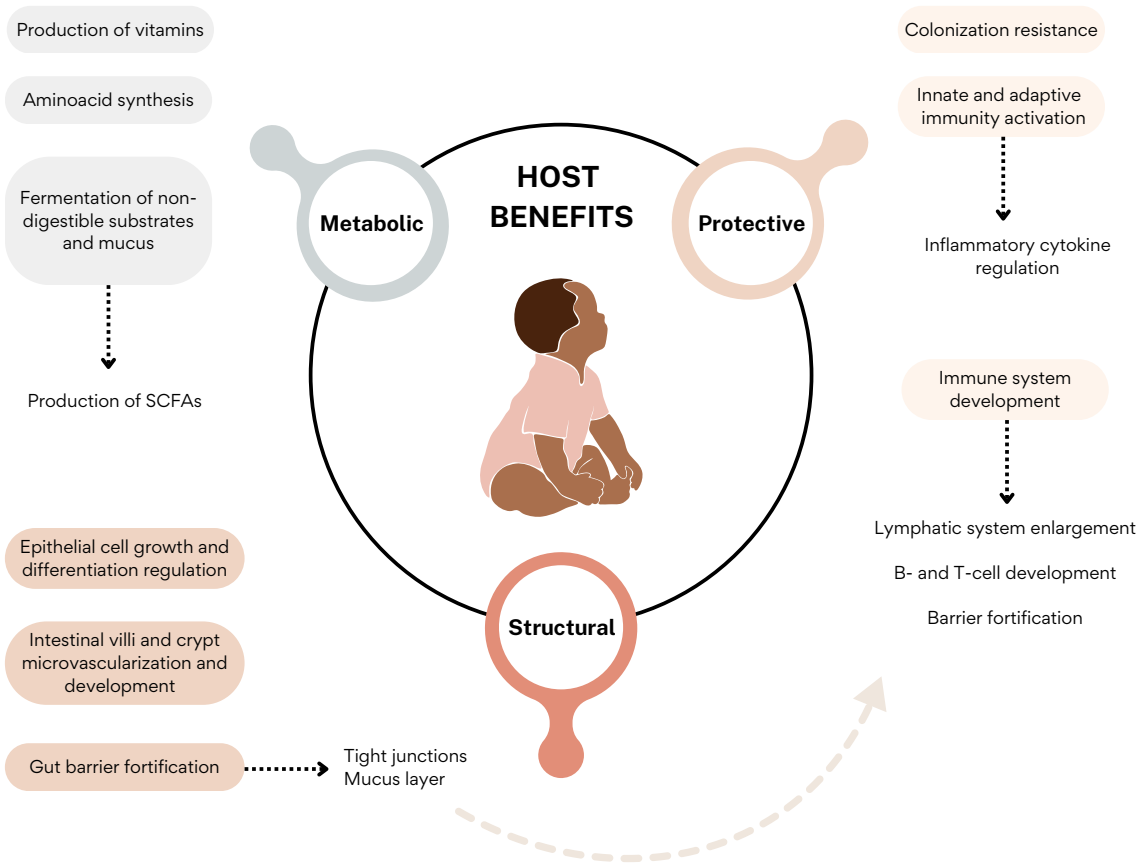


*Figure 5 Main functions and possible mechanisms of the human gut microbiota*

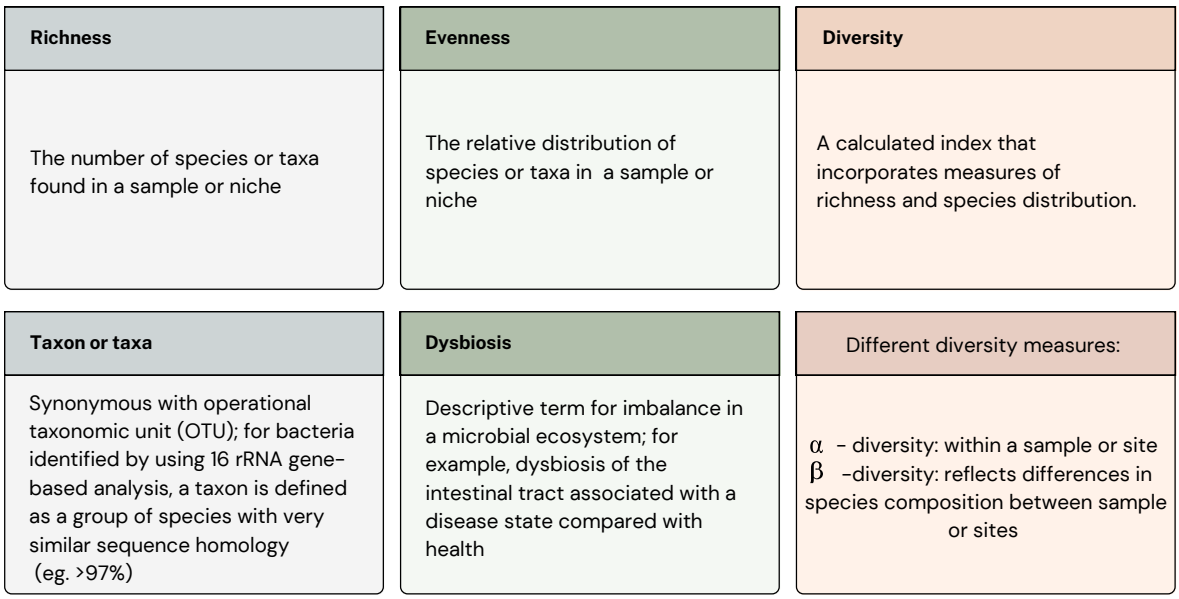


*Figure 6 Key definitions. Challenges to identifying a healthy gut microbiota persist due to its plasticity and interindividual differences in microbiota composition. Still, certain measures such as diversity and richness are used as an indicator of a healthy* [(123)].

### 3.5.1 Gut microbiome development and the influence of infant diet

Although the discovery of the human microbiome only happened in the past decades, the ability of food to shape gut ecology was described more than a century ago [(124)]. Through advances in sequencing technology and bioinformatics, a much broader understanding of the influence of environmental factors particularly diet, has been gained [(125)]. Dietary components are needed not only for human health but for the survival and function of the trillions of microbes that inhabit the human intestines [(126)]. It has been suggested that dietary changes may account for over half of the microbiome variation in a person, while genetic variations only account for 12 % [(127)].

Although environmental and lifestyle factors continue to influence a person´s microbiome until old age, the earliest years of life appear crucial for microbiome colonization [128]. The composition of an individual's gut microbiota over time appears to be relatively stable throughout life, in contrast to the changes observed in the first three years of life [121]. Within the first months, the infant's gut becomes colonized with an estimated 500- 1000 species, reaching $10^{11}$ – $10^{12}$ per gram of feces, comparable to densities in an adult gut [129,130]. Several early life factors may influence gut microbiome composition, such as mode of delivery, breastfeeding and infant diet, antibiotic usage in pregnancy and early infancy as well as gestational age [131,132], as summarized in Figure 7.

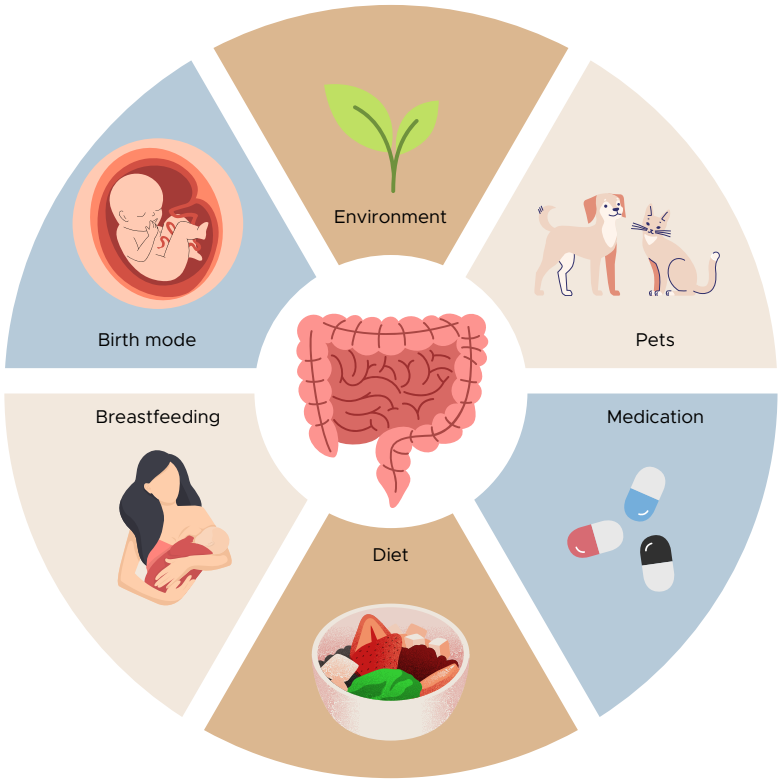


*Figure 7 Factors that may influence the composition of the gut microbiota in early life include, but ar not limited to: mode of birth, antibiotics and medication, mode of feeding, maternal diet, presence of furred pets, complementary diet, and local environment, including rural versus urban surroundings and geographical location.*

When life with microbes begins and whether the intrauterine environment is colonized in a healthy pregnancy or not have long been subjects of debate. Some studies have shown live microorganisms at intrauterine sites [133–135] , however contamination has been raised as a major concern in these studies and challenged the idea of microbial exposure in utero [136,137]. Results from our group support the hypothesis that fetal development in utero occurs with the absence of live bacteria in the amniotic fluid [138], and overall, the general consensus currently is supportive of the sterile-womb hypothesis [137].

During the time of birth, an infant is colonized with a vast number of maternal and environmental microbes [132]. Infants born with caesarean section (CS) have been shown to have differences in gut microbiota and immune response compared to vaginally delivered infants [139]. Infants delivered by the vaginal route are usually exposed to vaginal- and fecal bacteria as they move through the birth canal, typically resulting in colonization with *Bifidobacterium, Bacteroides, Lactobacillus, Prevotella, and Escherichia coli* [132]. In contrast, infants delivered by cesarean section may be exposed to bacteria from the adult skin flora and the hospital environment, with subsequent colonization with *Staphylococcus* and *Enterococcus* as first colonizers [140,141]. A recent meta-analysis compiling 1700 fecal metagenomes from nine recent studies showed distinct microbiota maturation trajectories between infants born vaginally or by CS, but with converging patterns after time [142]. Infants born via CS have been reported to lack certain species after birth; however, Bifidobacterium and Lactobacillus have been observed to colonize the infant´s gut within the first months, probably as a result of breastfeeding [143,144]. In a recent large study from the U.K. published in 2019, including 1679 samples of gut bacteria from nearly 600 healthy babies and 175

mothers, differences in microbiota composition between infants delivered vaginally or via CS varied greatly initially; however, the microbiota grew more similar, particularly after weaning [141].

Within the first weeks and months of life, exposure to new microbes, as well as increased colonization with different microbes, has been shown to increase microbiota diversity within the individual (alpha diversity). At the start of life, differences in gut microbiota composition between individuals (beta-diversity) are high. However, over time these differences seem to diminish, making the gut microbiota composition taxonomically more similar across infants [99], as illustrated in Figure 8.

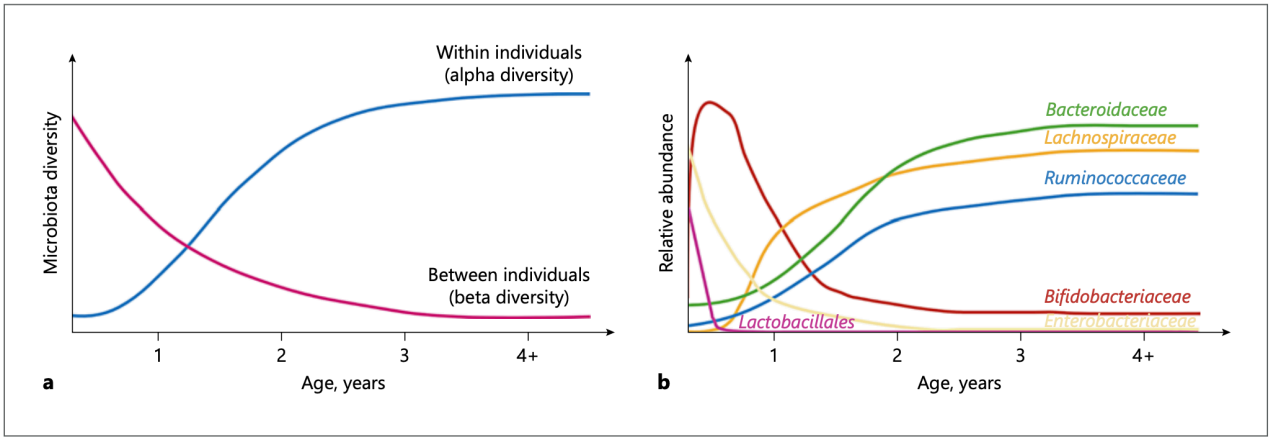


*Figure 8 Development of the gut microbiota in early life. a) Development of gut microbiota diversity with age. b) Development of the average gut microbiota composition with age, showing the relative abundance of the major microbiota families/ orders.* [99]

Diet is likely a key environmental factor that shapes the gut microbiota, also in the first year of life [145,146]. Especially the mode of milk-feeding after birth has been suggested to play a key role in determining microbiota trajectories in early infancy [99]. Recent studies have shown that breastmilk contains its own microbiota, in contrast to previous beliefs that

breastmilk was sterile [147,148]. Moreover, breastmilk has been shown not only to provide the infant with nutrients but also to supply gut microbes with dietary substrate- known as human milk oligosaccharides (HMO) [149]. These breastmilk carbohydrates, the third largest component of breastmilk, pass undigested to the colon, where they serve as nutrient sources for the gut microbiota [149]. Since specific species within the *Bifidobacterium* genus are specialized in degrading HMOs, they tend to dominate the gut in breastfed infants, and breastmilk has been shown to be a strong selective factor in shaping the gut microbiome in early life [147,150]. A gut microbiota lacking *Bifidobacterium*, and particularly the required enzymes for HMO degradation, has recently been linked to immune dysregulation and systemic and intestinal inflammation in a Swedish study of 208 infants using longitudinal systems immunology analyses and metagenomic profiling [151]. The gut microbiota of exclusively formula-fed infants has been characterized by a low presence of HMO-utilizing *Bifidobacterium* species and an abundance of *Clostridium* and *Enterobacteriaceae* species (e.g., *E.coli*) [152,153].

Since many microbes are passed from the mother to the infant, lifestyle-associated changes in microbiota over time can impact taxonomic composition in infants, as was illustrated in a recent study comparing fecal samples over a year from Hazda, hunter-gatherer communities in Tanzania, to those of 17 other populations around the world. The results revealed that *Bifidobacterium infantis,* an efficient metabolizer of HMOs, dominated the infant gut microbiome in non-industrialized communities, whereas *Bifidobacterium breve*, a species with reduced capacity to break down HMOs, was prevalent in infants living in industrialized

countries [154]. The consequence of a lack of *B.infantis* is largely unknown but has been implemented in immune dysregulation in early life [151].

The complementary feeding period marks a phase of significant change in gut microbiota. After weaning, the gut microbiota signature has been shown to become permanently established in healthy individuals [155]. It has also been shown that the major shift towards an adult-like gut microbial composition seems to happen when breastfeeding is discontinued, contrary to the idea that solid food introduction is the primary driver [156,157]. The effect of breastmilk on microbiota composition is still only partly understood, while even less is known about the effects of complementary foods on gut microbiota composition [158]. Significant changes in gut microbiota composition happen as the infant transitions to a solid diet [159,160]. Around the time of complementary food introduction, the gut microbiota shifts from a composition dominated by HMO-degrading *Proteobacteria* and *Bifidobacterium* to a composition characterized by increased *Bacteroides* and *Firmicutes* [146,161].
An observational study investigating the effect of complementary food on gut microbiota development in 227 Danish infants found that microbial composition and alpha diversity at 9 and 18 months was mainly affected by the introduction of food items with high fiber and protein content [162]. Dietary fiber, derived from plant foods such as grains, fruits, and vegetables, are indigestible by human enzymes and used only by gut microbes as energy sources [163,164]. Apart from dietary fiber and protein as crucial drivers of gut microbiome establishment, diversity of food introduced in the first year of life may influences the modulation of the microbiota composition as recently demonstrated in a study examining the day-to-day changes in the gut microbiota of 24 infants during the two-week period of

introduction to solid foods [165]. A progressive increase in both microbial richness (observed species) and diversity (Shannon index) was observed which were positively correlated to the variety of foods introduced in the infant's diet [165]. An earlier time of complementary food introduction has been linked to higher alpha diversity in infancy and a higher abundance of specific butyrate-producers [166].

While dietary changes are known to affect the adult gut microbiota, there is a knowledge gap of how the introduction and time of introduction of new dietary components into the diet of infants/young children and diet diversity affects the gut microbiota development.

### 3.5.2 Short Chain fatty acids and immune development

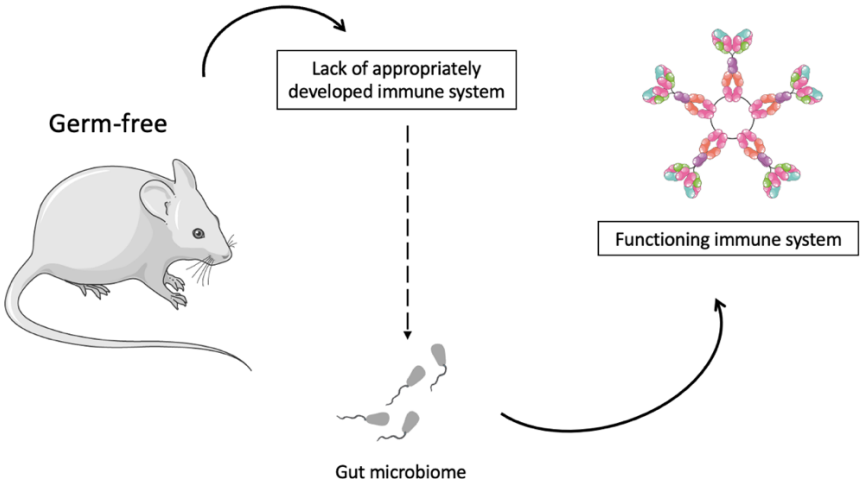


*Figure 9 Germ-free mouse models remain an important model system for studying host-microbe interactions. These animals show several important developmental and physiological differences, such as an altered immune system and a propensity to develop infections. Restoring a lacking gut microbiome will also repair immunological functions [168].*

The first experiments using germ-free rodents date back to the 1950s when it was discovered that life without a host microbiota is possible but comes with a number of physiological and immunological alterations (Figure 9) [167]. The immune system exists in an intertwined relationship with the microbes residing in the human body, and the influence of bacterial metabolites on immune development, specifically SCFAs has received particular attention [169]. Short-chain fatty acids are produced by gut microbes through fermentation of dietary fiber in various ratios, the most abundant ones being acetate, propionate, and butyrate [163,170].

Short-chain fatty acids have a number functions, such as regulating gut homeostasis and exerting important systemic functions, such as controlling immune- and inflammation responses [84,171]. Therefore, SCFAs are viewed as a crucial link between diet, gut microbiota, the immune system and host metabolism [172], and these effects can already take place during pregnancy [173]. Studies in the mouse model have demonstrated that increased levels of dietary fiber content during pregnancy, through modulation of microbial diversity and increased production of SCFAs, reduced the likelihood of allergic inflammation of the lungs in the offspring [174]. This process may be facilitated directly by SCFAs that cross the placenta and influence gene expression in the fetal lung and immune development [173].

The abundance of butyrate-producing bacteria strains depends on the availability of dietary substrates, particularly dietary fiber [175]. Dietary fiber not only acts as the main energy source for gut microbiota but can influence which bacteria are able to thrive in the intestine, and thereby control which metabolites are produced by the microbial community and at

what quantity [159]. Butyrate has been most widely studied for its potential anti-inflammatory effects and may be a key player in chronic disease prevention [172], through various mechanisms as illustrated in Figure 10. In this thesis, the focus will therefore be on butyrate, its temporal development in infancy, and its possible effect on atopic disease development.

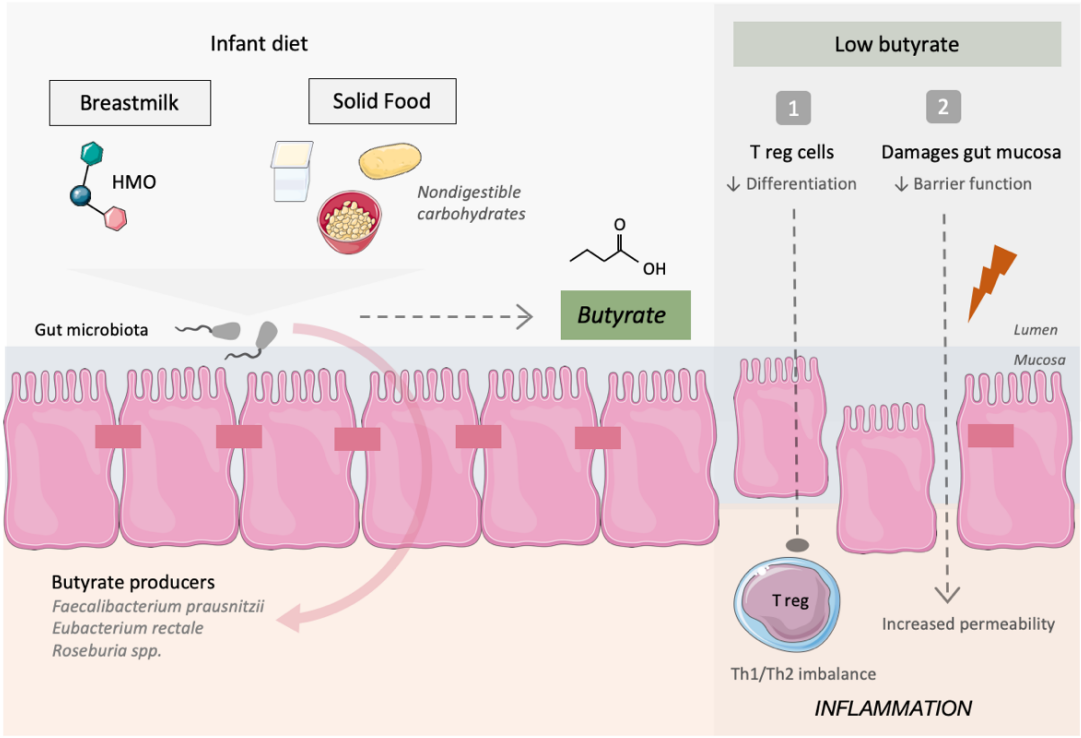


*Figure 10 Infant diet influences species composition of the gut microbiota. Low levels of butyrate can lead to decreased production of anti-inflammatory regulatory T cells. Low butyrate also exerts local responses to the gut mucosa and can increase the permeability of the gut mucosa and facilitate the translocation of pathogenic microorganisms and immunogenic components to the internal environment of the host, which might potentiate immune cell stimulation.*

Unlike acetate, which is produced by most gut bacteria, butyrate seems to be produced by a specific subset of bacteria. Particularly *Firmicutes* have been recognized for their ability to produce butyrate [176]. The composition of SCFAs have been shown to shift in infancy. In early infancy, when breastmilk serves as the only form of nutrition, *Bifidobacterium* and

*Bacteroides* dominate the gut, which leads to the production of acetate and propionate [150,177]. In contrast, in formula-fed infants, or infants fed complementary foods early, higher amounts of butyrate-producing bacteria have been observed earlier [166,178,179]. With changes in the infant diet and, conversely, the gut microbiome composition, SCFA production goes through important changes. However, how the introduction of complementary foods influences the infant gut microbiome composition and metabolic functions is still largely unknown.

## 3.6 The infant gut microbiome and atopic dermatitis development

Atopic diseases, including asthma, atopic dermatitis, food allergy, and allergic rhinitis often manifest in childhood, with atopic dermatitis commonly preceding food allergy during the first year of life, followed by asthma and/or allergic rhinitis from pre-school and school age. In this thesis, the focus will be on atopic dermatitis, since AD usually presents as the first atopic manifestation, with around 13 % presenting with eczema at 3 months and 17 % being diagnosed with possible atopic dermatitis by 12 months of age in our own study [180,181]. Atopic dermatitis has increased significantly in developed countries in the last few decades, mainly among children under one year of age, where the prevalence is around 20% [182–184]. Since most children will experience the onset of atopic disease in the first two years of life [185], it is important to find means by which one might prevent the onset of disease in infancy.

Particularly, the first year of life appears to be a key time period in which disturbances of microbial colonization may contribute to the development of atopic disease [186,187]. Studies

have found that infants with atopic dermatitis harbour different microbes in their gut compared to healthy infants and that these differences precede the development of atopy [152,188].

The balance between pro-inflammatory and anti-inflammatory factors is partly determined by microbial diversity, species composition [159], and SCFAs [189–191]. Particularly butyrate seems to play a crucial role in reducing inflammation, preventing the development of atopic dermatitis and alleviating eczema symptoms in infancy, as shown in studies by Roduit et al [94,192]. The gut of infants without eczema or mild eczema symptoms has been shown to contain higher amounts of butyrate-producing bacteria already at 6 months of age in a study of 39 infants [193], whereas faecal samples of patients with eczema have been shown to contain lower amounts [194]. In vitro studies show that butyrate can induce human tolerogenic DCs and regulatory T cell differentiation [195], as well as promote gut barrier function and help maintaining epithelial integrity [196].

Although butyrate seems to be implicated in the development of atopic disease, few studies have investigated the associations, and little is known about the underlying mechanisms.

# 4 Objective and specific aims of the thesis

Based on the hypothesis that atopic disease development in early childhood may be partly conveyed through gut modulation in early infancy, the overall objective of this thesis was to assess the impact of maternal and infant diet on the gut microbiome in early life and explore a possible impact of their metabolites on atopic dermatitis in infancy. The specific aims, as depicted in Figure 1, were:

1) To assess the maternal diet in mid-pregnancy and the diet of her offspring in the first year of life (Paper 1 & 3)
2) To explore if early complementary food introduction affected infant diet, including breastfeeding rates or food diversity in the first year of life (Paper 3)
3) To explore the temporal development of gut microbiome and SCFA production, and the potential impact of breastfeeding and time of solid food introduction in the first year of life (Paper 2 & 3)
4) To explore if butyrate at 12 months of age was associated with atopic dermatitis development in the first year of life (Paper 2 & poster)

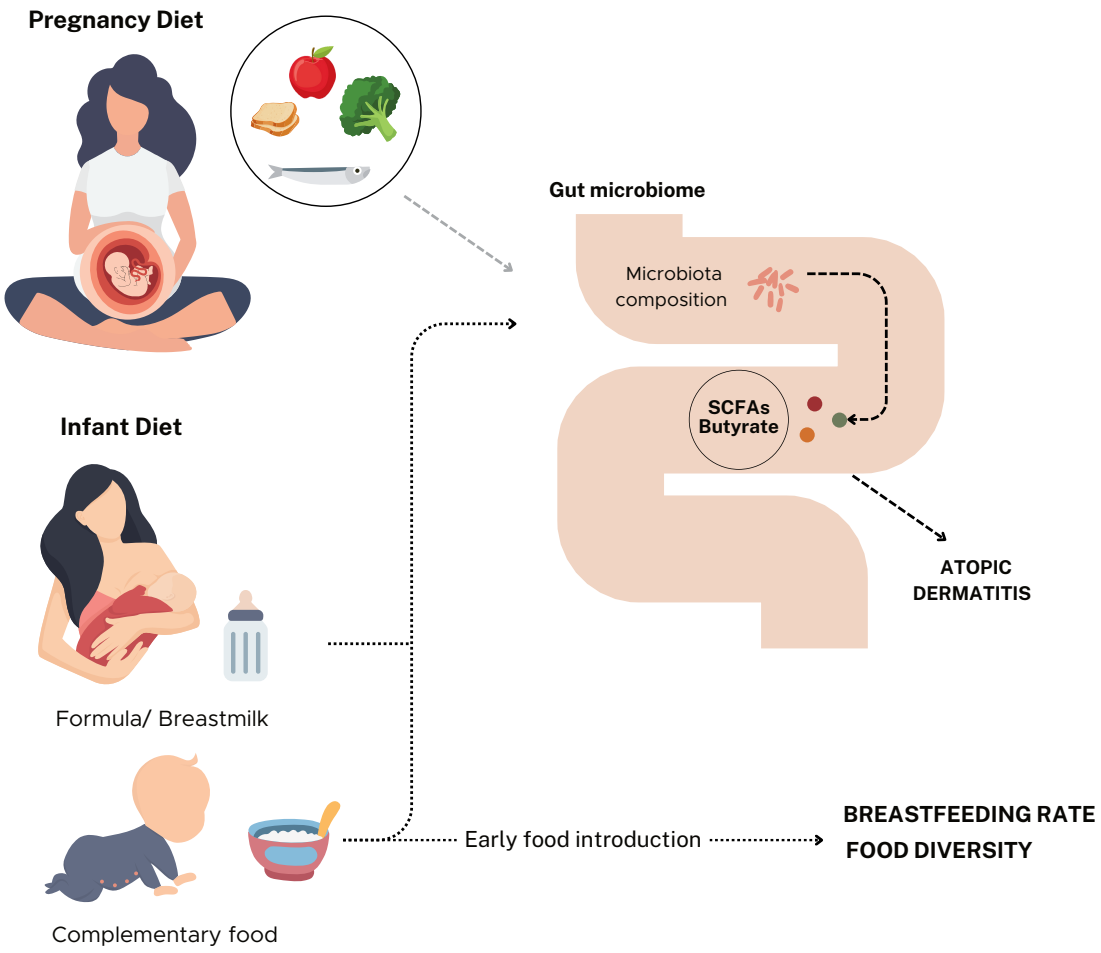


*Figure 11 Graphical abstract of the thesis aims and outcomes*

# 5 Materials and methods

## 5.1 Study design

This thesis uses data from the Preventing Atopic Dermatitis and Allergies (PreventADALL) study, a multicenter, prospective, interventional, population-based birth cohort study, that seeks to identify potential opportunities for intervention with the aim of reducing the burden of allergic disease. The study also explores the potential effects of the human microbiome and lifestyle factors, such as diet, on disease development from fetal life onwards. Study details, as well as characteristics of the Prevent-ADALL cohort, have been described in detail elsewhere [197]. The PreventADALL study plans to follow up included infants into adult age, and currently, the project period is defined from 2015-2044.
The two main arms of the study are: 1) exploratory with NCD outcomes, 2) randomized clinical trial seeking to explore primary prevention of AD, food allergy and other allergic disease outcomes.

## 5.2 Subjects

The study subjects in this thesis are all from the PreventADALL birth cohort and presented in Figure 12. A total of 2697 women with 2701 pregnancies were enrolled around 18-week pregnancy, 2149 in Norway and 552 in Sweden between December 9th 2014, and October 31st 2016. Four women were included twice with singleton pregnancies, and 17 were twin pregnancies. The majority were married (41.2%) or cohabiting (55.9%), 1.9% were single mothers, whereas 1% reported other living arrangements. Mean maternal and paternal age (min-max) was 32 (18-42) and 34 (21-72) years, respectively. Most parents were born in

Norway or Sweden (89% of mothers, 84 % of fathers). More than half of the women (57 %) and 48 % of fathers had more than four years of college or university education. A total of 11% of mothers and 21% of fathers had 9-10 years schooling as their highest educational level. At least one doctor-diagnosed allergic disease was reported by 42% of mothers, and at least two allergic diseases by 20.1%. A total of 19.8 % of mothers reported AD in contrast to only 10.2 % of the fathers.

The mean (SD) estimated fetal GA at enrolment was 18.7 (min-max 15.7 - 22.7) weeks. The mother-child cohort included 2383 mothers, of whom three were enrolled by the same mother but with two different pregnancies and 2397 children (52.7% boys) including 11 twins. One mother-child pair withdrew after randomisation. The mean (min-max) GA of newborns was 39.2 (35.6 - 42.9) weeks, and 16.4% were delivered by CS.

Figure 12 shows the PreventAdall study participants, as well as selected study groups for all 3 papers in this thesis.

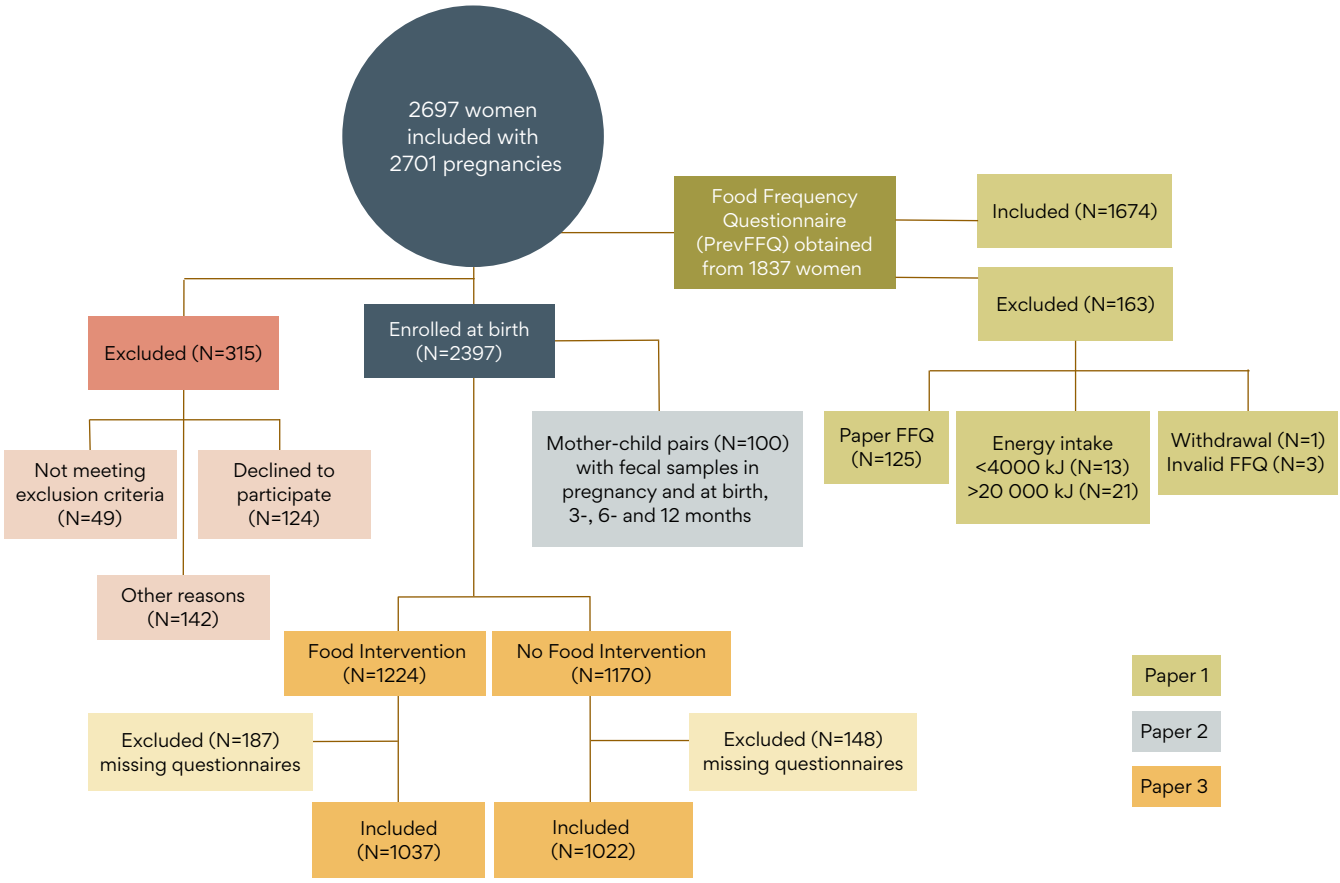


*Figure 12 The flow chart shows the number of women and children included in the PreventADALL study, as well as the study subjects in this thesis. The flowchart is color-coded to show the selected study populations of papers 1-3.*

The sub-population used for Aim 4 consisted of 100 mother-child pairs from the PreventADALL cohort. Infants were selected based on the availability of fecees samples at birth (meconium), 3 months, 6 months, and 12 months, as well as available data on skin examinations and doctor-diagnosed atopic dermatitis at the same time points.

# 6 Methods

### 6.1.1 Recruitment and enrolment

Women were recruited during the routine 18-week gestational age (GA) ultrasound examination at Oslo University Hospital, Østfold Hospital Trust (Norway), and Karolinska University Hospital (Stockholm, Sweden) between December 2014 and October 2016. A total of 2697 women with 2701 pregnancies and 2396 mother-child pairs were included in the study. Inclusion criteria were sufficient language skills (Scandinavian language) and gestational age (GA) of 16-22 weeks. Exclusion criteria were plans to move outside reasonable travel distance to study locations within the first year after birth, pregnancies with three or more fetuses, or severe fetal malformations or illnesses discovered at the 18-week ultrasound examination or before. Informed consent was given upon enrolment and inclusion of the infant at birth. Infants were preferably included in the study within 24 hours after birth, or as soon as possible thereafter. Newborn inclusion criteria were GA of at least 35 weeks. Exclusion criteria were severe neonatal disease, as well as plans to move outside reasonable travel distance to study locations within the first year after birth.

Samples were recorded in a fully traceable electronic biobank (MedInsight system) established in December 2014 at the Oslo University hospital. The study was approved by the Regional board for Medical Ethics – Health region South-East B in December 2014 (2014/518) and Stockholm, Sweden in March 2015 (2014/2242-31/4), as well as registered at clinicaltrial.gov *NCT02449850.*

### 6.1.2 Interventions

At birth infants were randomized into one of four similar-sized groups: (1) skin intervention only, (2) food intervention with early food introduction only, (3) both interventions, and (4) control with no intervention. Randomization was done in computer-generated clusters based on geographical residential areas and was changed in 3-month time frames. These were given by a statistician who had no participation in the subsequent trial's execution and analysis [71].

The skin intervention consisted of paraffin liquid oil baths (50 ml bath oil per 8L/water) and facial cream (Ceridal ®) at least 4 days/week, from 2 weeks of age through 8 months of age. The food intervention consisted of introducing four different foods between 3 and 4 months of age with the baby sucking on a parent's finger dipped in the food in question. Peanut butter was given for the first time at the 3-month follow-up visit. Cow´s milk was introduced ed one week later, followed by wheat in the third week, and loosely scrambled eggs in the fourth week. Parents were instructed to give each of these foods, starting with tastes, from their finger or a teaspoon, at least four days per week and until at least 6 months without any dose restriction, aiming at becoming part of the regular infant diet. For all infants the national recommendations of infant diet including breastfeeding, and skin care was advocated, except for the interventions.

Adverse events were recorded and investigated if necessary. In case of allergic reactions, study participants were offered direct access to the local pediatric department for relevant investigations and treatment.

### 6.1.3 Electronic questionnaires

Maternal health, sociodemographic- and lifestyle factors (physical activity, environment, stress, quality of life) were obtained through electronic questionnaires in pregnancy, developed in collaboration with the University Center for Information Technology (USIT) at the University of Oslo. The integrated electronic form (nettskjema.uio.no) tool administrating online data collection was used both for registering data obtained at the study visits by study personnel and the diaries/questionnaires completed by the mothers. Fully Pretty Good Privacy (PGP) encrypted answers to the project's secure infrastructure were ensured, including a set of virtual machines (VMs) for managing / post-processing data and dedicated secure storage within the central TSD system [197].

Detailed electronic questionnaires, including infant dietary data, were obtained at 3-, 6-, 9- and 12 months of infant age. Additionally, weekly electronic diaries (2-26 weeks of age) collected information on skin care, infant feeding, and symptoms of allergic diseases. An overview of the PreventADALL study relevant for this thesis is illustrated in Figure 13.

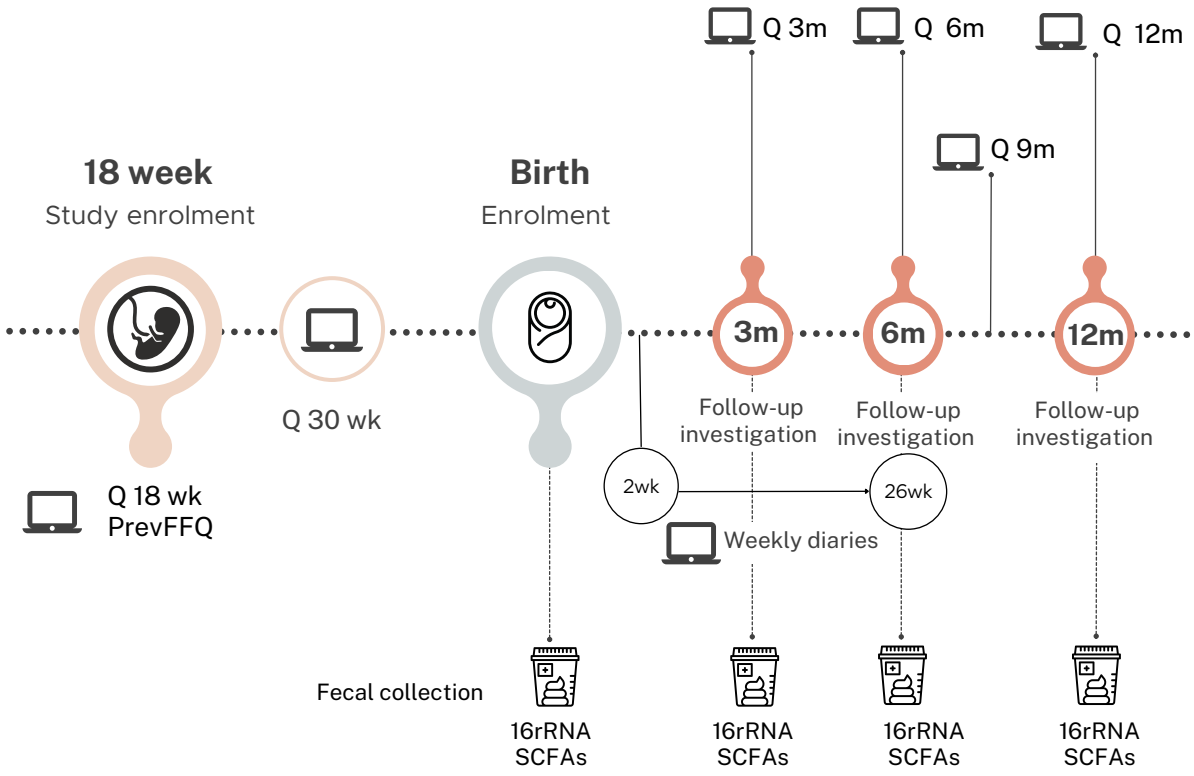


*Figure 13 The PreventADALL study overview is shown for the period included in this thesis, starting at enrolment in pregnancy and following the child through the first year of life. Q= electronic questionnaire, wk=weeks, m=months, PrevFFQ=PreventADALL Food Frequency Questionnaire, Weekly diaries are electronic questionnaires sent out weekly to caregivers from week 2-26*

### 6.1.4 Dietary Assessment in Pregnancy

The dietary assessment method was developed specifically for a Norwegian population with its food habits and meal patterns. Therefore, diets in pregnancy were assessed in the Norwegian part of the PreventADALL cohort only. A web-based semi-quantitative food frequency questionnaire (PrevFFQ), capturing habitual dietary intake during the first 4-5 months of pregnancy was sent via a link per e-mail shortly after study inclusion. The FFQ used in the PreventADALL study was previously validated in a population of women, using doubly labelled water and multiple 24-h-recalls [198].

The PrevFFQ consisted of 279 questions on the frequency and amount of about 280 food items, grouped according to main food groups and meal patterns. Frequency categories were used in increasing order: not at all, times per month, week, or day. Amounts were given in portion sizes, standard units, spoons, cups, glasses, etc. For food items where portion size may be particularly difficult to estimate, the PrevFFQ included pictures of 4 different portion sizes. Intake of vitamin or mineral supplements was also assessed. In case a participant neglected to answer a question, an automated follow up comment would prompt an answer, assuring a complete set of values in all questionnaires. The estimated time used for filling in the FFQ form was about 30 – 45 minutes, however, with large individual variations. Some participants used less than half an hour, and others used more than an hour. Figure 14 shows an example of illustrations provided in the PrevFFQ.

The data in the FFQ was transferred to the food and nutrient calculation system, Kostberegningssystemet (KBS), version 7.3, at the University of Oslo, Department of Nutrition, where all estimations of food and nutrient intakes were performed in KBS food composition database AE18 [199].

The first 125 enrolled women who received a paper version of the FFQ were eliminated to assure no discrepancies in methods between paper- vs electronic FFQs. Women who reported unlikely energy intakes (< 4000 kJ/day and > 20 000 kJ/day) were also excluded, as well as 3 invalid FFQ and one study withdrawal, giving a total of 1674 eligible study participants [200]. Because 154 out of the 1674 women did not answer the 18-week general questionnaire, some background information was only available for 1520 women.

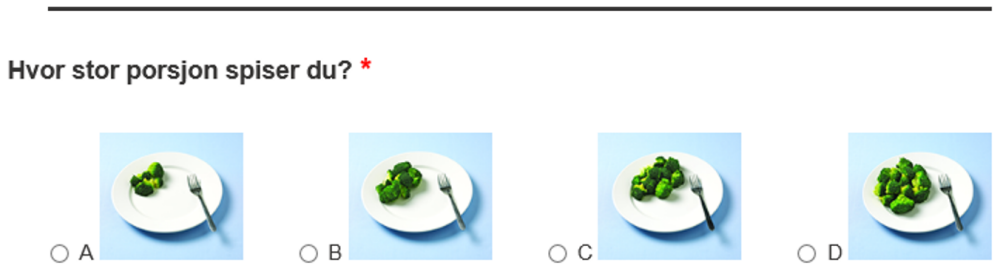


*Figure 14 Illustrations are included to provide help for the participants when filling out the electronic PrevFFQ. We included portion-size pictures for food items that do not have a natural unit or where portion sizes may vary considerably. Translation of text in the image: How large is your portion-size?*

### 6.1.5 Diet Assessment in Infancy

Infant dietary data were obtained from electronic weekly diaries in weeks 2-26 (weekly diaries) and electronic questionnaires at 3, 6, 9, and 12 months. Breastfeeding was assessed in the weekly diaries, as well as at 3, 6, 9, and 12 months of age by parents reporting for the last 3 months if the infant had received breastmilk and the time of breastfeeding cessation. Breastfeeding data from the weekly diaries were combined with the 3 monthly questionnaires, assuring a more complete dataset in the first 6 months of life. The time of complementary food introduction was assessed based on the weekly diaries. Specific questions about breastfeeding are illustrated in the supplement section, detailing the questions asked at 3,6, 9 and 12 months, as well as the complementary foods introduced, including porridge types, dairy products and bread/cookies/waffles/cakes and other baked goods, fruit or berries, root vegetables (such as potato, turnip, carrot, parsnip), other vegetables, peanut (as a spread or incorporated in other foods), pure egg (e.g., fried, cooked, scrambled, eggnog), egg in other foods (e.g., gratin, waffles, baked goods, paste, or similar), fatty fish (salmon, trout, mackerel, pike, halibut, eel), other fish, shellfish, poultry meat, other meat. We also asked about how much of the infant food was home cooked

versus commercially prepared (industrially processed), as well as the content of organic food in the infant's diet.

All questions about diet were mandatory, assuring a complete set of values in all questionnaires. An overview of dietary data assessment at each time point is depicted in Figure 15.

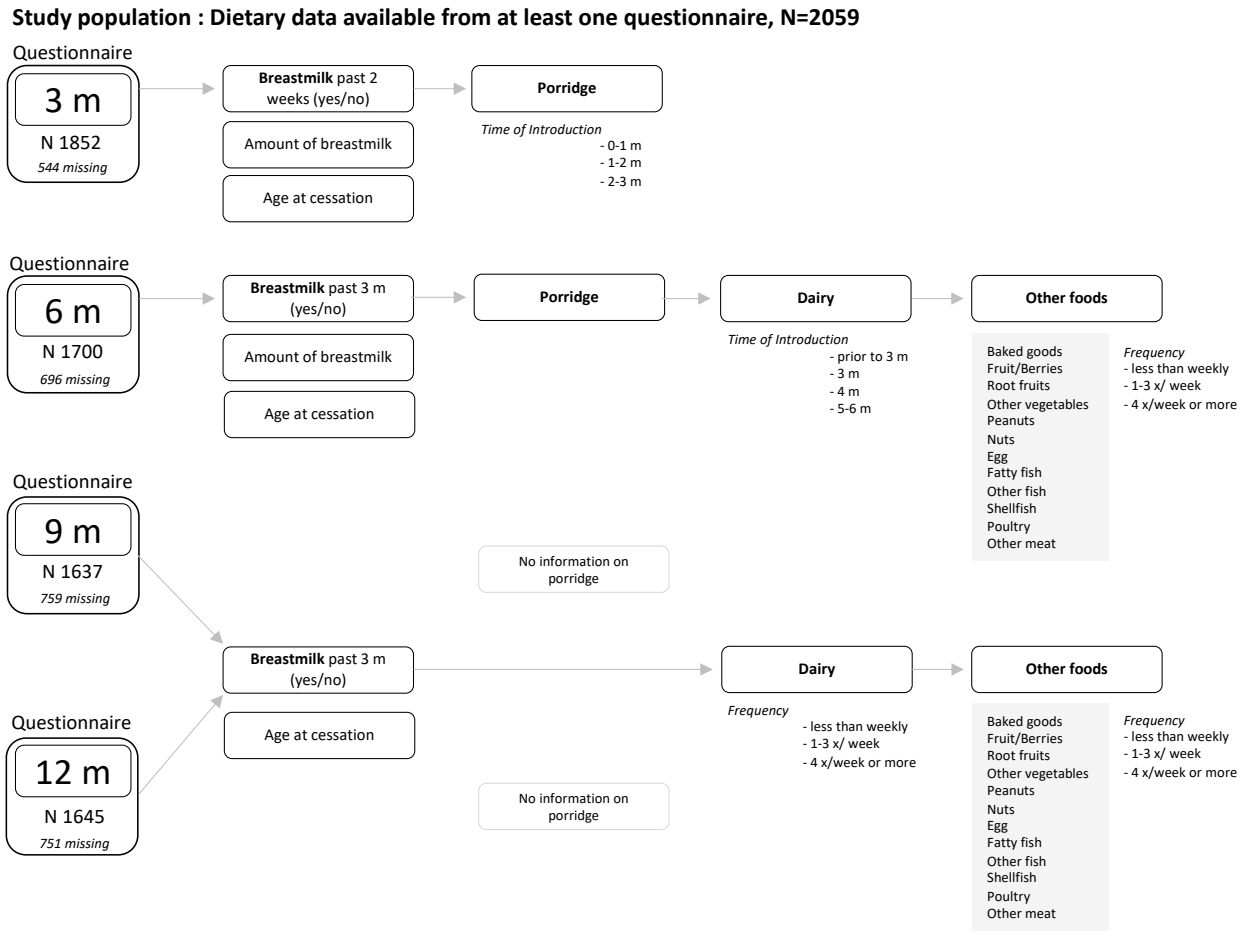


*Figure 15 Flowchart showing the number of questionnaires available, and missing, at each time point (3 months, 6 months, 9 months, and 12 months) and information collected at each point.*

### 6.1.6 Skin assessment at 3-, 6- and 12 months investigations

Trained healthcare personnel, blinded to randomization groups, performed all clinical investigations, including skin examinations. Parents were instructed not to bathe the infants or use any emollients within 24 hours prior to the examination. The infant´s skin was evaluated by visual inspection and palpation. For the diagnosis of AD, we used the UK Working Party diagnostic criteria at 3, 6 and 12 months investigation [201,202]. Severity is not included as an outcome in this thesis. From 12 months and onwards the Hanifin and Rajka diagnostic criteria were used additionally. As AD may be difficult to diagnose in early infancy according to diagnostic criteria, especially due to the lack of infant's ability to scratch themselves, eczema was used as a proxy for AD. Eczema was defined as the presence of eczematous lesions, verified by a medical doctor, excluding differential diagnoses to AD. Positive parental history of atopy was defined as doctors-diagnosed AD, allergic rhinitis, food allergies, and/or asthma.

### 6.1.7 Stool sampling

Fecal samples were collected from infants at birth, 3 months, 6 months, and 12 months of age. Parents were given an instruction leaflet for home sampling of feces using a paper collection device. Parents were instructed to place one spoon full of feces into each tube and then shake the tube for feces to be solubilized. The feces tubes were placed into transport containers and return envelopes. Parents were instructed to send the samples within the same day, however, if sampling was done in the evening, the samples were refrigerated overnight and mailed the next day. Upon receipt of the specimen, the feces tubes were

immediately placed in a -20 C freezer and samples were then transferred to a -80 C for long-term storage within one week.

Fecal samples used for 16S amplicons, reduced metagenome sequencing (RMS), and short-chain fatty acid analysis were diluted 1:10 in stool DNA stabilizer (PSP Spin Stool DNA Plus Kit, Invitek Molecular, Berlin, Germany). Fecal samples analyzed with shotgun sequencing were stored without buffer.

### 6.1.8 rRNA sequencing

Both mechanical and chemical lysis was used to disrupt bacterial cells from the fecal samples. Bacterial composition was established using 16S rRNA gene amplification. Amplification of the V3 to V4 region of 16 rRNA by PRK341F and PRK806R primers was used to derive taxonomic composition. [203] The amplicons were indexed with 16 forward and 30 reverse PRK-modified primers with Illumina indexes. Sequencing of the 16 S amplicons and shotgun was performed with the Illumina MiSeq Platform with the Illumina MiSeq Regent Kit V3 (Illumina Inc, San Diego, CA, USA). We used the following method to process data with 16SrRNA amplicons.

Metagenomic sequencing (RMS) was used on a subset of samples to determine the functions of the bacterial components by unveiling their genomic information. These functions included the bacterial networks' ability to produce SCFAs and catabolize complex sugars. For the shotgun processing, the Nextera XT DNA library preparation kit was used. The metagenome was fragmented randomly into DNA fragments, covering the whole

metagenome, and then assembled into genomes. The genomic information was further used as a database for metaproteomic data, to explore protein expression related to gut function.

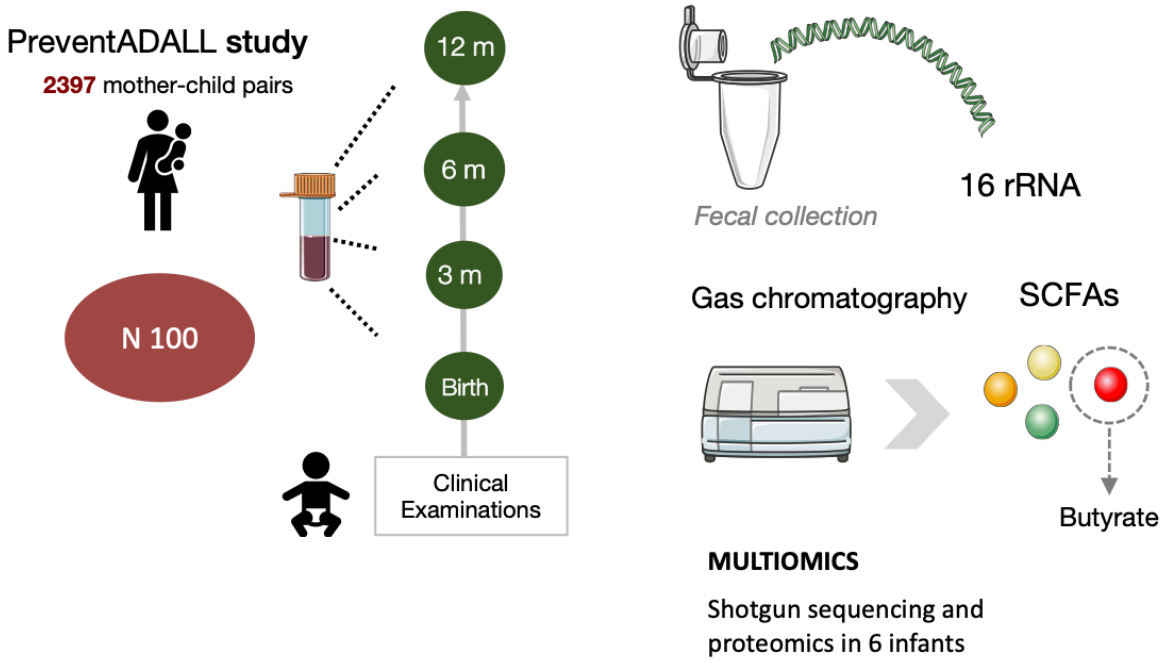


*Figure 16 Flowchart of study participants and samples for Aim 3 and Aim 4*

### 6.1.9 Short-chain fatty acid analysis

Aliquots were diluted 1:1 with MilliQ-water, and then 1:1 with an internal standard, containing 2% formic acid with 500µM 2-methylvaleric acid. This was done to lower the sample pH and increase the protonation of the SCFAs, and to reduce their interactions with other substances within the sample. The 2-methylvaleric acid was used as an internal standard to validate the quantification. Samples were centrifuged and the supernatant was filtered for 5 minutes, before being transferred to a gas chromatograph (GC) with ramping temperatures. The chromatogram peaks were used to detect the SCFAs and quantified

based on a standard curve of SCFAs with known concentrations. The chromatograms were processed with the Chromeleon 7 software. Figure 17 illustrates the study subjects and analysis performed for Aim 3.

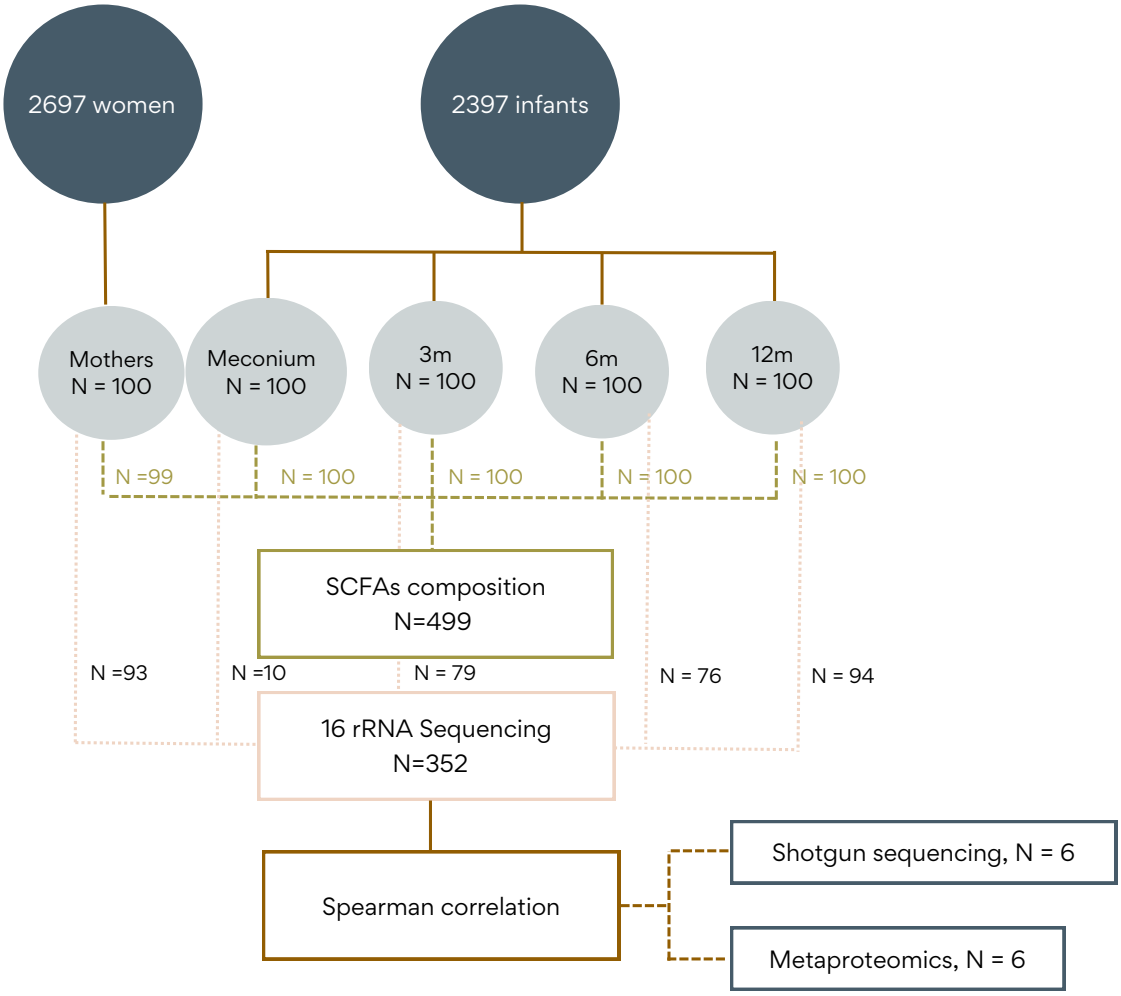


*Figure 17 Flowchart representing the study subjects, samples, and workflow of Aim 3. Fecal samples were analyzed from the same study subjects at four different time points (newborn, 3 months, 6 months, and 12 months) and their mothers in pregnancy. A multi-omics approach was used, including 16 rRNA sequencing, short-chain fatty acids (SCFAs) analysis, shotgun sequencing, and metaproteomics. The green line with adjacent numbers shows fecal samples analysed with gas chromatography for SCFAs composition. The pink line with adjacent numbers represents the number of fecal samples from each age group that were analyzed by 16S rRNA after rarefaction and filtering for poor-quality sequences*

### 6.1.10 Protein extraction and quantification

To remove bigger particles and human cells, fecal samples were filtered using a 20 μM filter and thereafter homogenized to disrupt remaining human cells and centrifuged to collect bacterial cells. The captured bacterial cells were then lysed with a buffer, freeing the intracellular proteins. The collected proteins were then purified and collected with an SDS gel, then stained, and cut into smaller pieces. The proteins' tertiary structure was dismantled by removing disulphide bonds through alkalizing and trypsin was added to transform the proteins to smaller peptides. The samples were thereafter desalted using C18 ZipTips, and the resulting peptides were dried and analyzed on a nanoLC-MS/MS system (Dionex Ultimate 3000 UHPLC; Thermo Scientific, Bremen, Germany, connected to a Q-Exactive mass spectrometer).

## 6.2 Definitions and outcomes

### 6.2.1 Definitions relating to pregnancy and infant diet

***Recommended Intake*** **(RI)** is defined using the NNR 2023 definition: “The average daily dietary nutrient intake level that is sufficient to meet the nutrient requirements of nearly all (usually 97.5%) individuals in a particular life-stage group in the general population. RI can be used as a guide for daily intake by individuals. Usually used to plan diets for groups and individuals.”

**Average Requirement (AR)** is defined using the NNR 2023 definition “The average daily dietary nutrient intake level that is estimated to meet the requirements of half of the individuals in a particular life-stage group in the general population. Average Requirements is usually used to assess adequacy of nutrient intake of groups and people and may be used in planning for groups.”

***Diet diversity*** can be measured by counting individual foods, food groups, or foods within a group, and can be assessed over a period ranging from the previous 24 hours, or a 7-day period, or intake over a year. In this study, the diet diversity score was calculated based on the number of different food groups consumed the last three months before 6-, 9-, and 12 months of age and was derived from the following food groups: bread and other baked goods, milk, fermented dairy products, cheese, other dairy products, fruits and berries, root vegetables, other vegetables, peanuts, other nuts, egg (pure and egg in other foods), fatty fish, other fish, shellfish, poultry, and other meat. The maximum achievable score at 9- and 12 months was 16. At 6 months, the maximum score was 10 since intervention foods,

including all dairy products, peanuts, and eggs, were excluded from the dietary diversity score. Porridge was not included in the score, as information on porridge consumption at 9- and 12-months was not included in the electronic questionnaires. The dietary diversity score did not consider frequency or portion sizes. Figure 18 details all 16 food categories used in the diet diversity score.


*Figure 18 All 16 food categories included in the diet diversity score at 9 and 12 months. At 6 months interventional foods including peanuts, egg and all dairy products were excluded from the score*

***Complementary food introduction*** is defined as any food or drink other than breast milk, formula, and water.

***Breastfeeding duration*** is defined as the number of months an infant was breastfed. Breastfeeding rates included exclusively breastfed infants and partially breastfed infants (receiving breastmilk and other sources of nutrition).

### 6.2.2 Food intervention (FI) group and No food intervention (NFI) group

In this thesis for Aim 2, the FI group consists of study participants in the PreventADALL study, from both the food intervention only group and the combined intervention group. In contrast, the NFI group in this thesis consists of study participants from the control group and the skin intervention group.

### 6.2.3 Atopic dermatitis

In this thesis AD was defined as the presence of eczematous lesions observed by trained study personnel at the clinical investigations, excluding differential diagnosis to atopic dermatitis by a trained medical doctor.

## 6.3 Statistical methods

Categorical variables are presented as numbers and percentages. Continuous variables are presented as means, SD, and minimum (min) – maximum (max). We also report median values with interquartile range (IQR) and 5$^{th}$ and 95$^{th}$ percentiles of proportions for normally and non-normally distributed variables.

Dietary data in pregnancy was exported from the KBS database and imported to SPSS (Statistical Package for the Social Sciences). All the analyses were performed using IBM© SPSS© statistics version 25 (Chicago, IL, U.S.A.). Descriptive analyses of energy intake, macro- and micronutrient intake was conducted. Normal distribution of variables was investigated through visual inspection of histograms and p-plots and by using the Kolmogorov-Smirnov test. Descriptive statistics were used to investigate adherence to the recommendations on frequency of consumption of micro- and macronutrients. Differences between categorical variables were analysed by Chi-Square test and numerical data by One-Way ANOVA tests. One-way between groups ANOVA was performed to test whether educational level would influence intake of micro- and macronutrients and food groups. The assumption of normality and homogeneity of variances was tested for each variable.

To analyse breastfeeding rates in the first year of life, we used Cox- regression. Kaplan-Meier curves were estimated for the FI, and NFI groups and differences in cessation rates between intervention groups were compared using the log rank test. These analyses were performed using IBM© SPSS© statistics version 25 (Chicago, IL, U.S.A.).

We used R version 3.6.0 for dietary diversity analysis, including complete case (i.e., answered the dietary questionnaire at a given time point) and modified intention to treat analysis (i.e., all randomised). For dietary diversity assessment at each time point between the intervention groups we analysed dichotomous endpoints using mixed effects logistic regression with the interventions and their interaction as fixed effects and randomisation period and residential postal code as random effects.

For the intention to treat analysis of diet diversity, missing primary outcome data were imputed using multiple imputations by chained equations. The number of multiple imputations was 15, and the scalar giving the number of iterations was 20. Complete case analysis was conducted as a sensitivity analysis.

The relationship between gut bacteria and SCFAs was investigated using Spearman correlation, and all shown correlations have a false discovery rate (FDR) corrected p-value of less than 0.05. We used ASCA-ANOVA to determine if delivery mode, gender, age, and breastfeeding from 3 to 12 months were factors associated with the microbiota composition.

## 6.4 Ethical considerations

Given the interventional nature of the study's design, which involved frequent study visits, bio-sampling, and electronic questionnaires, it was imperative that both women and their partners possessed a comprehensive understanding of the study's scope and requirements. To ensure this, the enrolment process, which typically occurred around the 18th week of gestational age (GA), commenced with expectant mothers reading a comprehensive four-page consent form. Following this, study personnel engaged in detailed conversations with both parents, whenever possible, to explain the study's implications and answer any questions. The index mother then signed the informed consent form. Upon the birth of their babies, parents were once again approached by study personnel, who reiterated the study's details and provided an opportunity for reflection and discussion regarding participation. Subsequently, both the mother and father or co-mother would sign a new consent form on behalf of the child. It was only after this step that randomization allocation information was disclosed to the parents. Potential ethical challenges by not following the recommended time of introducing allergenic foods was discussed thoroughly with the relevant ethical committee for medical research and resulted in a required first tasting of peanut at the clinic at 3 months of age. Clear contingency plans for possible adverse effects of early food introduction were outline in advance of starting the interventions and approved by the ethical committee. Throughout the children's follow-up period, parents had open access to the study personnel. They were actively encouraged to reach out to the study team if they suspected any development of allergic diseases in their child, with the assurance that appropriate medical measures would be taken, and they were continuously reminded that participation in all study-related investigations and data collections was entirely voluntary.

# 7 Results and summary of the papers

## 7.1 Maternal diet in mid-pregnancy and the diet in the offspring in the first year of life (Paper I and III)

### 7.1.1 Maternal diet in mid-pregnancy

Mean (SD) age of the population was 32,5 (4.1) years. For pre-pregnancy body mass index (BMI), mean (SD) was 24,6 (3.5) kg/$m^2$. A total of 90.8 % of women had a Scandinavian background, 37.1 % had previous deliveries, 61.1% had a university-level education of 4 years or more, 81.0% reported a full-time job, 97.1 % were married or cohabitating and 52.7% had a yearly household income of > 1 000 000 NOK. Use of tobacco- or nicotine-containing products any time before or during pregnancy was reported by 29.9 %. Current prevalence for nicotine products in 18-week pregnancy was 0.5% for smoking and 0.5% for snus. Other baseline characteristics are presented in Table 1. The background characteristics in the selected cohort did not differ significantly from the 2701 pregnant women in the whole Prevent-ADALL population.

The median intake, as well as interquartile ranges (IQR) and $5^{th}$ and $9^{th}$ percentiles of different foods and food groups is presented as g/day in Table 2.

*Table 1 General background characteristics of the present cohort and the total PreventAdall study cohort*

| **Anthropometric characteristics in present cohort** *(N=1674)*<br>*Height measured at inclusion<br>**Pre-pregnancy | | | | **PreventADALL study cohort**<br>*(N 2701)* |
|---|---|---|---|---|
| | **Mean (SD)** | **Min** | **Max** | **Mean (SD)** |
| **Age mother** (years) | 32.5 (4.1) | 21.0 | 48.0 | 32.3 (4.2) |
| **Height mother** (cm)* | 168.2 (6.3) | 147.0 | 187.0 | 168.0 (6.2) |
| **Weight mother** (kg)** | 65.5 (11.1) | 42.0 | 124.0 | 65.5 (11.4) |
| **BMI **** | 24.6 (3.5) | 17.2 | 41.4 | 24.9 (3.8) |
| | **Categories** | **N** | **%** | *(N 2349) %* |
| **Mother Nordic origin** *(N 1520)* | | 1379 | 90.8 | 90.1 |
| **Marital status** | *Married* | 604 | 39.7 | 41.2 |
| *(N=1520)* | *Cohabitants* | 872 | 57.4 | 55.9 |
| | *Single* | 26 | 1.7 | 1.9 |
| | *Other* | 18 | 1.2 | 1.0 |
| **Maternal education level** | *Preliminary school only (9/10y)* | 9 | 0.6 | 0.8 |
| *(N=1513)* | *High school only* | 100 | 6.6 | 10.2 |
| | *Higher education <4y* | 478 | 31.6 | 32.4 |
| | *Higher education 4 y or more* | 882 | 58.3 | 53.7 |
| | *PhD* | 43 | 2.8 | 2.9 |
| | *Other education* | 1 | 0.1 | 0.1 |
| **Paternal education level** | *Preliminary school only (9/10y)* | 16 | 1.1 | 1.3 |
| *(N=1470)* | *High school only* | 232 | 15.8 | 19.4 |
| | *Higher education <4y* | 453 | 30.8 | 30.0 |
| | *Higher education 4 y or more* | 700 | 47.6 | 44.7 |
| | *PhD* | 54 | 3.7 | 3.4 |
| | *Other education* | 2 | 0.1 | 0.3 |
| | *None of the above* | 13 | 0.9 | 0.9 |
| **Maternal Work** | *Fulltime* | 1299 | 81.0 | 73.3 |
| *(N=1604)* | *Part-time* | 129 | 8.0 | 8.0 |
| | *Student* | 103 | 6.4 | 5.5 |
| | *Housewife/ homemaker* | 16 | 1.0 | 0.9 |
| | *Job-seeker/unemployed* | 22 | 1.4 | 1.2 |
| | *Disabled* | 10 | 0.6 | 0.3 |
| | *Other* | 25 | 1.6 | 1.4 |
| **Gross income household (NOK)** | *Below 300 000* | 15 | 1.0 | 1.3 |
| *(N=1520)* | *300 000 -600 000* | 142 | 9.3 | 13.0 |
| | *600 000- 1 000 000* | 562 | 37.0 | 40.8 |
| | *1 000 000 – 1 400 000* | 572 | 37.8 | 31.6 |
| | *> 1 400 000* | 210 | 13.8 | 11.5 |
| | *Did not want to answer* | 17 | 1.1 | 1.7 |
| **Previous pregnancies (***N1520)* | *Yes* | 810 | 53.3 | 55.0 |
| **Previous deliveries** | *0* | 246 | 30.4 | 27.6 |
| *(N=810)* | *1* | 455 | 56.2 | 57.4 |
| | *2* | 96 | 11.9 | 13.2 |
| | *3* | 8 | 1.0 | 1.3 |
| | *4* | 3 | 0.2 | 0.4 |
| | *6* | 1 | 0.1 | 0.1 |
| | *8 or more* | 1 | 0.1 | 0.1 |
| **Living environment** | *City, densely populated* | 678 | 44.6 | 39.0 |
| *(N=1520)* | *City, less densely populated* | 608 | 40.0 | 37.5 |
| | *Suburb* | 113 | 7.4 | 15.9 |
| | *Village* | 90 | 5.9 | 2.2 |
| | *Countryside, outside village* | 31 | 2.0 | 5.4 |
| **Tobacco use (previous and/or current)** | *Ever* | 455 | 29.9 | 27.9 |
| *(N=1520)* | *Smoking* | 362 | 23.8 | |
| | *Snus* | 369 | 24.3 | 22.5 |

*Table 2 Intake of foods and food groups (g/day) in 1674 women in mid-pregnancy*

| Variable | Median | IQR | 5th Perc | 95th Perc |
|---|---|---|---|---|
| **Bread** | **140** | 114 | 35 | 311 |
| **Cereals** | **130** | 116 | 34 | 336 |
| **Cake** | **20** | 24 | 3 | 63 |
| **Potatoes** | **36** | 43 | 5 | 117 |
| **Vegetables** | **363** | 261 | 141 | 802 |
| **Fruit, berries** | **430** | 327 | 143 | 1008 |
| *Fresh* | **221** | 188 | 61 | 623 |
| *Jam, conserves* | **12** | 20 | 0.2 | 52 |
| *Juice, smoothie* | **144** | 240 | 6 | 501 |
| *Nuts, olives, seeds* | **7** | 15 | 0 | 50 |
| **Meat** | **112** | 78 | 28 | 225 |
| *Red meat* | **74** | 55 | 17 | 157 |
| *Poultry* | **33** | 39 | 2 | 97 |
| **Fish and seafood** | **77** | 64 | 17 | 182 |
| *Low-fat, half-fat* | **12** | 19 | 0 | 44 |
| *Fat* | **20** | 19 | 1 | 58 |
| *Fish Products* | **1** | 4 | 0 | 13 |
| *unspecified* | **6** | 12 | 0 | 30 |
| *Shellfish, entrails* | **2** | 4 | 0 | 14 |
| *Fish spread* | **4** | 15 | 0 | 43 |
| *Sushi* | **14** | 28 | 0 | 67 |
| **Egg** | **20** | 21 | 3 | 64 |
| **Dairy products** | **345** | 326 | 53 | 920 |
| *Milk, yoghurt* | **316** | 318 | 35 | 881 |
| *Full cream milk* | **0** | 2 | 0 | 143 |
| *Semi-skim milk* | **10** | 58 | 0 | 308 |
| *Skim milk* | **0** | 2 | 0 | 300 |
| *Milk unspecified* | **8** | 11 | 0 | 25 |
| *Yoghurt* | **62** | 97 | 0 | 301 |
| *Milk flavored* | **5** | 29 | 0 | 128 |
| *Cultured milk* | **0** | 2 | 0 | 57 |
| *Quark* | **0** | 9 | 0 | 49 |
| *(Ice-, sour-) cream* | **25** | 30 | 4 | 92 |
| **Cheese** | **29** | 24 | 8 | 75 |
| **Butter, margarine** | **27** | 29 | 5 | 77 |
| *Margarine* | **1** | 1 | 0 | 3 |
| *Low-fat margarine* | **0** | 11 | 0 | 40 |
| *Butter* | **1** | 13 | 0 | 41 |
| *unspecified* | **3** | 3 | 1 | 11 |
| *Oil, other fat* | **1** | 1 | 0 | 3 |
| *Mayonnaise, dressing* | **4** | 10 | 0 | 28 |
| **Sugar, sweets** | **19** | 23 | 3 | 68 |
| **Beverages** | | | | |
| *Coffee* | **203** | 385 | 0 | 878 |
| *Tea* | **92** | 231 | 0 | 768 |
| *Juice, soft-drink* | **19** | 18 | 0 | 291 |
| *Juice, soft-drink (sugar-free)* | **19** | 143 | 0 | 701 |
| *Wine, beer (alcohol-free)* | **5** | 36 | 0 | 143 |
| *Beer* | **0** | 5 | 0 | 143 |
| *Wine, Liquor* | **0** | 3 | 0 | 64 |
| *Wine* | **0** | 2 | 0 | 63 |
| *Liquor* | **0** | 0 | 0 | 1 |
| *Drinks* | **0** | 0 | 0 | 7 |

In Table 3 estimated daily intakes of macronutrients, fiber and different food groups are presented in Table 3, and Table 4 shows the intakes of fat in pregnancy.

*Table 3 Estimated daily intake of macronutrients, salt, and selected foods (N=1674)*

| **Macronutrient** | *Unit* | ***Median*** | ***IQR*** | *5th Perc* | *95thPerc* | **RIR (NNR 2023)** |
|---|---|---|---|---|---|---|
| **Carbohydrates** | E% | 45.7 | 42.3-49.2 | 35.9 | 54.7 | 45-60 E% |
| **Protein** | E% | 16.5 | 15.1-18.1 | 13.1 | 20.5 | 10-20 E% |
| **Total fat** | E% | 34.5 | 31.2-37.8 | 26.3 | 43.6 | 25-40 E% |
| *Saturated* | E% | 12.5 | 10.9-14.1 | *8.7* | 17.0 | *max* 10 E% |
| *Monosaturated fat* | E% | 12.7 | 11.2-14.3 | 9.3 | 17.3 | 10-20 E% |
| *Polyunsaturated fat* | E% | 5.7 | 5.0-6.8 | 4.0 | 9.2 | 5-10 E% |
| **Fiber** | g/day | 32.2 | *25.0-41.3* | *16.8* | *55.5* | *min* 25-35 |
| **Food group** | | | | | | **NFG** |
| **Fruit and berries** | g/day | 221 | 141-328 | 61 | 623 | *min* 250 g/day |
| **Vegetables** | g/day | 363 | 253-514 | 141 | 802 | *min* 250 g/day |
| **Fish+Seafood** | g/week | 539 | 188-791 | 121 | 1274 | 300-450 g/week* |
| **Fatty fish** | g/week | 136 | 71-206 | 7 | 404 | min 200 g/week** |
| **Red meat** | g/week | 516 | 339-723 | 116 | 1100 | *max* 500 g/week* |
| **Salt** | g/day | 7.2 | 5.7-8.9 | 3.9 | 12.2 | *Max 6 g/day* |
| **Coffee** | g/day | 203 | 21-405 | 0 | 878 | 170-340 g/day [c] |
| **Alcohol** | g/day | 0.1 | 0.0-0.8 | 0.0 | 9.3 | 0 g/day |

NNR2023 (Nordic Nutrition Recommendations 2023), the NFG (Norwegian Food Based Dietary Guidelines) refer to only Norway;
RIR: recommended intake range; E%: percentage of total energy intake per day;
*Recommendations given in gram per week; ** included in the total amount of fish and seafood per week;
[c] Recommendations based on reference values established by the Department of Nutrition [(204)]. Recommended daily intake is 1-2 cups/day. 1 cup is equivalent to 170 g coffee. Caffeine content was calculated based on European Food Safety Authority (EFSA) guidelines [(205)]: 44.5 mg caffeine/ 100 g black coffee.

*Table 4 Intake of fat (g/day) in mid pregnancy*

| Variable | *Unit* | Median | IQR | Min | Max | 5th | 95th |
|---|---|---|---|---|---|---|---|
| **Fat** | *g/day* | **92.4** | 45.6 | 29.1 | 232.8 | 50.5 | 161.7 |
| **Saturated** | *g/day* | **33.5** | 18.0 | 7.9 | 95.3 | 17.7 | 61.0 |
| **Trans** | *g/day* | **0.77** | 0.48 | 0.06 | 4.04 | 0.32 | 1.66 |
| **Monounsaturated** | *g/day* | **43.3** | 17.8 | 10.4 | 98.4 | 18.4 | 60.9 |
| **Polyunsaturated** | *g/day* | **15.7** | 9.0 | 3.9 | 71.3 | 8.0 | 31.1 |
| **Cholesterol** | *g/day* | **307** | 162 | 67 | 1218 | 155 | 585 |
| **Omega 3** | *g/day* | **3.9** | 2.6 | 0.6 | 15.8 | 1.8 | 8.1 |
| **Omega 6** | *g/day* | **11.8** | 7.1 | 3.5 | 54.8 | 6.0 | 24.1 |

The estimated daily median intake of iron, folate and magnesium were lower than recommended, while Vitamin A, tochoperhol and phosphorus were at least twice that of the recommended daily intake. Inadequate intake of essential micronutrients was observed in 54 % for folate, 50 % for iron, 41 % for selenium, 36 % for calcium, 29 % for vitamin D, and 24% for iodine. Daily estimated intakes of micronutrients are presented in Table 5.

*Table 5 Daily dietary intake of vitamins and nutrients (N=1674), IQR: Interquartile range, RI:Recommended intake for pregnant women, AR Adequate intake based on observed intakes in healthy people or approximations from experimental studies, used when an RI cannot be determined*

| **Variable** | *Unit* | **Median** | **IQR** | **5th Perc** | **95th Perc** | *RI (NNR 2023)* |
|---|---|---|---|---|---|---|
| **Vitamin A** | *RE [a]* | **1694** | 1101 | 661 | 3313 | **800** |
| **Vitamin D** | *µg* | **13.6** | 12.1 | 4.2 | 32.6 | **10** |
| **Iron** | *mg[b]* | **15.1** | 10.0 | 7.3 | 92.0 | **26 *** |
| **Zinc** | *mg [c]* | **15.5** | 14.8 | 7.7 | 43.4 | **11.3*** |
| **Thiamine** | *mg* | **2.36** | 1.78 | 1.1 | 5.2 | **1.5** |
| **Riboflavin** | *mg* | **2.8** | 2.2 | 1.3 | 6.2 | **1.9** |
| **Niacin** | *NE [d]* | **30.0** | 23.1 | 14.2 | 65.7 | **17.0** |
| **Vitamin B 6** | *mg* | **2.7** | 2.3 | 1.3 | 6.6 | **1.9** |
| **Folate** | *µg* | **480** | 275 | 236 | 921 | **600** |
| **Vitamin C** | *mg* | **207** | 136 | 86 | 425 | **105** |
| **Calcium** | *mg* | **1045** | 558 | 495 | 1960 | **950** |
| **Copper** | *mg* | **1.83** | 1.94 | 0.87 | 5.60 | **1.0** |
| | *Unit* | **Median** | **IQR** | **5th Perc** | **95th Perc** | *AR (NNR 2023)* |
| **Vitamin B 12** | *µg* | **7.9** | 4.1 | 4.0 | 14.5 | **4.5** |
| **Tocopherol** | *α-TE* | **25.1** | 17.2 | 10.2 | 48.8 | **10** |
| **Iodine** | *µg* | **256** | 189 | 103 | 563 | **200** |
| **Magnesium** | *mg* | **463** | 206 | 260 | 770 | **280** |
| **Potassium** | *g* | **4.73** | 2.10 | 2.68 | 7.75 | **3.1** |
| **Selenium** | *µg* | **69** | 52 | 31 | 162 | **60** |
| **Phosphorus** | *mg* | **1876** | 820 | 1060 | 3108 | **700** |
| **Manganese** | *mg* | **0.0** | 2.5 | 0.0 | 5.0 | **3** |

[a] Retinol equivalents; 1 retinol equivalent (re) = 1 µg retinol = 12 µg β-carotene.
α-tocopherol equivalents; 1 α-tocopherol equivalent (α-te) = 1 mg α-tocopherol
[b] Meal composition influences the utilization of dietary iron. Availability increases if the diet contains abundant amounts of vitamin C and meat or fish daily, and it is decreased with simultaneous intake of polyphenols or phytic acid.
[c] The utilization of zinc is negatively influenced by phytic acid and positively influenced by animal protein. The recommended intakes are valid for a mixed animal/vegetable diet. For vegetarian cereal-based diets, a 25%–30% higher intake is recommended
[d] Niacin equivalent; 1 niacin equivalent (ne) = 1 mg niacin = 60 mg tryptophan
*Assuming a mixed animal/vegetable diet with a phytic acid intake of about 600 mg/d.

### 7.1.2 Infant diet

We included 2059 infants with available dietary data from at least one questionnaire at 3-, 6-,9- and 12 months of age, as well as weekly electronic diaries from weeks 2 to 6 months. Breastfeeding at 3 months was reported in 95% of the women in Norway and 92% in Sweden, with the corresponding rates of 95% and 88% at 6 months and 51% and 22% at 12 months. At 3 months 67% of infants were exclusively breastfed, and 3 % were exclusively breastfed at 6 months in the NFI group. Among infants randomized to no food intervention, the mean age of any complementary food introduction was 18.3 weeks (CI 18.1-18.5 weeks), while infants randomized to food intervention introduced complementary foods from 12 weeks of age.

Overall, a total of 31 (1.3 %) infants were given porridge before 3 months of age and 134 (7.9 %) at 3 months. At 4 months 777 (45.3 %) were introduced to porridge, and 436 (25.6 %) at 5 months. By 6 months of age a total of 1378/1700 (81.1 %) infants had been given porridge. The most frequently introduced porridge before 6 months was oat 718 (62.2 %), wheat 689 (40.5 %), and corn 579 (34.1 %). The following porridge types were given less frequently: millet 312 (18.3 %), whole meal 226 (13.3 %), spelt 70 (4.1 %), Sinlac 71 (4.2 %), other type 87 (5.1 %). The most common foods to be given by 6 months of age were fruit/berries (92.5 %), root vegetables (86.8 %), and other vegetables (67.4 %).

Dairy (in any form) was given to 39.9 % at 6 months, 75.9 % at 9 months and 88.1 % at 12 months. Intakes of fish, meat and poultry increased significantly from 6 to 9 months of age and were consumed by most infants by 12 months of age (see Table 6-Table 8).

Commercially processed foods constituted a big part of an infant's diet, with 45.4 % receiving mostly commercially processed or only commercially processed food at 6 months, 47.3 % at 9 months and 31.8 % at 12 months. The proportion of infants receiving mostly organic food was 30.4 % at 6 months, 25.8 % at 9 months and 16.9 % at 12 months.

Infants in the NFI group were significantly less exposed to the interventional foods (dairy products, peanuts, egg foods, and pure egg at 6 months compared to infants randomized to the FI group ($p < 0.001$) as shown in Table 6. Similarly, a lower percentage of infants in the NFI group consumed these foods compared to infants randomized to the FI group ($p < 0.001$), as shown in Table 7 and Table 8. The intervention did not have a significant impact on the intake of other complementary foods, except for a lower number of infants consuming poultry in the FI group at 6 months (p 0.032) and a higher number of infants consuming bread and other baked goods at 9 months (p 0.043). The consumption of commercially prepared infant foods versus home-cooked foods and organic foods was similar in both groups, except for a higher number of infants in the FI group consuming little or no organic food at 12 months.

*Table 6 Intake of complementary foods at 6 months in the FI and NFI group*

| *Dairy products* | **NFI group** | **FI group** | **Whole group** |
|---|---|---|---|
| | ***N 843*** | **N 857** | ***N 1700*** |
| | *N 327 missing* | *N 368 missing* | |
| **Cow's milk** | 93 (11.0 %) | 551 (64.3 %) * | 644 (37.9 %) |
| **Lactose free milk** | 4 (0.5 %) | 28 (3.3 %) * | 32 (1.9 %) |
| **Unpasteurized cow milk** | 2 (0.2 %) | 1 (0.1 %) | 3 (0.02%) |
| **Pro/pre-biotic dairy products** | 14 (1.7 %) | 58 (6.8 %) * | 72 (4.2 %) |
| **Yoghurt** | 99 (11.7 %) | 222 (25.9 %) * | 321 (18.9 %) |
| **Cheese** | 52 (6.2 %) | 69 (8.1 %) * | 121 (7.1 %) |
| **Others** | 70 (8.3 %) | 116 (13.5 %) * | 186 (10.9%) |
| *Other foods* | | | |
| **Bread, pastry, cakes** | 180 (21.4 %) | 238 (27.8 %) | 418 (24.6 %) |
| **Fruit/ Berries** | 777 (92.2 %) | 796 (92.9 %) | 1573 (92.5 %) |
| **Root vegetables** | 749 (88.8 %) | 727 (84.8 %) | 1476 (86.8 %) |
| **Other vegetables** | 584 (69.3 %) | 561 (65.5 %) | 1145 (67.4 %) |
| **Peanuts** | 98 (11.6 %) | 725 (84.6 %) * | 823 (48.4 %) |
| **Other nuts** | 33 (3.9 %) | 60 (7.0 %) * | 93 (5.5 %) |
| **Egg foods** | 92 (10.9 %) | 189 (22.1 %) * | 281 (16.5 %) |
| **Egg pure** | 178 (21.1 %) | 706 (82.4 %) * | 884 (52.0 %) |
| **Fatty fish** | 159 (18.9 %) | 148 (17.3 %) | 308 (18.1 %) |
| **Fish other** | 100 (11.9 %) | 105 (12.3 %) | 205 (12.1 %) |
| **Shellfish** | 11 (1.3 %) | 21 (2.5 %) | 32 (1.9 %) |
| **Poultry** | 153 (18.1 %) | 130 (15.2 %) ** | 283 (16.6 %) |
| **Meat other** | 182 (21.6 %) | 167 (19.5 %) | 349 (20.5 %) |
| *Homecooked vs commercially prepared infant foods* | | | |
| **Only homecooked** | 76 (9.0 %) | 77 (9.0 %) | 153 (9.0 %) |
| **> homecooked vs commercial** | 180 (21.3 %) | 187 (21.8 %) | 367 (21.6 %) |
| **Homecooked = commercial** | 191 (22.7 %) | 189 (22.1 %) | 380 (22.4 %) |
| **> commercial vs homecooked** | 282 (33.5 %) | 306 (35.7 %) | 588 (34.6 %) |
| **Only commercially prepared** | 97 (11.5 %) | 86 (10.0 %) | 183 (10.8 %) |
| **Don't know** | 17 (2.0 %) | 12 (1.4 %) | 29 (1.7 %) |
| *Amount of organic food* | | | |
| **Very little/ none** | 122 (14.8 %) | 139 (16.4 %) | 261 (15.6 %) |
| **Small amount** | 177 (21.4 %) | 189 (22.4 %) | 366 (21.9 %) |
| **Half** | 192 (23.2 %) | 198 (23.4 %) | 390 (23.3 %) |
| **Majority** | 264 (32.0 %) | 244 (28.9 %) | 508 (30.5 %) |
| **Don't know** | 71 (8.6 %) | 75 (8.9 %) | 146 (8.7 %) |

*p<0.001, ** p<0.05 We used chi-square test to assess differences of complementary food intake between groups. "> Home cooked" refers to the infant diet consisting of more home-cooked foods compared to commercially prepared infant foods, and vice versa for "> Commercially prepared"

*Table 7 Intake of complementary foods at 9 months in the FI and NFI group*

| *Dairy products* | **NFI group** | **FI group** | **Whole group** |
|---|---|---|---|
| | ***N 821*** | **N 816** | ***N 1637*** |
| | *N 349 missing* | *N 409 missing* | |
| **Cow's milk** | 245 (29.8 %) | 520 (63.7 %) * | 765 (46.7 %) |
| **Lactose free milk** | 30 (3.7 %) | 53 (6.5 %) * | 83 (5.1 %) |
| **Unpasteurized cow milk** | 5 (0.6 %) | 13 (1.6 %) * | 18 (1.1 %) |
| **Pro/pre-biotic dairy products** | 51 (6.2 %) | 103 (12.6 %) * | 154 (9.4 %) |
| **Yoghurt** | 333 (40.6 %) | 487 (59.7 %) * | 820 (50.1 %) |
| **Cheese** | 402 (49.0 %) | 502 (61.5 %) * | 904 (55.2 %) |
| **Others** | 294 (35.8 %) | 392 (48.0 %) * | 686 (41.9 %) |
| *Other foods* | | | |
| **Bread, pastry, cakes** | 721 (87.8 %) | 730 (89.5 %) ** | 1451 (88.6 %) |
| **Fruit/ Berries** | 810 (98.7 %) | 811 (99.4 %) | 1621 (99.0 %) |
| **Root vegetables** | 811 (98.8 %) | 809 (99.1 %) | 1620 (99.0 %) |
| **Other vegetables** | 798 (97.2 %) | 792 (97.1 %) | 1590 (97.1 %) |
| **Peanuts** | 181 (22.0 %) | 588 (72.1 %) * | 769 (47.0 %) |
| **Other nuts** | 125 (15.2 %) | 167 (20.5 %) * | 292 (17.8 %) |
| **Egg foods** | 519 (63.2 %) | 616 (75.5 %) * | 1135 (69.3 %) |
| **Egg pure** | 523 (63.7 %) | 667 (81.7 %) * | 1190 (72.7 %) |
| **Fatty fish** | 704 (85.7 %) | 700 (85.8 %) | 1404 (85.8 %) |
| **Fish other** | 571 (69.5 %) | 572 (70.1 %) | 1143 (69.8 %) |
| **Shellfish** | 95 (11.6 %) | 105 (12.9 %) | 200 (12.2 %) |
| **Poultry** | 666 (81.1 %) | 661 (81.0 %) | 1327 (81.1 %) |
| **Meat other** | 735 (89.5 %) | 727 (89.1 %) | 1462 (89.3 %) |
| *Homecooked vs commercially prepared infant foods* | | | |
| **Only homecooked** | 32 (3.9 %) | 32 (3.9 %) | 64 (3.9 %) |
| **> homecooked vs commercial** | 231 (28.1 %) | 197 (24.1 %) ** | 396 (24.2 %) |
| **Homecooked = commercial** | 226 (27.5 %) | 198 (24.3 %) ** | 403 (24.6 %) |
| **> commercial vs homecooked** | 291 (35.3 %) | 342 (41.9 %) * | 691 (42.2 %) |
| **Only commercially prepared** | 27 (5.2 %) | 47 (5.8 %) | 83 (5.1 %) |
| *Amount of organic food* | | | |
| **Very little/ none** | 78 (9.7 %) | 88 (10.8 %) | 155 (9.5 %) |
| **Small amount** | 272 (33.7 %) | 229 (28.1 %) * | 468 (28.6 %) |
| **Half** | 235 (29.1 %) | 239 (29.3 %) | 494 (30.2 %) |
| **Majority** | 179 (22.2 %) | 206 (25.2 %) ** | 423 (25.8 %) |
| **Don't know** | 43 (5.3 %) | 54 (6.6 %) | 97 (5.9 %) |

*p<0.001, ** p<0.05 We used chi-square test to assess differences of complementary food intake between groups. "> Home cooked" refers to the infant diet consisting of more home-cooked foods compared to commercially prepared infant foods, and vice versa for "> Commercially prepared"

*Table 8 Intake of complementary foods at 12 months in the FI and NFI group*

| *Dairy products* | **Other** | **Food** | **Whole group** |
|---|---|---|---|
| | ***N 831*** | **N 814** | ***N 1645*** |
| | *N 339missing* | *N 411 missing* | |
| **Cow's milk** | 484 (58.2 %) | 602 (74.0 %) * | 1086 (66.0 %) |
| **Lactose free milk** | 58 (7.0 %) | 68 (8.4 %) | 126 (7.7%) |
| **Unpasteurized cow milk** | 9 (1.1 %) | 16 (2.0 %) | 25 (1.5 %) |
| **Pro/pre-biotic dairy products** | 162 (19.5 %) | 185 (22.7 %) ** | 347 (21.1 %) |
| **Yoghurt** | 668 (80.4 %) | 693 (85.1 %) * | 1361 (82.7 %) |
| **Cheese** | 712 (85.7 %) | 718 (88.2 %) | 1430 (86.9 %) |
| **Others** | 484 (58.2 %) | 514 (63.1 %) * | 998 (60.7 %) |
| *Other foods* | | | |
| **Bread, pastry, cakes** | 796 (95.8 %) | 790 (97.1 5) | 1586 (96.4%) |
| **Fruit/ Berries** | 827 (99.5 %) | 806 (99.0 %) | 1633 (99.3 %) |
| **Root vegetables** | 825 (99.3 %) | 809 (99.4 %) | 1634 (99.3 %) |
| **Other Vegetables** | 810 (97.5 %) | 798 (98.0 %) | 1608 (97.8 %) |
| **Peanuts** | 281 (33.8 %) | 552 (67.8 %) * | 833 (50.6 %) |
| **Other nuts** | 203 (24.4 %) | 283 (34.8 %) * | 486 (29.5 %) |
| **Egg foods** | 722 (86.9 %) | 732 (89.9 %) ** | 1454 (88.4 %) |
| **Egg pure** | 670 (80.6 %) | 720 (88.5 %) * | 1390 (84.5 %) |
| **Fatty fish** | 776 (93.4 %) | 759 (93.2 %) | 1535 (93.3 %) |
| **Fish other** | 701 (84.4 %) | 684 (84.0 %) | 1384 (84.1 %) |
| **Shellfish** | 225 (27.1 %) | 239 (29.4 %) | 464 (28.2 %) |
| **Poultry** | 734 (88.3 %) | 712 (87.5 %) | 1446 (87.9 %) |
| **Meat other** | 779 (93.7 %) | 765 (94.0 %) | 1544 (93.9 %) |
| *Homecooked vs commercially prepared infant foods* | | | |
| **Only homecooked** | 32 (3.8 %) | 27 (3.3 %) | 59 (3.6 %) |
| **> homecooked vs commeercial** | 250 (30.1 %) | 296 (36.4 %) * | 578 (35.1 %) |
| **Homecooked = commercial** | 237 (28.5 %) | 226 (27.8 %) | 484 (29.4 %) |
| **> commercial vs homecooked** | 300 (36.1 %) | 251 (30.8 %) ** | 493 (30.0 %) |
| **Only commercially prepared** | 24 (2.9 %) | 14 (1.7 %) | 29 (1.8 %) |
| *Amount of organic food* | | | |
| **Very little/ none** | 87 (10.5 %) | 117 (14.4 %) * | 204 (12.4 %) |
| **Small amount** | 317 (38.2 %) | 297 (36.5 %) | 614 (37.4 %) |
| **Half** | 233 (28.1 %) | 228 (28.0 %) | 461 (28.1 %) |
| **Majority** | 145 (17.5 %) | 133 (16.3 %) | 278 (16.9 %) |
| **Don't know** | 47 (5.7 %) | 39 (4.8 %) | 86 (5.2 %) |

*p<0.001, ** p<0.05 We used chi-square test to assess differences of complementary food intake between groups. "> Home cooked" refers to the infant diet consisting of more home-cooked foods compared to commercially prepared infant foods, and vice versa for "> Commercially prepared"

## 7.2 Impact of early food introduction on breastfeeding and diet diversity (Paper III)

Early food intervention did not affect breastfeeding rates, as illustrated in Figure 19. Figure 19 Percentage of breastfed infants from birth until 12 months of age in the FI vs NFI groupBreastfeeding rates from birth until 12 months were found to be similar between the FI (early introduction of peanut, milk, wheat, and egg) and NFI group.

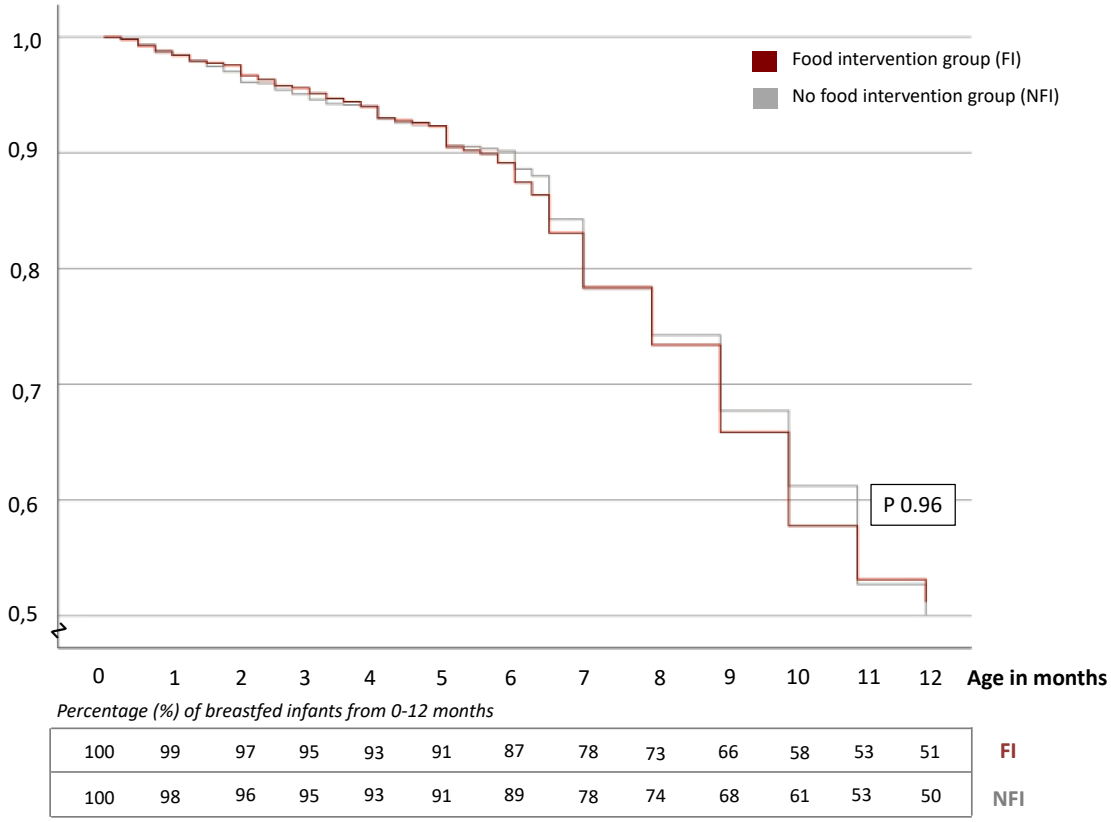


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*Figure 19 Percentage of breastfed infants from birth until 12 months of age in the FI vs NFI group*

Excluding the interventional foods, the mean diet diversity score (number of food categories consumed) was similar between the FI and NFI groups at 6 months (3.4 (SD 1.9) vs. 3.5 (SD

1.9), respectively). However, the diet diversity score was significantly higher in the FI group compared to the NFI group at 9 and 12 months of age (p<0.001), as shown in Figure 20 and Table 9. When including the interventional foods, the dietary diversity score was 11.1 (SD 2.3) in the FI group vs. 9.7 (SD 2.4) in the NFI group at 9 months and 12.9 (SD 1.2) vs. 12.1 (2.1) at 12 months.

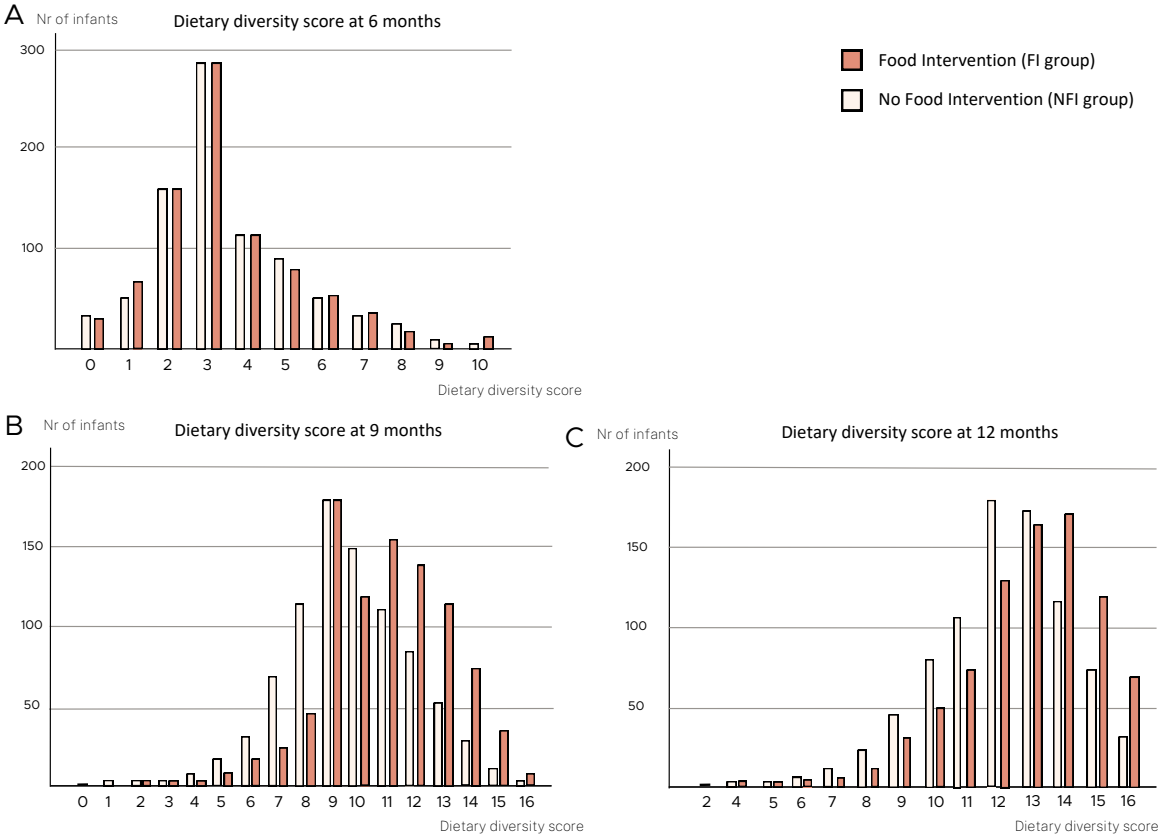


*Figure 20 Dietary diversity score at 6, 9 and 12 months in the FI and NFI group*

*Table 9 Effect of early interventional food introduction on dietary diversity score*

| | 6 months | | | 9 months | | | 12 months | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Effect estimate* | *CI* | *p value* | *Effect estimate* | *CI* | *p value* | *Effect estimate* | *CI* | *p value* |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Linear regression, multiple imputation | **0.01** | -0.18 to 0.20 | 0.95 | **1.39** | 1.16 to 1.62 | <0.001 | **0.70** | 0.50 to 0.90 | <0.001 |
| Linear regression, complete case- no imputation | **-0.28** | -0.71 to 0.15 | 0.77 | **1.41** | 1.18 to 1.64 | <0.001 | **0.78** | 0.58 to 0.98 | <0.001 |

## 7.3 The temporal development of gut microbiota composition and SCFA production and impact of infant diet (Paper II and III)

The number of unique species identified in the infant gut microbiota increased with increasing age, as shown in Figure 21. In meconium, the first stool after birth, 26 species were detected, increasing to 37 at 3 months, 46 at 6 months, and 72 at 12 months, compared to 183 unique species in the gut microbiome of the mothers. The same trend of increased diversity with age was observed using the Shannon-Wiener and Simpson's index, with the mothers' gut microbiota displaying the highest diversity index, as shown in Figure 22.

The infant gut microbiota reached its highest diversity at 12 months of age, with a most of unique species belonging to the Clostridiales order, Figure 21. Between the ages of 6 and 12 months, there was a significant increase in Clostridiales, which accounted for approximately 67% of the gut bacteria at 12 months. Within the Clostridiales, the prominent genera/families were Faecalibacterium (13.1%), *Ruminococcus gnavus* group (9.4%), and *Eubacterium rectale* group (7.3%). *Faecalibacterium* was found to be significantly higher than the *E. rectale* group, see Figure 23.

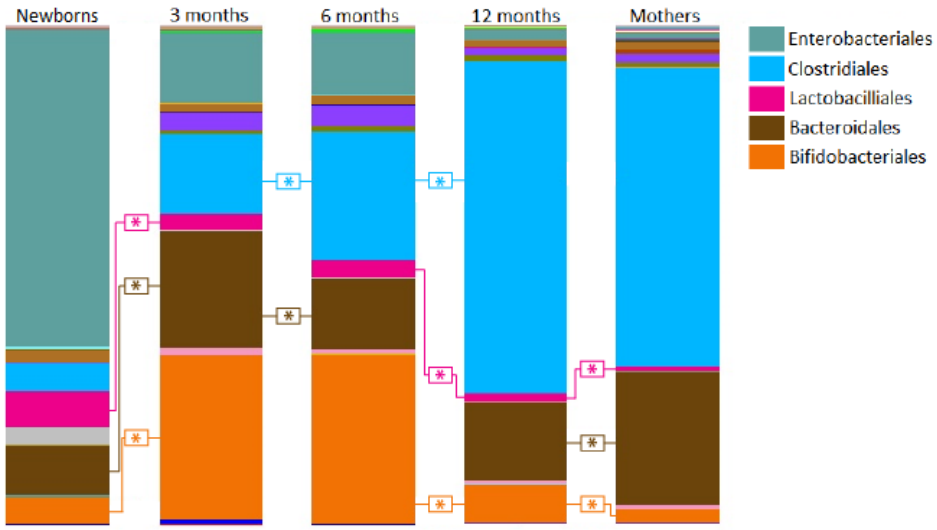


*Figure 21 Taxonomic composition. The bar chart shows the relative abundance (%) of bacterial orders acquired from sequencing. * p-value< 0.05*

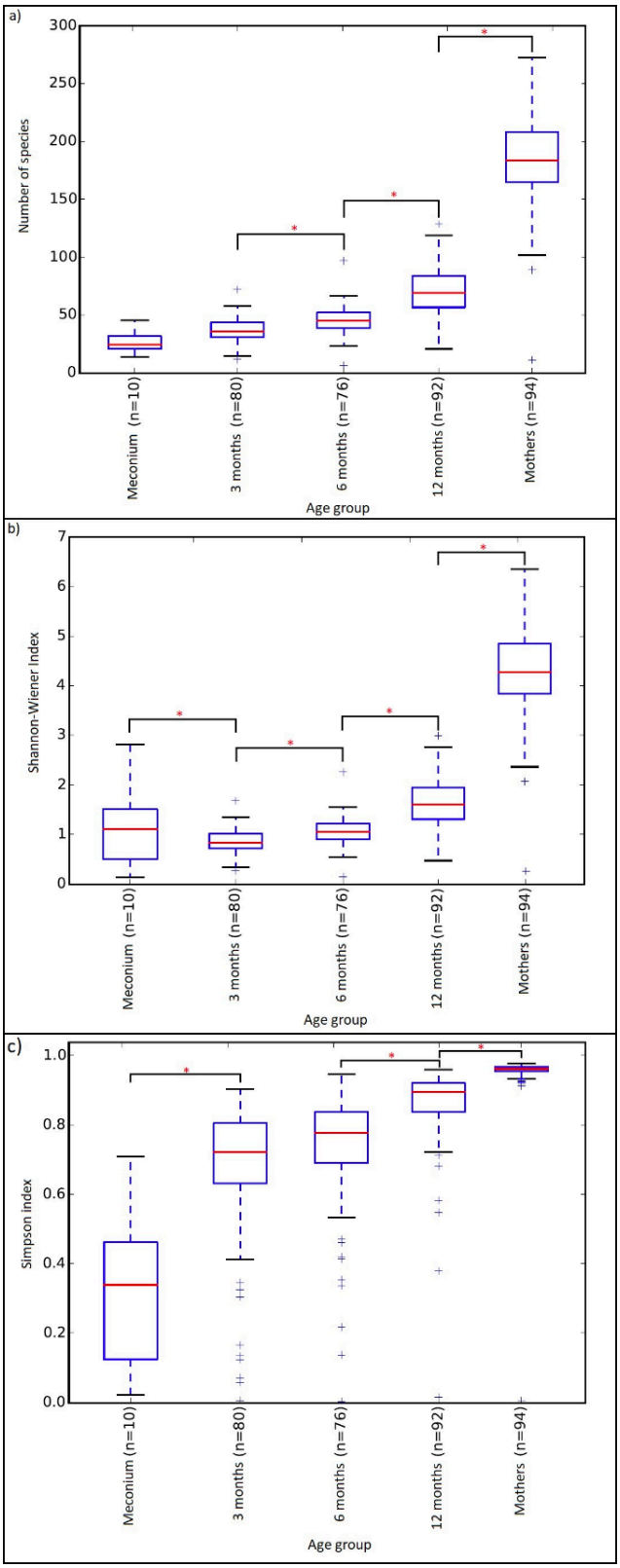


*Figure 22 Alpha diversity. The illustration gives an overview of observed species (A), Shannon-Wiener index (B), and inverse Simpsons-index (C). *p,0.05 (paired t-test)*

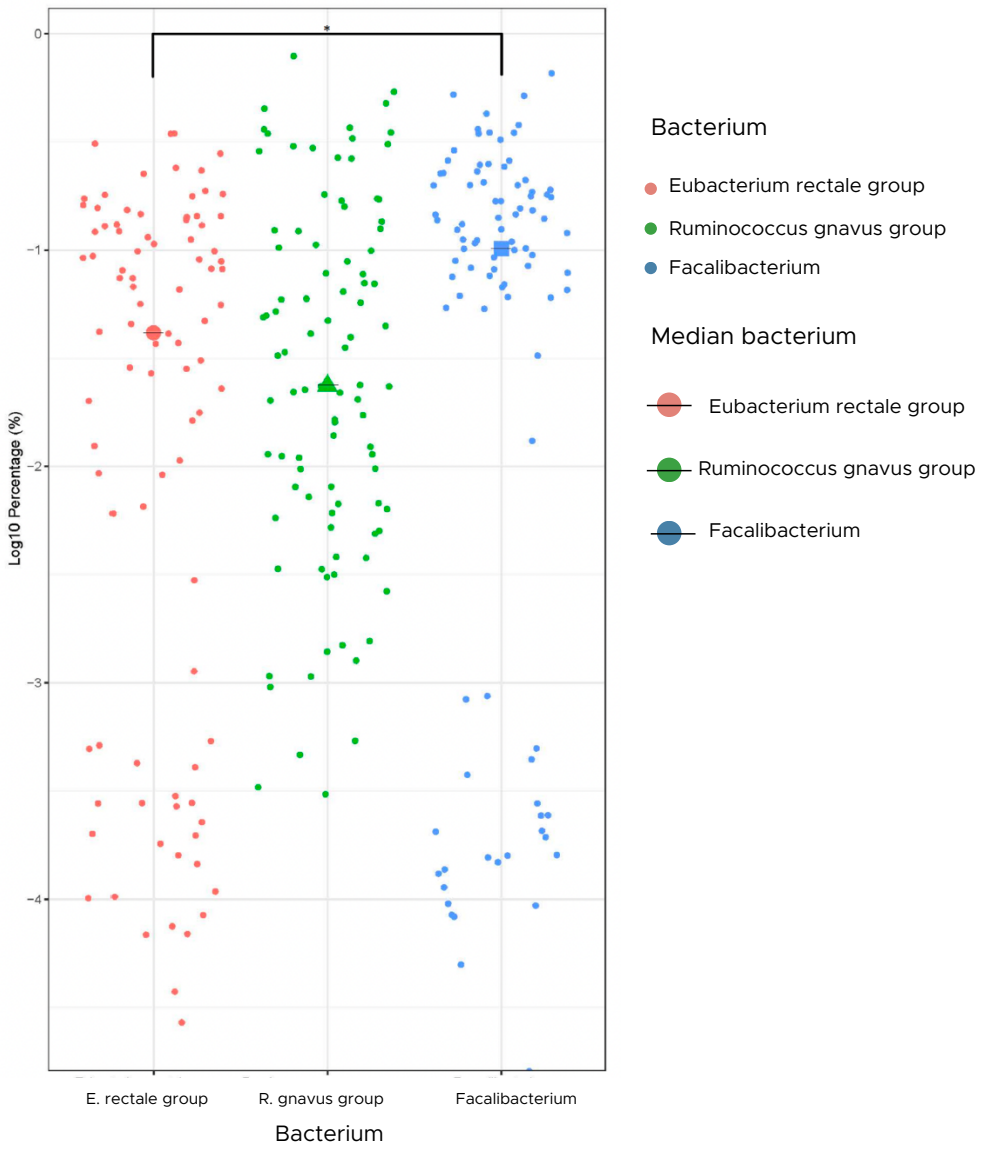


*Figure 23 The dot plot shows the relative abundance (Log10 of percentage abundance) and median of the E. rectale group, R. gnavus group, and Faecalibacterium at 12 month of age in 100 infants. The * shows a significant difference in rel. abundance (p,0.05) between bacteria*

Age of the infant, delivery mode, and breastfeeding at 3 months of age had significant impacts on the microbiota, as shown in Table 10. Infant age was the main factor contributing to the variation in infant microbiota composition. Vaginal delivery was associated with higher levels of *Bifidobacterium* and *Bacteroides,* whereas CS was associated with increased levels of *Clostridia*. Breastfeeding at 3 months was associated with higher levels of

*Bifidobacterium*, while not being breastfed at 3 months was associated with higher levels of *Bacteroides*. Younger age was associated with higher levels of *Bifidobacterium* and *Escherichia/Shigella*, while *Clostridia* was more prevalent in infants at 12 months and their mothers.

*Table 10 Maternal and infant factors associated with operational taxonomic units (OTUs)*

| | *Effect (%)* | **p-value** |
|---|---|---|
| Infant gender | *0.26* | **0.25** |
| Delivery mode | *1.06* | **0.01** |
| Breastfeeding 3-6 months | *1.12* | **0.01** |
| Breastfeeding 6-9 months | *0.60* | **0.73** |
| Breastfeeding 9-12 months | *0.72* | **0.05** |
| Infant age | *26.63* | **0.001** |

In all age groups, as well as in mothers, acetate was the dominant SCFA (Figure 24). The relative abundance of acetate in infants decreased from 3 months (90.1 ± 7.9%), to 12 months (67.4 ± 5.1%). Proprionate increased significantly between 3 and 12 months, with an overall ratio of 11.2 ± 1.3% at 12 months of age. The relative abundance of butyrate increased four-fold from 6 to 12 months of age ($p < 0.05$, Kruskall-Wallis-Dunn's test, FDR corrected with Benjamini-Hochberg). At 12 months of age butyrate represented 18.9 ± 2% of the total SCFAs.

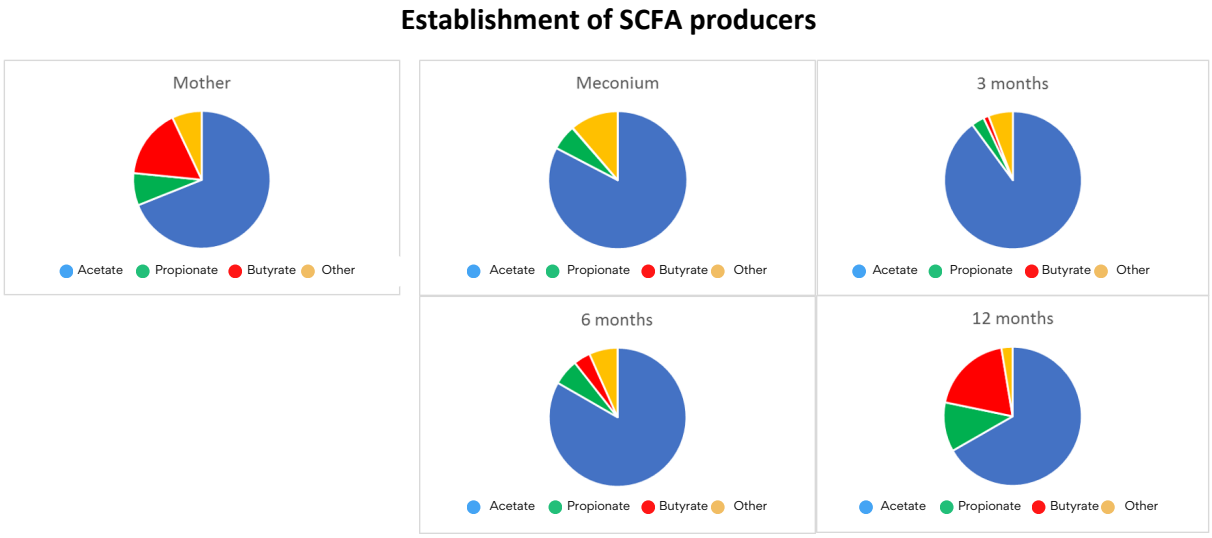


*Figure 24 Short-chain fatty acid composition for the respective age groups. The dominant orders of bacteria and SCFAs in their respective colors are displayed as pie charts.*

We found two distinct bacterial networks at 12 months of infant age. The first network was characterized by a positive correlation between *Eubacterium rectale* and the relative amount of butyrate. The second network was defined by a negative correlation between *Ruminococcus gnavus* and the relative amount of butyrate (see Figure 25).

Within the E. rectale network, E. rectale was the most abundant species with positive correlation to both the relative amount of butyrate and other bacteria such as Roseburia, Lachnospiraceaea NK4A136, and Lachnospira. All bacteria within the E. rectale network were negatively correlated with R. gnavus. In contrast, R. gnavus showed a negative correlation with the relative amount of butyrate and a positive correlation with Erysipelatoclostridium, Veillonella, and Clostridium innocuum. Enterococcus and Escherichia/Shigella also showed a

positive correlation with members of the R. gnavus network. All bacteria related to the R. gnavus network negatively correlated with butyrate (see Figure 25).

Based on the cumulative sum of the relative abundance of bacteria that had a positive or negative correlation with butyrate, as well as a positive correlation between the bacteria themselves, 43 infants were classified with the *E. rectale* network, 27 infants with the *R. gnavus* network, 19 infants with neither network and 5 infants with both networks at 12 months of age ($p < 0.05$, chi-square test). No significant correlations were observed between the presence of the *E. rectale* and *R. gnavus* networks at 12 months of age and delivery mode, gender, breastmilk feeding, or introduction to solid foods ($p > 0.05$, chi-square test).

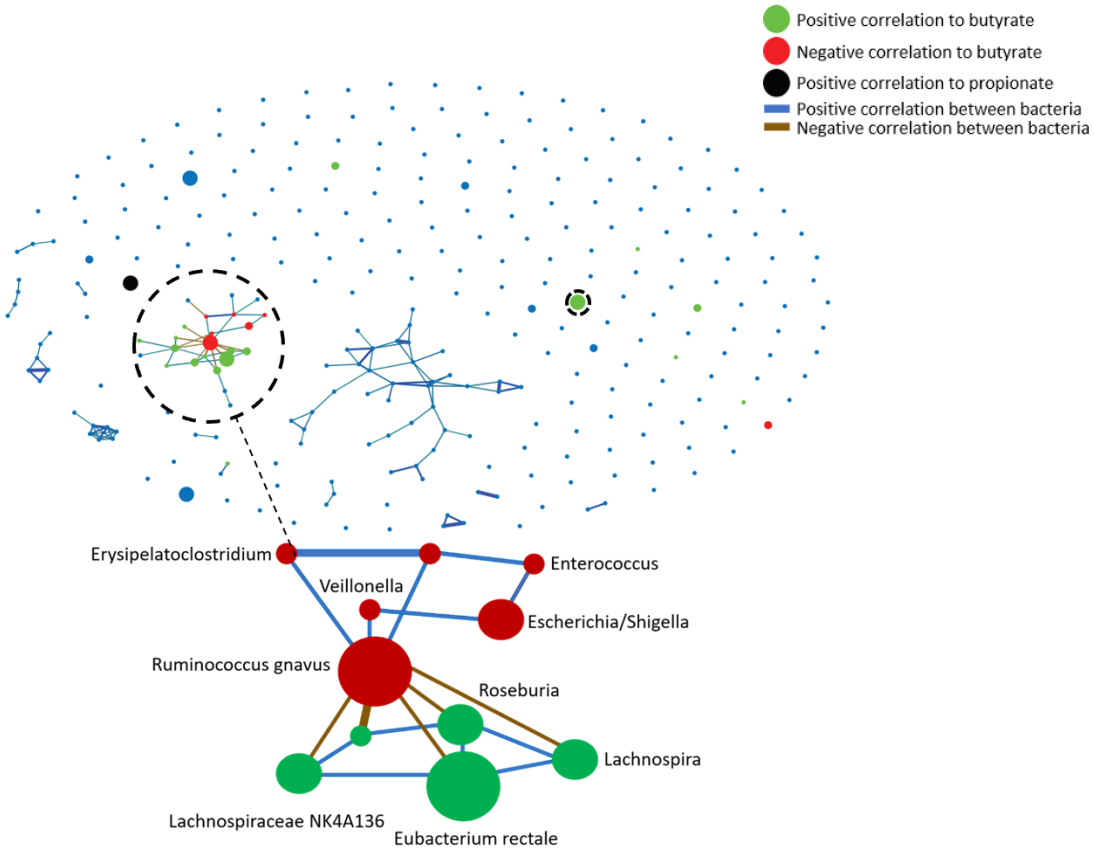

*Figure 25 At 12 months, we analyzed the correlation between bacteria and SCFAs. Nodes represented OTUs from 16rRNA, with colors indicating their correlation to SCFAs. Node size reflected bacteria abundance. Line thickness represented correlation strength. Blue lines indicate positive correlations, brown lines indicate negative correlations. Prominent nodes were highlighted with their OUT taxonomy. A green circle with positive associations to butyrate but no correlation to other bacteria was noted*

We further performed metaproteome analysis in 6 infants based on their relative abundance of *E. rectale* and *R. gnavus* networks and butyrate at 12 months. We identified the necessary enzymes involved in converting acetyl-CoA to butyrate through the butyryl-CoA:AcetateA-transferase pathway. The butyrate-associated bacteria *E. rectale, F. prausnitzii*, and *Roseburia* expressed several of these enzymes. We found that the enzymes enoyl-CoA, 3-hydroxybutyryl-CoA dehydratase, and butyryl-CoA:Acetate CoA-transferase were exclusively present in fecal samples of infants with the *E. rectale* network (as shown in Figure 26).
The conversion from propanoyl-phosphate to propionate was only detected and mapped to Bifidobacteria species such as *B. breve, B. pseudocatenulatum, B. longum*, and *B. bifidum*.
In terms of acetate production, our analysis revealed that *F. prausnitzii, E. halli, Blautia*, *Lachnospiraceae, B. breve, B. pseudocatenulatum, B. longum*, and *B. bifidum* are potential acetate producers.

In terms of proteins detected, we found glycoside hydrolases (GHs), which potentially degrades a variety of dietary fibers, including hemicellulose (GH43 and GH51), starch/glycogen (GH13 and GH77), cellobiose/chitobiose (GH94), and fucose (GH29), in addition to broad specific glucosidases(GH2 and GH3), which were potentially expressed by E. rectale (Figure 8). Glycosidee hydrolases mapped to R. gnavus consisted of GHs related to mucin (GH33 and GH101), fucose (GH29, GH95, and GH151), human milk oligosaccharides

(GH20), mannose (GH26), starch (GH31), sucrose (GH32), and a broad-specific glucosidase (GH3).

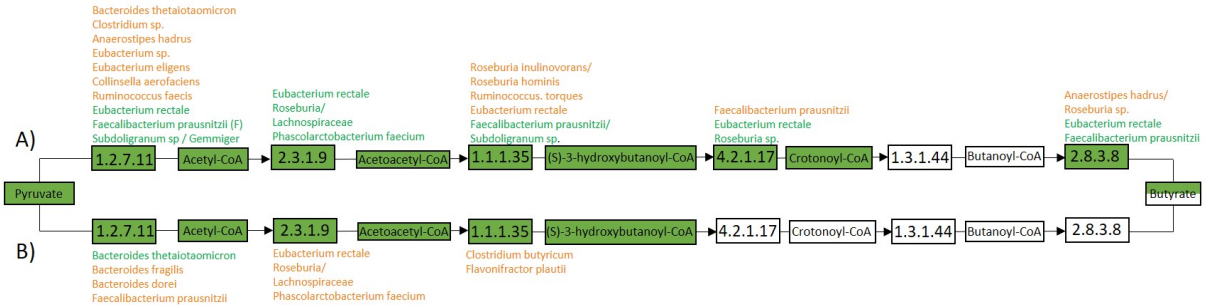


*Figure 26 Protein presence related to butyrate production. The figure gives an overview of bacterial proteins (E.C. number) in relation to the butyrate pathway butyryl-CoA:Acetate CoA-transferase. The figure shows proteins detected (green box) in infants with the E. rectale network (A), or R. gnavus network (B). Bacterial taxonomy is shown next to each E.C. number, representing the bacterial source of the given protein. The bacterial sources are divided by two di erent colors, where orange represents detection in three or fewer samples in (A) or two or fewer in (B), and green represents detection in four or more in (A) or three or more in (B).*

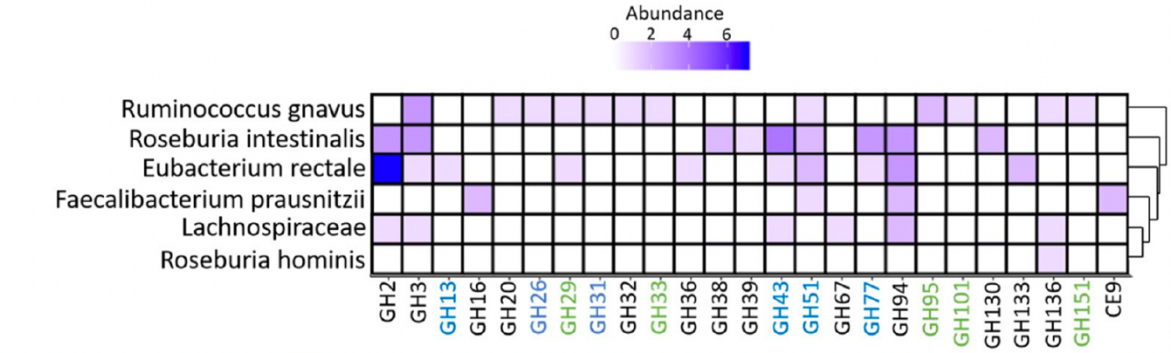


*Figure 27 Expressed glycoside hydrolases/carbohydrate esterases. The figure shows proteins expressed within the glycoside hydrolase and carbohydrate esterase groups expressed based on the contiguous sequences assembled from shotgun sequencing and protein expression derived from nanoLC-Orbitrap tandem mass spectrometry (MS/MS). The abundance represents the number of unique proteins expressed from a given taxa within the glycoside hydrolases (GH) or carbohydrate esterase (CE) group.The y-axis shows the relevant taxonomies, and the dendrogram represents the Euclidian distance between the taxonomic groups based on GH and CE expression. The plot was created using ggplot2 [18]. GH numbers related to mucus and fucose degradation are marked in green, and degradation of starch,glycogen, and hemicellulose are marked in blue*

## 7.4 Butyrate at 12 months of age and atopic dermatitis development in the first year of life (Paper II and poster)

The relative abundance of butyrate was assessed in fecal samples of 100 infants at 12 months of age, all of whom also had skin examinations at 3 months, 6 months and 12 months. Eczema was observed in 13 infants at 3 months, 24 at 6 months and 16 at 12 months of age.

The characteristics of the study group overall, as well as based upon the butyrate proportion (%) of SCFA ≤ or > the 75$^{th}$ percentile (23.4 %) is described in Table 11.

*Table 11 Background characteristics of 100 infants with butyrate and skin assessments at 2, 6 and 12 months of age are shown for the total group, as well as based on the relative butyrate levesl ≤and ≥ 75th percentile(23.4% of total SCFA composition)*

| *Characteristics* | *Total (N100)* | *Butyrate <75P (N 75)* | *Butyrate >75P (N 25)* |
|---|---|---|---|
| *Allergic disease mother N (%)* | *56 (60.9 %) N92* | *43 (63.2 %) N68* | *13 (54.2 %) N24* |
| *Allergic disease father N (%)* | *48 (52.7 %) N91* | *41 (59.4%) N69* | *7 (31.8 %) N22* |
| *Vaginal delivery N (%)* | *78 (78 %)* | *60 (80 %)* | *18 (72 %)* |
| *Caesarian section N (%)* | *22 (22 %)* | *15 (20 %)* | *7 (28 %)* |
| *Male gender N (%)* | *51 (51 %)* | *32 (42.7 %)* | *19 (76 %)* |
| *Birth weight (g) mean, (SD, min-max)* | *3577 (529, 1935-5632)* | *3528.8 (558, 1935-5632)* | *3722 (409, 3108-4750)* |
| *Butyrate (%) median (SD, min-max)* | *15.6 (11.2, 0-59)* | *11.4 (5.5, 0- 22.8)* | *30.2 (10,23.7-59)* |
| *Eczema 3 months N (%) N100* | *13 (13 %)* | *12 (16 %)* | *1 (4 %)* |
| *Eczema 6 months N (%)* | *24 (24 %)* | *24 (32 %)* | *0 (0 %)* |
| *Eczema 12 months N (%)* | *16 (16 %)* | *15 (20 %)* | *1 (4 %)* |

The median (SD, min-max) of fecal butyrate proportion at 12 months was 15.6 % (11.2 %, 0.0 -59.0%). We observed that infants with higher butyrate levels had significantly lower rates of eczema (Figure 28). Infants with fecal butyrate levels above the 75$^{th}$ percentile were significantly less likely to have eczema in the first year of life (p=0.001), see Figure 28.

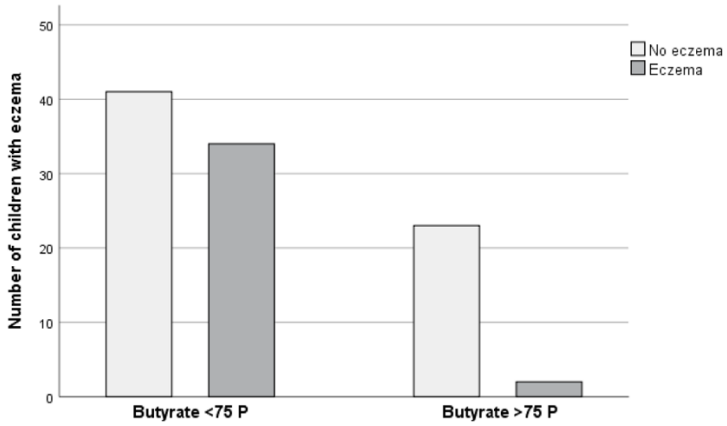


*Figure 28 Eczema observed among 100 infants in the first year of life at 3,6 and/or 12 months with low and high butyrate levels (<75 Percentile and >75 Percentile) and eczema development in the first year of life. p 0.001 (chi square)*

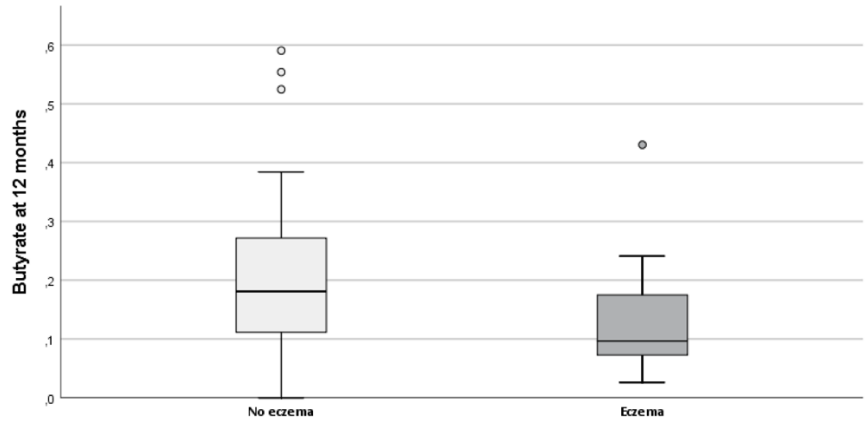


*Figure 29 Fecal butyrate ratio at 12 months in infants with compared to without observed eczema at 3,6 and/or 12 months of age*

The relative butyrate SCFA ratio was significantly associated with eczema in the first year of life in direct logistsic regression analyses $\chi^2$ (1, N100) = 13.22, p< 0.001.

# 8 Discussion and main findings

## 8.1 Maternal diet in mid-pregnancy

Our assessment of dietary intake during pregnancy in a mother-child cohort from the general Norwegian population showed that while many participants had satisfactory intakes of healthy food items such as vegetables (76%), fish (81%), and high intakes of fiber (78%), more than half of the subjects in the cohort reported consumption of excessive amounts of red meat, salt, and saturated fatty acids, surpassing the recommended limits. Additionally, almost a quarter of women reported an alcohol intake of >1g/day in pregnancy, and 19% exceeded recommended daily caffeine levels. High reported alcohol and coffee consumption was associated with educational level. Inadequate intake of essential micronutrients was observed in 54 % for folate, 50 % for iron, 41 % for selenium, 36 % for calcium, 29 % for vitamin D, and 24% for iodine.

Reported fiber intake in our study is higher than in a systematic review and meta-analysis by Blumfield et al. of pregnant women in high-income countries (including Europe, USA, Canada, Australia, New Zealand, and Japan), which found that fiber intake during pregnancy was below recommendations for all included countries with a mean intake level at 18.7 g/day (4.4 SD) [(42)]. In the large prospective Norwegian Mother, Father, and Child Cohort study (MoBa study), including 85 898 women recruited between 1998 and 2008, however, fiber intakes were similar to ours, with a mean fiber intake of 31.0 g/day (SD 10.5) [(206)]. The excess intake of red meat observed in our study was also found in the MoBa study, where only 45% of the pregnant women followed the recommendation for red and processed meat [(54)]. Our finding of excessive saturated fat intake with a median of 33.5 (IQR 18) g/day is

comparable to other studies. Blumfield et al. reported that mean saturated fat intake for all high-income countries collectively was 32.2 ± 9.1 g/day during pregnancy, with all included regions reporting saturated fat intakes above recommended levels [42].

Almost a quarter of women in our study reported alcohol intake >1g/day. A global meta-analysis showed that approximately 10% of women reported alcohol intake during pregnancy, with the highest prevalence in European countries [46]. Alcohol consumption in pregnancy was also reported in 36% of women in the MoBa study [207] and by 23% after week 12 in a study from Oslo, including 1749 women answering a longitudinal questionnaire in 2000 [208]. In contrast, results from the Norwegian Fit for Delivery randomized controlled trial involving 575 Norwegian women showed that 27% reported consuming alcohol weekly before pregnancy, while none reported alcohol intake in early pregnancy [209]. It is not entirely clear why the findings in the Norwegian Fit for Delivery study differed from ours, as both studies used similar FFQs, and had comparable study populations. However, it is possible that their population may have been more health conscious and more likely to adhere to national guidelines, since their intervention included antenatal nutrition counselling and exercise classes. Our study found that daily caffeine intake, with 41% of women exceeding recommended coffee intake, was higher than in the MoBa study, where 10 % of women exceeded recommended caffeine levels [210].

The insufficient intakes of essential micronutrients such as iron, folic acid, calcium, and vitamin D among the pregnant women in our study has also been consistently observed in other countries. A systematic review from 2013 of micronutrient intakes, including 90

studies (n=126 242) on the diets of pregnant women in high-income countries, revealed that the mean intake of folate, iron, and vitamin D was below the recommended levels in every geographic region studied [42]. Iodine deficiencies in pregnancy continue to prevail in certain areas of Europe [211], including Norway. However, compared to our results of 24 % of pregnant women with inadequate reported iodine intake, other studies have reported higher numbers of women at risk of iodine deficiency, such as 46 % of young women in a large Norwegian dietary survey from 2010 [212], and 55 % of pregnant women in the Oslo area with iodine intakes below estimated average requirements [44]. Moreover, there has been a growing concern about poor vitamin D status during pregnancy, with documented insufficiency ranging from 50% to 65 % in the Mediterranean region [213]. A Norwegian study including 855 healthy pregnant women found that 34 % had Vitamin D insufficiency, assessed through 25(OH)D in the third trimester [214] and only 18% of women supplemented with the recommended 10 mcg daily. In another Norwegian study including 2960 women, 61 % of women had Vitamin D intake below the recommended intake [215]. Similar results have been observed in Sweden, with low intake levels of vitamin D and vitamin D deficiency in most women by the third trimester [216]. Over half of pregnant women in our study are at risk for folate insufficiency. Inadequate folate intake during pregnancy has also been documented in high-income countries, with average intake levels between 13% and 63 % lower than recommendations, despite increased energy intake and weight gain being reported [217]. According to data from the MoBA study only 10.22 % of women reported taking supplements containing folic acid from 1 month preconception and through the first 3 months of pregnancy [218].

The screening of diet in the PreventADALL study is the most recent and updated dietary data in pregnant women in Norway. Dietary data collection in large cohort studies is an essential instrument to inform public health recommendations and detect disease associations [219]. Women in the PreventADALL study are older, less often smokers, and have higher educational levels compared with the general pregnant population in Norway, which might limit the generalizability of our results and increased the bias of dietary reporting. A large body of evidence points to the influence of social class on dietary and lifestyle choices [220,221]. Higher level of education is a known independent factor affecting food choice [222]. A total of 83,9 % of women completed a higher education in our study, compared to an average of 55,8 % in the general female population in Norway. Their financial and social resources may have provided for better means to support a healthy lifestyle [220]. Marital status is also known to affect a person´s lifestyle. Most of our study participants were married or cohabitants, with only 1,6 % reporting to be single. Results from a large mother-child cohort study (MoBa) in Norway has previously shown that single mothers tend to have lower dietary quality and include more smokers than women who live with a partner [223].

## 8.2 Infant diet

### 8.2.1 Breastfeeding

We reported a high proportion of breastfeeding throughout the first year of life, with 99 % of infants being breastfed at 1 month, 95% at 3 months, 88% at 6 months, 67 % at 9 months and 51% at 12 months. Exclusive breastfeeding rates among the the No Food Intervention group were 67% at 3 months and 3 % at 6 months.

Breastfeeding rates in our study are higher compared to other nationwide studies, such as the latest Spedkost survey from 2019, including 2182 infants, which found that 85 % were breastfed at 3 months, 78 % at 6 months, and 48 % at 12 months [224]. Breastfeeding rates in our study also exceeded those of another national survey on infant feeding in 2013, conducted by Statistics Norway, including 2500 participants, with breastfeeding rates of 86 % at 3 months, 55 % at 9 months, and 35 % at 12 months. The 29621 infants from the Norwegian Mother, Father and Child Cohort Study (MoBa) also had lower rates of any breastfeeding, as well as another Norwegian study including 1490 infants born in Norway in a three-week period in 2006 [225], as listed in Table 12. Although there are some differences among studies in Norway, overall rates of any breastfeeding have remained relatively steady over the past decade as evidenced by comparing recent numbers with the Spedkost study in 2006/7, where 80% were breastfed at 6 months and 46 % at 12 months. However, the proportion of infants breastfed at 12 months in 1999 was markedly lower at 36 % [226] compared to the results from the PreventADALL study.

Exclusive breastfeeding rates at 6 months in our study are similar to the latest Spedkost survey and MoBa study, which both reported rates of 2%, and lower than the Spedkost survey from 2006/7 reporting an exclusive breastfeeding rate of 9 % [226]. Revheim et al observed stable exclusive breastfeeding rates at 6 weeks among 28503 infants extracted from medical records of Norwegian public health care centres from 2010- 2018. However, exclusive breastfeeding rates at 6 months declined from 28 % in 2010 to 11 % in 2010 [227]. Low exclusive breastfeeding rates at 6 months have previously also been reported in other Nordic countries. A review including studies from Denmark, Finland, Iceland, Norway, and

Sweden found that low proportions (8-12 %) of infants were exclusively breastfed at six months. [76] Similarly, data from the UK Infant Feeding Survey from 2010 showed that 17% of infants born in 2010 were exclusively breastfed at 3 months, 12% at 4 months, and only 1% at 6 months [228].

*Table 12 Reported breastfeeding rates (%) in the PreventADALL study compared to relevant larger studies in Norway and Sweden*

| | ***Breastfeeding*** | *1m* | *2m* | *3m* | *4m* | *5m* | *6m* | *9m* | *12m* |
|---|---|---|---|---|---|---|---|---|---|
| **Norway** | | | | | | | | | |
| **PreventADALL** | *Any breastfeeding %* | **99** | **96** | **95** | **93** | **91** | **88** | **67** | **51** |
| | *Exclusive breastfeeding %* | **-** | **-** | **67** | **-** | **-** | **3** | **-** | **-** |
| Spedkost 2018-2019 [88,229] | *Any breastfeeding %* | 93 | 89 | 85 | 82 | 79 | 78 | 63 | 48 |
| | *Exclusive breastfeeding** | 81 | 74 | 64 | 39 | 13 | 2 | - | - |
| MOBA study [230] | *Any breastfeeding %* | 97 | 94 | 91 | 87 | 84 | 80 | - | - |
| | *Exclusive breastfeeding %* | 85 | 79 | 71 | 44 | 17 | 2 | - | - |
| Spedkost 2006-2007 [226] | *Any breastfeeding %* | 95 | 91 | 88 | 85 | 82 | 80 | 63 | 46 |
| | *Exclusive breastfeeding %* | 82 | 73 | 63 | 46 | 25 | 9 | - | - |
| Kristiansen et al[225] | *Any breastfeeding %* | 96 | 92 | 89 | 86 | 84 | 82 | 55 | 46 |
| | *Exclusive breastfeeding %* | 84 | 75 | 65 | 48 | 26 | 10 | - | - |
| 2013 Statistics [73] | *Any breastfeeding %* | 93 | 89 | 86 | 81 | 78 | 71 | 55 | 35 |
| | *Exclusive breastfeeding %* | 79 | 73 | 65 | 44 | 25 | 3 | - | - |
| **Sweden** | | | | | | | | | |
| 2012 National statistics | *Any breastfeeding %* | - | 87 | - | 76 | - | 63 | 34 | 16 |
| | *Exclusive breastfeeding %* | - | 67 | - | 51 | - | 11 | - | - |
| 2017 National board of | *Any breastfeeding %* | - | 84 | - | 73 | - | 63 | 44 | 27 |
| Health and Welfare [231] | *Exclusive breastfeeding %* | - | 62 | - | 50 | - | 13 | - | - |

*number on exclusive breastfeeding reported for the No Food Intervention group

Our study results indicate that breastfeeding rates have again experienced an incline in the past decade, with a particularly notable increase of any breastfeeding from 3 - 9 months. It is not clear why breastfeeding rates in our study were higher than other studies. Except for a slightly higher maternal age in our study, background characteristics, including higher maternal educational status, were similar across the studies. Sociodemographic factors have been shown to influence breastfeeding rates, with a mother´s level of education being a predictor for the length of exclusive breastfeeding. [232,233] However, exclusive breastfeeding rates in our study were low, despite a higher maternal educational level.

One may speculate that parents may believe that prolonged breastfeeding could prevent atopic disease, and that may have influenced a mother's decision to breastfeed for a longer duration.

### 8.2.2 Complementary feeding and time of complementary food introduction

We showed that infants not subject to early food intervention, introduced complementary foods at a mean age of 18.3 weeks. Almost half of our study population was introduced to porridge, already at 4 months, which was the most common first infant food. Infants randomized to early food intervention were more likely to consume peanuts, egg, and dairy products, also later in infancy and had a higher diet diversity at 9 and 12 months compared to infants following regular weaning advice.

Our results on the time of complementary food introduction are in line with recent data from the Norwegian nationwide Spedkost study, which showed that a total of 62 % of infants were introduced to complementary foods at 4 months and 98 % by 6 months [224]. Another Norwegian study based on the RCT Early Food for Future Health found that 5% of the 715 infants were introduced to solid food before 4 months of age, while 14% were not introduced to solid food at 5.5 months of age [234]. In Norway, the introduction of complementary food prior to 4 months has decreased over the past two decades, from 21 % in 1998 to 11 % in 2006, 7% in 2013, and 6 % in 2019, which is slightly lower than in our cohort [226,234].

The time of complementary food introduction varies between countries. In most European countries complementary foods are introduced before 6 months [235]. Schiess et al reported data on the timing of introduction of complementary food in infants born between 2002-2004 in five EU countries (Belgium, Germany, Italy, Poland and Spain) [87]. Complementary foods were introduced earlier in formula-fed infants than in breastfed infants, with significant differences between countries. Approximately 37% of formula-fed infants and 17% of breastfed infants received complementary food earlier than that at 4 months, with >75% versus >50% receiving complementary food at 5 months and 96% versus 87% receiving complementary food at 6 months for formula-fed and breast-fed infants, respectively [87]. In contrast, a cross-sectional analysis of 1482 children from the US found that 16.3 % of infants were introduced to complementary food before 4 months, while 45 % had not yet been introduced to complementary food at 6 months of age [236].

We did not ask about reasons for early complementary food introduction. Our cohort consisted of 64 % first-time mothers [237], which could one reason for earlier complementary food introduction. Mothers provide various arguments for introducing complementary food early. A systematic review, including studies from Europe, Australia, and the US, found that the most common reasons were related to infant weight gain, sleep issues, and infant contentment [238].

Apart from porridge, vegetables (including root vegetables) and fruit were the most common foods to be introduced before six months. In our study, 93 % of infants consumed fruit, 87% consumed root vegetables, and 67 % consumed vegetables at 6 months. The

Spedkost 2019 survey also found various positive changes in eating patterns over the past decade, such as increased consumption of fruit and vegetables and higher intake of homemade porridge and wholegrain bread [229]. Multiple observational studies have documented that introduction of vegetables early in the weaning process leads to a higher acceptance and intake of these foods also years later.[239] An observational study (n=203) in France found that infants introduced to vegetables early were more likely to accept new vegetables, and new food acceptance increased with the number of foods introduced in the early weaning period.[240] Similar findings from a UK study including 7866 mothers and infants show that infants who were given more home-cooked fruit and vegetables at 6 months had higher intakes of fruit and vegetables at 7 years of age.[241] Overall, we believe that the trend towards early introduction of vegetables and fruit observed in our study could be beneficial.

Dairy products were introduced to 60% for the first time after 6 months of age, which is similar to an observational study from Sweden including 11 081 infants showing that 30.2 % received cow milk before six months [242]. The TEDDY study, including 7366 infants from the United States, Finland, Germany, and Sweden, found that the consumption of regular cow's milk as a drink or mixed in food was common, regardless of country [243]. Since cows' milk is a poor source of iron and contains an excess amount of protein, fat, and energy, it is not advised as a main drink before 12 months of age, although small volumes may be added to complementary foods [55]. The WHO states that if infants 6–11 months of age are fed animal milks, full fat milk should be used [59]. Milk and other dairy intake by 12 months were higher in the food intervention groups compared to no-food intervention groups, in whom

approximately half of infants received cow's milk by 12 months and 79.1 % yogurt, which is consistent with the findings of Spedkost 3, where 46 % were given cow's milk as a drink and 73 % yogurt by 12 months.

Our results further showed that infants randomized to the early food introduction continued to regularly ingest interventional foods even after the intervention period had ended. This finding would suggest that encouraging parents to introduce allergenic foods early will make it more likely to maintain intake levels throughout the first year of life, which would be beneficial to maintaining tolerance and preventing food allergies long-term. More importantly, it is likely that more parents have become aware of the benefits of earlier introduction to allergenic foods as a means of reducing the risk of food allergies. Comparison of results from the Spedkost studies, with 24 % of parents avoiding the introduction of allergenic foods, such as nuts, diary, and citrus fruits in 2019 vs 45 % in 2007, might indicate a shift in parents' perception over the past decade. The increase in educational level from 2007 to 2019 in the Spedkost study population may also have influenced the results [224].

Around 9% of infants received only home-cooked food, and 11% only commercially available foods up to 9 months, whereas 48% of our the PreventADALL infants were given more commercially prepared than homemade food at 6 and 9 months. The findings are unsurprising as porridge is a common first food, and infants in Norway commonly consume commercial options due to their fortification with iron [229]. Similar trends have been observed in other European countries [228], as well as in the US [244]. In the UK, national feeding data showed that a higher percentage of infants aged 4-6 months were being fed

commercially prepared baby food compared to homemade baby food (38% versus 28%) [228], and almost half (45%) of mothers with 8-10-month-old infants used commercially prepared baby foods at least once a day. A German birth cohort study found that 94.4% of infants consumed at least one commercially prepared baby food product within a 3-day period, while only 5.6% exclusively consumed homemade complementary food [245]. In the United States, a national cross-sectional feeding survey conducted in 2002 showed that between 73% and 95% of infants aged 4-12 months consumed commercially produced foods [244].

The benefits of commercial, also called “ready-made” infant foods, versus homemade foods, depend on the quality of each option. Well-prepared homemade foods can offer a greater variety of flavour and textures and higher energy density [246]. Previous studies have found that diet diversity was higher in infants receiving homemade foods [247]. However, homemade foods can be unsuitable if they contain excessive sugar or salt, and cooking methods can influence nutrient content. Studies have shown a lack of vegetable variety in commercially prepared foods, with a dominance of sweet vegetables [248,249].

The Norwegian Directorate of Health's recommendations from 2001 advising exclusive breastfeeding for 6 months was controversial since research data could not support the health benefit of delayed introduction of other types of foods. It seems necessary to highlight that early introduction of solid foods should not come at the expense of breastfeeding, and improving global breastfeeding initiation and continuation should be an ongoing effort. The recent Lancet Breastfeeding series highlights the inadequate progress in improving breastfeeding practices globally, mainly due to marketing strategies by

commercial milk formula industries [250]. Breastfeeding is associated with protection against infections and reduced risk of hospitalization, as shown in the Norwegian Mother, Father and Child cohort study (MoBa study) including 70 511 participants [251]. However, the protective effects of breast milk remained even if the child receives complementary food from the age of 4 months [251]. In Norway, after several years of discussions, the revision that followed in 2016 ended up with a divided recommendation, opening for solid food introduction from 4 months of age [73]. The European Food Safety Authority (EFSA) panel recently suggested that available data on the nutritional adequacy of 6 months of exclusive breastfeeding is insufficient and, therefore, the timing of complementary food introduction needs to be decided on an individual basis [63]. Furthermore, there seems to be no convincing evidence that the introduction of complementary food is linked with any adverse or beneficial health effects. On the contrary, earlier introduction of specific foods has shown benefits, as several trials have shown over the last decade, summarized in a recent systematic review and meta-analysis by Scarpone et al [86]. Results from our study [71] showed a 60% lower relative risk of food allergy at 3 years of age if the child was introduced to peanuts, eggs, milk, and wheat from 3-4 months of age compared to following the national recommendations (exclusive breastfeeding to 6 moths of age). The possible benefit of early food introduction on allergic disease development point in the same direction as two other well-known studies, the Learning Early about Peanut Allergy (LEAP) trial from 2015, and the Enquiring About Tolerance (EAT) trial [70,72]. The LEAP study found that peanut consumption from 4 to 10 months of age decreased peanut allergy in high-risk infants. The EAT trial, which introduced multiple allergenic foods to a general cohort of breastfed infants from 3 months of age, found a significant reduction in food allergy in infants who adhered to the food

intervention. The possible benefits of early food introduction are important to consider when assessing current infant feeding guidelines and have recently been echoed in guidelines from Sweden, encouraging small tastes of complementary food from four months of age, as long as it does not compete with breastfeeding [82].

There is no doubt that breastfeeding is important for the child's health [252]. However, recommendations on exclusive breastfeeding for 6 months are largely based on observational studies [73]. Advice on the duration of exclusive breastfeeding should ideally be based on RCTs rather than observational studies due to risk of bias and reverse causation in the latter study design.

## 8.3 The impact of early complementary food introduction on breastfeeding, and dietary diversity (Aim2)

The high breastfeeding rates of around 86% at 6 months and 50% at 12 months of age in our study were not affected by the food intervention. Earlier introduction of complementary foods was associated with a higher dietary diversity score at 9 and 12 months.

The observation in our study that early food introduction did not affect breastfeeding rates is similar to findings in the EAT study, with 97% of mothers continuing to breastfeed while introducing allergenic foods, and breastfeeding rates in the study group surpassed those in the UK by a significant margin [253]. These results provided evidence that introducing solid foods early on did not have any negative effects on the duration of breastfeeding. Similarly,

the LEAP trial, which randomized 640 atopic infants aged 4 to 11 months to regular consumption or avoidance of peanuts, found no difference in breastfeeding rates between the two groups [89]. These findings align with a Swedish observational study involving 1177 mother-infant pairs, which also found no association between breastfeeding duration and the age at which complementary foods were introduced [254].

Findings from other observational studies, however, have raised concern about the possible negative effects of complementary food introduction on breastfeeding. A US study from 2008, including 2970 infants at birth, found that 41% of infants that were consuming complementary foods at 4 months were more likely to have discontinued breastfeeding at 6 months (70% vs 34%) [255]. Although this study did not report the length of maternity leave, it may be a reason for earlier breastfeeding cessation and introduction of complementary foods in the US, as another recent US study including 12301 women showed that 66 % of women took less than 3 months of leave after giving birth and these women also reported shorter length of breastfeeding [256]. In the retrospective "Swedish Pregnancy Planning Study" 1251 mothers completed three questionnaires up to one year after birth. The study's main findings revealed that half of the infants were introduced to "tiny tastings" at four months, and the earlier these were introduced, the sooner the infants consumed larger amounts of food. Infants' age upon introduction was identified as having a negative effect on breastfeeding duration [257]. A German study including 366 infants found that those with a higher intake of complementary foods were fully breastfed for five weeks less than those with lower complementary food consumption, and overall breastfeeding rates were 10 weeks shorter. This study, however, quantified intake consumption of foods and did not

assess the time when complementary foods were introduced on breastfeeding rates [245]. A recently published meta-analysis of data from 3 UK-based cohorts, including 10407 infants, reported that earlier introduction of complementary foods was associated with a shorter duration of breastfeeding. The analysis, including the Avon Longitudinal Study of Parents and Children (born 1990-1991), the Southampton Woman's Survey (1998-2008) and Infant Feeding Survey (2010), found that introduction before 4 months was associated with breastfeeding cessation before 6 months in all three cohorts, with little effect of adjustment for maternal sociodemographic characteristics [258].

Importantly, only RCTs can avoid the issue of reverse causality associated with cohort studies. There are now high-quality RCTs that demonstrate that early complementary food introduction does not adversely affect breastfeeding.

Although few studies have assessed the time of complementary food introduction on diet diversity, we are not the first to show an association between early introduction and higher diet diversity in later infancy. In an observational study from the US including 2907 infants, the introduction of complementary foods by 4 months was the only factor significantly associated with the infants' consumption of a varied diet at 9 months, from 3 food groups: cereals, fruits and vegetables, and meats or meat substitutes (including fish, egg, peanuts and soy) [255]. However, our study is, to the best of our knowledge, the first RCT assessing the time of complementary food introduction on diet diversity.

## 8.4 The temporal development of the infant gut microbiome and SCFA production and the potential impact of breastfeeding and time of complementary food introduction in the first year of life (Aim 3)

In the longitudinal sampling of feces in 100 infants from birth until 12 months of age, we observed that *Bifidobacterium* dominated the gut until 6 months of age, with a shift to Firmicutes at 12 months of age. The average number of unique species increased with age and reached the highest diversity at 12 months, with *Clostridiales* representing 67% of the microbiota composition at 12 months. Within the *Clostridiales*, the most prominent genera were *Faecalibacterium, Ruminococcus gnavus (R. gnavus)* and *Eubacterium rectale (E. rectale)*, while the representation of *Bifidobacterium* declined from 32% at 6 months to 8% at 12 months. The delivery method significantly influenced the initial colonization of gut bacteria. In terms of SCFA development we observed a significant increase in fecal proprionate, and a 4-fold increase in butyrate levels from 6 to 12 months of age. Approximately one in four children had a low abundance of *E. rectale* and *Faecalibacterium prausnitzii* and, in turn, low fecal butyrate. Infants with low butyrate associated with the presence of *R. gnavus.* Breastfeeding at 3 months was associated with microbiota composition, especially with *Bifidobacterium*, while no associations were found at 6 and 12 months of age. Despite the early abundance of *Bacteroides* during infancy, an inverse correlation was observed with breastfeeding at 3 months. Time of solid food introduction did not explain the distinct bacterial networks observed in the infant gut at 12 months. The proteomic analysis of the *E. rectale* bacterial network revealed several glucoside hydrolases, with the ability to degrade dietary fiber. In contrast, infants with low butyrate and higher

abundance of *R. gnavus* bacterial networks expressed different glycoside hydrolases, related to mucin and HMO.

Our finding that *Bifidobacterium* dominates the infant gut in the first months of life is well established [259,260], since an infant's diet is initially compromised of only breastmilk and/or formula. In line with our results, a Swedish cohort of 83 children showed that the diversification of gut microbiome is mostly associated with age and phylum distribution becoming more similar to mothers at 12 months [261]. A longitudinal study from the UK which performed 16 rRNA sequencing of 500 stool samples collected from 6 weeks until 12 months of age and shotgun metagenomic sequencing in 350 samples, found an increase of microbes such as *Ruminococcus* and *Faecalibacterium* over the first year of life, while bacteria such as *Bacteroides, Bifidobacterium, Escherichia coli, Staphylococcus* and *Klebsiella* decreased [262].

Our finding of two distinct bacterial networks represented by *E. rectale* (in 43 infants) or *R. gnavus* (in 27 infants) and their negative correlation to each other is novel to the best of our knowledge. The presence of *R. gnavus* in the infant gut, however, has also been observed in other studies. In a study of 25 infants followed until 2 years of age, *R. gnavus* was recorded in all but 3 children [263]. In a twin cohort study increased abundance of *R. gnavus* was observed before the onset of allergic manifestations and was associated with respiratory allergies or respiratory allergies coexistent with AD [264]. In the same study mice fed *R. gnavus* developed airway hyper-responsiveness and had histologic evidence of airway inflammation [264]. In another mouse study, however, administration of a specific *R. gnavus*

strain alleviated allergic disease-associated parameters (e.g., transepidermal water loss, total IgE levels, and skin inflammation) through increased regulatory T-cell counts and SCFA production [265]. These differences highlight the fact that different *R. gnavus* strains can either elicit pro- or anti-inflammatory responses [266]. In the adult gut microbiota, *R. gnavus* has been positively associated with inflammatory bowel disease in a number of studies [267], although a causal relationship remains to be demonstrated. The presence of *R. gnavus* and its related bacteria, coupled with low butyrate levels in a significant number of infants in our study, could have clinical implications and will be investigated in a continuation of this project.

Our finding that vaginal delivery is associated with *Bifidobacterium* and *Bacteroides*, while caesarean section was associated with *Clostridiales*, is in agreement with other large-scale studies [132,141]. Recently published data from our study have demonstrated that infants born through vaginal delivery exhibit early colonization by members of the Bacteroidaceae family, while infants delivered via CS display a delayed colonization pattern [268].

Infants in our study had a lower relative abundance of fecal butyrate at 6 months, compared to a study including 43 healthy Estonian infants and 25 healthy Swedish infants; however, with a larger increase between 6 and 12 months [269]. It is likely that the later time of complementary food introduction in our study might have caused the slower emergence of butyrate. The large increase of butyrate in the present study was associated with two distinct members of the *Clostridiales, E. rectale* and *Faecalibacterium prausnitzii*, with both being considered an indicator of human health [270]. Other studies have also confirmed that

species from *Eubacterium*, *Faecalibacterium* and *Roseburia* compromise a significant portion of butyrate producers [271,272].

We found no significant difference in the relative abundance of SCFAs between 12 months old infants and their mothers, which is in line with other studies have showing that pre-weaned infants exhibit the highest total SCFAs levels and that fecal SCFAs levels decrease with age [273], suggesting that 12 months old children already possess a similar SCFAs profile to adults. However, using fecal samples as proxies for SCFAs production might be misleading, as fecal SCFAs levels only represent the non-absorbed SCFAs that are produced [274]. Therefore, the inverse relationship between SCFAs levels and age may indicate a lower rate of absorption in infants, rather than a decrease in SCFAs production by the gut community.

In our study, breastfeeding at 3 months was associated with *Bifidobacterium*, however, this association was no longer present at 6 and 12 months of age. The transition from an infant-like gut microbiota to an adult-like one is marked by two significant events: the weaning of breast milk and the introduction of complementary foods [275]. It is well-established that *Bifidobacterium* tends to dominate the gut microbiota in pre-weaned infants due to their specialized ability to break down milk oligosaccharides (HMO) [276]. The large Environmental Determinants of Type 1 Diabetes in the Young study (TEDDY) showed in a longitudinal sampling of 903 children that the gut microbiome composition was largely determined by breastfeeding and that breastmilk was most significantly associated with *Bifidobacterium* [277]. Moreover, the receipt of breastmilk explained the observed variance from 3 to 14 months of life, and infants who were breastfed had a lower diversity compared to infants no

longer receiving breastmilk due to the dominance of *Bifidobacterium* in breastfed infants [277]. Although a higher microbial diversity is associated with health in adults, these findings cannot be inferred to infants since the microbial composition and immune functions differ significantly from adults [278]. It has been speculated that the lower diversity in breastfed infants due to the predomination of *Bifidobacterium* is beneficial for babies' health [279]. In a meta-analysis of 7 microbiome studies, including 1825 stool samples from 684 infants, a shorter duration of exclusive breastfeeding was linked to increased gut microbiota age, as well as an earlier abundance of bacterial families other than Bifidobacteriaceae [280]. In contrast, a longer duration of exclusive breastfeeding was associated with a higher abundance of *Bifidobacterium* and a more stable microbiota composition in the first months of life. The authors hypothesized that the initial gut microbiota composition in infants not breastfed, might not be proportionate to their immunological maturity [280]. Similarly, infants who were no longer breastfed at 3 months in the CHILD cohort study had higher microbiota diversity and maturity [281]. Recently an RCT study demonstrated that supplementing infants with *Bifidobacterium infantis* showed promise in mitigating the effects of systemic inflammation [151], providing possible mechanistic explanations for the observed correlation that a lack of *Bifidobacteria* is associated with immune dysregulation and immune-mediated disease in early life [282,283].

We did not detect an association between breastfeeding from 6 to 12 months of age and microbiota composition. This is somewhat surprising, as breastfeeding rates decreased significantly from 88% at 6 months to 51 % at 12 months, and other studies have shown that the cessation of breastfeeding appears to have the most pronounced impact on microbiota

composition in infancy [156]. A study in 107 healthy infant-mother pairs found that the proportion of daily breastmilk intake was associated with changes in the infant gut bacterial community in a dose-dependent matter, even after the introduction of solid foods and suppression of Firmicutes in infants receiving breastmilk was noted. [177].

We are not the first to show a negative association between breastfeeding at 3 months and *Bacteroides.* The meta-analysis by Ho et al. found that *Bacteroides* had an increased abundance in infants not exclusively breastfed in all 7 studies included [280]. A study including 91 term infants showed that *Bacteroides* was the third most abundant genus in breastfed infants at 40 days but decreased up to 3 months [279]. A study including 15 breastfed infants and 6 formula fed infants, on the other hand, found that *Bacteroides* was associated with breastfeeding [284]. However, the small sample size, might limit the generalizability of the results. The observed negative association between *Bacteroides* and breastmilk in our study might be attributed to their interactions with *Bifidobacterium*. Given that *Bifidobacterium* is consistently supplied with breastmilk, it has the potential to hinder the growth of *Bacteroides* by producing antimicrobial compounds and outcompete *Bacteroides* in the utilization of HMOs [285,286].

We found that neither breastmilk feeding nor time of introduction to complementary foods explained the difference in abundance of *E. rectale* or *R. gnavus* and it is unclear what caused the different gut community states at 12 months. Other studies have shown that a dietary shift towards complementary foods results in modifications to the gut microbiota, characterized by active recruitment of *Clostridiales, Bacteroidetes,* and a reduction in

*Bifidobacterium* populations [156]. A study assessing fecal microbiota in 605 European infants (Sweden, Scotland, Germany, Italy and Spain) 4 weeks after the introduction to complementary foods found that *Bifidobacteria* still dominated the gut, however, the microbiota composition had diversified [146].

Although time of complementary food introduction showed no significant effect on microbiota composition in our study, it is still likely that dietary factors played an important part in the different microbiota compositions observed, based on the results of our metaproteomic analysis. In the infant gut, bacteria compete for dietary substrate while also relying on a symbiotic relationship to extract energy from carbohydrate fermentation [287]. Breastmilk and infant formula, constituting the first infant foods, provide glycan sources such as lactose, HMOs and/ or galacto- and fructo-oligosaccharides. With the gradual introduction of complementary foods, the range of complex plant-derived carbohydrates from grains, fruits and vegetables expands, to include cellulose, hemicellulose, pectin and starches, thereby increasing the diversity of available nutrients for the gut bacteria [287,288]. We have previously shown that, coupled with dietary fiber introduced to the infant, the carbon source utilization potential changed and the microbiotas capability of degrading dietary polysaccharides increased [289]. The difference in detected proteins in our metaproteome analysis of the *E.rectale* and *R.gnavus* associated networks is likely influenced by different dietary sources. However, we did not include different complementary foods in our analysis and can, therefore, only indirectly assume that dietary factors played an important role. Other studies have also observed that the transition to solid foods induces a more mature microbiota, structured to metabolize plant-derived

polysaccharides, with genes responsible for complex carbohydrate and starch degradation, as well as vitamin production [161,290]. A Swiss study including 175 fecal samples found that the presence of *E. rectale* and *Faecalibacterium prausnitzii* increased after the introduction of solid foods and in line with our own results, they found that butyrate levels in infants correlated with the presence of these bacteria [291].

Other studies have shown that the transition from an exclusively milk-based diet to one consisting of complementary foods causes a transition to a more adult-like microbiota [275]. Microbial richness in infancy seems to be driven by food items with higher fiber and protein content [101,158]. Moreover, diet diversity has been shown to influence multiple gut microbial diversity metrics in a recent large study by Xiao et al including 1916 adults [292]. Higher dietary diversity and increased fiber intake could protect against allergic disease development by modulating the gut microbiota [293,294]. Therefore, our finding that early introduction of complementary food led to a higher dietary diversity at 9 and 12 months of age, could in turn lead to a richer gut microbial community affecting both SCFA production and influencing immune tolerance.

## 8.5 Short-chain fatty acids and atopic dermatitis development

In a pilot including 100 children, high fecal butyrate levels at 1 year of age was associated with a reduced the risk of developing AD in the first year of life.

Our findings that higher butyrate levels were associated with lower risk of AD are in line with previous studies assessing potential associations between gut butyrate and AD

development. Nylund et al. characterized the microbiota signatures in 11 healthy infants and 28 infants with varying extents of eczema and found that the severity of AD correlated with the abundance of butyrate-producing bacteria in 6-month-old infants. Specifically, they found that infants with mild eczema had 1.6-fold higher diversity index values, and two-fold higher butyrate-producing bacteria than infants with more severe eczema [193]. Another study from Singapore including 63 infants found low levels of butyrate and impaired microbial trajectories with colonization of *E.coli* and *K. pneumoniae* as early as 3 weeks in infants who later developed eczema. The underlying hypothesis being that this colonization pattern might compete and delay for butyrate and propionate-producing bacteria [295]. In a Korean study of 132 children, including 90 with AD, Song et al. found lower levels of fecal butyrate concentration among patients with AD, which was possibly linked with dysbiosis of *F. prausnitzii* composition and gut epithelial inflammation [194]. Low levels of butyrate in 3-month-old infants were shown to precede the development of eczema in another study, which classified feces samples in 33 children based on the absence or presence of eczema in the first two years of life [296]. Similar findings have also been observed for other allergic disease manifestations. A study involving 105 infants found that children who developed atopic sensitization later in life lacked bacterial species involved with butyrate production and key genes encoding for carbohydrate fermentation at 3 months of age [297]. In contrast, a clinical trial using non-digestible oligosaccharides in infant formula in 138 high-risk infants found that higher levels of fecal butyrate at 3 months of age but lower levels of butyrate at 6 months of age were associated with eczema at 18 months [298].

Differences in infant fecal SCFA levels between those who develop AD and those who do not could be an important link between gut microbiota, the mucosal immune system, and

eczema development. Short-chain fatty acids might prevent the development of atopic disease in various ways. Studies have shown that specifically butyrate and acetate, can directly enhance the production of regulatory T-cells in the colon of mice [299,300].
The extent to which butyrate is produced depends on a number of factors, such as the abundance of butyrate-producing bacteria, but also the availability of dietary substrate, and the gut environment [301]. It is likely that diet, both directly and through microbiota interactions and production of butyrate, may prevent the development of atopic disease. Roduit et al. showed in 1041 infants from the Protection against Allergy-Study in Rural Environments (PASTURE) birth cohort study that the diversity of complementary foods in the first year of life was significantly associated with a risk reduction of having AD with onset after the first year of life, and yogurt introduced in the first year of life, reduced the risk for AD [94]. The PASTURE study, as well as others, further showed a strong risk reduction for the development of AD and asthma later in childhood when vegetables, fruit, butter, and yogurt were given to the infant in the first year of life [94,302,303]. A prospective study from the Netherlands including 2558 infants found that a delay in the introduction of cow milk products was associated with a higher risk for AD. In addition, a delayed introduction of other food products was associated with an increased risk for atopy development at the age of 2 years. [304]

Roduit et al. conducted a follow-up study from the same cohort, including 301 children, looking at the association between fecal SCFA at one year of age and the association with early life exposures, especially diet, and allergy and asthma up to six years of life [192]. The underlying hypothesis being that the protective effects observed could be attributed to

SCFAs, which could be derived either from the consumption of dairy products containing SCFAs or from the microbial fermentation of dietary products. They found that in addition to yogurt, the introduction of fish in the 1st year of life and vegetables and/ or fruits in the first 6 months were associated with an increased level of butyrate. Infants with the highest levels of fecal butyrate and propionate (95$^{th}$ percentile) at one year of age had less atopic sensitization and asthma between three and six years. Moreover, children with the highest levels of butyrate were less likely to report a diagnosis of food allergy or allergic rhinitis. However, in this study, no difference between the high and low levels of SCFAs in terms of AD development was observed [(192)]. Thorburn et al. showed that a high-fiber diet altered the composition of the microbiota, increased SCFA production (particularly acetate), and protected against the development of allergic airways disease in adult mice, as well as reduced airways symptoms in the offspring of 61 pregnant women, 42 of whom had asthma [(173)]. The protective effects of butyrate in early life are not limited to allergic disease development but also seem to influence other immune-mediated illnesses, such as type 1 diabetes [(277)].

In support of the underlying hypothesis for the aims of this thesis, Trompette et al. recently used a murine skin model to show that butyrate plays a crucial role in promoting skin barrier function, emphasizing the important role of the gut-skin axis in the prevention of allergic disease [(305)]. Specifically, the study showed that mice fed a high-fiber diet or given oral SCFA, particularly butyrate, experienced a decrease in allergen-induced breaches in the skin barrier, reduced allergen entry and sensitization and, consequently, less inflammation in the skin [(305)].

There are limitations to our pilot study, particularly with butyrate levels measured at 12 months and AD outcomes observed throughout the first year of life. Thus, we might have observed an association, but this study design cannot ascertain possible causation. However, with available longitudinal data, this may be investigated in future PreventADALL studies with a considerably larger study population.

# 9 Methodological considerations

The prospective design of the PreventADALL study involving a large number of participants from a general population and the systematic collection of detailed questionnaires is a major strength of the study. The repeated weekly electronic diaries and 3-monthly questionnaires limit the likelihood of recall bias and ensure accuracy of data. Our study had three different study sites with close collaboration ensuring standardisation of all clinical and other data collection methods. Also, the high rate of infants attending the follow-up investigations is a strength of the study.

The PreventADALL FFQ was previously certified as a valid tool for assessing the diet of women during pregnancy, according to energy, nutrients, and food items [198]. However, all FFQs are subject to large between-person errors and evaluation studies have highlighted the risk for weakened associations with nutritional health outcomes when using this form of dietary recall [306]. Still, it remains the most-used instrument, especially in large cohorts. Biomarkers have emerged as potential complementary tools to self-reported dietary data. Their use is, however, not recommended for nutritional assessment in cohort studies to

date, due to a lack of validation studies. Therefore the need to rely on self-reported data remains [307]. Dietary data should in general be interpreted cautiously, due to insufficient information about composition and complexity of foods and food practices. Nutritional research studies are predisposed to social desirability bias; a tendency of study participants to answer questions in a way that will be viewed favourably by others. Overreporting of good habits that do not reflect participant's actual diet and lifestyle choices, is likely to have affected our dietary reporting as well. This study focuses on the dietary habits of Norwegian women; however, these data may not be representable for pregnant women in other countries, and therefore, the implications may differ in other settings.

While self-reporting can introduce response bias, we took steps to address this concern in our infant data analysis. Our results using imputed data for missing diet diversity and sensitivity analysis yielded similar results. Regarding diet diversity assessment, the EAACI task force recommends food recall periods within the first year of life and repeated measures of intake, as was done in our study, instead of just recording consumption of a food group at only one time point. [93] Moreover, consideration must be given to diet diversity being affected by ethnic backgrounds, which could lead to unmeasurable characteristics [93]. Overall, more research and validation studies are needed to improve diet diversity as a measuring tool and to assess its usability for various clinical outcomes and contexts [90].

In terms of microbiome analysis, we were able to longitudinally sample feces in the same 100 infants at birth, 3 months, 6 months, and 12 months of age, and their mothers at 18 weeks of pregnancy. A strength of the study is that we not only performed 16s rRNA

sequencing but utilized reduced metagenome sequencing to detect and deduce functions from the bacterial composition. Furthermore, whole metagenome shotgun sequencing was performed on a subset of biological samples to predict bacterial pathways with respect to SCFAs production, and metaproteomics was used to observe their potential ability to catabolize complex carbohydrates. Through an in-depth approach like this we believe that a deeper understanding of infants' gut bacterial composition and function can be gained. Since the human gut microbiome composition shows large intra-individual differences in humans, there will always be confounding factors that might significantly influence the results, making it challenging to draw conclusions from small-scale studies. [308]

# 10 Main conclusions

1. **To assess the maternal diet in mid-pregnancy and the diet of her offspring in the first year of life**

Reported dietary intake during pregnancy was appropriate for vegetables, fish, and fiber, while more than half reported excessive consumption of red meat, salt, and saturated fatty acids. Inadequate intake of essential micronutrients, such as folate, iron, selenium, calcium and iodine were reported in in 1/4 to 1/2 of pregnant women.

Breastfeeding rates throughout the first year of life, with 99 % of infants being breastfed at 1 month, 95% at 3 months, 88% at 6 months, 67 % at 9 months, and 51% at 12 months, while exclusive breastfeeding…in the No Food Intervention group.

2. **To explore if early complementary food introduction affected infant diets including breastfeeding rates or diet diversity in the first year of life**

Early complementary food introduction increased diet diversity at 9 and 12 months but did not affect breastfeeding rates or duration.

3. **To explore the temporal development of gut microbiome and SCFA production, and the potential impact of breastfeeding and time of solid food introduction in the first year of life**

Gut microbiome diversity increased from birth through 3, 6 and 12 months of age, with *Bifidobacterium* dominating until 6 months of age, and *Clostridiales* representing 2/3 of the microbiota composition at 12 months. Both dietary factors and changes in microbiota composition influenced fecal SCFAs, particularly butyrate, which increased 4-fold from 6 to 12 months. We observed that *Eubacterium rectale* and *Faecalibacterium prasunitzii* are potential key bacterial members responsible for butyrate production in the infant gut, while *Ruminococcus gnavus* was associated with low butyrate levels. Breastfeeding at 3 months was positively associated with *Bifidobacterium* and negatively associated with Bacteroides, while no associations were observed with breastfeeding at 6 and 12 months of age. Time of solid food introduction did not explain the distinct bacterial networks observed in the infant gut at 12 months.

4. **To explore if butyrate at 12 months of age is associated with atopic dermatitis development in the first year of life**

High fecal butyrate levels at 1 year of age were associated with a reduced risk of developing atopic dermatitis in the first year of life.

# 11 Clinical implications and future perspectives

Nutrition is one of the most easily modifiable environmental factors during early life- including the mother´s diet in pregnancy. Our study underscores the need for targeted interventions to improve the nutritional status of pregnant women in the general Norwegian population. Adequate dietary advice to women before and during pregnancy seems a promising strategy for preventing chronic diseases in future generations. By addressing the specific areas of inadequate nutrient intake and promoting healthier food choices, we can support the overall health and well-being of both mothers and their children.

In our study, early food intervention did not negatively affect breastfeeding rates but increased dietary diversity in infancy. We, therefore, believe that our results indicate a possible positive impact of early food introduction beyond just allergic disease prevention.

The Directorate of Health states that they have no plans to update the current guidelines but await a new update from the World Health Organization. This way of thinking assumes that optimal infant nutrition is the same regardless of where in the world infants live and under which circumstances and conditions. In low-income countries, long-term exclusive breastfeeding is an important way to prevent infectious diseases and malnutrition. In high-income countries, however, children may face other health challenges, such as increased risk of allergic disease. At the same time, they are less likely to experience a deficiency of macronutrients in the complementary feeding period [55]. Complementary food for infants in Norway is nutritious, clean, and does not pose a health threat. Therefore, a varied diet should be encouraged while also promoting breastfeeding.

Naturally, the gradual increase of solid foods should follow an infant´s cues. Most babies show a clear interest in food around 4-6 months of age and would find both pleasure and benefit from tasting food at this stage. Starting the gradual process of introducing complementary foods around this period has the added effect of giving a baby plenty of time to practice before the need for food and nutrients becomes greater. Parents should also be encouraged to think of variety/diversity as a central theme of their child´s diet, offering a variety from different food groups, such as fruit, vegetables, grains, nuts, dairy products, and fish. Renal- and gastrointestinal functions are sufficiently mature to metabolize complementary foods at 4 months [309]. Both neuromotor development and apparent interest in non-milk foods will differ between children. Therefore it would make more sense to determine an appropriate age range for introducing solid food, rather than a definite time point [63].

Moreover, given how well microbes in the gut respond to diet, eating a variety of foods is most likely the best way to increase microbial diversity early on. Since microbiome signatures are established in the first three years of life, this marks the time when interventions and active strategies to manipulate the evolving gut microbiota will likely yield the greatest results. These findings should also be considered when assessing the ideal age to start complementary feeding of infants, particularly in high-income countries such as Norway.

Our results support the hypothesis that butyrate is a key component influencing immune processes in early life. Our data further suggest that there is a link between diet, the

metabolic potential of the gut microbiome and atopic disease development. More studies are needed to identify keystone species of a healthy infant's gut microbiota and, most importantly, their metabolic properties. Further studies on the role of diet, gut microbiota, and metabolites will likely lead to a new approach to our understanding of atopic disease development and its treatment and prevention. Although prevention is unlikely to be achieved with a single approach, and strategies will probably be customized to individuals, several hopeful means have emerged, such as a targeted manipulation of the microbiome. It has been suggested that dietary SCFA therapy might hold promising potential to prevent the development of allergic disease, however this has not been tested in infants [310]. Moving forward, it will be crucial to assess which diet-specific features most effectively promote a healthy microbiome and use this information to update evidence-based dietary recommendations. More studies, including intervention studies in the complementary feeding period, using detailed dietary recordings coupled with measurements of the gut microbiota (microbiome and metabolome profiling) are needed. Dietary diversity might become a central variable; and a consensus on how to define dietary diversity needs to be reached. More studies are needed to assess how diet diversity can be used and modified to impact the microbiome.

Our understanding of the role of microbiota in health and disease continues to evolve at a rapid speed. The key moving forward will be to bridge the gaps in analysis and interpretation and incorporate existing data into a systems biology context of humans and their environment. The PreventADALL study is a prospective study planned to follow the birth cohort until adulthood, and a 6-7 year follow-up was started in the fall of 2022.

Fecal microbiome data, as well as SCFA assessment has been performed in over 1000 infants and analysis are currently ongoing. The association between dietary factors, and the gut microbiomes potential to cause and prevent disease will be investigated in a large part of our study population from birth and throughout childhood.

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# I

food & nutrition research

ORIGINAL ARTICLE

# Food and nutrient intake and adherence to dietary recommendations during pregnancy: a Nordic mother–child population-based cohort

Carina Madelen Saunders[1,2]*, Eva Maria Rehbinder[2,3], Karin C. Lødrup Carlsen[1,2], Malén Gudbrandsgard[1], Kai-Håkon Carlsen[1,2], Guttorm Haugen[2,4], Gunilla Hedlin[5,6], Christine Monceyron Jonassen[7,8], Katrine Dønvold Sjøborg[9], Linn Landrø[3], Björn Nordlund[5,6], Knut Rudi[8], Håvard O.Skjerven[1,2], Cilla Söderhäll[5,6], Anne Cathrine Staff[2,4], Riyas Vettukattil[1,2] and Monica Hauger Carlsen[10] on behalf of the PreventADALL study group

[1]Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway;
[2]Faculty of Medicine, Institute of Clinical Medicine, University of Oslo, Oslo, Norway;
[3]Department of Dermatology, Oslo University Hospital, Oslo, Norway;
[4]Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway;
[5]Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden;
[6]Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden;
[7]Genetic Unit, Centre for Laboratory Medicine, Østfold Hospital Trust, Kalnes, Norway;
[8]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway;
[9]Department of Obstetrics and Gynaecology, Østfold Hospital Trust, Kalnes, Norway;
[10]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

**Popular scientific summary**

- This population-based study showed that pregnant women had a high intake of fiber, vegetables, and fish.
- Median intake of red meat, salt, and saturated fat was higher than recommended in most pregnancies.
- Over half of the pregnant women likely had a folate intake below the recommended intake.
- Intake of alcohol and coffee exceeded the recommended levels in almost half of the participants, and increased with an increase in the level of education.
- Intervention strategies may be considered to improve maternal nutrition in pregnancy.

*Abstract*

***Background:*** A woman's food intake during pregnancy has important implications not only for herself but also for the future health and well-being of her child. Suboptimal dietary quality has been consistently reported in many high-income countries, reflecting poor adherence to dietary guidelines.
***Objective:*** This study aimed to explore the intake of food and nutrients in a cohort of pregnant women in Norway and their adherence to Nordic Nutrition Recommendations (NNR) and Norwegian food-based guidelines (NFG).
***Design:*** We investigated the dietary intake in 1,674 pregnant women from the mother–child birth cohort, PreventADALL, recruited at approximately 18-week gestational age. Dietary intake was assessed by an electronic validated food frequency questionnaire (PrevFFQ) in the first half of pregnancy.
***Results:*** Total fat intake was within the recommended intake (RI) range in most women; however, the contribution of saturated fatty acids to the total energy intake was above RI in the majority (85.2%) of women. Carbohydrate intake was below RI in 43.9% of the women, and 69.5% exceeded the RI of salt. Intakes of fiber, vegetables, and fish were high in a large part of the population. Many women had a high probability of inadequate intakes of the following key micronutrients during pregnancy: folate (54.4%), iron (49.6%), calcium (36.2%), vitamin D (28.7%), iodine (24.4%), and selenium (41.3%). A total of 22.8% women reported an alcohol intake of >1 g/day, and 4.4% reported an alcohol intake of >10 g/day. Women with

higher educational levels showed a tendency towards healthier eating habits, except for higher intakes of alcohol and coffee, compared to women with lower educational level.
***Discussion:*** Excessive saturated fat intake and limited intake of many important micronutrients during pregnancy were common, potentially increasing the risk for adverse pregnancy and birth outcomes.
***Conclusions:*** This study highlights the need for improved nutritional guidance to pregnant women across all educational levels.

Keywords: *nutrients; dietary intake; Nordic diet; fetal programming; food intake; nutritional recommendations*

To access the supplementary material, please visit the article landing page

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A healthy, nutrient-rich, and energy-appropriate diet during pregnancy is crucial for optimal development and growth of the fetus (1). Nutrient requirement is considerably increased during pregnancy and stands in contrast to the recommended modest increase in total energy intake throughout all three trimesters (1, 2). Malnutrition in women and children is a major global health issue, and pregnant women are at increased risk of micro- and macronutrient deficiency (3).

The significance of dietary intake and lifestyle factors during pregnancy has been accentuated with the increasing knowledge of fetal programming on later health outcomes (4–6). Women with a Western diet, characterized by high amounts of saturated fat, sugars, processed foods, and low amounts of fiber (7), during pregnancy are at an increased risk of delivering a child with lower birth weight, whereas adherence to Mediterranean dietary patterns, with high intakes of fruits, vegetables, and fish, has been associated with a decreased risk of delivering infants with lower birth weight (8, 9). Moreover, maternal micro- and macronutrient intake may directly influence the offspring's organ development, function, and metabolism (10).

In high-income countries, maternal malnutrition tends to manifest itself as a combination of macronutrient overnutrition and micronutrient undernutrition (11). The unfavorable profile of macronutrient intake is commonly reflected by low intake of complex carbohydrates, and high intakes of fat, saturated fat, and salt during pregnancy. Due to the increase in lower-quality diets, inadequate intakes of folate, iron, and vitamin D during pregnancy have consistently been reported in the United States, the United Kingdom, and other European countries (12, 13). Iodine deficiency among pregnant ladies is prevalent in many parts of Europe (14). Although women are advised to abstain from alcohol consumption during pregnancy, about a quarter of women in the general European population reported alcohol use during pregnancy (15).

Nordic countries have for several decades collaborated in developing guidelines for the intake of nutrients, resulting in the Nordic Nutrition Recommendations (NNR) (16). The NNR are intended to support health and prevent diet-associated diseases by setting dietary recommendations for the intake of energy-providing nutrients, intake of micronutrients, as well as fiber and alcohol intake. In 2011, the Norwegian Council for Nutrition published the Norwegian food-based dietary guidelines (NFG), which specify healthy food and lifestyle choices; highlight the need for an increased intake of fruit, vegetables, and whole-grains; and emphasize the importance of limiting the intake of processed and red meat; opting for low-salt foods; and avoiding all forms of alcohol throughout pregnancy (17). The recommended intake (RI) levels for a number of micronutrients are increased in pregnancy. Pregnant women are therefore advised to consume foods with high nutrient density, to ensure adequate intake levels of particularly folate, iron, calcium, vitamin D, and iodine. Evaluation of the diet in a population should thus include both the nutrient- and food-based dietary recommendations.

Assessing dietary intake in large cohort studies is essential for addressing potential diet–disease associations and informing the public about necessary health recommendations (18). Studies reporting on pregnant women's adherence to dietary guidelines in Nordic countries are few (19, 20). Therefore, the aim of the present study was to explore the intake of food and nutrients, from both diet and supplements, in a recently established cohort of pregnant women recruited from a general population, the Preventing Atopic dermatitis and Allergies in children (PreventADALL) study, and to evaluate their adherence to the NNR and the NFG. In addition, the study aimed to assess coffee and alcohol intake, as well as to evaluate the impact of educational level on dietary intake.

## Study design and methods

### *Cohort description*

Nutritional data in the present study is derived from the PreventADALL study, a multi-center, prospective, interventional, general population-based mother–child birth cohort study, aimed at the primary prevention of allergic

disease. Study design, recruitment, and inclusion criteria are described in detail elsewhere (21).

*Participants and eligibility criteria*

Women were recruited from December 2014 to October 2016 by postal invitation from the three participating centers (Oslo University Hospital, Østfold Hospital Trust, and Karolinska Institutet, Stockholm) in connection with their first routine ultrasound examination around gestational week 18. During this period, pregnant women were invited to participate in the study with the following inclusion criteria: singleton and twin pregnancies between weeks 16 and 22 and sufficient Scandinavian language skills. Exclusion criteria were severe maternal or fetal disease. A total of 2,697 pregnant women were enrolled in Norway and Sweden, four of these contributing with two pregnancies.

Study enrollment, baseline interview, and height and weight measures were performed by trained personnel. Weight was recorded to the nearest 100 g by a digital scale, and height to the nearest 1 mm by a stadiometer. Maternal health, pre-pregnancy weight, and sociodemographic and lifestyle factors were obtained through electronic questionnaires, developed in collaboration with the University Center for Information Technology (USIT) at the University of Oslo. The participants received the first general electronic questionnaires within a week after inclusion with an automatic reminder in case of no response. The women were asked to specify the week of gestational age when completing the questionnaires. All women signed a written informed consent prior to study enrollment.

The PreventADALL study was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway (2014/518) and in Sweden (2014/2242-31/4), and the study was registered at ClinicalTrials.gov (number NCT02449850).

*Dietary assessment*

The present dietary assessment was carried out in the Norwegian part of the PreventADALL cohort only, because the dietary assessment method was developed specifically for Norwegian food habits and meal patterns. The pregnant women were given access to the web-based, semi-quantitative food frequency questionnaire (PrevFFQ) via a link sent to their respective e-mails shortly after study inclusion. The PrevFFQ was designed to capture the habitual dietary intake during the first 4–5 months of pregnancy. The FFQ used in the PreventADALL study was previously validated in a population of women, using doubly labeled water and multiple 24-h-recalls (22). The PrevFFQ consisted of 279 questions on the frequency and amount of intake of about 280 food items, grouped according to the main food groups and meal patterns. Frequency categories were used in the increasing order: not at all, times per month, week or day. Amounts were given in portion sizes: standard units, spoons, cups, glasses, etc. The PrevFFQ included pictures of four different portion sizes for food items where portion size may be particularly difficult to estimate. The PrevFFQ included questions about the intake of vitamin or mineral supplements. Omission to answer a question would be followed by an automated comment, prompting the participant to submit an answer. All questions about diet in the FFQ were mandatory, assuring a complete set of values in all questionnaires. The data in the FFQ were transferred to the food and nutrient calculation system, Kostberegningssystemet (KBS), version 7.3, at the Department of Nutrition, University of Oslo, where all estimations of food and nutrient intakes were performed in KBS food composition database AE18. We included only women who answered the electronic questionnaires, excluding the first 125 enrolled women who received a paper version of the FFQ, thereby eliminating potential differences in results due to discrepancies in methods (paper vs. electronic FFQ). The possibility of double participation by four women who were enrolled with two pregnancies was eliminated, as the questionnaire from the first pregnancy was paper-based and therefore excluded. Women who reported unlikely energy intakes (<4,000 kJ/day and >20,000 kJ/day) were also excluded, as well as three invalid FFQs and one study withdrawal, giving a total of 1,674 eligible study participants (23). A study overview and selection criteria are presented in Figure 1. A total of 154 out of the 1,674 women did not answer the 18-week general questionnaire. Therefore, some background information was only available for 1,520 women.

The NNR 2012 defined RI as 'the amount of a nutrient that meets the known requirement and maintains good nutritional status among practically all healthy individuals in a particular life stage or gender group' (16). Hence, we present the proportion of women that had intakes within the recommended intake range (RIR) or above the RI, ensuring minimal probability for inadequacy. We did not assess the proportion of the group with relatively high probability of inadequate intake, defined as the proportion below average requirement (AR), as AR levels are not defined for pregnancy in the NNR. We included supplement intake when assessing the micronutrient intake through frequency estimations to increase the reliability of the results (24).

*Statistical analyses*

Data were exported from the KBS database and imported to SPSS (Statistical Package for the Social Sciences). All the analyses were performed using IBM© SPSS© statistics version 25 (Chicago, IL, USA). We conducted descriptive analyses of food intake, as well as energy intake, and macro- and micronutrient intake. Normal

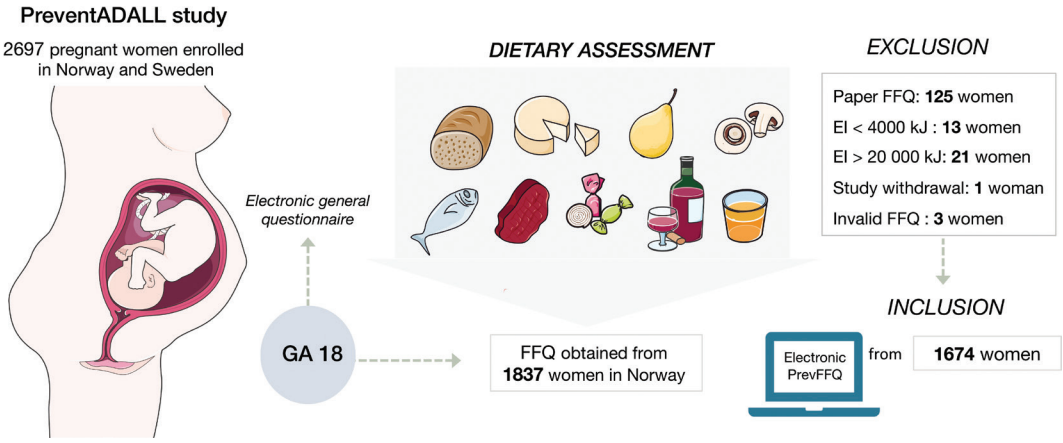


*Fig. 1.* Schematic overview of study and subjects, GA: gestational age, FFQ: Food Frequency Questionnaire. Participants with energy intakes (EI) < 4,000 and > 20,000 kilojoules (kJ) were excluded.

distribution of variables was investigated through visual inspection of histograms and *p*-plots and by using the Kolmogorov–Smirnov test. Variables are described as means and standard deviations (SD) or median values with interquartile range (IQR) and 5th and 95th percentiles of proportions for normally and non-normally distributed variables, respectively. Descriptive statistics were used to investigate adherence to the recommendations on the frequency of consumption of micro- and macronutrients. Differences between categorical variables were analyzed by Chi-Square test and numerical data by One-Way ANOVA tests. One-way between groups ANOVA was performed to test whether educational level would influence the intake of micro- and macronutrients and food groups. The assumption of normality and homogeneity of variances was tested for each variable.

## Results

Mean (SD) age of the population was 32.5 (4.1) years, and the mean (SD) pre-pregnancy body mass index (BMI) was 24.6 (3.5) kg/m$^2$. Other baseline characteristics are presented in Supplementary Table 1 for 1,520/1,674 respondents. Most (90.8%) women had a Scandinavian background, 37.1% had one or more previous deliveries, 61.1% had a university-level education of more than 4 years, 81.0% reported a full-time job, 97.1% were married or cohabitating, and 52.7% had a yearly household income of >1,000,000 NOK.

### *Intakes and adherence to recommendations for macronutrients and the main food groups*

The participants' intake of macronutrients and selected foods and beverages per day, as well as the proportion of participants with intakes in line with the recommendations, are presented in Table 1.

Median (IQR) energy intake was 10,082 (4,139) kJ. Total fat intake was above RIR (25–40 E%) in 14.0% of the women, and saturated fatty acid intake was above RI (max 10 E%) in 85.2% of the women. Average intake of fats is presented in Supplementary Table 2. Protein RIR (10–20 E%) was met by 92.9%, carbohydrate RIR (45–60 E%) by 55.6%, and fiber RI (> 25 g/day) by 77.6% of the women.

The recommendations for vegetable intake (>250 g/day) was met by 75.6% and fresh fruit intake (>250 g/day) by 42.1% of women. A total of 52.2% exceeded the recommended intake for red meat (max 500 g/week), and 60.3% exceeded the recommended intake of fish and seafood (300–450 g/week). Daily salt intake was above recommendations (6 g/day) in 69.5% of women. The average intakes of other food groups and beverages are included in Supplementary Table 3.

### Adherence to recommendations for micronutrients

Table 2 shows the participant's estimated average daily intake of micronutrients compared to the RI levels (NNR 2012). Micronutrients deemed especially important during pregnancy and the intake of other micronutrients are listed in Supplementary Table 4. An intake below RI was seen in a high percentage of women for the following micronutrients: 54.4% for folate (500 µg/day), 49.6% for iron (15 mg/day), 36.2% for calcium (900 mg/day), 24.4% for iodine (175 µg/day), 41.3% for selenium (60 µg/day), and 28.7% for vitamin D (10 µg/day).

### Alcohol and coffee

Almost a quarter (22.8%) of the women reported an alcohol intake of >1 g/day, 4.4% an intake of >10 g/day, and 1% an intake of >15 g/day. Women reported their coffee consumption in cups. One cup was defined as 170 ml, equal



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*Table 1.* Estimated daily intake of macronutrients, salt, and selected foods, compared to dietary recommendations (N = 1,674)

| Macronutrient | Unit | Median | Interquartile range | 5th Percentile | 95th Percentile | Recommended intake range (RIR) (Nordic Nutrition Recommendations 2012 [NNR 12]) | Below RIR (%) | Above RIR (%) | Within RIR (%) |
|---|---|---|---|---|---|---|---|---|---|
| **Carbohydrates** | E% | 45.7 | 42.3–49.2 | 35.9 | 54.7 | 45–60 E% | 43.9 | 0.5 | 55.6 |
| **Protein** | E% | 16.5 | 15.1–18.1 | 13.1 | 20.5 | 10–20 E% | 0.2 | 6.9 | 92.9 |
| **Total fat** | E% | 34.5 | 31.2–37.8 | 26.3 | 43.6 | 25–40 E% | 2.9 | 14.0 | 83.1 |
| **Saturated** | E% | 12.5 | 10.9–14.1 | 8.7 | 17.0 | max 10 E% | 14.8 | 85.2 | 14.8 |
| **Monosaturated fat** | E% | 12.7 | 11.2–14.3 | 9.3 | 17.3 | 10–20 E% | 9.4 | 0.8 | 89.8 |
| **Polyunsaturated fat** | E% | 5.7 | 5.0–6.8 | 4.0 | 9.2 | 5–10 E% | 23.7 | 3.3 | 73.0 |
| **Fiber** | g/day | 32.2 | 25.0–41.3 | 16.8 | 55.5 | min 25–35 | 22.4 | 41.6 | 36.0 |
| **Food group** | | | | | | **Recommended intake range (RIR) (Norwegian Food Based Dietary Guidelines [NFG] 2011)** | **Adherence to NFG (%)** | | |
| **Fruit and berries** | g/day | 221 | 141–328 | 61 | 623 | min 250 g/day | 42.1 | | |
| **Vegetables** | g/day | 363 | 253–514 | 141 | 802 | min 250 g/day | 75.6 | | |
| **Fish + seafood** | g/week | 539 | 188–791 | 121 | 1,274 | 300–450 g/week[a] | 19.5 (60.3% >RIR) | | |
| **Fatty fish** | g/week | 136 | 71–206 | 7 | 404 | min 200 g/week[b] | 27.7 | | |
| **Red meat** | g/week | 516 | 339–723 | 116 | 1,100 | max 500 g/week[a] | 47.8 | | |
| **Salt** | g/day | 7.2 | 5.7–8.9 | 3.9 | 12.2 | Max 6 g/day | 30.5 | | |
| **Coffee** | g/day | 203 | 21–405 | 0 | 878 | 170–340 g/day[c] | 59.1 | | |
| **Alcohol** | g/day | 0.1 | 0.0–0.8 | 0.0 | 9.3 | 0 g/day | 56.5 | | |

NNR12 (Nordic Nutrition Recommendations 2012), the NFG (Norwegian Food Based Dietary Guidelines) refer to only Norway; E%: percentage of total energy intake per day;
[a]Recommendations given in gram per week.
[b]Included in the total amount of fish and seafood per week.
[c]Recommendations based on reference values established by the Department of Nutrition (25). Recommended daily intake is 1–2 cups/day. One cup is equivalent to 170 g coffee. Caffeine content was calculated based on European Food Safety Authority (EFSA) guidelines (26): 44.5 mg caffeine/100 g black coffee.

*Table 2.* Estimated daily intakes of selected micronutrients, compared to Nordic Nutrition Recommendations (NNR) (*N* = 1,674)

| Variable | Unit | Median | Interquartile range | 5th Percentile | 95th Percentile | Recommended intake (NNR) | Percentage with intakes | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Below RI | Above RI |
| Vitamin A | RE[a] | 1,694 | 1,101 | 661 | 3,313 | 800 RE[a]/day | 9.6 | 90.4 |
| Vitamin C | mg | 207 | 136 | 86 | 425 | 85 mg/day | 4.4 | 95.6 |
| Vitamin D | µg | 13.6 | 12.1 | 4.2 | 32.6 | 10 µg/day | 28.7 | 71.3 |
| Vitamin B 12 | µg | 7.9 | 4.1 | 4.0 | 14.5 | 2 µg/day | 0.3 | 99.7 |
| Iodine | µg | 256 | 189 | 103 | 563 | 175 µg/day | 24.4 | 75.6 |
| Folate | µg | 480 | 275 | 236 | 921 | 500 µg/day | 54.4 | 45.6 |
| Zinc | mg[b] | 15.5 | 14.8 | 7.7 | 43.4 | 9 mg/day | 10.2 | 89.8 |
| Calcium | mg | 1,045 | 558 | 495 | 1,960 | 900 mg/day | 36.2 | 63.8 |
| Selenium | µg | 69 | 52 | 31 | 162 | 60 µg/day | 41.3 | 58.7 |
| Iron | mg[c] | 15.1 | 10.0 | 7.3 | 92.0 | >15[d] mg/day | 49.6 | 50.4 |

RI: Recommended intake (NNR 2012) in pregnancy.
[a]Retinol equivalents: 1 retinol equivalent (re) = 1 µg retinol = 12 µg β-carotene. α-tocopherol equivalents: 1 α-tocopherol equivalent (α-te) = 1 mg rrr α-tocopherol.
[b]The utilization of zinc is negatively influenced by phytic acid and positively influenced by animal protein. The recommended intakes are valid for a mixed animal/vegetable diet. For vegetarian cereal-based diets, a 25–30% higher intake is recommended.
[c]Meal composition influences the utilization of dietary iron. Availability increases if the diet contains abundant amounts of vitamin C and meat or fish daily, and it is decreased with simultaneous intake of polyphenols or phytic acid.
[d]Increased need of iron intake during second and third trimesters in pregnancy.

to 170 g of coffee. Median coffee intake was 203 g/day, and 40.9% of the women reported a daily coffee consumption above the recommended 340 g (27). Median caffeine intake from coffee was 90 mg/day, and 19.4% exceeded a daily intake of 200 mg of caffeine (RI max 200 g/day).

### Education level

Post hoc comparisons using the Tukey's HSD test indicated that the median intake of red meat was significantly higher in participants with high school degree only and those with university education ≤ 4 years, compared to women with university education > 4 years ($P = 0.015$). We found a significantly higher intake of fiber ($P = 0.002$), vegetables ($P = 0.001$), and omega-3 ($P = 0.001$) in women with university education > 4 years compared to women with high-school degree only. Coffee consumption increased with an increase in the educational level and was significantly higher in women with a university degree > 4 years compared to high school only ($P = 0.005$). Alcohol intake differed significantly between different educational groups ($P = 0.004$, Kruskal–Wallis test). Women with a university education > 4 years had a significantly higher alcohol intake ($P = 0.016$, adjusted with Bonferroni) compared to those with lower educational level. We found no associations between educational levels and micronutrient intakes. Income levels did not significantly associate with dietary or alcohol and coffee intake.

## Discussion

Our assessment of dietary intake during pregnancy in a mother–child cohort from the general Norwegian population showed that a large number of participants had satisfactory intakes of healthy food items, such as fiber, vegetables, and fish. However, a significant part of our population was at risk for macro- and micronutrient inadequacy, with particularly low adherence levels for folate, iron, selenium, calcium, vitamin D, and iodine. Self-reported red meat, salt, and saturated fatty acid intake exceeded recommendations in more than half of the subjects in the cohort, and together with alcohol and coffee consumption, they were associated with higher educational level.

### *Macronutrient- and food intakes*

The satisfactory intake of healthy food items by most women in our study is in line with a previous study showing that Nordic diets are commonly characterized by a high consumption of milk and dairy products, moderate to high consumption of meat, and moderate consumption of fruit and vegetables (16). Appropriate birthweight (weight between the 10th and the 90th percentiles) may be facilitated by a maternal plant-food-based dietary pattern, with high intakes of fruit and vegetables, low-fat dairy, and lean meats throughout pregnancy (28, 29). The high vegetable and fiber intake in our cohort, in line with a previous study showing that Norwegian women tend to increase their fruit and vegetable consumption from pre-pregnancy to early pregnancy (30), will likely have positive health effects for both mother and child. A higher intake of fruits and vegetables is also associated with increased infant growth up to 6 months of age (31). Our study was not designed to assess pre-pregnancy dietary habits, but a heightened motivation in pregnancy might have played a role in increasing vegetable consumption in our study population, resulting in the reported high intakes of vegetables. Although estimated vegetable and fiber intake was satisfactory at the group level, more than half of the women in our study population did not meet the recommended daily intake of fruit (250 g/d). Total fish and seafood intake in our cohort was higher than in a study with 119 pregnant Norwegian women reporting a total fish intake of 39 g/day, which is only half of the intake found in our study (32). The high intake of fish and seafood is likely to be beneficial also for the offspring, as maternal fish intake in pregnancy has been associated with positive fetal neurodevelopmental outcomes and reduced levels of allergic disease (33, 34). In spite of its nutritional benefits, fish is also a known source of mercury and other environmental toxins, which can have a negative impact on fetal development (35). Therefore, the benefits of fish intake above the recommended intake levels, as seen in many women in our study, need to be weighed against the potential detrimental effects. The high intake of red meat, salt, and saturated fat in our study is consistent with data from a large meta-analysis concluding that the overall fat and saturated fat intake is above the recommended levels in most pregnant women in high-income countries, whereas carbohydrate intake is below the recommended levels(12). The majority of women in our study had salt intakes above RI levels, which is in line with a Canadian study comprising 1,533 pregnant women reporting sodium intakes above the recommended levels in 85% of the participants (36). Excessive consumption of saturated fat and low intakes of omega 3 fatty acids have been linked to adverse health outcomes in both mother and child (37, 38). Knudsen et al. showed in a large cohort of pregnant Danish women that high intakes of red- and processed meat and high fat dairy was associated with an increased risk of having a child small for gestational age (9).

### *Micronutrient intake*

Our findings of low adherence to RI for some micronutrients is in line with the consistently reported rise of lower-quality diets in many industrialized countries, leading to an inadequate intake of particularly iron, folic acid, calcium, and vitamin D among pregnant women (39, 40). The potentially inadequate intake of vitamin D, folate, and iron is supported by similar findings in 118 pregnant



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Finnish women (41). Significant folate deficiencies were observed in about half of the population of 204 Italian pregnant women and women of childbearing age (42). A low reported folate intake, such as observed in our study, is in line with inadequate periconceptional use of folic acid supplement in Oslo (43) and could potentially have serious consequences for the developing fetus (44). Inadequate folate intakes in pregnancy have also been described in Sweden and other European countries (12, 45). Pregnant Norwegian women are advised to use supplementation with 400 mg of folic acid/day, starting 1 month before conception until 2–3 months of pregnancy (46), as food is not fortified with folic acid. More than half of our study population reported a daily fruit consumption below the RI. It is likely that an inadequate intake of fruit contributes to low folate intakes in many of the pregnant women. Although the prevalence of iodine deficiency among pregnant women in Europe has been well-known for decades, intake still seems to be insufficient in many countries, including Norway (47). A quarter of the women in our study reported iodine intakes below the RI, in line with a representative Norwegian population (Norkost 3), showing unsatisfactory iodine intakes in 46% of women from the general population (48). Other studies have revealed mild-to-moderate iodine deficiencies in a large number of pregnant Norwegian women (49), likely because diet alone seems to be insufficient in maintaining adequate iodine concentrations (50). Iodine deficiency can cause severe adverse effects in the child, such as impaired cognitive outcomes and delayed neurodevelopment (51, 52). Reported vitamin D intake was higher in our study (13.6 µg/day) compared to a large Norwegian cohort study of more than 40,108 pregnant women conducted approximately 15 years ago, in which 63% did not reach the RI level of 10 µg/day, in spite of high vitamin D supplementation rates (19). Public health efforts in the past decade might have increased the awareness of Vitamin D and its importance, and contributed to higher intake rates in our study. Although previous studies have generally reported sufficient maternal calcium intakes in the United States, the United Kingdom, and Europe, we found a significant number of women at risk for potentially inadequate intakes (12). Low selenium intakes have also previously been reported in Western countries, particularly in inhabitants of Northern Europe (53). In a study of 230 pregnant women in the United Kingdom, the majority had an overall low selenium status (54). Low maternal selenium levels in pregnancy may adversely affect the child's psychomotor development (55, 56). Adequate maternal zinc intake is also crucial for normal fetal development (57). Most women in our study reported zinc intakes above the RI, which is in agreement with previous reports from other European countries (12).

*Coffee and alcohol*

Daily caffeine intake was higher in our study than in the MoBa study, which assessed caffeine intake in 59,123 pregnant women in Norway and found that 10% of the women exceeded the caffeine level of 200 mg/day (41). The high rate of women exceeding recommendations for coffee intake in our study (41%) is a matter of concern. Maternal caffeine intake in pregnancy has been associated with reduced birthweight in relation to gestational weight in the Norwegian MoBa study, a large prospective observational cohort study (58), in line with other studies (59–61). Although women in our study tended to give up lifestyle choices that could harm their unborn child, such as the consumption of nicotine-containing products (62), we found a high number of women reporting alcohol intake in the first 4–5 months of pregnancy. The adverse effects of alcohol on the fetus are well known and an important reason for many women to abstain from alcohol during pregnancy (63). Severe effects on the fetus are seen with an alcohol intake above 24–48 g/day; however, even lower levels of alcohol consumption can adversely affect the developing fetus. Due to uncertainty of the threshold level for negative health effects in the offspring, it is recommended to totally abstain from alcohol in pregnancy (64). A large meta-analysis concluded that approximately 10% of women globally report alcohol intake in pregnancy. The highest prevalence was seen in European countries, where up to a quarter of women consumed alcohol in pregnancy (15). The large Norwegian MoBa study reported alcohol consumption among 31.8% of women in the first trimester and among 9.7% in the second trimester. The majority of these women consumed alcoholic beverages less than once a month (65). Contrary to these findings, a recent study comprising 575 Norwegian women found that 27% reported weekly alcohol intake before pregnancy, compared with none in early pregnancy (66). We found a higher alcohol consumption in women with higher educational level, which is comparable to the findings of the MoBa study (65). Similarly, a Danish study in 100,000 pregnant women found that binge drinking in the pre-recognized part of pregnancy was more common among well-educated women (67). It has been suggested that alcohol consumption in pregnancy might increase around the globe in the coming years, which could cause a surge of alcohol-related birth defects (68). Our findings therefore warrant special attention.

Apart from a higher intake of alcohol and coffee, our results suggest that women with a higher educational level eat more healthy foods, such as whole-grain foods, vegetables, foods rich in omega 3, and have a reduced intake of red meat. The tendency toward better dietary quality in these women was not surprising, as higher education independently influences better food choice (69). A large body of evidence points to the influence of social class

on diet and lifestyle (70, 71). Similar to our findings, a Spanish study assessing dietary intake and compliance to national food guidelines found that less educated women reported lower intakes of omega-3 fatty acids, and higher intakes of saturated fatty acids (72). Greater financial and social resources possibly provide for better means to support a healthy lifestyle, although we found no association between income and dietary intake in our study (70).

## Strengths and limitations

A major strength of the present study is the robust sample size from a general population, which ensures a large variation of dietary habits. All FFQs are subject to large between-person errors. In addition, self-reported data are prone to systematic bias and methodological challenges (73), such as social desirability bias. This bias regularly occurs because study participants tend to answer questions in a way that will be viewed favorably by others, which leads to overreporting of good dietary habits and underreporting of unhealthy habits. Consequently, overreporting intakes of fiber and healthy food items, such as vegetables and fruit, is a common issue with FFQs. In spite of its limitations, the FFQ remains the most-used instrument, especially in large cohorts.

Women in the PreventADALL study are older, wealthier, better educated, and less often smokers and single, compared with the general pregnant population in Norway, which might limit the generalizability of our results (74, 75). Moreover, our dietary intake data may not be representable for pregnant women in other countries or other ethnicities, and therefore the implications may differ in other settings.

## Conclusion

Dietary intake in a large cohort of Norwegian pregnant women in a mother–child birth cohort showed low adherence to recommendations with regard to saturated fat, total carbohydrates, folate, iron, calcium, vitamin D, and iodine. Women with a university degree showed a tendency towards healthier eating habits, except for higher intakes of alcohol and coffee. Thus, our results highlight the role of education in dietary decision-making.

A sufficient nutrient supply in pregnancy is crucial for ensuring favorable maternal and fetal health outcomes. Intervention strategies, aimed at educating pregnant women and encouraging healthful dietary choices, may therefore be needed for women at all educational levels.

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## Conflict of interest and funding

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## Clinical trial registration

The ClinicalTrials.gov registration number for this study is NCT02449850.

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***Carina Madelen Saunders**
Division of Paediatric and Adolescent Medicine
Oslo University Hospital
Postboks 4950, Nydalen
NO-0424 Oslo
Norway
E-mail: carinamadelensaunders@gmail.com

# 3 months

## BARNETS KOST OG ERNÆRING

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «3. Hvor gammelt er barnet?»: Ca. 3 måneder

### 5. a) Hvordan ernæres barnet ditt nå (siste 2 uker)? *

**How is your child currently fed (past 2 weeks) ?**

Kryss for det ene alternativet som passer best (se bort fra evt. vitamintilskudd). Morsmelkerstatning regnes som annen mat.

- ○ Kun amming — Only breastfed
- ○ Kun morsmelk, også fra flaske — Only breastmilk, also from a bottle
- ○ Amming/morsmelk og annen mat (inklusive morsmelkerstatning) — Breastfed/breastmilk and other food (including formula)
- ○ Kun annen mat (inklusive morsmelkerstatning) — Only other foods (including formula)
- ○ Vet ikke — Don't know

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. a) Hvordan ernæres barnet ditt nå (siste 2 uker)?»: Kun annen mat (inklusive morsmelkerstatning), Amming/morsmelk og annen mat (inklusive morsmelkerstatning)

### 5. a) i. Hvilken annen mat får barnet nå (siste 2 uker)? *

**What other foods is your child currently fed? (past 2 weeks) ?**

Kryss for alle aktuelle, uavhengig av hvor mye barnet får.

- ☐ Morsmelkerstatning — Formula
- ☐ Annen fast føde (ikke fra flaske) — Other solid foods (not from a bottle)
- ☐ Annet — Other

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. a) Hvordan ernæres barnet ditt nå (siste 2 uker)?»: Amming/morsmelk og annen mat (inklusive morsmelkerstatning)

### 5. a) ii. Hvor stor del av barnets kost/ernæring er morsmelk? *

**How much of the child's diet constitutes breastmilk?**

Velg det alternativet som passer best.

- ○ Største del av kosten — Most of the diet
- ○ Omtrent like mye som annen kost — Equal amounts as other foods
- ○ En liten del av kosten/bare for kos — A small part of the diet/ only for comfort

# 6 months

**5. b) De siste 3 månedene, har barnet fått morsmelk/blitt ammet?** *

In the past 3 months was your child fed breastmilk/ been breastfed?

- ○ Ja, får morsmelk/ammer fortsatt — Yes, is fed breastmilk/ still breastfeeds
- ○ Ja, men ikke nå lenger — Yes, but not anymore
- ○ Nei — No

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. b) De siste 3 månedene, har barnet fått morsmelk/blitt ammet?»: Ja, får morsmelk/ammer fortsatt

**5. b) i. Hvor stor del av barnets kost/ernæring er morsmelk nå (siste 2 uker)?** *

Velg det alternativet som passer best.

How much of the child's diet constitutes breastmilk? (past 2 weeks)

- ○ Får ikke morsmelk — Isn't fed breastmilk
- ○ En liten del av kosten/bare for kos — A small part of the diet/ only for comfort
- ○ Omtrent halvparten av barnets kost — Approximately half of the child's diet
- ○ Største del av kosten — Most of the diet
- ○ Får kun morsmelk — Only breastmilk

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. b) De siste 3 månedene, har barnet fått morsmelk/blitt ammet?»: Ja, men ikke nå lenger

**5. b) ii. Hvor mange måneder var barnet da det sluttet å amme/få morsmelk?** *

Velg det alternativet som passer best.

How many months was your child when it wasn't fed breastmilk anymore?

Velg

Indicate the appropriate alternative

## 9, 12 months

**5. c) De siste 3 månedene, har barnet fått morsmelk/blitt ammet?** *

In the past 3 months, was your child fed breastmilk/ been breastfed?

- Ja, får morsmelk/ammer fortsatt — Yes, still being fed breastmilk/ still breastfeeds
- Ja, men ikke nå lenger — Yes, but not anymore
- Nei — No

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. c) De siste 3 månedene, har barnet fått morsmelk/blitt ammet?»: Ja, men ikke nå lenger

**5. c) i. Hvor mange måneder gammelt var barnet da det sluttet å amme/få morsmelk?** *

Velg det alternativet som passer best.

How many months was your child when it wasn't fed breastmilk anymore?

Velg ... Indicate the appropriate alternative

## 12 months

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. d) De siste 3 månedene, har barnet fått morsmelk/blitt ammet?»: Ja, får morsmelk/ammer fortsatt

**5. d) ii. Når ammes barnet vanligvis?** *

At what times is the child usually brestfed ?

Kryss for det som passer best.

- Ammes kun på kvelden/natten — Breastfeeds only in the evening/ at night
- Ammes kun på morgenen/dagen — Breastfeds only in the morning/ in the day
- Ammes både på natten og dagen — Breastfeeds both in the day and night
- Vet ikke — Don't know

# 3, 6 months

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «3. Hvor gammelt er barnet?»: Ca. 3 måneder

**5. a) iii. De siste 3 månedene, har barnet fått grøt/velling? *** In the past 3 months, was your child fed porridge?

Kryss av for alle aktuelle alternativer

- [ ] Nei — No
- [ ] Ja, ved 0-1 mnd. alder — Yes, at 0-1 months
- [ ] Ja, ved 1-2 mnd. alder — Yes, at 1-2 months
- [ ] Ja, ved 2-3 mnd. alder — Yes, at 2-3 months

**6. a) De siste 3 månedene, har barnet fått grøt/velling? ***

Kryss av for alle aktuelle alternativer

- [ ] Nei — No
- [ ] Ja, ved 3 mnd. alder — Yes, at 3 months
- [ ] Ja, ved 4 mnd. alder — Yes, at 4 months
- [ ] Ja, ved 5 - 6 mnd. alder — Yes, at 5-6 months

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. a) iii. De siste 3 månedene, har barnet fått grøt/velling?»: Ja, ved 1-2 mnd. alder, Ja, ved 2-3 mnd. alder, Ja, ved 0-1 mnd. alder

**5. a) iv. Hvor ofte får barnet grøt/velling, og hva er denne laget av?** How often is yoru child fed porridge, and what is it made of?

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. a) iii. De siste 3 månedene, har barnet fått grøt/velling?»: Ja, ved 1-2 mnd. alder, Ja, ved 2-3 mnd. alder, Ja, ved 0-1 mnd. alder

Kryss for alle typer grøt det som passer best for perioden der barnet har fått grøt/velling

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. a) iii. De siste 3 månedene, har barnet fått grøt/velling?»: Ja, ved 1-2 mnd. alder, Ja, ved 2-3 mnd. alder, Ja, ved 0-1 mnd. alder Not at all  Less than weekly.  1-3 days/week.  4days/week or more.  Don't know

| | | Ikke i det hele tatt | Sjeldnere enn ukentlig | 1-3 dager i uken | 4 dager i uken eller mer | Vet ikke |
|---|---|---|---|---|---|---|
| Ris | Rice | ○ | ○ | ○ | ○ | ○ |
| Hirse | Millet | ○ | ○ | ○ | ○ | ○ |
| Havre | Oats | ○ | ○ | ○ | ○ | ○ |
| Mais | Corn | ○ | ○ | ○ | ○ | ○ |
| Hvete | Wheat | ○ | ○ | ○ | ○ | ○ |
| Fullkorn | Whole meal | ○ | ○ | ○ | ○ | ○ |
| Spelt | Spelt | ○ | ○ | ○ | ○ | ○ |
| Sinlacgrøt | Sinlac | ○ | ○ | ○ | ○ | ○ |
| Annen type | Other type | ○ | ○ | ○ | ○ | ○ |

# 6 months

## 6. a) ii. Har barnet ditt fått følgende meieriprodukter?

Was your child fed the following dairy products?

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «5. b) i. Hvor stor del av barnets kost/ernæring er morsmelk nå (siste 2 uker)?»: Største del av kosten, En liten del av kosten/bare for kos, Omtrent halvparten av barnets kost, Får ikke morsmelk

| | Ikke i det hele tatt | Startet før 3 mnd. alder | Startet ved 3 mnd. alder | Startet ved 4 mnd. alder | Startet ved 5 - 6 mnd. alder | Vet ikke |
|---|---|---|---|---|---|---|
| Vanlig kumelk (hel melk, lett melk, ekstra lett melk, skummet melk, styrk, fløte etc.) | Not at all | Started before 3 months | Started at 3 months | Started at 4 months | Started at 5-6 months | Don't know |
| Laktosefri melk | ○ | ○ | ○ | ○ | ○ | ○ |
| Upasteurisert melk | ○ | ○ | ○ | ○ | ○ | ○ |
| Biola, cultura og/eller andre produkter med pro/pre-biotika | ○ | ○ | ○ | ○ | ○ | ○ |
| Youghurt og/eller andre syrnede melkeprodukter | ○ | ○ | ○ | ○ | ○ | ○ |
| Ost/prim | ○ | ○ | ○ | ○ | ○ | ○ |
| Andre meieriprodukter | ○ | ○ | ○ | ○ | ○ | ○ |

Normal cow's milk
Lactose free milk
Unpasteurized milk
Products with pro/pre-biotics
Yogurt and/or other fermented dairy products
Cheese/Prim
Other dairy products

# 9, 12 months

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «3. Hvor gammelt er barnet?»: Ca. 12 måneder, Mer enn 13 måneder, Ca. 9 måneder

## 6. b) De siste 3 månedene, hvor ofte har barnet ditt fått følgende meieriprodukter?

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «3. Hvor gammelt er barnet?»: Ca. 12 måneder, Mer enn 13 måneder, Ca. 9 måneder

Not at all Less than weekly 1-3 days/week4 days/week or more Don't know

| | Ikke i det hele tatt | Sjeldnere enn ukentlig | 1-3 dager i uken | 4 dager i uken eller mer | Vet ikke |
|---|---|---|---|---|---|
| Vanlig kumelk (hel melk, lett melk, ekstra lett melk, skummet melk, styrk, fløte etc.) | ○ | ○ | ○ | ○ | ○ |
| Laktosefri melk | ○ | ○ | ○ | ○ | ○ |
| Upasteurisert melk | ○ | ○ | ○ | ○ | ○ |
| Biola, cultura og/eller andre produkter med pro/pre-biotika | ○ | ○ | ○ | ○ | ○ |
| Youghurt og/eller andre syrnede melkeprodukter | ○ | ○ | ○ | ○ | ○ |
| Ost/prim | ○ | ○ | ○ | ○ | ○ |
| Andre meieriprodukter | ○ | ○ | ○ | ○ | ○ |

Normal cow's milk
Lactose free milk
Unpasteurized milk
Biola and/or other products with pro/pre-biotics
Yogurt and or other fermented dairy products
Cheese/Prim
Other dairy products

# 6, 9, 12 months

## 6. d) De siste 3 månedene, hvor vanlig er det for barnet å spise:

How often is your child fed the following foods (last 2 weeks)?

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «3. Hvor gammelt er barnet?»: Ca. 12 måneder, Mer enn 13 måneder, Ca. 6 måneder, Ca. 9 måneder

Velg alternativene som passer best for hver sort mat, uansett om det er i ren form eller som pure, oppbløtt, mos, juice eller annet.
Det kommer oppfølgingsspørsmål om eventuelle matprodukter som ikke inngår i kostholdet.
Eks: spiser barnet glutenfritt skal du fortsatt krysse av kategorien for Brød/kjeks/vafler/kaker annet bakverk, oppfølgingsspørsmålene vil avklare om det er glutenfritt eller ikke.

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «3. Hvor gammelt er barnet?»: Ca. 12 måneder, Mer enn 13 måneder, Ca. 6 måneder, Ca. 9 måneder

| | Ikke i det hele tatt<br>Not at all | Sjeldnere enn ukentlig<br>Less than weekly | 1-3 dager i uken<br>1-3 days/week | 4 dager i uken eller mer<br>4 days/week or more | Vet ikke<br>Don't know |
|---|---|---|---|---|---|
| Brød/kjeks/vafler/kaker annet bakverk<br>Bread/cookies/waffles/cakes and other baked goods | ○ | ○ | ○ | ○ | ○ |
| Frukt eller bær<br>Fruit or berries | ○ | ○ | ○ | ○ | ○ |
| Rotfrukter (som f. eks potet, kålrot, gulrot, nepe, pastinakk)<br>Rootfruits (e.g., potato, turnip, carrot, parsnip | ○ | ○ | ○ | ○ | ○ |
| Andre grønnsaker<br>Other vegetables | ○ | ○ | ○ | ○ | ○ |
| Peanøtter (pålegg, eller i andre matvarer)<br>Peanuts (as spread or incorporated in other foods) | ○ | ○ | ○ | ○ | ○ |
| Nøtter utenom peanøtter (pålegg eller i andre matvarer)<br>pure egg (e.g., fried, cooked, scrambled, eggnog) | ○ | ○ | ○ | ○ | ○ |
| Egg i ren form (f.eks stekt/kokt egg, eggerøre, eggedosis)<br>egg in other foods (e.g., gratin, waffles, baked goods, paste, or similar) | ○ | ○ | ○ | ○ | ○ |
| Egg i annen mat (f.eks grateng, vafler, bakst, posteier o.l.)<br>fatty fish (salmon, trout, mackerel, pike, halibut, eel), | ○ | ○ | ○ | ○ | ○ |
| Fet fisk (laks, ørret, makrell, gjedde, kveite, ål)<br>Other fish | ○ | ○ | ○ | ○ | ○ |
| Annen fisk<br>Shellfish | ○ | ○ | ○ | ○ | ○ |
| Skalldyr<br>Poultry meat | ○ | ○ | ○ | ○ | ○ |
| Kjøtt fra fjærkre<br>Other meat | ○ | ○ | ○ | ○ | ○ |
| Annet kjøtt | ○ | ○ | ○ | ○ | ○ |

# 6, 9, 12 months

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «3. Hvor gammelt er barnet?»: Ca. 12 måneder, Mer enn 13 måneder, Ca. 6 måneder, Ca. 9 måneder

**6. e) Hvor stor andel av barnets kost er hjemmelaget? *** What is the proportion of homemade food in the child's diet?

Prefabrikert er f.eks: barnematglass, grøtpulver, ferdige smoothies, juicer.

- ○ Kun hjemmelaget — Only homemade
- ○ Mer hjemmelaget enn prefabrikert — More homemade than commercially prepared
- ○ Like mye hjemmelaget og prefabrikert — Same amount homemade and commercially prepared
- ○ Mer prefabrikert enn hjemmelaget — More commercially prepared than homemade
- ○ Kun prefabrikert — Only commercially prepared
- ○ Vet ikke — Don't know

Dette elementet vises dersom et av følgende alternativer er valgt på spørsmål «6. e) Hvor stor andel av barnets kost er hjemmelaget?»: Mer prefabrikert enn hjemmelaget, Kun prefabrikert, Mer hjemmelaget enn prefabrikert, Like mye hjemmelaget og prefabrikert, Kun hjemmelaget

**6. e) i. Hvor stor del av kosten er økologisk produsert? *** What proportion of the child's diet is organically produced ?

- ○ Svært lite eller ingenting — Very little
- ○ En liten andel av kosten — A small part of the diet
- ○ Omtrent halvparten av kosten — Approximately half
- ○ Mesteparten av kosten — Most of the diet
- ○ Vet ikke — Don't know

II

genes

MDPI

*Article*

# Butyrate Levels in the Transition from an Infant- to an Adult-Like Gut Microbiota Correlate with Bacterial Networks Associated with *Eubacterium Rectale* and *Ruminococcus Gnavus*

**Morten Nilsen [1,*], Carina Madelen Saunders [2,3,*], Inga Leena Angell [1], Magnus Ø. Arntzen [1], Karin C. Lødrup Carlsen [2,3], Kai-Håkon Carlsen [2,3], Guttorm Haugen [3,4], Live Heldal Hagen [1], Monica H. Carlsen [5], Gunilla Hedlin [6,7], Christine Monceyron Jonassen [1,8], Björn Nordlund [6,7], Eva Maria Rehbinder [3,9], Håvard O. Skjerven [2,3], Lars Snipen [1], Anne Cathrine Staff [3,4], Riyas Vettukattil [2,3] and Knut Rudi [1]**

1 Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1430 Ås, Norway; inga.angell@nmbu.no (I.L.A.); magnus.arntzen@nmbu.no (M.Ø.A.); live.hagen@nmbu.no (L.H.H.); christine.monceyron.jonassen@so-hf.no (C.M.J.); lars.snipen@nmbu.no (L.S.); knut.rudi@nmbu.no (K.R.)
2 Division of Paediatric and Adolescent Medicine, Oslo University Hospital, 0450 Oslo, Norway; k.c.l.carlsen@medisin.uio.no (K.C.L.C.); k.h.carlsen@medisin.uio.no (K.-H.C.); h.o.skjerven@medisin.uio.no (H.O.S.); m.r.vettukattil@medisin.uio.no (R.V.)
3 Faculty of Medicine, Institute of Clinical Medicine, University of Oslo, 0318 Oslo, Norway; guttorm.haugen@medisin.uio.no (G.H.); e.m.rehbinder@medisin.uio.no (E.M.R.); a.c.staff@medisin.uio.no (A.C.S.)
4 Division of Obstetrics and Gynaecology, Oslo University Hospital, 0450 Oslo, Norway
5 Department of Nutrition, Faculty of Medicine, Institute of Basic Medical Sciences, University of Oslo, 0405 Oslo, Norway; m.h.carlsen@medisin.uio.no
6 Astrid Lindgren Children's Hospital, Karolinska University Hospital, 17176 Stockholm, Sweden; gunilla.hedlin@ki.se (G.H.); bjorn.nordlund@ki.se (B.N.)
7 Department of Women's and Children's Health, Karolinska Institutet, 17176 Stockholm, Sweden
8 Genetic Unit, Centre for Laboratory Medicine, Østfold Hospital Trust, 1714 Kalnes, Norway
9 Department of Dermatology, Oslo University Hospital, 0424 Oslo, Norway
* Correspondence: morten.nilsen@nmbu.no (M.N.); c.m.saunders@medisin.uio.no (C.M.S.)

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**Abstract:** Relatively little is known about the ecological forces shaping the gut microbiota composition during infancy. Therefore, the objective of the present study was to identify the nutrient utilization- and short-chain fatty acid (SCFA) production potential of gut microbes in infants during the first year of life. Stool samples were obtained from mothers at 18 weeks of pregnancy and from infants at birth (first stool) at 3, 6, and 12-months of age from the general population-based PreventADALL cohort. We identified the taxonomic and SCFA composition in 100 mother-child pairs. The SCFA production and substrate utilization potential of gut microbes were observed by multiomics (shotgun sequencing and proteomics) on six infants. We found a four-fold increase in relative butyrate levels from 6 to 12 months of infant age. The increase was correlated to *Eubacterium rectale* and its bacterial network, and *Faecalibacterium prausnitzii* relative abundance, while low butyrate at 12 months was correlated to *Ruminococcus gnavus* and its associated network of bacteria. Both *E. rectale* and *F. prausnitzii* expressed enzymes needed for butyrate production and enzymes related to dietary fiber degradation, while *R. gnavus* expressed mucus-, fucose, and human milk oligosaccharides (HMO)-related degradation enzymes. Therefore, we believe that the presence of *E. rectale*, its network, and *F. prausnitzii* are key bacteria in the transition from an infant- to an adult-like gut microbiota with respect to butyrate production. Our results indicate that the transition from an infant- to an adult-like gut microbiota with respect to butyrate producing bacteria, occurs between 6 and 12 months of infant age. The bacteria

associated with the increased butyrate ratio/levels were *E. rectale* and *F. prausnitzii*, which potentially utilize a variety of dietary fibers based on the glycoside hydrolases (GHs) expressed. *R. gnavus* with a negative association to butyrate potentially utilizes mucin, fucose, and HMO components. This knowledge could have future importance in understanding how microbial metabolites can impact infant health and development.

**Keywords:** gut microbiota; infant; short-chain fatty acids; metaproteomics

## 1. Introduction

The temporal development of the gut microbiota during infancy is essential for immunological and developmental programming [1]. Although there has been some debate on whether colonization happens before birth, based on bacterial findings in placentas [2], umbilical cords [3], and meconium [4], recent studies, including our own [5], have challenged this view in support of the sterile womb hypothesis [5,6]. Regardless of when colonization occurs, the bacterial diversity in vaginally delivered newborns largely represent bacteria from the mother's natural vaginal flora, such as *Lactobacillus*, *Bifidobacterium*, *Prevotella*, and *Sneathia* [5,7], as well as from the maternal gut flora [8]. The earliest colonization often consists of Proteobacteria, which is likely involved in facilitating a suitable environment for anaerobic bacteria by depleting the gut of oxygen [7]. The oxygen depletion increases the amount of *Bacteroides*, *Clostridium*, and *Bifidobacterium*. *Bifidobacterium* dominates from an early stage until weaning, due to their capacity to break down human milk oligosaccharides [7,9,10]. After weaning, the infant's gut microbiota composition starts to resemble the adult gut microbiota in terms of anaerobes, such as *Clostridium* and *Bacteroides* [11].

A vital group of metabolites are the short-chain fatty acids (SCFAs), which are produced by gut microbes and which influence immune modulation, control anabolic processes, serve as an energy source for colonocytes, and are precursor metabolites for lipogenesis and gluconeogenesis [12,13]. Butyrate is essential in regulating immune responses by controlling inflammation responses and serving as the main energy source for gut epithelial cells [14,15]. Although the importance of SCFAs has been well established, we lack detailed knowledge about their longitudinal development in the first year of life and the bacteria responsible for their production.

The objective of the present study was to identify the nutrient utilization and the SCFA production potential of gut microbes in infants during the first year of life. First, we characterized the taxonomy of gut bacteria in pregnant women and their infants through the first 12 months of life. We then identified SCFA composition in pregnant women and their infants through the first year of life and correlated the SCFA composition with gut bacteria and influencing factors. Finally, we assessed the mechanism for potential polysaccharide utilization and SCFA production potential through metaproteomics.

## 2. Materials and Methods

We analyzed samples from the general population-based cohort, the Preventing Atopic Dermatitis and ALLergy (PreventADALL) study [16] that included 2397 mother-child pairs from Norway and Sweden. The primary aim of PreventADALL was to prevent allergic disease development, and secondarily to assess early life factors involved in the development of non-communicable diseases. Fecal samples from this study were collected at the enrollment of the mother at approximately 18 weeks of pregnancy, and in the infant at birth (meconium), 3, 6, and 12 months of age. In the present study, we used a multiomics approach to analyze fecal samples in the first 100 mother-child pairs that had infant stool samples available from at least three out of four sampling time points. The included children, of whom 51 were boys, were born at 39.6 ± 1.6 weeks of gestation with a birthweight of 3577 ± 529 g. A total of 22 infants were delivered via caesarean section and 78 vaginally. Eighty-three

infants were breastfed between 3 and 6 months (nine missing), 73 infants between 6 and 9 months (four missing), and 49 between 9 and 12 months (10 missing).

Informed written consent from all pregnant mothers was received upon inclusion, and from both parents upon inclusion of the newborn child. The PreventADALL study has been approved by the Regional Ethical Committee (REK) for Medical and Health Research Ethics in South-Eastern Norway (2014/518) as well as in Sweden (2015/4:3) by the Regional Ethical Trial Committee of Stockholm.

Stool samples were diluted 1:10 in stool DNA stabilizer (PSP Spin Stool DNA Plus Kit, Invitek Molecular, Berlin, Germany) and stored at −80 °C prior to analysis. Gas chromatography was used to determine SCFA composition. Taxonomic composition was derived by amplification of the V3 to V4 region of 16S rRNA by PRK341F and PRK806R primers [17]. The amplicons were indexed using a combination of 16 forward and 30 reverse PRK modified primers with illumina indexes. The pooled library was thereafter sequenced on the Illumina MiSeq platform using Illumina's MiSeq Reagent Kit v3 (Illumina Inc, San Diego, CA, USA). We used ASCA-ANOVA to determine if delivery mode, gender, age, and breastfeeding from 3 to 12 months were factors associated with the microbiota composition. The relationship between gut bacteria and SCFAs was investigated using Spearman correlation, and all shown correlations have a false discovery rate (FDR) corrected p-value of less than 0.05. Intracellular proteins from bacteria were extracted and analyzed in technical duplicates from six children at 12 months of age. A metagenome sequencing was performed to detect bacteria present in the stool samples and to reconstruct their genomes, unveiling their genomic information. This genomic information was further used as a database for metaproteomics data in order to observe protein expression related to gut function. The workflow of the study is illustrated in Figure 1, and detailed methods are available in supplementary materials and methods.

Sequencing data is available in the NCBI SRA database with identifier PRJNA609319. Proteome data has been uploaded via ProteomeXchange with identified PXD017844. Shotgun data are available upon request in TSD UiO (University of Oslo).

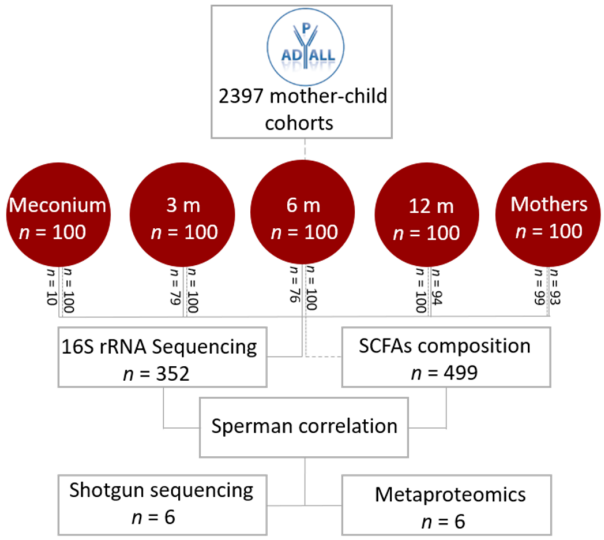


**Figure 1.** Flowchart of the study. The figure represents the workflow of the project. Sampling was performed by the Oslo University Hospital/University of Oslo, Østfold Hospital Trust and Karolinska Institutet, Stockholm [16]. In this study, we analyzed fecal samples from children in four different age groups (newborn, 3 months, 6 months, and 12 months) and their respective mothers, using a multiomics approach, which included 16S rRNA sequencing, short-chain fatty acids, shotgun sequencing, and metaproteomics. The stippled line represents the number (*n*) of fecal samples from infants analyzed at the different age groups at gas chromatography for short-chain fatty acid composition, and the full line represents the number of fecal samples from each age group that were analyzed by 16S rRNA after rarefaction and filtering for poor quality sequences.

## 3. Results

### 3.1. Taxonomic Composition

Quality filtering and cut-off from raw illumina sequencing reads resulted in 352 samples with sufficient quality for downstream analysis. The cut-off was set to 5000 sequences per sample, as the observed species rarefaction curve showed saturation at approximately 5000 sequences for all infant age groups (Supplementary Figure S1).

The average number of unique species increased with age; 26 species were detected in meconium (first stool after birth), 37 at 3 months, 46 at 6 months, 72 at 12 months, and 183 in the mothers. The same pattern, characterized by the increased α-diversity with infant age, was also shown by Shannon–Wiener and Simpson's index. Here, the mothers' gut microbiota displayed the highest α-diversity index (Supplementary Figure S2). β-diversity showed a distinct clustering of the gut microbiota by the different age groups (Supplementary Figure S3).

The infant gut microbiota showed the highest diversity at 12 months of age with the highest number of unique species, mostly derived from *Clostridiales* (Figure 2). *Clostridiales* increased significantly ($p < 0.05$, Kruskal–Wallis–Dunn's test, FDR corrected by the Benjamini–Hochberg method) between the ages of 6 and 12 months, and represented approximately 67% of the gut bacteria at 12 months. The prominent genera/families within the *Clostridiales* were *Faecalibacterium* (13.1%), *Ruminococcus gnavus* group (9.4%), and *Eubacterium rectale* group (7.3%) (Figure 3). *Faecalibacterium* was significantly higher than the *E. rectale* group ($p < 0.05$, Wilcoxon rank sum, FDR by the Benjamini–Hochberg method).

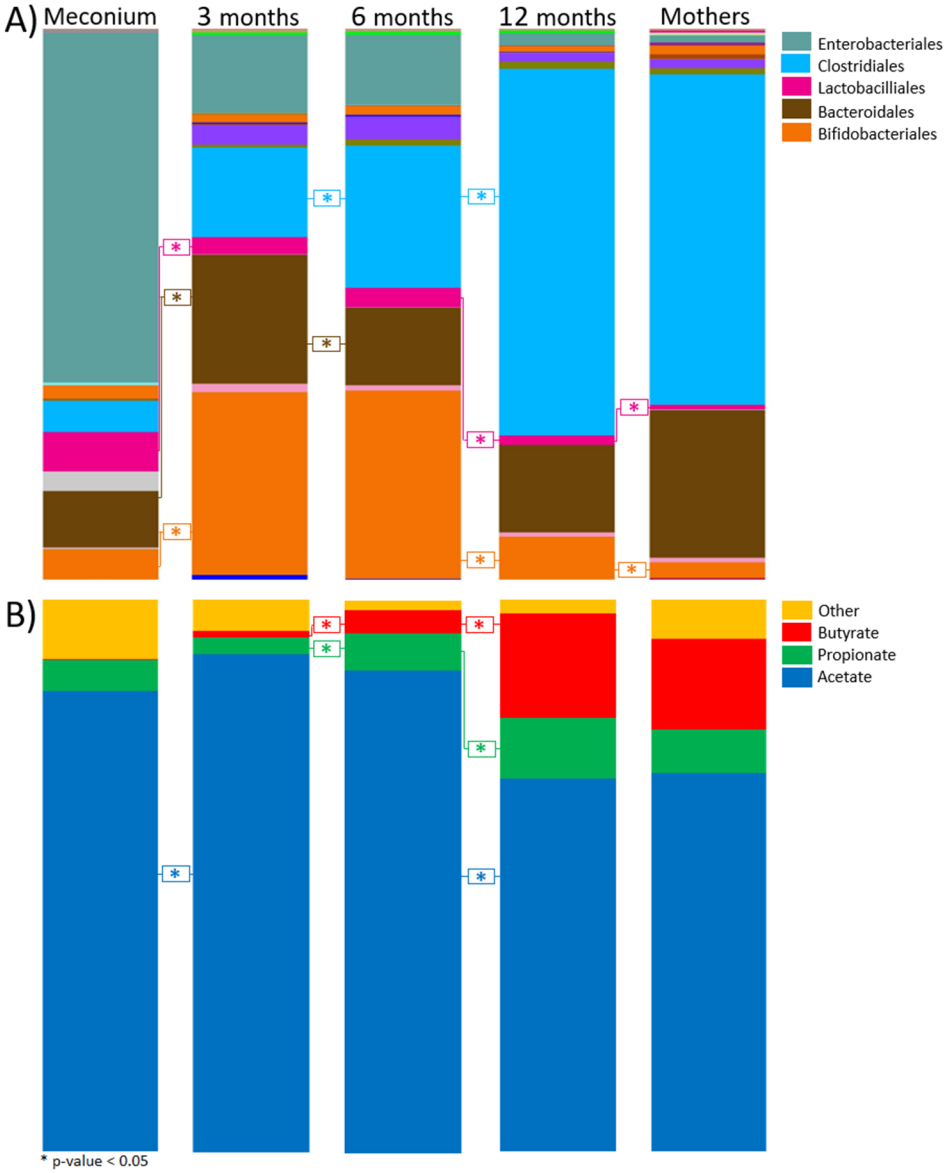


**Figure 2.** Taxonomic and short-chain fatty acid (SCFA) composition. The bar chart shows the relative abundance (%) of bacterial orders acquired from sequencing processed by the QIIME pipeline (**A**) and SCFAs composition (**B**) for the respective age groups. The dominant orders of bacteria and SCFAs in their respective colors are displayed on the top right. The asterisks (*) represent a $p$-value < 0.05, determined by Kruskal–Wallis–Dunn's test, FDR correct by the Benjamini–Hochberg method. The exact p-values are shown Supplementary Tables S1 and S2. SCFAs illustrated represent percent based on average total SCFAs detected. SCFAs included in the "other" group are isobutyrate, isovalerate, and valerate.

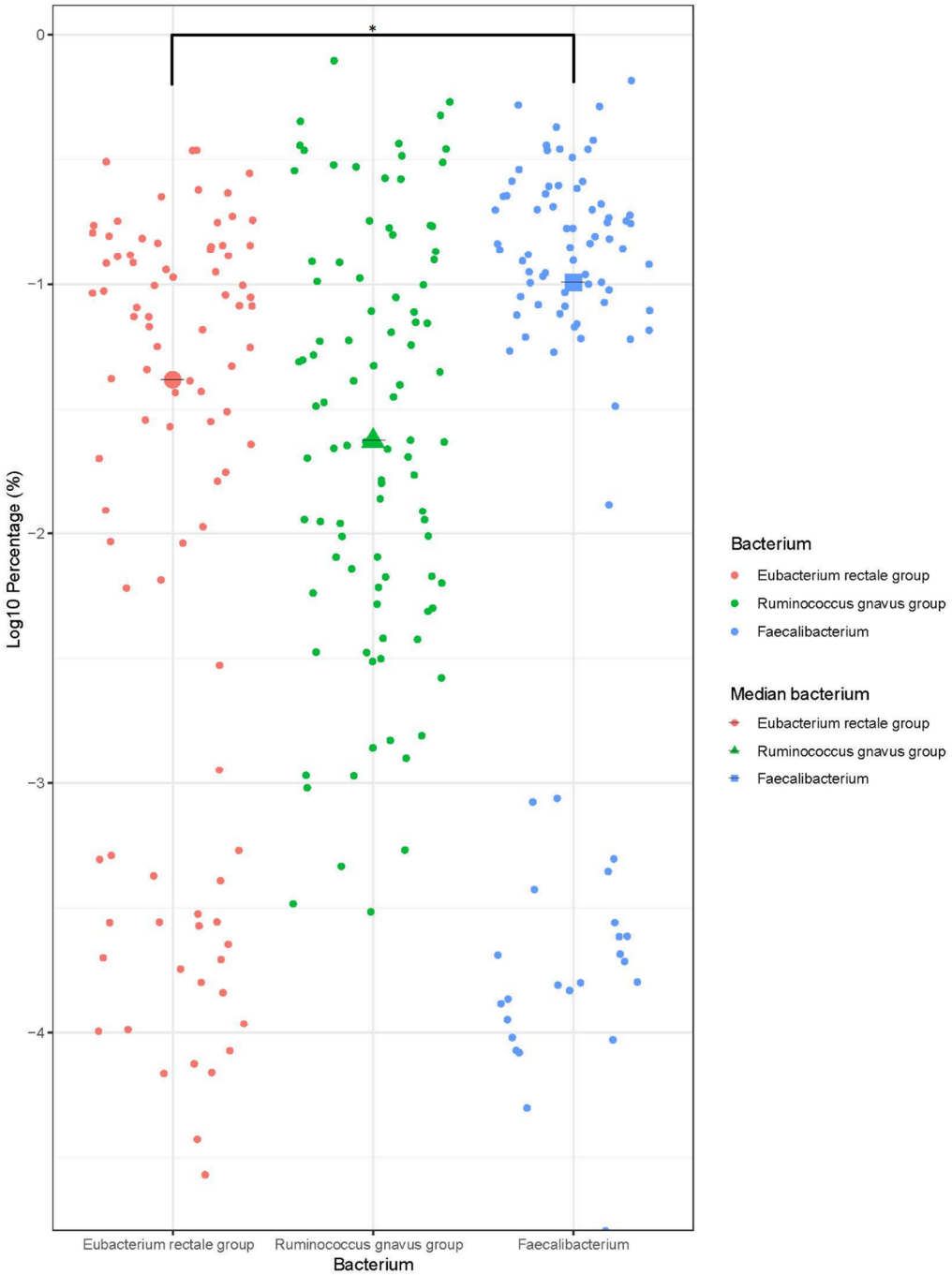


**Figure 3.** Relative abundance of *E. rectale*, *R. gnavus*, and *Faecalibacterium*, at 12 months. The dot plot shows the relative abundance (Log10 of percentage abundance) and median of the *E. rectale* group, *R. gnavus* group, and *Faecalibacterium*, at 12 months of age for all infants. The asterisk (*) represents a significant difference in relative abundance ($p < 0.05$, Wilcoxon rank sum test, FDR correct by Benjamini–Hochberg method) between the bacteria.

### 3.2. Maternal and Infant Factors Association with Microbiota

Age, delivery mode, and breastfeeding at 3 months of age showed significant associations with microbiota, with increasing age explaining the majority of the variance (Table 1). Vaginal delivery was associated with *Bifidobacterium* and *Bacteroides*, while *Clostridia* were associated with caesarean section (Figure 4A). At 3 months, breastfeeding was associated with *Bifidobacterium*, while no breastfeeding was associated with *Bacteroides* (Figure 4B). *Bifidobacterium* and *Escherichia/Shigella* were associated with young age, while *Clostridia* were associated with infants at 12 months and mothers (Figure 4C).

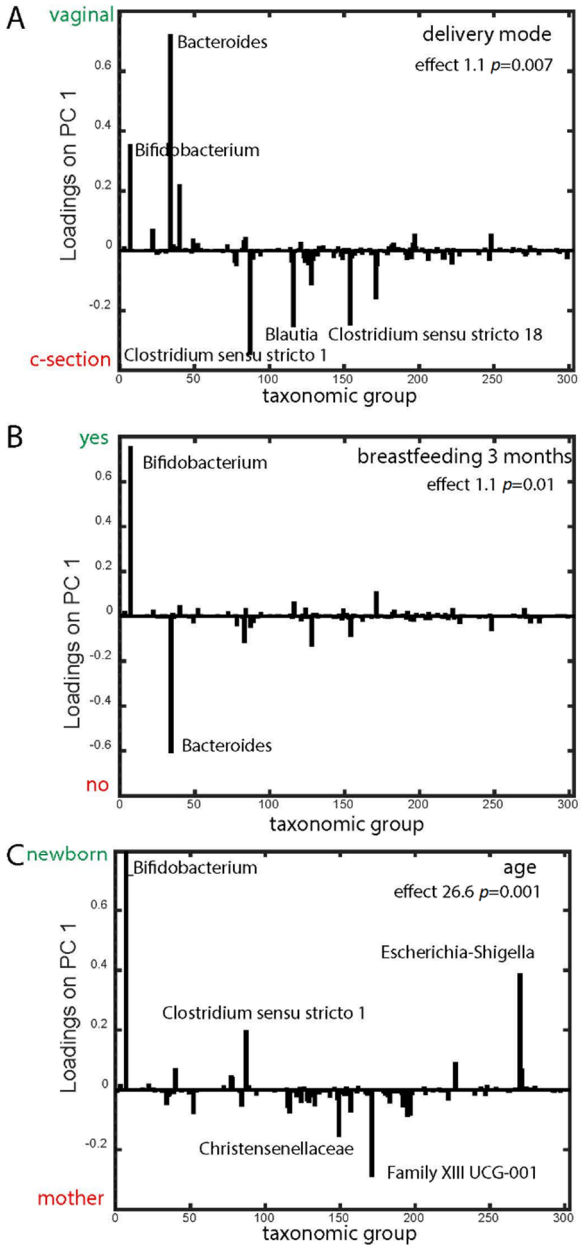


**Figure 4.** Metadata association with microbiota. ANOVA-simultaneous component analysis (ASCA-ANOVA) was used to determine the association of microbiota to known factors of the children. The Principal Component Analysis (PCA) plot shows the effect (Y-axis) of delivery method: vaginal and C-section (**A**), breastfeeding between 3 and 6 months of age (**B**), and age (**C**) based on the taxonomic groups acquired from 16S rRNA sequencing (X-axis). (**C**) The Y-axis is a gradient of age, where the effect points towards a young age or old age, showing the outer points of the scale: newborns and mother.

**Table 1.** Maternal and infant factors associations to operational taxonomic units (OTUs). The table shows the effect (%) and p-value for delivery mode, gender, and breastfeeding, between 3 and 6 months, 6 and 9 months, and 9 and 12 months, and infant age from 0–12 months.

| Term | Effect (%) | *P*-Value |
|---|---|---|
| Delivery mode | 1.06 | 0.01 |
| Infant gender | 0.26 | 0.25 |
| Breastfeeding 3–6 months | 1.12 | 0.01 |
| Breastfeeding 6–9 months | 0.60 | 0.73 |
| Breastfeeding 9–12 months | 0.72 | 0.05 |
| Infant age | 26.63 | 0.001 |

### 3.3. SCFAs Composition

Acetate was the dominant SCFA in mothers and in all infant age groups. The highest relative abundance of acetate in infants was observed at 3 months of age (90.1 ± 7.9%), while the lowest observed was at 12 months (67.4 ± 5.1%) (Figure 2). Propionate was present in all age groups and increased significantly between 3 and 12 months (Supplementary Table S2), with an overall ratio of 11.2 ± 1.3% at 12 months of age. Similar to propionate, butyrate increased significantly between 3 and 12 months of age. We detected a four-fold increase of the relative abundance of butyrate between 6 and 12 months of age ($p < 0.05$, Kruskall–Wallis–Dunn's test, FDR corrected with Benjamini–Hochberg), with butyrate representing 18.9 ± 2% of the total SCFAs at 12 months. In general, the absolute values of SCFA in relation to 16S rRNA gene copy number decreased with increasing age (Supplementary Figure S4A).

### 3.4. Bacterial and SCFA Correlation

At 3 months of age, we observed *Anaeroglobus, Anaerotruncus, Ruminococcus torques*¸ and *Eubacterium xylanophilum* to have a positive correlation to the relative amount of butyrate (Supplementary Figure S5A). However, at 6 months of age, we observed a positive correlation between *Sphingomonas* and the relative amount of propionate, while *Stenotrophomonas* had a positive correlation to the relative amount of both acetate and propionate (Supplementary Figure S5B). *Haemophilus* in mothers were positively correlated to the relative amount of butyrate, while *Akkermansia* were negatively correlated (Supplementary Figure S5C). At 12 months of infant age, we observed a correlation pattern revealing two distinct bacterial networks, one characterized by *Eubacterium rectale's* positive correlation to the relative amount of butyrate and the other defined by *Ruminococcus gnavus'* negative correlation to the relative amount of butyrate (Figure 5).

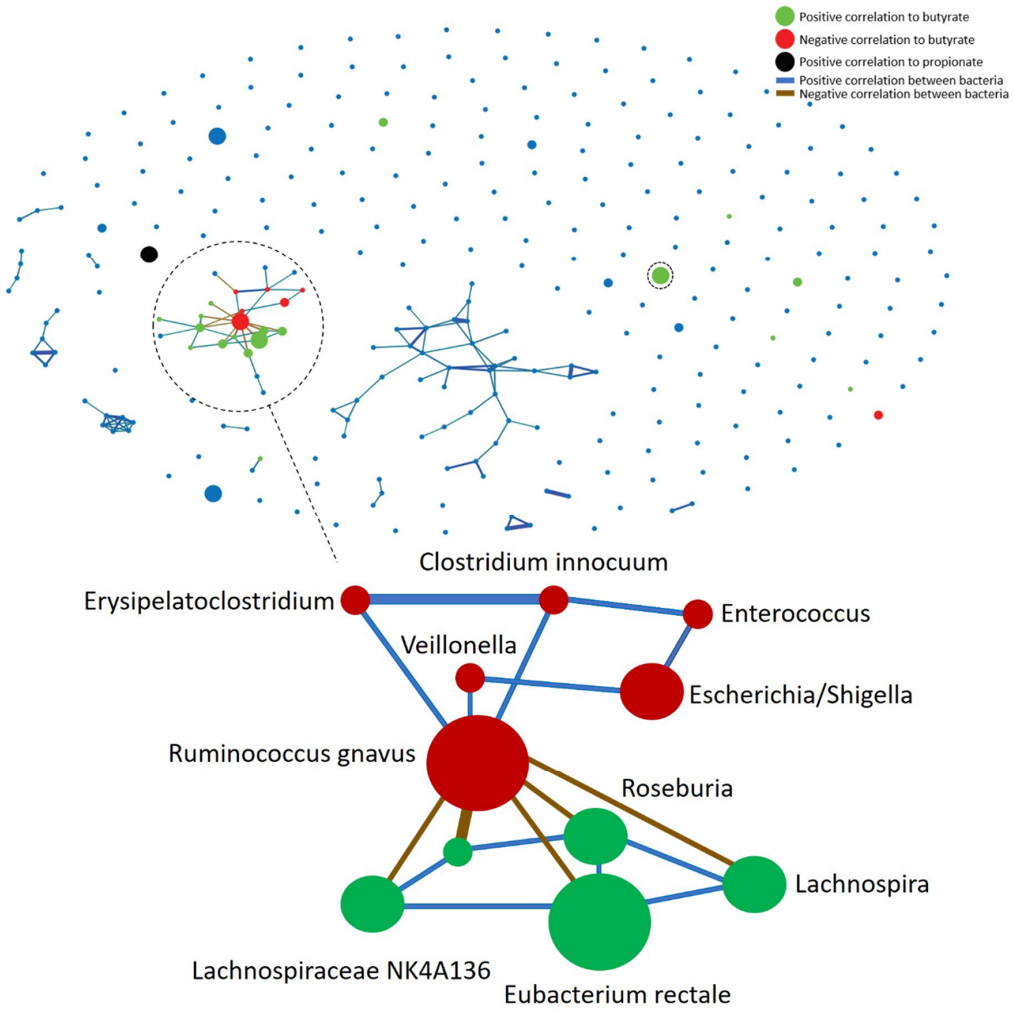


**Figure 5.** Bacterial and SCFA correlations at 12 months. The illustration shows all OTUs from 16S rRNA represented as nodes, with color indicating their correlation to SCFAs; blue = no correlation, red = negative correlation to butyrate, green = positive correlation to butyrate, and black = positive correlation to propionate. The three different node sizes represent the general abundance of the respective bacteria. The thickness of the lines between nodes represents a correlation between the bacteria, of which a thick line is a strong correlation. Blue lines indicate a positive correlation between the bacteria, while brown lines indicate a negative correlation. Prominent nodes in the networks are highlighted with their respective OTU taxonomy. The highlighted green circle with positive association to butyrate but without correlations to other bacteria was assigned to *Faecalibacterium*.

*E. rectale* represented the most abundant species in the network, correlating positively with both the relative amount of butyrate and the bacteria *Roseburia, Lachnospiraceaea* NK4A136, and *Lachnospira*. Bacteria in the *E. rectale* network were all negatively correlated to *R. gnavus*. *R. gnavus* showed a negative correlation to the relative amount of butyrate and a positive correlation to *Erysipelatoclostridium*, *Veillonella*, and *Clostridium innocuum*, with *Enterococcus* and *Escherichia/Shigella* also showing a positive correlation to members of the *R. gnavus* network. All bacteria related to the *R. gnavus* network had a negative correlation to butyrate (Figure 5). Infants with the *E. rectale* network had significantly higher absolute abundance of butyrate per 16S gene copy ($p < 0.05$, Mann–Whitney–Wilcoxon test) than infants with the *R. gnavus* network at 12 months of age (Supplementary Figure S4B).

Infants were divided into groups based on the cumulative sum of the relative abundance of bacteria with a positive or negative correlation to butyrate, and a positive correlation between the

bacteria. This resulted in 43 infants being categorized with the *E. rectale* network, 27 with the *R. gnavus* network, 19 with none, and 5 with both at 12 months of infant age ($p < 0.05$, chi-square test).

The presence of *R. gnavus* in infants with a high relative abundance of the *R. gnavus* network at 12 months of age was not significantly associated with the presence of *R. gnavus* at 6 months of age ($p > 0.05$, chi-square test). However, the presence of *R. gnavus* in infants at 6 months of age was significantly associated with the *R. gnavus* presence at 3 months of age ($p < 0.05$, chi-square test). The eight infants with a high relative abundance of *R. gnavus* at 3 months had a high relative abundance of the *E. rectale* network at 12 months of age, with one of the infants still having a high relative abundance of the *R. gnavus* network (>10% of the microbiota) as well.

The presence of the *E. rectale*- and *R. gnavus* networks at 12 months of infant age did not significantly correlate to delivery mode, gender, breastmilk feeding, or introduction to solid foods ($p > 0.05$, chi-square test) (Supplementary Table S3).

### *3.5. Metaproteome Analysis of E. Rectale and R. Gnavus Network Associated Bacteria*

Intracellular bacterial proteins were extracted and analyzed in technical duplicates from six infants, excluding one, based on their relative abundance of *E. rectale*, *R. gnavus*, and butyrate at 12 months of infant age. Of the 2212 detected protein IDs, 511 were observed to be significantly different in abundance between infants having a high relative abundance of either *E. rectale* or *R. gnavus* (Figure 6) ($p < 0.05$, t-test with permutation-based FDR and with missing values imputed). In addition, 253 protein IDs were only detected in infants with the *E. rectale* network and 80 detected only in infants with the *R. gnavus* network.

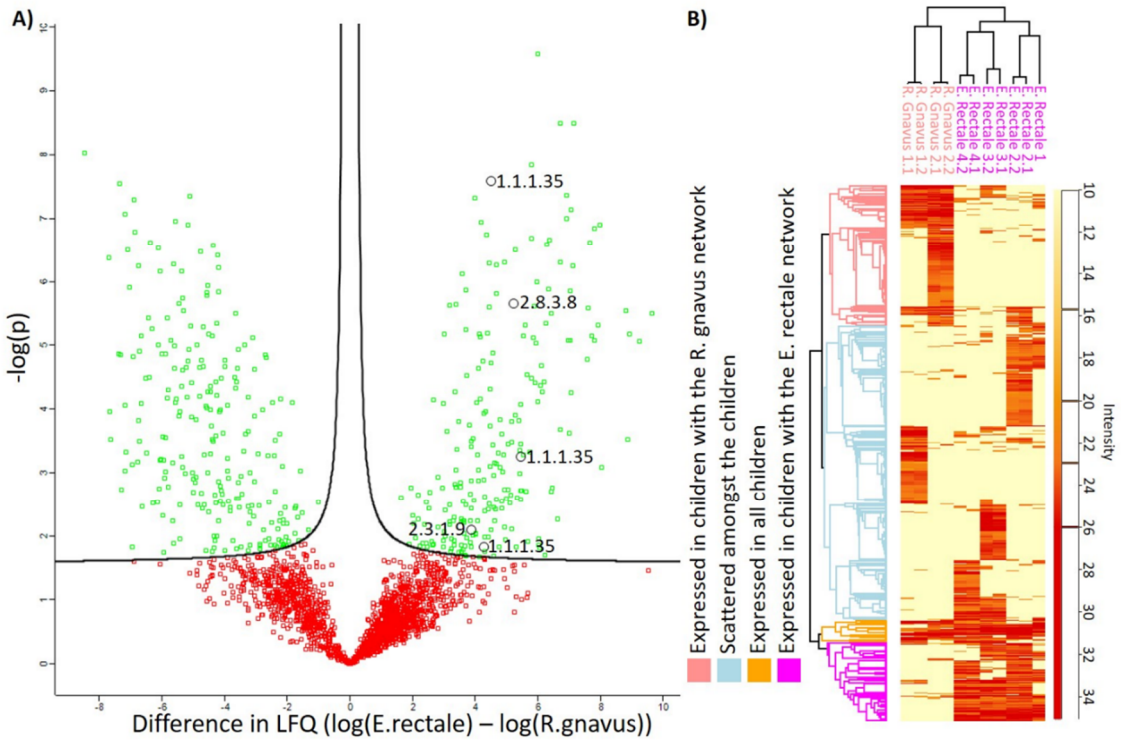


**Figure 6.** Protein expression. (**A**) shows a Volcano-plot highlighting (green) proteins differentially expressed between children with *E. rectale* and the *R. gnavus* community. The volcano plot was created using the Perseus software. Proteins marked as black circles represent significant differential expression in proteins related to the butyrate pathway with their respective Enzyme Commission (E.C.) numbers.

(**B**) shows the Log2 Label-Free Quantification (LFQ) intensity of the proteins, with missing values imputed with the constant 10. The intensity is represented as a gradient from light yellow (not detected) to red (highly abundant). Clustering is shown on the left, including four clusters, expressed in children with the *R. gnavus* network, *E. rectale* network, scattered, or expressed in all. A dendrogram is included at the top, to show the similarities and dissimilarities between the technical replicates and the children with *E. rectale*- or *R. gnavus*-dominated communities, with their respective technical duplicate (e.g., "*R. gnavus* 1.2" annotates child number one with the *R. gnavus* network and technical duplicate two). *E. rectale* infant 1 did not have any technical duplicates.

We detected the enzymes needed to convert acetyl-CoA to butyrate via the butyryl-CoA:Acetate CoA-transferase pathway, with several of the enzymes being mapped to the butyrate-associated bacteria *E. rectale*, *F. prausnitzii*, and *Roseburia* based on their genomic information. Enoyl-CoA, 3-hydroxybutyryl-CoA dehydratase, and butyryl-CoA:Acetate CoA-transferase were only detected in fecal samples of infants with the *E. rectale* network (Figure 7). We did not detect enzymes related to propionate production to be mapped to the propionate associated bacteria, *Bacteroides*, apart from an enzyme needed for acetyl-CoA to malonyl-CoA conversion (Supplementary Figure S6). However, we detected several enzymes in relation to propionate production that were mapped to *Eubacterium halli*, *Lachnospiraceae*, and *F. prausnitzii*. Conversion from propanoyl-phosphate to propionate was only detected and mapped to *Bifidobacteria* (*breve, pseudocatenulatum, longum,* and *bifidum*). As for enzymes related to acetate production, we detected *F. prausnitzii, E. halli, Blautia, Lachnospiraceae, B. breve, B. pseudocatenulatum, B. longum,* and *B. bifidum* to be potential acetate producers.

Of the proteins detected, we observed glycoside hydrolases (GHs), which potentially degraded a variety of dietary fibers, including hemicellulose (GH43 and GH51), starch/glycogen (GH13 and GH77), cellobiose/chitobiose (GH94), and fucose (GH29), in addition to broad specific glucosidases (GH2 and GH3), which were potentially expressed by *E. rectale* (Figure 8). GHs mapped to *R. gnavus* consisted of GHs related to mucin (GH33 and GH101), fucose (GH29, GH95, and GH151), human milk oligosaccharides (GH20), mannose (GH26), starch (GH31), sucrose (GH32), and a broad-specific glucosidase (GH3).

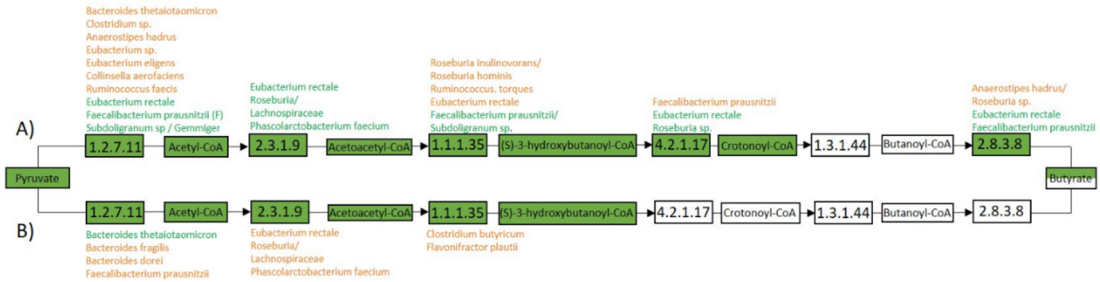


**Figure 7.** Protein presence related to butyrate production. The figure gives an overview of bacterial proteins (E.C. number) in relation to the butyrate pathway butyryl-CoA:Acetate CoA-transferase. The figure shows proteins detected (green box) in infants with the *E. rectale* network (**A**), or *R. gnavus* network (**B**). Bacterial taxonomy is shown next to each E.C. number, representing the bacterial source of the given protein. The bacterial sources are divided by two different colors, where orange represents detection in three or fewer samples in (**A**) or two or fewer in (**B**), and green represents detection in four or more in (**A**) or three or more in (**B**).

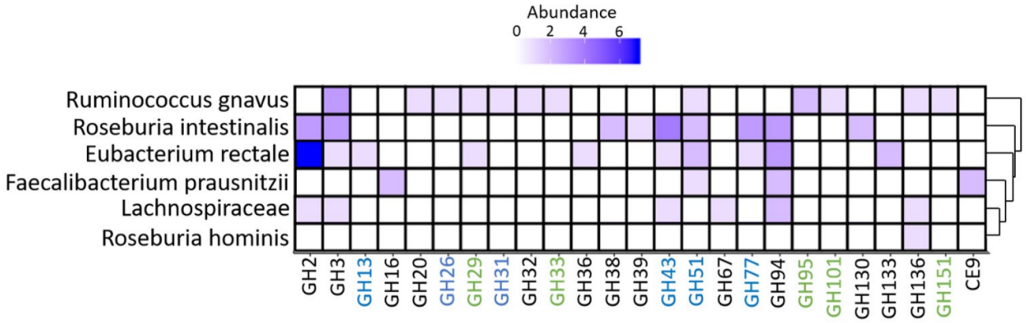


**Figure 8.** Expressed glycoside hydrolases/carbohydrate esterases. The figure shows proteins expressed within the glycoside hydrolase and carbohydrate esterase groups expressed based on the contiguous sequences assembled from shotgun sequencing and protein expression derived from nanoLC-Orbitrap tandem mass spectrometry (MS/MS). The abundance represents the number of unique proteins expressed from a given taxa within the glycoside hydrolases (GH) or carbohydrate esterase (CE) group. The y-axis shows the relevant taxonomies, and the dendrogram represents the Euclidian distance between the taxonomic groups based on GH and CE expression. The plot was created using ggplot2 [18]. GH numbers related to mucus and fucose degradation are marked in green, and degradation of starch, glycogen, and hemicellulose are marked in blue.

## 4. Discussion

The highest relative abundance of butyrate was observed at 12 months of infant age with the presence of the *E. rectale* network and *F. prausnitzii*, suggesting an additive effect in butyrate production. Based on our results, we believe that the *E. rectale* network co-occurring with *F. prausnitzii* could play a key role in the elevated relative abundance of butyrate. Earlier studies have observed that both *F. prausnitzii* and *E. rectale* are important butyrate producers in the adult gut [19], which suggests that the transition from 6 to 12 months of infant age may represent a crucial transition stage with respect to establishment of butyrate producers. We only detected one enzyme in relation to propionate production by *Bacteroides*, which may reflect a lower resolution of detected enzymes from other pathways due to selecting samples with a high relative abundance of butyrate, *E. rectale*, and *R. gnavus*.

Infants with a low relative abundance of butyrate at 12 months had a high relative abundance of the *Ruminococcus gnavus* network. *R. gnavus* can potentially degrade protein-linked human milk oligosaccharides (HMOs) [20], in addition to utilizing mucin glycans from the host [21]. Their potential ability to degrade HMOs and host glycans might explain their early presence in the gut. In addition, *R. gnavus* has been observed to be overrepresented in infants with a higher risk of developing allergic disease, including allergic rhinitis, asthma, and atopic dermatitis [22]. The potential pathogenicity of *R. gnavus* is suggested to be related to mucin degradation [23], by trans-sialidase or fucosidase, depending on the strain [21,24]. Concurrently, we observed glycoside hydrolases related to mucus- and HMO degradation to potentially be expressed by *R. gnavus*. As only 8.4% of the PreventADALL infants had documented atopic dermatitis at 12 months of age [25], we did not investigate microbiota-atopy associations, as the current study is underpowered to address these questions. We cannot exclude expression of other GHs, as the method used in the current study is only able to analyze intracellular proteins. In addition, we could not identify polysaccharide utilization loci (PUL), nor complete phosphotransferase system (PTS); therefore, the expressed proteins might not necessarily reflect the bacteria's metabolism.

The infants in our study had lower relative abundance of butyrate at 6 months (4.1%) than Estonian (12.3%) and Swedish (7.8%) infants [26], but had the largest increase in butyrate ratio between 6 and 12 months, while Estonian infants had the lowest. The earlier presence and slower emergence of butyrate in Estonian infants could potentially be linked to an earlier introduction of fibers, which may result in lower allergy occurrence [27], but this needs to be explored further.

The bacterial successional patterns in infants in the present study were similar to previous observations in other studies, with *Bifidobacterium* dominating the gut up to 6 months of age and then shifting to Firmicutes dominance at 12 months [28,29]. *E. rectale* has previously been observed already from 6 months of age, but increases its prevalence and abundance at 12 months of infant age [30], which coincides with our results. *R. gnavus* has been observed to be prevalent already at 1 month of age [31]. This early presence might be a result of its potential to degrade HMOs and host glycans. The abundance of *R. gnavus* has been shown to increase with age, particularly in allergic infants [22]. We observed an increase in *R. gnavus* prevalence with age; however, we did not find its presence at 12 months of age to be dependent on its presence at 3 months of age.

The association of *Bacteroides* with vaginal delivery was recently observed in both The Environmental Determinants of Diabetes in the Young (TEDDY) and the Baby Biome studies (BBS) [8,32], and association between *Bifidobacteria* and vaginal delivery has also been observed earlier [33]. Cesarean-section is often associated with a higher abundance of Firmicutes, which correspond with our observation of *Clostridiales* being associated with cesarean-section [34]. The observed negative association of *Bacteroides* with breastfeeding at 3 months of age has not been described previously and could possibly be explained by the fact that our study population was sampled from a general population, unlike most other large-scale studies. The maternal and infant factors assessed in this study did not explain why some infants had either the *E. rectale*- or the *R. gnavus* network at 1 year. Breastmilk feeding and introduction to solid foods are categorical variables, and do not reflect the intake amount or other foods introduced. The breastfeeding variable does not represent exclusive breastfeeding for the given time frame. We therefore cannot rule out that infant diet might have played a role in the presence of the networks.

## 5. Conclusions

In conclusion, we observed a four-fold increase in the relative abundance of butyrate from 6 to 12 months of infant age, with the *Eubacterium rectale*- and *Ruminococcus gnavus* networks being positively and negatively correlated to butyrate, respectively. Furthermore, we detected expressed enzymes from the butyryl-CoA:Acetate CoA-transferase pathway that might originate from *E. rectale* and *Faecalibacterium prausnitzii*. In addition, glycoside hydrolases related to dietary fiber degradation were potentially expressed by *E. rectale*. We believe these results suggest that the transition from 6 to 12 months of infant age represents a crucial transition stage with respect to establishment of key butyrate-producing bacteria.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4425/11/11/1245/s1. Supplementary 1: Materials and Methods. Figure S1: Rarefaction curve of observed species, Figure S2: $\alpha$ diversity, Figure S3: $\beta$-Diversity, Figure S4: Total short-chain fatty acids per 16S rRNA gene copy, Figure S5: Bacterial correlation to SCFA in all age groups, Figure S6: Expressed enzymes related to the propionate production pathway. Table S1: *p*-values Kruskall-Wallis-Dunn's test for bacterial orders, Table S2: *p*-values Kruskall-Wallis Dunn's test for SCFAs, Table S3: Maternal and infant factors associated with the presence of either *E. rectale* or the *R. gnavus* network in infants.

**Author Contributions:** Conceptualization, M.N. and K.R.; methodology, I.L.A., M.Ø.A., L.H.H., M.N., and K.R.; software, L.H.H., L.S. and R.V.; validation, M.A., K.C.L.C., L.S. and K.R.; investigation, M.N., M.Ø.A. and K.R.; data curation, R.V.; writing—original draft preparation, M.N. and C.M.S.; writing—review and editing, C.M.S., I.L.A., M.Ø.A., K.C.L.C., K.-H.C., G.H. (Guttorm Haugen), L.H.H., M.H.C., G.H. (Gunilla Hedlin), C.M.J., B.N., E.M.R., H.O.S., L.S., A.C.S., R.V., M.N., and K.R.; visualization, M.N. and M.Ø.A.; supervision, K.C.L.C., L.S., M.Ø.A. and K.R.; project administration, K.C.L.C., K.-H.C., G.H. (Gunilla Hedlin), G.H. (Gunilla Hedlin), C.M.J., B.N., E.M.R., H.O.S. and K.R. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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**Materials and Methods Supplementary**

**Sample information**

Fecal samples used in this study derived from the first 100 mother-child cohorts who attended at least three of the four follow-up investigations in the PreventADALL study. Infants were randomized to one of four groups by skin and food interventions[1]. From the 100 mother-child pairs, 99 pairs had available longitudinal sampling data from at least three of the four infant samples and from their respective mother approximately 18 weeks into pregnancy. Thirteen samples were mislabeled, although within the correct age group, so these samples were included in the 16S rRNA and SCFAs analysis as these were not analyzed longitudinally within each mother-child pair, but as a population of age groups over time.

**Sample preparation**

All fecal samples were diluted 1:10 in stool DNA stabilizer (PSP Spin Stool DNA Plus Kit, Invitek Molecular, Berlin, Germany) and stored at -80°C prior to analysis. Fecal samples were homogenized and pulse centrifuged at 1200 rpm for 8 seconds for easier extraction. From the 1:10 diluted samples, 300µl and 100µl aliquots were used for 16S rRNA sequencing and SCFA composition, respectively.

**Short-chain fatty acid analysis**

The aliquots were diluted 1:1 with MilliQ-water, and then 1:1 with an internal standard, containing 2% formic acid with 500µM 2-methylvaleric acid. Samples were centrifuged at 13 000 rpm for 10 minutes. The supernatant was filtered with 0.2µM filter columns (VWR, USA) at 10 000 rpm for 5 minutes. The eluate was transferred to gas chromatograph (GC) vials (VWR, USA) and applied to the gas chromatograph (Trace 1310 equipped with an autosampler, ThermoFisher Scientific) with ramping temperatures from 90°C to 150°C for 6 minutes and 150°C to 245°C for 1.9 minutes. 0.2µl was applied with a split injection to a Topaz 4.0mm drilled uniliner (Restek), using helium as the carrier gas with 2.5ml/min column flow, 3 ml/min purge flow and 200 ml/min split flow. The column used was a Stabilwax DA 30m, 0.25mm ID, 0.25µM (Restek), with a flame ionization detector analyzing the analytes. The chromatograms were processed with the Chromeleon 7 software.

A standard with 300µM acetic acid, 12µM propionic acid, 8µM isobutyric acid, 12µM butyric acid, 8µM isovaleric acid, 8µM valeric acid, 25µM internal standard and 1% formic acid was applied twice in between every 10$^{th}$ sample to detect shifts or variabilities. All acids used were purchased from Sigma-Aldrich, Germany.

**16.S rRNA sequencing**

Bacterial cells in fecal sample aliquots were disrupted using 0.2g <106µm acid-washed glass beads (Sigma-Aldrich, Germany), 0.2g 425-600µm acid-washed glass beads (Sigma-Aldrich, Germany) and 2×2.5-3.5mm acid-washed glass beads before being processed twice on a FastPrep 96 (MP Biomedicals, USA) at 1800rpm for 40 seconds. The samples were centrifuged at 13 000 rpm for 5 minutes before DNA was extracted using LGC Mag Midi Nucleic acid extraction kit (LGC genomics, UK). The V3 to V4 region of 16S rRNA was amplified using PRK341F and PRK806R primers[2] at 95°C for 15 minutes followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, before a final step at 72°C for 7 minutes. Cycles were increased to 30 for meconium. Reactions contained 2µl DNA template with 1× HotFirePol Blend Master Mix Ready to Load (Solis BioDyne, Germany) and 0.2µm PRK forward and reverse primers. Samples were purified using 1× Sera Mag beads to the DNA volume, following AMPure's protocol on a Biomek 3000 (Beckman Coulter, USA).

Index PCR was performed with a combination of 16 forward and 30 reverse modified PRK primers with Illumina indexes. Samples were amplified at 95°C for 5 minutes followed by 10 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 45 seconds, before a final step of 72°C for 7

minutes. Each reaction consisted of 1× FirePol Master Mix Ready to Load (Solis BioDyne, Germany), 0.2μM forward & reverse primers, nuclease free-water (VWR, USA) and 1μl DNA. The DNA concentration was quantified following Qubit's protocol, normalized and pooled on a Biomek 3000. The pooled sample was split in two for quantification and sequencing. Samples for quantification were first subjected to droplet generation using BioRad QX200™ – Droplet Generator, before being amplified at 95°C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds before the last two steps at 4°C for 5 minutes and 90°C for 5 minutes before quantification on BioRad QX200 – Droplet Reader. The reactions contained 1× Super mix for EvaGreen (BioRad, USA), 0.2μM Illumina colony forward & reverse primer, 2.4μl DNA template and PCR water. The second part of the sample was diluted to 6 pM DNA with 15% PhiX following Illumina's instructions, with the exception of using nuclease-free water instead of Tris and sequenced on Illumina MiSeq.

**Quantitative Insights Into Microbial Ecology (QIIME)**

The 16S rRNA data were analyzed with Quantitative Insights Into Microbial Ecology (QIIME) pipeline[3]. QIIME v.1.9.1 was used to assemble forward and reverse reads and split them into their respective samples. Usearch v8 was used to check reads for chimeras, and OTUs with a 97% or higher 16S rRNA identity were created and assigned taxonomy by the SILVA 128 database[4]. Two sequencing runs were performed resulting in 30 878 312 ssDNA fragments. The cut-off was set at 5 000 dsDNA fragments, resulting in 352 samples with sufficient depth and quality. This was distributed as follows: meconium n=10, 3 months n=79, 6 months n=76, 12 months n=94 and mother n=93.

**Shotgun metagenome sequencing**

Six samples were selected for Shotgun sequencing, based on the presence and absence of *E. rectale, R. gnavus* and butyrate. The samples were processed using the Nextera XT DNA Library Preparation Kit (Illumina Inc, San Diego, CA, USA), following the manufacturer's instructions and sequenced on the Illumina MiSeq platform twice, resulting in approximately 4.7 Gb.

**Processing shotgun data**

Human DNA was removed using bowtie2 v2.3.5.1 [5] and samtools v1.9 [6], and reads were trimmed using trimmomatic v0.36 [7], with the following parameters: Leading: 10, Trailing: 10, Slidingwindow: 5:20 and minlen: 32. Assembly was performed using MetaSPADES v3.13.1 [8], and the contigs created qualitatively assessed using MetaQuast v5.0.2[9]. Prodigal v2.6.3 [10] was used for gene prediction, whereas the InterProScan v5.39-77.0 [11] consortium predicted proteins and pathways. The predicted proteins from shotgun sequencing were further used as a sample-specific protein sequence database for the metaproteomic analysis. Contig taxonomy was annotated by a customized Kraken2 database [12], which involved the standard database with inclusion of *Ruminococcus gnavus* ATCC 29147, *Bacteroides uniformis* AF14-42, *Eubacterium rectale* AF36-2BH retrieved from NCBI.

**Protein extraction and quantification**

Intracellular proteins were extracted from the gut bacteria derived from the same six children used for shotgun sequencing. All samples were run in technical duplicates, excluding one due to an insufficient amount of fecal material.

An indirect double filtering process was performed on 0.2g fecal material. Fecal samples were suspended in 10mL TBS (Tris-based saline buffer), before being filtered through a 20μm filter (Merck™ Nylon-Net Steriflip™ Vacuum Filter Unit, Fisher Scientific), homogenized at 30 000 rpm for 60s (VDI12, VWR), centrifuged at 4000g for 10 minutes and thereafter resuspended in 10mL TBS and filtered through a 0.22μm nitrocellulose filter [13]. The filter was cut into smaller pieces, mixed with lysis buffer, and bacteria were mechanically lysed as explained in the 16S rRNA sequencing section.

The lysis buffer contained 50mM Tris-HCl, 200mM NaCl, 0.1% Triton-X100, 10mM Dithiothreitol (DTT) and 4% Sodium Dodecyl Sulfate (SDS). Protein quantification was performed using the BCA-DC kit, following the manufacturer's protocol, before applying 40µg protein to an SDS-gel. The gel was run for approximately 2 minutes. The proteins were fixed using 50% methanol and 10% glacial acetic acid for 1 hour with gentle agitation, and thereafter stained using 0.1% Coomassie Brilliant Blue R-250 with 50% methanol and 10% glacial acetic acid, and destained with 40% methanol and 10% glacial acetic acid.

**In-gel reduction, alkylation and digestion**

The gel band containing proteins was cut into approximately 1×1mm pieces and de-colored with Milli-Q water (MQ) and incubated at 15 minutes in room temperature (RT). MQ was removed and 50% acetonitrile (ACN) with 25 mM ammonium bicarbonate was added and incubated 15 minutes at RT. 100% ACN was added and incubated for 5 minutes at RT, before it was removed and then air-dried. Reduction and alkylation were performed by adding 10mM DTT with 100 mM ammonium bicarbonate for 30 minutes at 56ºC. The samples were cooled before the solution was removed and 55mM iodoacetamine (IAA) with 100mM ammonium bicarbonate was added and incubated in the dark for 30 minutes in RT. The IAA solution was removed, and 100% ACN was added and incubated for 5 minutes in RT before the solution was removed and air-dried for 2 minutes. For digestion 40ng of Trypsin solution was added and gel-pieces were incubated overnight at 37ºC, thereafter 1% trifluoroacetic acid (TFA) was added and the gel-pieces were sonicated in a water bath for 15 minutes. The resulting peptides were desalted using $C_{18}$ solid phase ZipTips according to the manufacturer's instructions.

**nanoLC-Orbitrap MS/MS**

Dried peptides were dissolved in a solution (0.1% TFA and 2% ACN in water) and analyzed on a nanoLC-MS/MS system (Dionex Ultimate 3000 UHPLC; Thermo Scientific, Bremen, Germany) connected to a Q-Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). In brief, peptides were loaded onto a trap column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. × 5 mm) and then backflushed onto a 50 cm × 70 µm analytical column (Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. × 50 cm, nanoViper). A 120 min gradient from 3.2 to 36% solution B (99.9 % ACN, 0.1% formic acid) was used for separation of the proteins, at a flow rate of 300 nl/min. The Q-Exactive mass spectrometer was set up as follows (Top5 method): a full scan (300-1600 m/z) at R=70.000 was followed by (up to) 12 MS2 scans at R=17500, using an NCE setting of 28. Singly charged precursors were excluded for MSMS, as were precursors with z>5. Dynamic exclusion was set to 20 seconds.

The MS raw files were analyzed, identified and quantified using MaxQuant version 1.6.6.0, with the MaxLFQ algorithm [14,15] implemented for label-free quantitative detection of proteins. In brief, the raw files were searched against the sample-specific protein sequence database and against the human genome (*Homo sapiens*, 73952 sequences). The sequences database was complemented with common contaminants, such as human keratin, trypsin and bovine serum albumin, as well as reversed sequences of all protein entries to estimate the false discovery rate. Oxidation of methionine's, protein N-terminal acetylation, deamination of asparagine and glutamine, and conversion of glutamine to pyro-glutamic acid were used as variable modifications, while carbamidomethylating of cysteine residues was used as a fixed modification. Two missed cleavages of trypsin were allowed, and all identifications were filtered in order to achieve a protein false discovery rate of 1% using the target-decoy strategy in Perseus version 1.6.6.0 [16], resulting in 2215 proteins. Functional annotation of the proteins was determined by a combination of the InterProScan database [11], Pfam database [17] and manual search using protein BLAST. Proteins were annotated as GH or CE from the DBCan meta server v8 [18] and all GH and CE annotated equally by at least two of the three databases were included (DIAMOND, HMMER, Hotpep).

**Statistical analysis**

Statistical analysis was performed in Rstudio [19] and MatLab [20]. Kruskall-Wallis-Dunns test was performed with R version 3.4.3 and R-package PMCMR plus (2018). Correlation of bacterial profiles to SCFAs was performed by using Spearman correlations, with a p-value less than 0.05, FDR corrected using the Benjamini-Hochberg method in the MatLab programming environment [20]. Correlation of the metadata to 16S using ASCA-ANOVA was performed in the MatLab environment, while metadata correlation to children with the bacterial networks was performed using a chi-square test.

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## Supplementary Figures and Tables

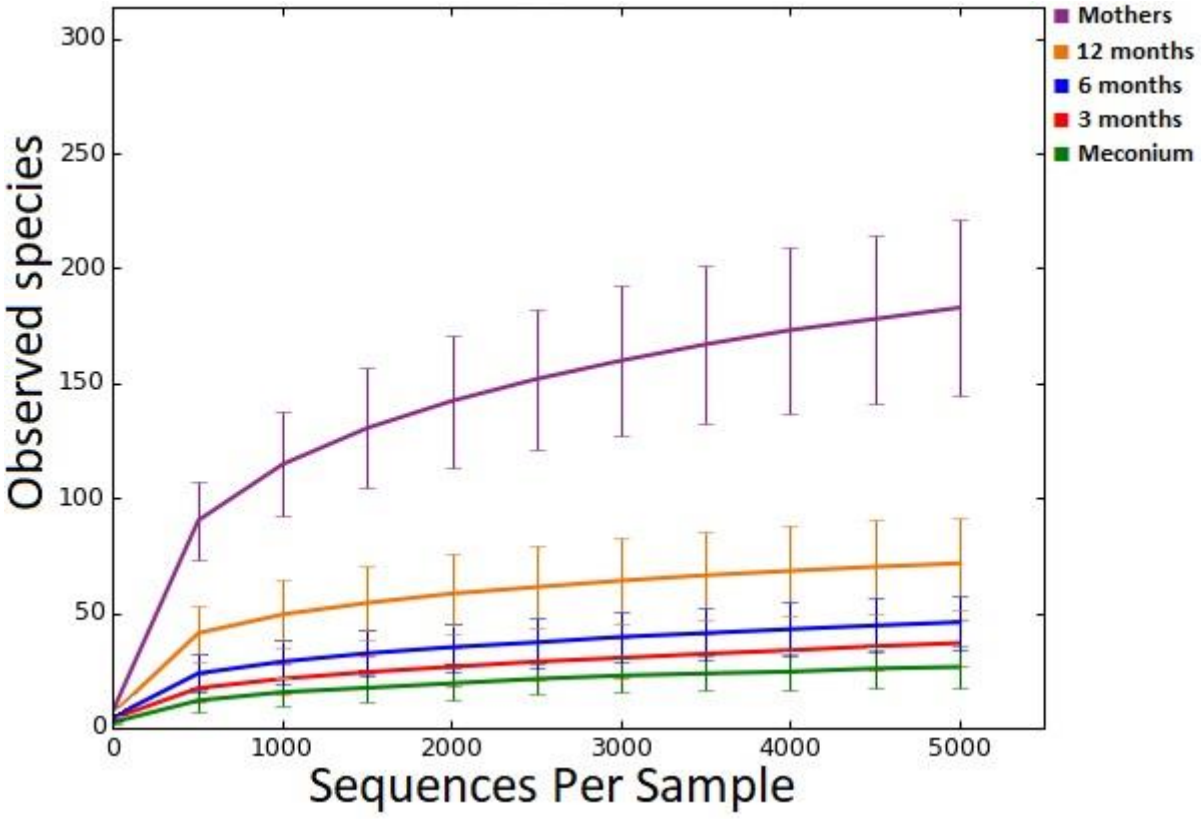


**Figure S1.** Rarefaction curve of observed species. The illustration shows the amount of observed species based on sequences per sample (SPS) for the different infant age groups and mothers. The cut-off was set to 5 000 SPS. SPS was saturated at approximately 5 000 for infants, but it was not fully established in mothers.

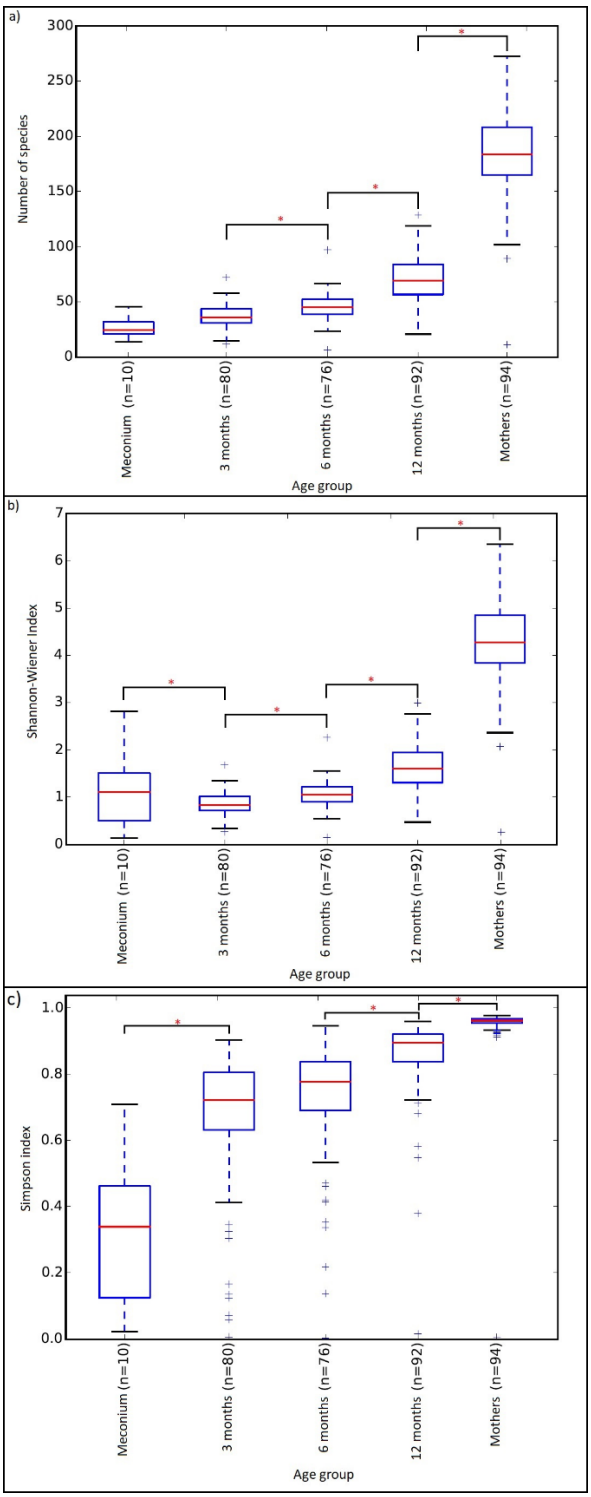


**Figure S2.** Alpha diversity. The illustration gives an overview of observed species (A), Shannon-Wiener index (B), and inverse Simpsons-index (C). Asterisks represents a p-value < 0.05 (paired t-test).

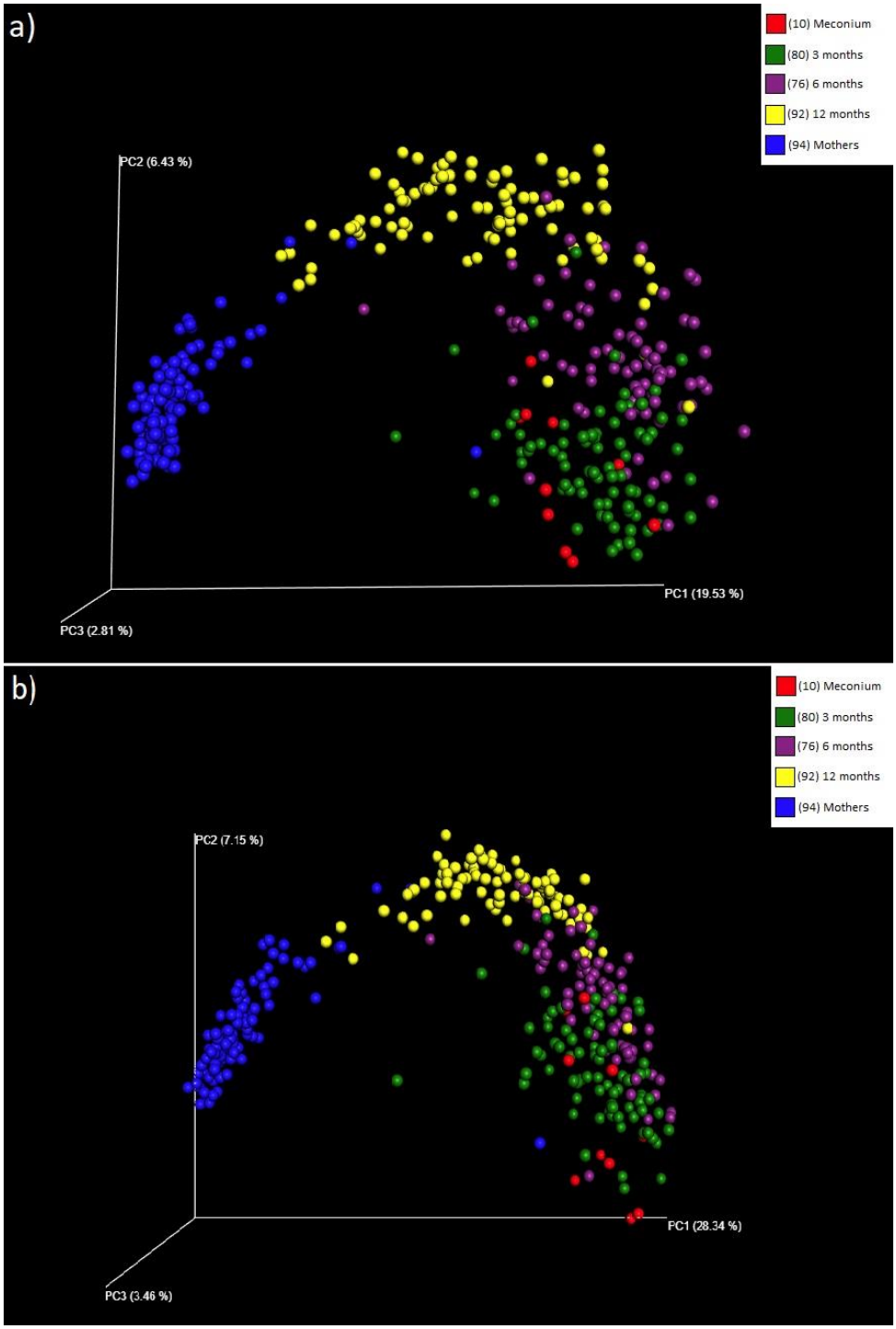


**Figure S3.** Beta-Diversity. This figure illustrates the Binary-Jaccard (A) and Unweighted Unifrac (B) indexes. The PCoA plot shows the OTU dissimilarities between the different infant age groups and mothers. The age groups are divided by colors, shown at the top right, with the number of samples per age group in parentheses.

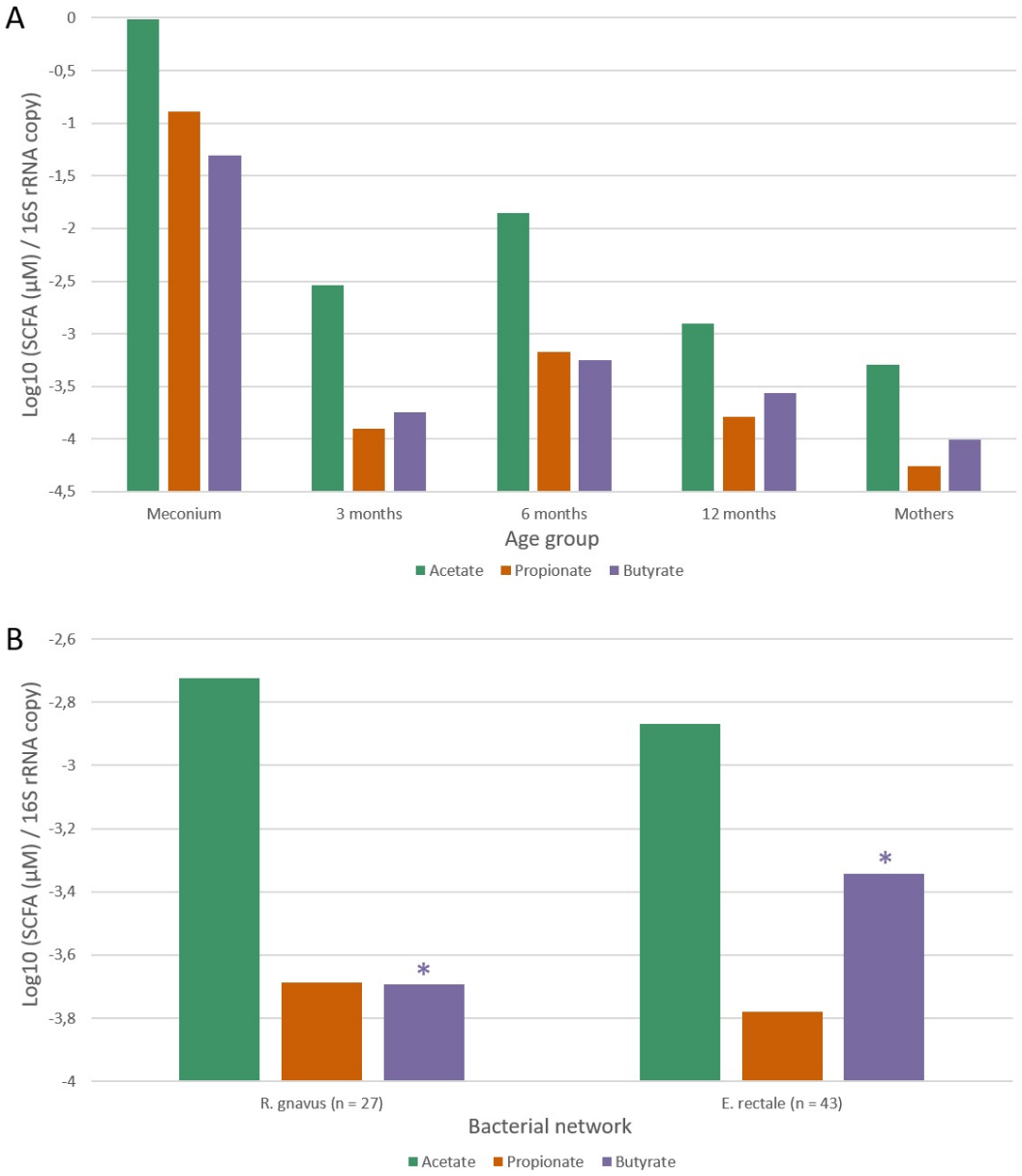


**Figure S4.** Total short-chain fatty acids per 16S rRNA gene copy. The bar-chart illustrates the logarithmic (log10) abundance of short-chain fatty acids per 16S rRNA gene copy in the infant for all age groups (A) and by the two dominant bacterial networks with a positive or negative correlation to butyrate (B). The bacterial load for each sample was determined by calculating 16S copy number based on Cq-values resulting from qPCR. Asterisks represent a significant difference ($p<0.05$, Mann-Whitney-Wilcoxon test). Asterisks are not included in A) as all SCFAs were significant between the age groups.

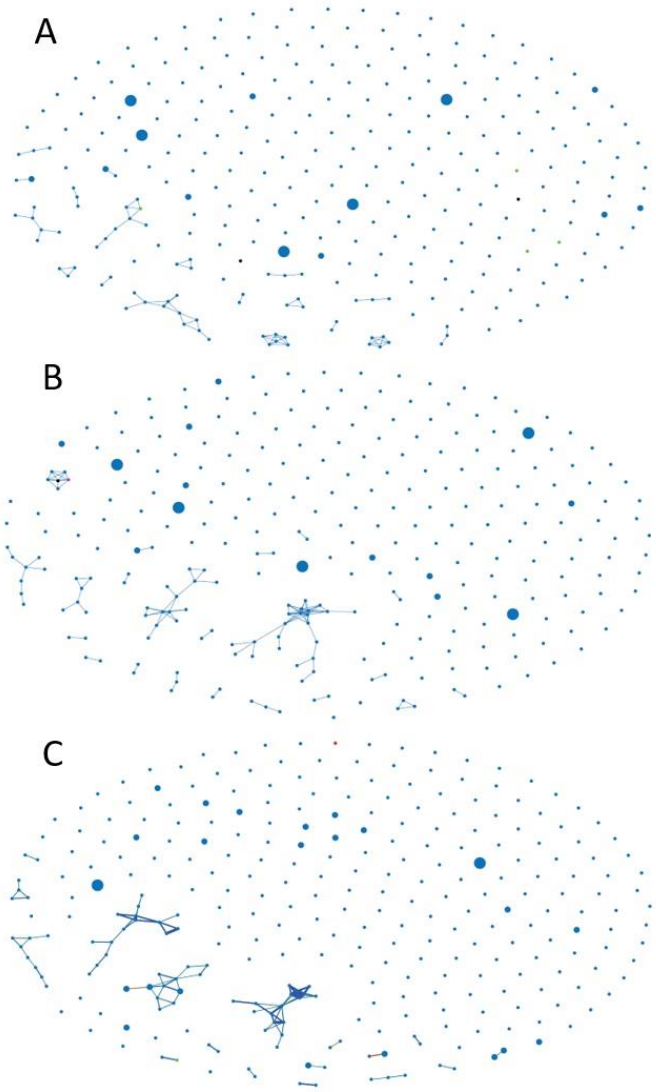


**Figure S5.** Bacterial correlation to SCFA in all age groups. The figure illustrates the correlation pattern between bacteria and relative amount of SCFAs for 3 months of age (A), 6 months of age (B), and mothers (C). The illustration shows all OTUs from 16S rRNA represented as nodes, with color indicating their correlation to SCFAs; Blue = no correlation, red = negative correlation to butyrate, green = positive correlation to butyrate, black = positive correlation to propionate and purple represents a positive correlation to both propionate and acetate. The three different node sizes represent the general abundance of the respective bacteria. The thickness of the lines between nodes represents a correlation between the bacteria, of which a thick line is a strong correlation. Blue lines indicate a positive correlation between the bacteria, while brown indicate a negative correlation.

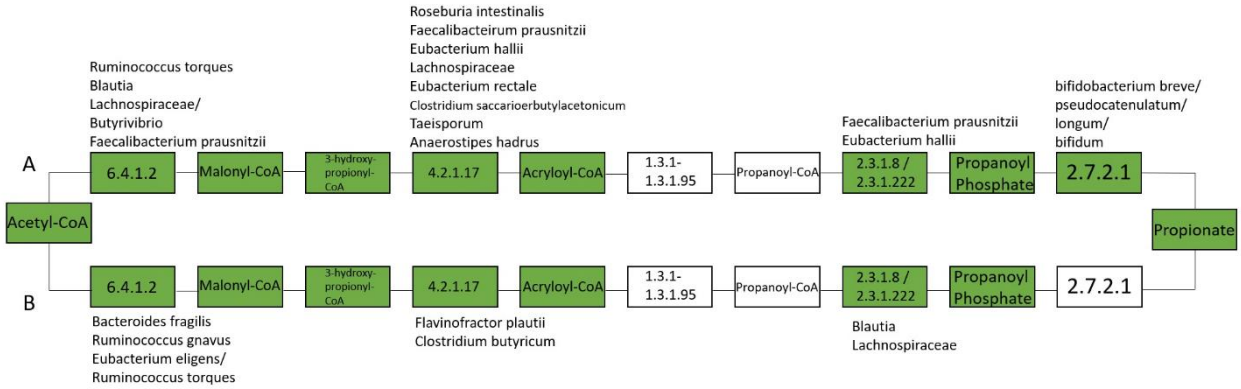


**Figure S6.** Expressed enzymes related to the propionate production pathway. The figure illustrates the expressed bacterial proteins (represented by E.C numbers) related to propionate production. The figures show proteins detected (green box) in infants with the *E. rectale* network (A), or *R. gnavus* network (B). Bacterial taxonomy is shown next to each E.C number, representing the bacterial source of the given protein.

**Table S1.** P-values Kruskall-Wallis-Dunn's test for bacterial orders. The table shows p-values for the major bacterial orders between each age group. All values in bold represent a p-value < 0.05.

| | **Bacteroidales** | | | |
|---|---|---|---|---|
| | **Meconium** | **3 months** | **6 months** | **12 months** |
| **Meconium** | - | **-** | - | - |
| **3 months** | **0.0084** | - | **-** | - |
| **6 months** | 0.1693 | **0.0071** | - | - |
| **12 months** | 0.0622 | 0.0615 | 0.2676 | - |
| **Mothers** | **0.0001** | **0.0087** | **$4.4\times10^{-08}$** | **$2.4\times10^{-06}$** |
| | **Bifidobacteriales** | | | |
| | **Meconium** | **3 months** | **6 months** | **12 months** |
| **Meconium** | - | **-** | - | - |
| **3 months** | **$3.6\times10^{-05}$** | **-** | - | **-** |
| **6 months** | **$3.6\times10^{-05}$** | 0.963 | - | **-** |
| **12 months** | 0.077 | **$3.3\times10^{-07}$** | **$4.4\times10^{-07}$** | **-** |
| **Mothers** | 0.963 | **$<2\times10^{-16}$** | **$<2\times10^{-16}$** | **$8.1\times10^{-05}$** |
| | **Clostridiales** | | | |
| | **Meconium** | **3 months** | **6 months** | **12 months** |
| **Meconium** | - | **-** | **-** | **-** |
| **3 months** | 0.2271 | **-** | **-** | **-** |
| **6 months** | **0.0135** | **0.0069** | **-** | **-** |
| **12 months** | **$1.3\times10^{-10}$** | **$<2\times10^{-16}$** | **$<2\times10^{-16}$** | **-** |
| **Mothers** | **$1.5\times10^{-08}$** | **$<2\times10^{-16}$** | **$2.2\times10^{-11}$** | 0.0829 |
| | **Enterobacteriales** | | | |
| | **Meconium** | **3 months** | **6 months** | **12 months** |
| **Meconium** | **-** | **-** | **-** | **-** |
| **3 months** | 0.9509 | **-** | **-** | **-** |
| **6 months** | 0.9509 | 0.7665 | **-** | **-** |
| **12 months** | **0.0037** | **$3.3\times10^{-10}$** | **$2.0\times10^{-11}$** | **-** |
| **Mothers** | **$1.8\times10^{-06}$** | **$<2\times10^{-16}$** | **$<2\times10^{-16}$** | **$2.5\times10^{-05}$** |
| | **Lactobacilliales** | | | |
| | **Meconium** | **3 months** | **6 months** | **12 months** |
| **Meconium** | **-** | **-** | **-** | **-** |

| | | | | |
|---|---|---|---|---|
| **3 months** | **0.00932** | **-** | **-** | **-** |
| **6 months** | **0.00337** | 0.48377 | **-** | **-** |
| **12 months** | 0.41882 | **0.00026** | **$1.1\times10^{-05}$** | **-** |
| **Mothers** | 0.62474 | **$1.0\times10^{-11}$** | **$8.0\times10^{-14}$** | **0.00190** |

**Table S2.** P-values Kruskall-Wallis Dunn's test for SCFAs. The table shows p-values for the major SCFAs between each age group. All values in bold represent a p-value < 0.05.

| | **Acetate** | | | |
|---|---|---|---|---|
| | **Meconium** | **3 months** | **6 months** | **12 months** |
| **Meconium** | - | - | - | - |
| **3 months** | **0.011** | - | - | - |
| **6 months** | **0.010** | 0.947 | - | - |
| **12 months** | **$<2\times10^{-16}$** | **$<2\times10^{-16}$** | **$<2\times10^{-16}$** | - |
| **Mothers** | **$<2\times10^{-16}$** | **$1.3\times10^{-14}$** | **$1.8\times10^{-14}$** | 0.524 |
| | **Propionate** | | | |
| | **Meconium** | **3 months** | **6 months** | **12 months** |
| **Meconium** | **-** | - | - | - |
| **3 months** | 0.133 | - | - | - |
| **6 months** | **$9.7\times10^{-05}$** | **4-$9\times10^{-08}$** | - | - |
| **12 months** | **$2.1\times10^{-10}$** | **$4.2\times10^{-15}$** | **0.017** | - |
| **Mothers** | **$1.9\times10^{-10}$** | **$4.2\times10^{-15}$** | **0.016** | 0.954 |
| | **Butyrate** | | | |
| | **Meconium** | **3 months** | **6 months** | **12 months** |
| **Meconium** | **-** | **-** | **-** | **-** |
| **3 months** | 0.152 | **-** | **-** | **-** |
| **6 months** | **$<2\times10^{-16}$** | **$3.8\times10^{-16}$** | **-** | - |
| **12 months** | **$<2\times10^{-16}$** | **$<2\times10^{-16}$** | **0.041** | - |
| **Mothers** | **$<2\times10^{-16}$** | **$<2\times10^{-16}$** | 0.654 | 0.102 |

**Table s3.** Maternal and infant factors associated with the presence of either *E. rectale* or the *R. gnavus* network in infants. The table gives an overview of the different metadata: delivery mode, breastfeeding between 3 – 12 months and solid food introduction (3 – 6m) with their respective p-value (chi-square test).

| | | **Infants with the E. rectale network (n/%)** | **Infants with R. gnavus network (n/%)** | **p-value** |
|---|---|---|---|---|
| **Delivery Mode** | Vaginal | 30/81.1 | 16/69.6 | .43 |
| | C-Section | 7/18.9 | 6/26.1 | |
| | Missing | 0/0 | 1/4.3 | |
| **Gender** | Boy | 21/56.8 | 9/39.1 | .66 |
| | Girl | 16/43.2 | 13/56.5 | |
| | Missing | 0/0 | 1/4.3 | |
| **Breastfeeding at 6 months** | Yes | 30/81.1 | 19/82.61 | .81 |
| | No | 4/10.81 | 2/8.7 | |
| | Missing | 3/8,1 | 2/8.7 | |
| **Breastfeeding at 9 months** | Yes | 27/73 | 17/73.9 | .75 |
| | No | 8/21.6 | 4/17.4 | |
| | Missing | 2/5.41 | 2/8.7 | |
| **Breastfeeding at 12 months** | Yes | 19/51.4 | 10/43.5 | .87 |
| | No | 14/37.8 | 10/43.5 | |
| | Missing | 4/10.8 | 3/13.0 | |
| **Solids 3m** | Yes | 2/5.88 | 4/17.4 | .45 |
| | No | 31/91.2 | 16/65.6 | |

| | | | | |
|---|---|---|---|---|
| | Missing | 1/2.94 | 3/13.0 | |
| | Yes | 18/48.65 | 12/52.2 | |
| **Solids 4m** | No | 15/40.54 | 8/34.8 | .36 |
| | Missing | 4/10.81 | 3/13.0 | |
| | Yes | 28/75.68 | 18/78.3 | |
| **Solids 5-6m** | No | 5/13.5 | 2/8.7 | .60 |
| | Missing | 4/10.81 | 3/13.0 | |

III

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# Feeding Practices and Dietary Diversity in the First Year of Life: PreventADALL, a Scandinavian Randomized Controlled Trial and Birth Cohort Study

Carina Madelen Saunders [1,2,*], Eva Maria Rehbinder [1,3], Karin C. Lødrup Carlsen [1,2], Christine Monceyron Jonassen [4,5], Marissa LeBlanc [6], Björn Nordlund [7,8], Håvard Ove Skjerven [1,2], Cilla Söderhäll [7,8], Riyas Vettukattil [1,2], Monica Hauger Carlsen [9]

[1] *University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway;* [2] *Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway;* [3] *Department of Dermatology, Oslo University Hospital, Oslo, Norway;* [4] *Genetic Unit, Centre for Laboratory Medicine, Østfold Hospital Trust, Kalnes, Norway;* [5] *Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of LifeSciences, Ås, Norway;* [6] *Oslo University Hospital and University of Oslo, Oslo, Norway, Oslo Centre for Biostatistics and Epidemiology;* [7] *Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden;* [8] *Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden;* [9] *Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway*

A B S T R A C T

**Background:** Breastmik is considered the optimal source of nutrition in early infancy. However, recommendations and practices for when and how complementary food should be introduced in the first year of life vary worldwide. Early introduction of allergenic foods may prevent food allergies, but if early food introduction influences infant feeding practices is less known.

**Objectives:** We sought to assess infant feeding practices in the first year of life and to determine if early interventional food introduction influences breastfeeding and dietary diversity.

**Methods:** Dietary intake was assessed in infants from the population-based clinical trial Preventing Atopic Dermatitis and ALLergies (PreventADALL) in children study. A total of 2397 infants were cluster-randomized at birth into 4 different groups: *1*) control, *2*) skin intervention, *3*) introduction to 4 allergenic foods between 3 and 4 mo of age: peanut, cow milk, wheat, and egg, as small tastings until 6 mo, and *4*) combined skin and food interventions. Dietary data were available from at least one of the 3-, 6-, 9-, and 12-mo questionnaires in 2059 infants. In the present analysis, groups 1 and 2 constitute the No Food Intervention group, whereas groups 3 and 4 constitute the Food Intervention group.

We used the log-rank test and Cox regression to assess the impact of food intervention on age of breastfeeding cessation. Mixed effects logistic regression was used to compare dietary diversity, defined as the number of food categories consumed, between intervention groups.

**Results:** At 3, 6, 9, and 12 mo, 95%, 88%, 67%, and 51% were breastfed, respectively, and breastfeeding duration was not affected by the food intervention. In the No Food Intervention group, mean age of complementary food introduction was 18.3 wk (confidence interval [CI]: 18.1, 18.5). In the Food Intervention group, the dietary diversity score was 1.39 units (CI: 1.16, 1.62) higher at 9 mo ($P < 0.001$) and 0.7 units (CI: 0.5, 0.9) higher at 12 mo ($P < 0.001$) compared to the No Food Intervention group.

**Conclusions:** Early food intervention did not affect breastfeeding rates and increased dietary diversity at 9 and 12 mo.

*Keywords*: breastfeeding, complementary food, solid food, food diversity, diet diversity, early food introduction, infant diet, infant feeding, infant feeding practices, PreventADALL

## Introduction

The first year of life is a critical period to establish feeding practices, taste preferences, and long-term dietary habits. Breastmilk is considered the optimal source of nutrition for infants [1,2]. Exclusive breastfeeding for ≥4 mo is recommended by European authorities [3,4] and for 6 mo by the American Academy of Pediatrics and WHO [5,6]. In Norway, exclusive

*Abbreviations:* FI group, Food Intervention group; NFI group, No Food Intervention group; PreventADALL, Preventing Atopic Dermatitis and ALLergies.

* Corresponding author. *E-mail address:* carinamadelensaunders@gmail.com (C.M. Saunders).

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breastfeeding is recommended for the first 6 mo of life, with an optional introduction of complementary foods (foods other than breastmilk or formula) from 4 mo, if breastmilk is insufficient [7, 8].

Compared to other countries, Norway has high rates of breastfeeding initiation at birth (98%), with 80% of infants still breastfeeding at 6 mo, and many continuing to breastfeed throughout the first year of life [9]. Most infants are introduced to complementary foods by 6 mo of age [10–12]. Breastfeeding rates in Sweden are lower than that in Norway but have been increasing in recent years, with 63% breastfeeding at 6 mo and 28% at 12 mo [13,14].

Throughout the years, recommendations regarding the introduction of complementary foods have changed and continue to be a topic of debate [15,16]. These challenges are reflected in great variations of infant feeding practices between industrialized countries [17]. Several randomized controlled trials (RCTs), including the Preventing Atopic Dermatitis and ALLergies (PreventADALL) trial, found evidence that earlier introduction of specific allergenic foods may prevent allergic sensitization and food allergies [18–21]. In response, a number of guidelines have been adapted [22–24].

A diverse diet and timely introduction of complementary foods may also ensure an adequate supply of nutrients and influence both short- and long-term health outcomes [25–27]. As long as complementary foods are given in an age-appropriate texture and are nutritionally adequate, there is currently no convincing evidence that early food introduction has adverse health effects [3]. Dietary diversity is defined as the number of different foods or food groups consumed over a given period [28]. Dietary diversity is linked to a higher nutrient intake and increased exposure to different food antigens [29]. A diverse diet could decrease the risk of developing food allergies [26] and may also have positive health effects by exposing the gut microbiota to various foods early on and thus increase microbial diversity [30–32].

Given the conflicting advice and cultural differences in infant feeding practices, more research into the timing of complementary food introduction is warranted. Therefore, the primary aim of this substudy, from the Scandinavian RCT and birth cohort PreventADALL, was to assess infant feeding practices, including the timing of complementary food introduction. The secondary aim was to determine if early food intervention (early exposure to small tastes of allergenic foods) impacted breastfeeding rates and dietary diversity in the first year of life.

## Methods

### Study design and study population

In the present study, we analyzed dietary data from infants in the PreventADALL RCT, a multicenter, prospective, general, population-based mother–child birth cohort, aiming at primary prevention of allergic disease. Study design, recruitment, inclusion criteria, and baseline characteristics are described in detail elsewhere [33]. Briefly, 2397 mother–child pairs were recruited from the general population of pregnant women around 18 wk of gestational age at Oslo University Hospital, Østfold Hospital Trust (Norway), and Karolinska University Hospital (Stockholm, Sweden) between December 2014 and October 2016. The children were included at birth, provided there was no severe illness and gestational age was ≥35.0 wks. Three children later withdrew from the study. Maternal health and sociodemographic and lifestyle factors were obtained through electronic questionnaires administered during pregnancy, and detailed electronic questionnaires, including infant dietary data, were obtained at 3 (77% response rate), 6 (71% response rate), 9 (68% response rate), and 12 (69% response rate) mo of age. We included all 2059 infants with at least one completed diet questionnaire.

Newborns were randomly assigned to 4 intervention groups: no intervention (control group), skin intervention, food intervention, or the combined interventions [34]. The control group was instructed to follow regular advice and national guidelines regarding weaning and skin care. The skin intervention consisted of a 5- to 10-minute emulsified oil bath and face cream (Ceridal) for ≥4 d/wk, from 2 wk through 8 mo of age. The food intervention consisted of small tastes of 4 different commonly allergenic foods between 3 and 4 mo of age. Peanut butter was given for the first time at the 3-mo follow-up visit. Cow milk was introduced 1 wk later, followed by wheat, and scrambled eggs in the fourth week of introduction. Parents were instructed to give each of these foods as a tiny taste, either from their finger or a teaspoon, ≥4 d/wk and until 6 mo without any dose restriction. The purpose of the early food intervention was to expose infants to small amounts of allergenic foods over time and was not intended to replace breastmilk.

The PreventADALL study was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway (2014/518) and in Stockholm, Sweden (2014/2242-31/4). The study was registered at clinicaltrials.gov (NCT02449850). Informed consent was given upon enrollment and inclusion of the infant at birth.

Dietary data were obtained through electronic weekly diaries (2–26 wk of age) and questionnaires (at 3, 6, 9, and 12 mo of age). Breastfeeding was assessed at 3, 6, 9, and 12 mo of age by parents reporting for the last 3 mo if the infant had received breastmilk and the time of breastfeeding cessation. If the infant was not exclusively breastfed, the amount of breastmilk consumed was categorized as most of the diet, the same amount as other food, or as a small part of the diet. At 6 mo, parents reported the amount of breastmilk consumed as no breastmilk, a small part of the diet, approximately half of the diet, or most of the diet. Consumption of porridge, how often it was given, at what age it was first introduced, and what it was made of (rice, millet, oat, corn, wheat, whole meal, spelt, Sinlac, and other types) was reported at 3 and 6 mo. Questions about dairy intake at 6, 9, and 12 mo included the time of first introduction and what kind of dairy products were given to the infant. At 6, 9, and 12 mo, parents were asked to indicate how often the infant consumed several solid foods. The categories included: bread/cookies/waffles/cakes and other baked goods, fruit or berries, root vegetables (such as potato, turnip, carrot, and parsnip), other vegetables, peanut (as a spread or incorporated in other foods), pure egg (eg, fried, cooked, scrambled, and eggnog), egg in other foods (eg, gratin, waffles, baked goods, paste, or similar), fatty fish (salmon, trout, mackerel, pike, halibut, and eel), other fish, shellfish, poultry meat, and other meat. We also asked about how much of the infant food was home cooked compared with commercially prepared (industrially processed), as well as the content of organic food in the infant's diet.

All questions about diet were mandatory, assuring a complete set of values in all questionnaires. Please see the Supplementary data section and Supplementary Figure 1 for more details on the infant diet questions.

## Definitions

In the present analysis, we included all infants who were randomly assigned to the food intervention (early exposure to small amounts of 4 allergenic foods [peanut, cow milk, wheat, and egg] as part of the study intervention described earlier [33]) into the Food Intervention (FI) group, whereas the No Food Intervention (NFI) group consisted of all study participants who were randomly assigned to no food intervention (control group and the skin intervention groups). For an overview of the study population, see Figure 1.

Complementary food introduction was defined as the age at which any food or drink other than breastmilk, formula, and water was given for the first time to the infant.

### *Outcomes*

Primary outcome: Breastfeeding duration was defined as the number of months an infant was breastfed. Breastfeeding rates included exclusively breastfed infants and partially breastfed infants (receiving breastmilk and other sources of nutrition).

Secondary outcome: The dietary diversity score is defined as the number of different food groups given to the infant during the last 3 months at 6, 9, and 12 mo of age. The calculated score was based on the following food groups: bread and other baked goods, milk, fermented dairy products, cheese, other dairy products, fruits and berries, root vegetables, other vegetables, peanuts, other nuts, egg (pure and egg in other foods), fatty fish, other fish, shellfish, poultry, and other meat. Summing the number of foods introduced at 9 and 12 mo, the maximum score was 16. At 6 months, the maximum score was 10 because intervention foods, including all dairy products, peanuts, and egg, were excluded from the dietary diversity score. Infant porridge was not included in the score because we did not include questions on infant porridge in the 9- and 12-mo questionnaires. The dietary diversity score did not consider frequency or portion sizes.

## Statistical analysis

Categorical variables are presented as numbers and percentages. Continuous variables, including frequency and intake of various foods, are presented as means, SD, and minimum (min) –

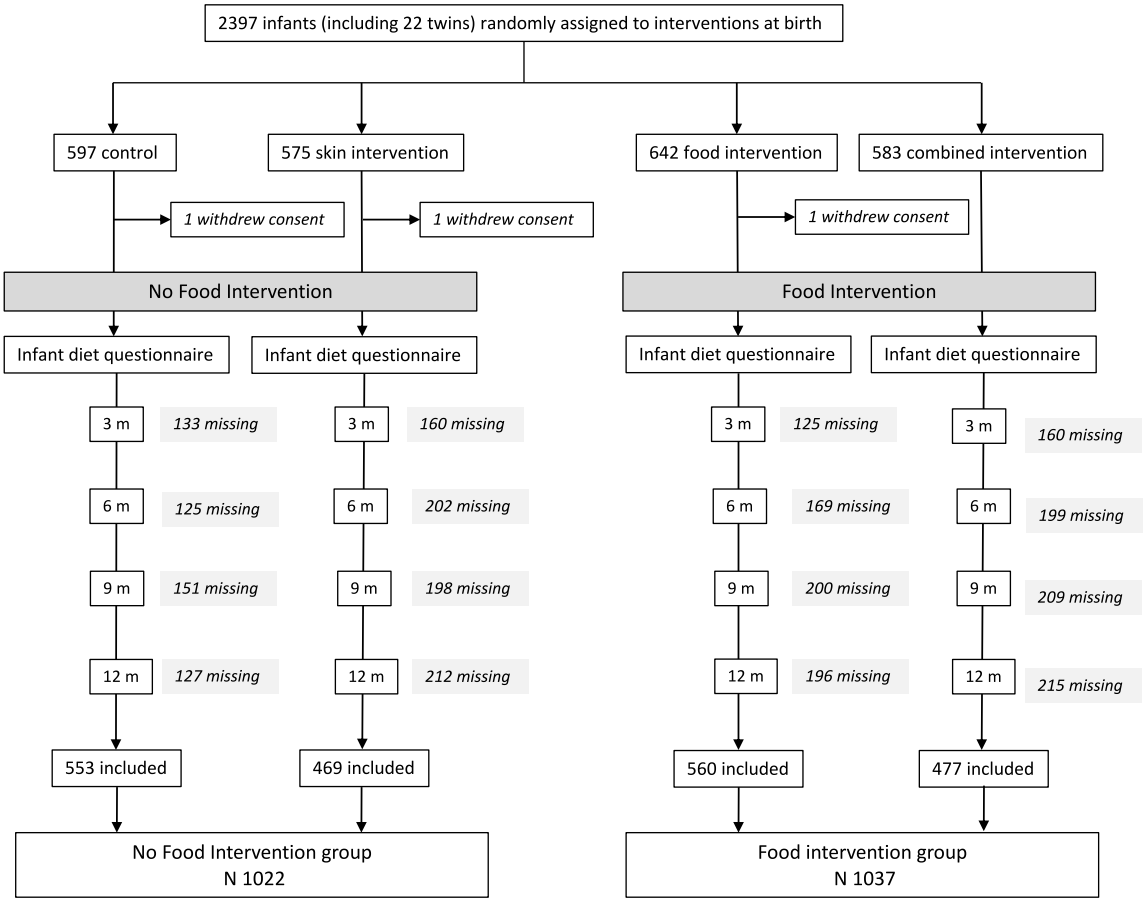


**FIGURE 1.** Overview of the PreventADALL study population, including withdrawals and randomization groups. We included all 2059 infants with at least one completed dietary questionnaire. In the present study, the Food Intervention group includes infants from both the food intervention and combined intervention group, whereas the No Food Intervention group includes infants from the control and skin intervention groups.

maximum (max). To analyze breastfeeding rates in the first year of life, we used Cox regression. Kaplan–Meier curves were estimated for the FI and NFI groups, and differences in cessation rates between intervention groups were compared using the log-rank test. The proportional hazards assumption was tested and met. These analyses were performed using IBM SPSS statistics version 25.

We used R version 3.6.0 for dietary diversity analysis, including complete case (ie, answered the dietary questionnaire at a given time point) and modified intention to treat analysis (ie, all randomly assigned). For dietary diversity assessment at each time point between the intervention groups, we analyzed dichotomous end points using mixed effects logistic regression with the interventions and their interaction as fixed effects and randomization period and residential postal code as random effects.

For the intention to treat analysis, missing primary outcome data were imputed using multiple imputations by chained equations. The number of multiple imputations was 15, and the scalar giving the number of iterations was 20. Complete case analysis was conducted as a sensitivity analysis.

## Results

The background characteristics of the 1022 infants in the FI group and 1037 infants in the NFI group (N1037) were similar, as shown in Table 1. We also assessed the background characteristics among our study population of 2059 infants and their parents and the remaining 338 study participants in the PreventADALL cohort, as seen in Supplementary Table 1. In our study population, 440 (21%) were from Sweden and 1619 (79%) from Norway.

### Breastfeeding and complementary feeding

In the Swedish population, breastfeeding rates at 3 mo and 6 mo were 92% and 80% compared with 95% and 88% in the Norwegian part of the study population, respectively. At 12 mo, 22% breastfed their children in Sweden and 51% in Norway.

In the NFI group, the mean age of complementary food introduction was 18.3 wk (CI: 18.1, 18.5 wk), whereas infants randomly assigned to food intervention were introduced complementary foods from 12 wk of age. Porridge was the most common first food, other than the interventional foods (peanut, cow milk, wheat, and egg). In the total study population, porridge was introduced in 9.6 % of infants by 3 mo of age, 45.7% at 4 mo, and 25.6% at 5 mo. By 6 mo of age, 81.1% infants had been given porridge. The most frequent porridges to be introduced before 6 mo were oat (62.2%), wheat (40.5%), and corn (34.1%). The following porridge types were given less frequently: millet (18.3%), whole meal (13.3%), spelt (4.1%), Sinlac (4.2%), and other types (5.1%).

The cumulative proportion of infants introduced to other complementary foods is listed in Table 2. The most common complementary foods given at 6 mo of age were fruit and berries, root vegetables, and other vegetables. Most infants consumed dairy (in any form) by 12 mo of age. The number of infants consuming fish, meat, and poultry increased from 6 to 9 mo and were consumed by most infants by 12 mo of age.

At 6 mo, 45% of infants were reported to consume mostly or only commercially prepared infant foods, whereas 9% consumed only home-cooked foods. By 12 mo, the proportion of home-cooked foods in the infant's diet increased. The proportion of infants mainly receiving organic food declined from 6 to 12 mo (Table 2).

**TABLE 1**
Baseline characteristics of study population[1]

| | | NFI group ($n$ = 1022) | FI group ($n$ = 1037) |
|---|---|---|---|
| Age of mother, y | | 32.4 (4.1, 21–48) | 32.6 (4.0, 20–44) |
| Age of father, y | | 34.8 (5.4, 23–72) | 34.8 (5.5, 21–72) |
| Male sex, infants | | 516 (51) | 562 (54) |
| Birth weight, g | | 3582 (489, 1794–5632) | 3573 (463, 2005–4900) |
| Birth length, cm | | 50.5 (2.2, 33–61) | 50,5 (2.0, 44–56) |
| Vaginal delivery | | 861 (84) | 865 (83) |
| Caesarian section | | 161 (16) | 172 (17) |
| Marital status[2] | Married | 385 (41) | 400 (42) |
| | Cohabitants | 530 (56) | 541 (56) |
| | Single | 19 (2) | 15 (2) |
| | Divorced/separated | 0 (0) | 0 (0) |
| | Other | 5 (<1) | 9 (1) |
| Maternal education level[3] | Primary school (9/10 y) | 6 (<1) | 5 (<1) |
| | High school only | 86 (9) | 96 (10) |
| | Higher education <4 y | 288 (31) | 302 (31) |
| | Higher education ≥4 y | 520 (56) | 535 (56) |
| | PhD | 32 (3) | 24 (3) |
| | Other education | 2 (<1) | 0 (0) |
| Paternal education level[4] | Primary school (9/10 y) | 10 (1) | 12 (1) |
| | High school only | 163 (18) | 169 (18) |
| | Higher education <4 y | 261 (29) | 283 (31) |
| | Higher education ≥4 y | 426 (47) | 423 (46) |
| | PhD | 32 (4) | 31 (3) |
| | Other education | 3 (<1) | 1 (<1) |
| Maternal work | Full time | 808 (79) | 821 (79) |
| | Part-time | 85 (8) | 83 (8) |
| | Student | 55 (5) | 60 (6) |
| | Housewife/ homemaker | 7 (<1) | 9 (<1) |
| | Jobseeker/ unemployed | 9 (<1) | 11 (1) |
| | Disabled | 3 (<1) | 7 (<1) |
| | Other | 13 (1) | 18 (2) |
| Household income (NOK)[5] | Below 300,000 | 9 (1) | 8 (1) |
| | 300,000–600,000 | 117 (13) | 112 (12) |
| | 600,000–1,000,000 | 383 (41) | 396 (41) |
| | 1,000,000–1,400,000 | 299 (32) | 319 (33) |
| | >1,400,000 | 119 (13) | 112 (12) |
| | Did not want to answer | 12 (1) | 18 (2) |
| Tobacco use (ever) | Smoking | 197 (22) | 213 (21) |
| | Snus | 205 (22) | 218 (23) |

Abbreviations: FI, Food Intervention; NFI, No Food Intervention.
[1] Values are means (SD, min-max) or $n$ (%) unless otherwise stated.
[2] Information available from $n$ = 939 (NFI)/$n$ = 965 (FI)
[3] $n$ = 934 (NFI)/$n$ = 962 (FI),
[4] $n$ = 906 (NFI)/$n$ = 924 (FI)
[5] $n$ = 939/$n$ = 965

**TABLE 2**
Proportion of infants who received various complementary foods at 6, 9, and 12 mo in the No Food Intervention and Food Intervention groups[1]

| | 6 mo (%) | | 9 mo (%) | | 12 mo (%) | |
|---|---|---|---|---|---|---|
| | NFI | FI | NFI | FI | NFI | FI |
| Cow milk | 11 | 64 * | 30 | 64 * | 58 | 74 * |
| Lactose free milk | <1 | 3 * | 4 | 7 * | 7 | 8 |
| Unpasteurized cow milk | <1 | <1 | <1 | 2 * | 1 | 2 |
| Pro/prebiotic dairy | 2 | 7 * | 6 | 13 * | 20 | 23 * |
| Yogurt | 12 | 26 * | 41 | 60 * | 80 | 85 * |
| Cheese | 6 | 8 * | 50 | 62 * | 86 | 88 |
| Other dairy products | 8 | 14 * | 36 | 48 * | 58 | 63 * |
| Bread and other baked goods | 21 | 28 | 88 | 90 * | 96 | 97 |
| Fruit and berries | 92 | 93 | 99 | 99 | 99 | 99 |
| Root vegetables | 89 | 85 | 99 | 99 | 99 | 99 |
| Vegetables | 69 | 66 | 97 | 97 | 98 | 98 |
| Peanuts | 12 | 85 * | 22 | 72 * | 34 | 68 * |
| Other nuts | 4 | 7 * | 15 | 21 * | 24 | 35 * |
| Egg foods | 11 | 22 * | 63 | 76 * | 87 | 90 * |
| Egg pure | 21 | 82 * | 64 | 82 * | 81 | 89 * |
| Fatty fish | 19 | 17 | 86 | 86 | 93 | 93 |
| Fish other | 12 | 12 | 70 | 70 | 84 | 84 |
| Shellfish | 1 | 3 | 12 | 13 | 27 | 29 |
| Poultry | 18 | 15 * | 81 | 81 | 88 | 88 |
| Meat other | 22 | 20 | 90 | 89 | 94 | 94 |
| Home-cooked vs commercially prepared | | | | | | |
| Only home-cooked | 9 | 9 | 4 | 4 | 4 | 3 |
| >Home-cooked[2] | 21 | 22 | 24 | 24 | 34 | 36 |
| Equal amounts | 23 | 22 | 25 | 24 | 31 | 28 |
| > Commercially prepared | 34 | 36 | 43 | 42 | 29 | 31 |
| Only commercially prepared | 12 | 10 | 4 | 6 | 2 | 2 |
| Amount of organic food | | | | | | |
| Very little/ none | 15 | 16 | 8 | 11 | 11 | 14 * |
| Small amount | 21 | 22 | 29 | 28 | 38 | 37 |
| Half | 23 | 23 | 29 | 28 | 28 | 28 |
| Majority | 32 | 29 | 26 | 25 | 18 | 16 |
| Don't know | 9 | 9 | 5 | 7 | 6 | 5 |

Abbreviations: FI, Food Intervention; NFI, No Food Intervention.
[1] Numbers are presented in %.
[2] > Home cooked refers to the infant diet consisting of more home-cooked foods than commercially prepared infant foods, and vice versa for > Commercially prepared.
* Indicates a significance $P < 0.05$ when comparing infants in the Food Intervention (FI) group with infants in the No Food intervention (NFI) group at each time point. We used chi-square test to assess differences of complementary food intake between groups.

## Impact of early food intervention on breastfeeding rates and dietary diversity

Breastfeeding rates from birth until 12 mo did not differ significantly between the FI (early introduction of peanut, milk, wheat, and egg) and the NFI group ($P = 0.96$), as shown in Figure 2.

Significantly fewer infants in the NFI group consumed dairy products, peanuts, other nuts, egg foods, and pure egg at 6 mo compared with infants in the FI group ($P < 0.001$). At 9 and 12 mo, the percentage of infants consuming these foods remained lower ($P < 0.001$) in the NFI group compared with the FI group (Table 2). Intakes of other complementary foods were not influenced by the intervention, except fewer infants in the FI group consumed poultry at 6 mo ($P = 0.032$) and more infants consumed bread and other baked goods at 9 mo ($P = 0.043$). Intake of commercially prepared infant foods compared with home-cooked foods was similar in both groups, as was the intake of organic foods, except more infants in the FI group consumed little or no organic food at 12 mo.

Excluding interventional foods, the mean dietary diversity score (number of food categories consumed) was similar in the FI compared with the NFI group at 6 mo (3.4 [SD 1.9] compared with 3.5 [SD 1.9], respectively). However, the dietary diversity score was significantly higher in the FI group compared with the NFI group at 9 and 12 mo of age ($P < 0.001$), as shown in Table 3 and Figure 3. The dietary diversity score, including interventional foods, was 11.1 (SD 2.3) in the FI group compared with 9.7 in the NFI group (SD 2.4) at 9 mo and 12.9 (SD 1.2) compared with 12.1 (SD 2.1) at 12 mo.

## Discussion

In a general population of >2000 infants, >85% of infants were breastfed throughout the first 6 mo of life, and half of the population was still breastfed at 12 mo. The mean age of complementary food introduction in the NFI group was 4.5 mo. Most infants were introduced to complementary foods, mainly fruits, vegetables, and porridge, between 4 and 6 mo. The food intervention did not affect breastfeeding rates, whereas the dietary diversity score at 9 and 12 mo was significantly higher in infants subjected to the early food intervention compared with the infants who were not.

Breastfeeding rates in our study are consistent with recent findings in Spedkost 3, a Norwegian nationwide survey from 2019, including 2182 infants [9,35], and higher than in another study from 2013, including 2500 infants, where 35% were breastfed at 12 mo [8,9]. Our results indicate that Norwegian breastfeeding rates have experienced an incline in the past decade, with a notable increase in breastfeeding from 3 to 9 mo [12]. In our study, breastfeeding rates were considerably lower in the Swedish study population, consistent with surveys based on medical records of children born in Sweden in 2017, where 44% were breastfed at 9 mo and 27% at 12 mo [36]. The shorter breastfeeding duration in Sweden might be a result of differences in parental leave policies [37].

A study from 2019 [38] of 11 European countries found that 56%–98% of infants were breastfed directly after birth and 38%–71% at 6 mo, with 10%–38% being exclusively breastfed at 6 mo. At 6 mo of age, Norway (71%), Sweden (61%), and Germany (57%) reported the highest rates of any breastfeeding.

Most infants in the NFI group were introduced to complementary foods between 4 and 6 mo of age, in line with the European Society for for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines [4] and the Spedkost 3 study in Norway, with 62% of infants introduced to complementary foods at 4 mo and 98% by 6 mo [9]. The rate of complementary food introduction before 4 mo has decreased from 21% in 1998 to 11% in 2006, 7% in 2013, and 6% in 2019 [12,39]. Complementary food is introduced earlier in other countries, with recent rates of complementary feeding before 4 mo being 21% in 2157 Dutch infants, 32% in the United States [40], 43% in the United Kingdom,

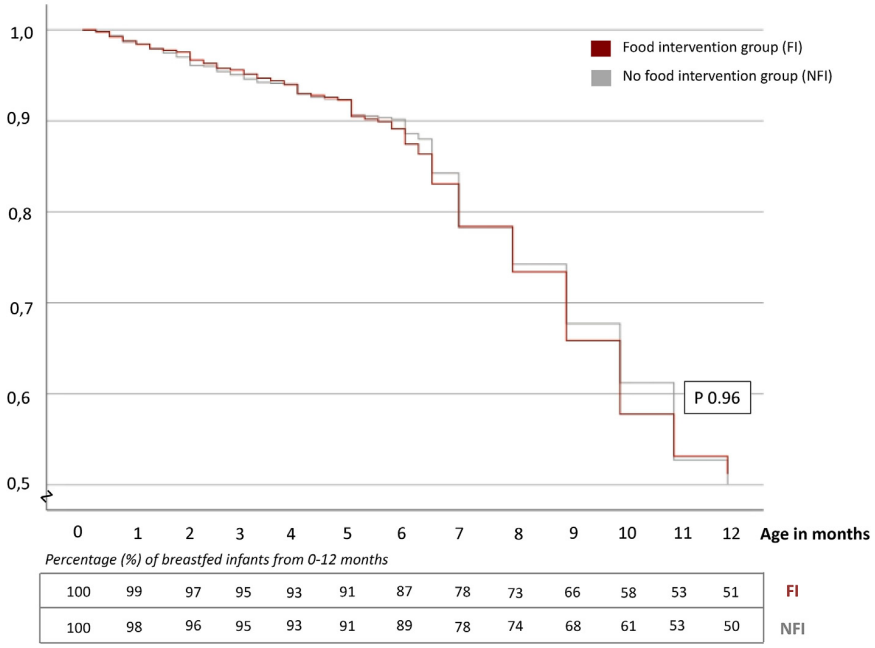


**FIGURE 2.** Kaplan–Meier curve showing no significant difference in breastfeeding rates (%) between the Food Intervention and No Food Intervention groups from 0 to 12 mo in 2059 infants. The table shows the percentage of breastfed infants each month in both groups from 0 to 12 mo.

**TABLE 3**
Effect of early interventional food introduction on dietary diversity score

| | 6 mo | | | 9 mo | | | 12 mo | | |
|---|---|---|---|---|---|---|---|---|---|
| | Effect estimate | CI | *P* value | Effect estimate | CI | *P* value | Effect estimate | CI | *P* value |
| Linear regression, multiple imputation | 0.01 | −0.18, 0.20 | 0.95 | 1.39 | 1.16, 1.62 | <0.001 | 0.70 | 0.50, 0.90 | <0.001 |
| Linear regression, complete case[1] | −0.28 | −0.71, 0.15 | 0.77 | 1.41 | 1.18, 1.64 | <0.001 | 0.78 | 0.58, 0.98 | <0.001 |

[1] no imputation

and 40% in New Zealand [41,42]. The intake of commercially prepared infant foods and organic food at 6 and 9 mo in our population is comparable to the findings in Spedkost 3 [9].

Breastfeeding rates in our study were not affected by the food intervention, similar to 2 other large early allergenic food introduction RCTs, the Enquiring About Tolerance study in a general population [20] and the Learning Early About Peanut Allergy trial in children at high risk of peanut allergy [43]. In contrast, a recent Swedish study of 1200 infants with dietary information reported at 1 y of age showed that earlier exposure to tiny tastings of complementary foods was associated with shorter breastfeeding length [44]. Another study, including 856 children from the Protection Against Allergy Study in Rural Environments/EFRAIM study, found no association between breastfeeding duration and diet diversity [45].

The finding that early introduction of complementary foods was associated with significantly increased dietary diversity at 9 and 12 mo of age is novel, to the best of our knowledge. Dietary diversity in infancy has gained significant attention because of its role in modulating allergic disease outcomes. Given the complexities and challenges of dietary diversity research, the European Academy of Allergy and Clinical Immunology Task Force recently provided an expert consensus regarding multiple aspects of diet diversity research and highlighted the need for more clinical trials with agreed definitions [26].

Major strengths of the present study include the prospective design and robust sample size from a general population and the systematic collection of detailed questionnaires, which limits the likelihood of recall bias and ensures high accuracy of timing of solid food introduction and milk-feeding practices [46]. Response bias may be an issue with self-reporting; however, we found similar results in our main analysis using imputed data for missing dietary diversity and in the sensitivity analysis, thereby strengthening our findings. Comparisons with previous national dietary surveys need to be made with caution because of differences in study cohort selection and questionnaires. Dietary diversity is a challenging area of research because single nutrients or foods are not eaten in isolation but are part of a complex interplay of factors. Considerations should also be given to diversity in diets being strongly impacted by ethnic traditions, leading to unmeasurable characteristics that could influence the outcome and interpretation of the results. The dietary diversity

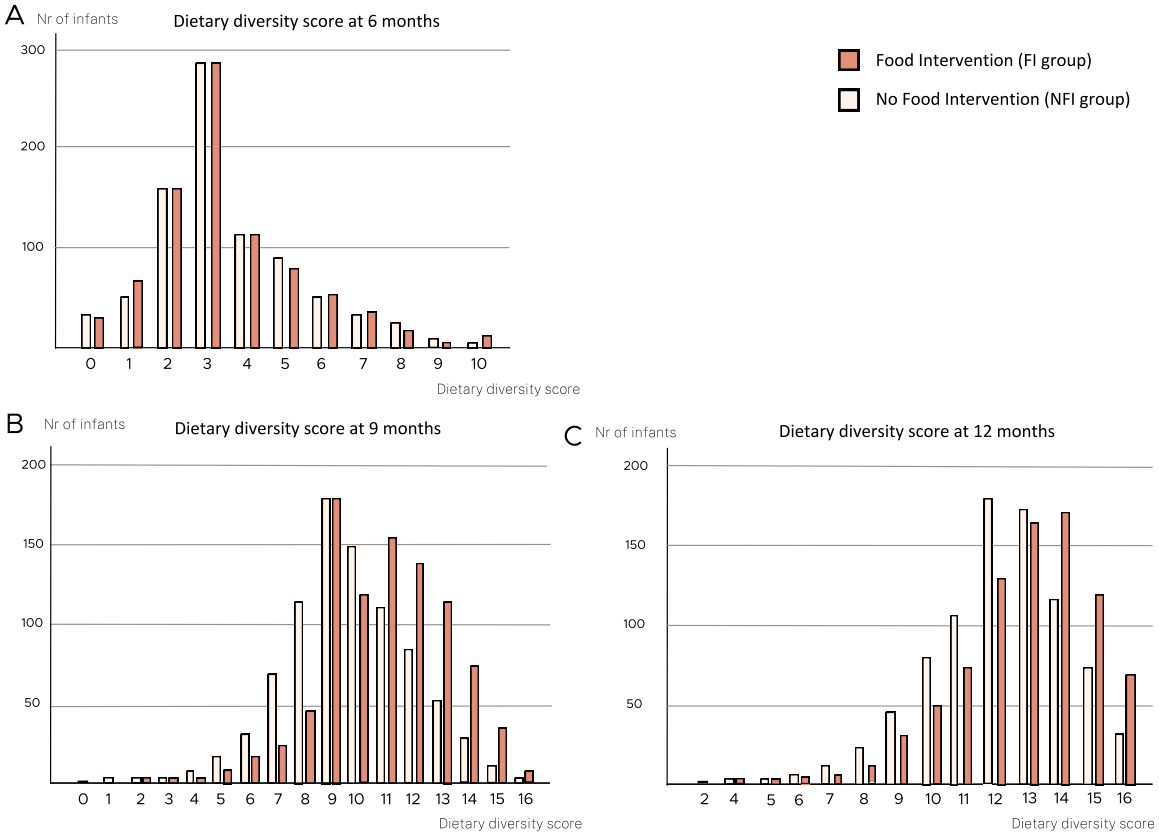


**FIGURE 3.** Dietary diversity score in infants categorized by Food Intervention compared with No Food Intervention groups at 6, 9, and 12 mo of age. The x-axis represents the total number of food items introduced. The y-axis presents the number of infants. (A) Dietary diversity score at 6 mo ($n$ = 1700). (B) Dietary diversity score at 9 mo ($n$ = 1637). (C) Dietary diversity score at 12 mo ($n$ = 1645).

score could have been influenced by certain limitations in our questionnaires that did not assess certain food groups, such as legumes, and the complementary foods given were not quantified. Our study population consisted of a larger proportion of infants with a first-degree relative with allergic disease, higher maternal educational level, and higher income than the general population of Norway and Sweden, which might have influenced feeding practices.

The Norwegian Directorate of Health recommends exclusive breastfeeding for 6 months, in line with the WHO [5,8]. These recommendations are particularly important in low-income countries where childhood malnutrition is prevalent. European infants are less likely to experience deficiency of nutrients in the complementary feeding period [4]. High-income countries face different challenges, including an increase in allergic diseases, a loss of gut microbial diversity, and impaired immune development. Potential mechanisms by which a higher dietary diversity could protect against allergic and other noncommunicable diseases is through modulation of the infant's gut microbiota, increased nutrient intake, and exposure to different food antigens [26,30,47].

An infant's readiness to start complementary feeding will depend on the individual's development [3,4]. Evidence suggests that renal and gastrointestinal functions are sufficiently mature at 4 mo to metabolize complementary foods [4]. Because neuromotor development and apparent interest in nonmilk foods differ among infants, the focus should be on determining an appropriate age range for complementary food introduction [3].

As breastfeeding is the best for the infant, early introduction of solid foods should not come at the expense of breastfeeding. The results from our study suggest that early complementary introduction is beneficial for increased dietary diversity with no negative impact on breastfeeding rates. Further studies are needed to address the potential impact on disease prevention, as well as potential underlying mechanisms.

In summary, in this general population-based mother–child birth cohort, most infants were breastfed throughout the first 6 mo of life, and half were still breastfed at 12 mo. The mean age of complementary food introduction was 4.5 mo, and most infants received complementary foods by 6 mo. Our data provide novel information that introducing small amounts of complementary foods from 3 mo of age increased dietary diversity but not at the expense of breastfeeding rates or breastfeeding duration.

## Author contributions

The authors' responsibilities were as follows – CMS, EMR, KLC, HOS, MHC: designed research; CMS, EMR, KLC, CMJ, MLB, BJ, HOS, CS, RV, MHC: conducted research; CMS, MLB, RV: analyzed data or performed statistical analysis; CMS: wrote paper, CMS, EMR, MHG: had primary responsibility for final content; and all authors: read and approved the final manuscript.

## Conflict of interest

CMS reports personal fees from Libero outside the submitted work. ML reports personal fees from MSD outside the submitted work. EMH reports personal fees from Sanofi-Genzyme, Novartis, Leo-Pharma, Perrigo, and The Norwegian Asthma and Allergy Association outside the submitted work. All other authors report no conflicts of interest.

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## Data availability

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2023.06.015.

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