Effects of early probiotic supplementation in a pediatric setting
Focus on body composition, metabolism and inflammation

Frida Karlsson Videhult
To my boys; Kristofer, Vidar & Ebbot

“Knowledge is invariably a matter of degree: you cannot put your finger upon even the simplest datum and say 'this we know'.”

T.S. Eliot

“What day is it?”
“It's today,” squeaked Piglet.
“My favourite day,” said Pooh.

A.A. Milne
# Table of Contents

**Table of Contents**

Table of Contents  
Abstract  
Abbreviations  
Original papers  
Populärvetenskaplig sammanfattning  

## Background

- Personal point of departure  
- The obesity “epidemic”  
- Early programming  
- Gut microbiota  
- Establishment of the gut microbiota  
- The environment influences the intestinal colonization pattern  
- Classification of gut microbiota  
- Diet influences gut microbiota composition and functions  
- The gut microbiota and its potential role in the obesity “epidemic”  
- Systemic low-grade inflammation  
- Probiotics  
- Probiotic effects  
- *Lactobacillus*  
- *Lactobacillus paracasei ssp paracasei F19*  
- Probiotic studies in childhood  
- Long-term effects following early probiotic supplementation  
- Growth and body composition  
- Dietary assessment  
- Metabolomics  
- Multivariate data analysis  

## Objectives


## Materials and methods

- Study population and study design  
- The intervention study (Paper I)  
- The follow-up (Paper II-IV)  
- Data collection  
- Anthropometric measurements  
- Biochemical measurements  
- Metabolomic analysis  
- Dietary registration and nutritional calculation  
- Physical activity  
- Statistical methods  

## Results

- Baseline characteristics  
- Growth, body composition and metabolic markers during weaning (Paper I)  
- Main results  
- Growth, body composition and metabolic markers at school age (Paper II)  
- Main results  
- Extended panel of metabolic and inflammatory markers (Paper III)  
- Main results  
- Long-term effects on the metabolome at 8-9 years of age (Paper IV)  
- Main results
Abstract

Background Once established, obesity and its health consequences are difficult to treat. An aberrant gut microbiota has been associated with several noncommunicable diseases, including obesity and its associated comorbidities. Probiotics, defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” has been proposed as one tool for healthy gut microbial modulation.

Aims To determine the short- and long-term effects on growth, body composition, metabolic and inflammatory markers following supplementation with the probiotic Lactobacillus paracasei ssp. paracasei F19 (LF19) during weaning.

Material and Methods Families with a healthy, newborn child in the area of Umeå city, Västerbotten County were invited to participate in an allergy prevention study during the years 2000 to 2003. One-hundred and seventy-nine infants were included, 89 were randomised to daily intake of cereals containing $10^8$ colony-forming units (CFU) of LF19 and 90 infants to cereals without LF19 (placebo) from 4 to 13 months of age. Weight, length, head circumference and body composition, as assessed by skinfold thickness, were examined at 4 (entry), 5.5, 6.5, 9, 12 and 13 months of age. Venous blood was drawn at 5.5 and 13 months. A total of 171 infants completed the intervention and were invited to a clinical follow-up at 8-9 years of age between 2009 and 2011, and 120 children participated. At the clinical visit weight, height and sagittal abdominal diameter (SAD) of the child and accompanying parents’ were measured. The body composition of the child was measured using a Dual Energy X-ray Absorptiometry (DXA)-scan. From the general check-ups at 4 years of age, data on weight and height were collected from medical records. The participating families filled out a four-day food record and a food frequency questionnaire (FFQ). To assess physical activity, the child wore a pedometer for 7 days. A venous blood sample was collected after overnight fasting for analysis of metabolic and inflammatory markers.

At 5.5, 13 months and 8-9 years of age total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB) and triacylglycerol (TAG) were analysed in serum. Low density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula. At 8-9 years serum samples were also analysed for glucose, insulin, aspartate transaminase (AST) and alanine transaminase (ALT) according to accredited methods at the Department of Clinical Chemistry, University Hospital of Umeå. Homeostatic Model Assessment (HOMA) index was calculated using the
equation (S-insulin in mU/L x P-glucose in mmol/L)/22.5. At that age an expanded panel of metabolic and inflammatory markers; serum high molecular weight (HMW) adiponectin and high-sensitivity C-reactive protein (hsCRP) were analysed using ELISA and plasma C-peptide, ghrelin, gastric inhibitory polypeptide (GIP), glucagon-like peptide 1 (GLP-1), glucagon, insulin, leptin, plasminogen activator inhibitor-1 (PAI-1), resistin and visfatin were analysed using Luminex (Bio-Rad Laboratories, Hercules, CA).

For characterisation of the metabolome, plasma samples in a subgroup (n=40) were analysed at 5.5 and 13 months of age by gas chromatography time-of-flight mass spectrometry (GC-TOF/MS) analysis. The plasma metabolome at the follow-up was assessed using untargeted GC-GC/MS on a Pegasus 4D (Leco Corp., St Joseph, MI, USA) with an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, GA, USA).

**Results** There were no differences between the LF19 and the placebo group regarding body weight, length/height at any of the eight assessments from 4 months to 8-9 years of age; nor were there any differences between the groups in body composition. In the LF19 group 19 % were overweight/obese, the corresponding number was 21 % in the placebo group (p=0.78). At 13 months of age, TC, LDL-C and ApoA1 tended to be lower in the LF19 group, however the differences did not reach statistical significance. The other analysed metabolic and inflammatory markers, both during the intervention and the follow-up did not differ between the two groups.

At 13 months of age lower levels of palmitoleic acid (p<0.04) and higher levels of putrescine (p<0.01) were seen in the LF19 compared to the placebo group. These differences did not persist at 8-9 years of age. At that age, we found statistically stronger models when comparing overweight/obese and normal weight children as well as in relation to sex.

When analysing the children according to weight class, BMI z-score was higher at every assessment from 4 months to 8-9 years of age in the overweight/obese children. The overweight/obese children had lower levels of HDL-C (p=0.02) and higher levels of insulin (p<0.01), HOMA index (p<0.01), C-peptide (p=0.01), leptin (p<0.01) and hsCRP (p<0.01).

**Conclusion** Early intervention with the probiotic LF19 exerted transient effects on the metabolome but did not affect growth, body composition, metabolic or inflammatory markers in a long-term perspective. Collectively, we found neither benefit nor harm on growth, body composition, metabolic or inflammatory markers following supplementation with LF19 during weaning.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>One dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>ALT</td>
<td>Aspartate transaminase</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Angiopoietin-like 4</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ApoA1</td>
<td>Apolipoprotein A1</td>
</tr>
<tr>
<td>ApoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>AST</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-Forming Units</td>
</tr>
<tr>
<td>CV</td>
<td>Cross validation</td>
</tr>
<tr>
<td>DA</td>
<td>Discriminant Analysis</td>
</tr>
<tr>
<td>DLW</td>
<td>Doubly labelled water</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual Energy X-ray Absorptiometry</td>
</tr>
<tr>
<td>EI</td>
<td>Energy Intake</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food frequency questionnaire</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GIP</td>
<td>Gastric Inhibitory Polypeptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like Polypeptide 1</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HMCR</td>
<td>Hierarchical Multivariate Curve Resolution</td>
</tr>
<tr>
<td>HMO</td>
<td>Human Milk Oligosaccharides</td>
</tr>
<tr>
<td>HMW</td>
<td>High Molecular Weight</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostatic Model Assessment</td>
</tr>
<tr>
<td>hsCRP</td>
<td>high sensitivity C-reactive protein</td>
</tr>
<tr>
<td>IOTF</td>
<td>International Obesity Task Force</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>NCD</td>
<td>Noncommunicable disease</td>
</tr>
<tr>
<td>OPLS</td>
<td>Orthogonal Projections to Latent Structures</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor-1</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized controlled trial</td>
</tr>
<tr>
<td>SAD</td>
<td>Sagittal Abdominal Diameter</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short Chain Fatty Acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TC</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Original papers

This thesis is based on the following papers, which will be referred to by the corresponding Roman numerals (I – IV):


IV. Karlsson Videhult F, Antti H, Hernell O, West CE. The plasma metabolome is influenced by body weight and sex already at school age. (Manuscript)

*Shared first authorship.

Papers I-III are reprinted with kind permission of the publishers: Cambridge University Press (paper I), Springer (paper II) and Taylor & Francis (paper III).
Populärvetenskaplig sammanfattning

Bakgrund

Övervikt och fetma är ett globalt växande problem bland barn och det saknas effektiva strategier för att förebygga och behandla övervikt. Den föreslagna kopplingen mellan sammansättningen av mikrobiotan i tarmen och risken att utveckla övervikt är en intressant teori som behöver utvärderas i kliniska studier. Att påverka tarmens mikrobiota har i djurstudier visat positiva resultat vid behandling av olika metabola störningar, men i dagsläget finns det få välgjorda studier på metabola sluteffekter av probiotikatillförsel hos människan. I en nyligen publicerad studie där möss gavs låga doser penicillin efter födseln eller under avvänjningen sågs övergående påverkan på tarmens mikrobiota. Dock fann man kvarstående metabola effekter, vilket föreslår en programmerande effekt av mikrobiota-manipulation tidigt i livet.

Probiotika definieras som levande mikroorganismer vilka när de tillförs i tillräcklig mängd kan ge hälsoeffekter hos värden. De anses ofarliga när de ges till friska individer och tillförs via livsmedel eller i form av t ex. kapslar eller droppar. Vanligast är laktobaciller och bifidobakterier och den mest påtagliga effekten av probiotika har setts vid virusorsakad magsjuka där specifika probiotikastammar kan förkorta sjukdomsperioden med ungefär ett dygn. Vid andra föreslagna och studerade sjukdomstillstånd finns i dagsläget inte tillräckligt med stöd för att kunna ge generella rekommendationer.

Djurstudier har visat att den probiotiska bakterien Lactobacillus paracasei ssp. paracasei F19 (LF19) minskar risken att utveckla övervikt och påverkar uttrycket av flera gener som styr ämnesomsättningen, vilket föreslår att LF19 skulle kunna påverka både ämnesomsättning och kroppssammansättning även hos människa.

Syfte

Att undersöka om dagligt intag av barngröt med eller utan tillsats av LF19 under avvänjningen från amning påverkar tillväxt, kroppssammansättning, ämnesomsättnings- och inflammationsmarkörer samt risken att utveckla övervikt och fetma i skolåldern.

Studiepopulation och metod

Familjer med friska, nyfödda barn i Umeå med omnejd bjöds in via barnhälsovården att delta i en allergi-preventions studie mellan åren 2000 och 2003. Ursprungsstudien omfattade 179 barn som randomiserades till ett dagligt...
intag av gröt med \((n=89)\) eller utan \((n=90)\) \(10^8\) colony-forming units (CFU) LF19 per portion från 4 till 13 månaders ålder. Det rekommenderade intaget var en grötoportion dagligen och vid 6 månaders ålder hade majoriteten av barnen uppnått den rekommenderade mängden. Föräldrarna ombads att inte ge några andra produkter med probiotika, inga andra kostråd gavs. LF19 valdes för att den uppfyllde kraven för en probiotisk bakterie. Totalt fullföljde 171 barn interventionsstudien, dessa barn kontaktades och erbjöds att delta i en uppföljningsstudie. Uppföljningen påbörjades 2009 och avslutades 2011, totalt följes 120 barn upp vid 8-9 års ålder till denna del av projektet som undersöker långtidseffekter avseende tillväxt och kroppssammansättning. Föräldrarna ombads att fylla i en enkät med frågor om kroniska sjukdomar, medicinering samt intag av produkter innehållande laktobaciller eller andra probiotiska bakterier.


Vid 5,5 och 13 månaders ålder samt vid 8-9 års ålder analyserades blodfettsprofil i blodprover. Vid 8-9 år analyserades även glukos, insulin och transaminaser (AST och ALT) i blodprover. Insulinkänslighet beräknades. Under uppföljningen analyserades även en utökad panel av metabol och inflammatoriska markörer.

Barnens kostvanor och kostintag beräknades med hjälp av ett födoämnesfrekvensformulär samt en fyra-dagars kostregistrering, varav två av fyra dagar var helgdagar. Typ och mängd mat som barnet åt registrerades och till hjälp hade familjerna ett häfte med bilder på portionsstorlekar. Familjerna ombads använda hushållsmått eller hushållsvåg vid registreringen. För att registrera fysisk aktivitet använde barnen en stegräknare under sju dagar.

**Resultat**

Under interventionen tolererades studiegröten bra och gav inga biverkningar. I arbete I fann vi att barnen i probiotikagruppen hade lägre nivåer av den mättade fettsyran palmitinsyra \((\text{C}16:0)\) och den enkelomättade fettsyran palmitoljesyra
(C16:1), dessa fettsyror är kopplade till visceral fetma hos barn. Vi fann högre nivåer av putrescine, en polyamine involverad i celltillväxt och differentiering samt även åldersrelaterade förändringar i ämnesomsättningen i båda grupperna \((n=40)\). Kroppssammansättning mätt med kaliper mätte visade ingen skillnad mellan grupperna, inte heller skiljde sig tillväxtmönster, dvs. vikt, längd och huvudomfång, eller blodfettsprofils sig åt mellan probiotika och placebo gruppen.

I arbete II till IV utvärderades långtidseffekter av den tidiga probiotikatillförseln när barnen var 8-9 år gamla. I arbete II respektive III sågs ingen effekt på vikt och längd, kroppssammansättning mätt med DXA, SAD eller de metabola och inflammatoriska markörerna. Totalt deltog 120 barn i uppföljningsstudien, av dessa kategoriserades 16 % som överviktiga och 4 % som feta.

Då palmitinsyra kopplats till bland annat hjärt-kärlsjukdom och palmitoljesyra korrelerar starkt med fetmaindex och bukfetma var det intressant att undersöka detta i skolålder. Om effekterna kvarstod skulle det tala för programmerande effekter av LF19 även hos människa. Således analyserades den metabola profilen \((n=112)\) i arbete IV. Trots att den multivariata modellen inte var statistiskt stark syntes gruppskillnader med avseende på unika metaboliter mellan probiotika och placebo gruppen. De skillnader som påvisats avseende palmitin- och palmitoljesyra i arbete I under interventionen kvarstod inte vid uppföljningen.

**Slutsats**

Att identifiera markörer som kan kopplas till risken att senare i livet utveckla sjukdomar som en följd av fetma är viktigt. I delarbete I såg vi att tillförsel av LF19 under avvänjningen från amning påverkade ämnesomsättningsprofilen i en subgrupp av de studerade barnen. Dessa skillnader fanns inte kvar när vi analyserade hela populationen vid 8-9 års ålder. Det var inga skillnader mellan probiotika och placebogruppen gällande tillväxt, kroppssammansättning eller metabola och inflammatoriska markörer. Detta talar för att föreslagna effekter vid tidig tillförsel av probiotika är mer komplexa än vad som tidigare framförts.

Våra resultat visar att tillförsel av LF19 tidigt i livet varken ger några direkta för- eller nackdelar avseende ämnesomsättning, tillväxt eller kroppssammansättning i ett långsiktigt perspektiv. Det är viktigt att framtida kliniska studier ytterligare belyser mikrobiotans roll i tidig metabol programmering.
Background

Personal point of departure

As a pediatric dietitian I have met overweight and obese children who constitute a group of patients where treatment involves modification of lifestyle factors, which is often challenging. This attracted my interest for this group of patients. Is it always a lack of compliance that results in the absence of positive results e.g. decreased weight gain or weight loss, or an improved metabolic profile? The aetiology of obesity might not be fully explained and the last couple of years the gut microbiota has been implicated in the development of overweight and/or obesity. I was introduced to the inspiring ELEFANT-study, primarily evaluating effects of early supplementation with the probiotic *Lactobacillus paracasei* ssp. *paracasei* F19 (LF19) on allergy, asthma and eczema prevalence (1, 2). However, since the gut microbiota has been suggested to affect inflammation, metabolism and the risk of developing overweight we also aimed at investigating possible long-term effects of LF19 on body composition, immune- and metabolic programming at school-age.

The obesity “epidemic”

The prevalence of obesity has reached epidemic proportions worldwide (3). Approximately 30% of children in the US were overweight or obese in 2012. During 1986-2001 the prevalence of overweight in Swedish schoolchildren living in Umeå, a University town in northern Sweden, doubled with an even higher increase among the children with severe overweight (4). In 2001, 18 % and 5 % of schoolchildren age 6-13 years were overweight and obese, respectively. A more recent study shows concordant results although a slight reduction in overweight/obesity with a prevalence of around 20 % in Swedish pre-schoolers was reported (5). Even though recent studies have shown that the incidence of overweight and obesity among children and adolescents is plateauing, the prevalence is still unacceptably high (6) and poses as a so-called “time bomb” for future demands on health care services since obese children have an increased risk of becoming obese adults (7). Obesity is often associated with comorbidities such as dyslipidemia, type 2 diabetes and the metabolic syndrome (8) leading to even worse health outcomes and higher demands on the health care system.
Factors associated with our modern lifestyle include excess energy intake (EI) and sedentary behaviours. These are considered major contributing reasons to the obesity “epidemic” (9) although a genetic predisposition exists (8). More recently, dysbiosis, i.e. “an imbalance in microbiota structure and/or function that disrupts host-microorganism homeostasis” (10), of the gut microbiota and reduced microbial diversity have been suggested to be central in several noncommunicable diseases (NCDs) (11) although causality is yet to be proven.

Over the last decade associations between the gut microbiota composition, host metabolism and obesity have been identified (12-14), which was first shown in experimental animal studies (15) and later also in humans (13, 14, 16). Early antibiotic exposure causing disruption of the gut microbiota has also been associated with increased risk of overweight and central adiposity in preadolescence (17) giving further support for this theory.

**Early programming**

Mounting evidence suggests that diseases such as obesity and type 2 diabetes in adulthood might originate from ‘programming events’ in early life (18), both prenatally (19) and postnatally (20). This implies that environmental influences during a critical time of developmental plasticity could provoke life-long health effects on the offspring. The hypothesis of the Developmental Origins of Health and Disease (DOHaD) evolved from early publications of Barker and colleagues following epidemiological studies focusing mainly on low birth weight and the risk of developing coronary heart disease later in life (21). The hypothesis sprung from the theory that undernutrition in utero leads to permanent changes in the body’s structure, function and metabolism thereby contributing to adult disease. The EarlyNutrition Project has recently proposed three hypotheses on early metabolic programming of adiposity and subsequent adverse health related disorders (22) (Figure 1). Early transient events may cause late onset of diseases, thus it has been suggested that developmental programming has epigenetic components (18). Since epigenetic processes are potentially reversible, interventions and strategies can be implemented once underlying mechanisms are understood.
Figure 1. Three hypotheses on early programming suggested by the EarlyNutrition Project; i, the fuel-mediated *in utero* hypothesis (i.e. maternal factors during pregnancy), ii, the mismatch hypothesis (i.e. fetal undernutrition followed by postnatal overnutrition) iii, the accelerated postnatal growth hypothesis (pertains to postnatal feeding practises).

**Gut microbiota**

The gut microbiota is important to an individual’s health (11). In humans, the gut microbiota consists of at least a trillion microorganisms, thus exceeding the body’s own cells by a tenfold (23). The microbiota is the collection of microbial populations residing in the host and the microbiome refers to the catalogue of these microbes and their genes. It is estimated that the microbiome contains 150-fold more genes than the host genome (24). The gut microbiota is central in many physiologic and immunologic processes e.g. in the defence against pathogens, and in the development of immunity and gut barrier functions (11). It is involved in many aspects of metabolism, including the production of bile acids, lipids, vitamins, choline and polyamines (25). The gut microbiota is also involved in the degradation of non-digestible polysaccharides (fermentation of resistant starch, oligosaccharides, inulin) thereby harvesting energy for the host from dietary compounds that are ingested, but not digested by the host (25, 26).

Both the host immune system and the microbiota are important for gut homeostasis, hence a complex interaction exists. If this interaction is disrupted, dysbiosis occurs which could potentially cause or contribute to
disease (11, 27-29). Through the rapid development of more sophisticated “omics” techniques, we are on our way to reveal and understand the true microbial diversity of the human intestine, the function of microbes and their role in health and disease.

Early shifts in the gut microbiome have been reported to precede the development of disorders with metabolic dysfunction, e.g. type I and type II diabetes, proposing potential means of improving an individual’s health by modulation of the gut microbiota (30). Focus of this research has been the period before and shortly after birth, however, the underlying mechanisms between the early-life environment and the development of a healthy metabolic phenotype are lacking. Modulation of the gut microbiota early in life, i.e. during its establishment might induce programming effects on the host. In a murine model, low doses of penicillin postnatally and at weaning transiently affected the gut microbiota however, metabolic effects remained after the treatment period (31) giving further support for this theory.

**Establishment of the gut microbiota**

The intestine has previously been considered sterile during fetal life, however, recent discoveries that bacterial DNA is presented both in the fetal-placental unit (32, 33) and meconium (32, 34) challenges this paradigm. It remains undecided how and when the infant gut is first exposed to and colonized by the first pioneering microbes. Using 16S ribosomal DNA and whole-genome shotgun sequencing, Aagaard et al (33) demonstrated the greatest resemblance between placental microbiome profiles and the human oral microbiome. The authors suggest an in utero interaction where a haematogenous spread of oral microbiota affects the placental microbiota establishment, thereby questioning the “sterile womb” view.

Critical time points during early life for the establishment of gut microbial communities are at birth, weaning and when complementary foods are introduced (35). The gut microbiota establishment is considered a gradual process; aerobic and facultative anaerobic bacteria are pioneer species that are gradually outnumbered by obligate anaerobes as the child approaches adulthood (36, 37). As the gut microbiota matures with increasing age of the neonate the colonization resistance becomes stronger and creates a barrier against establishment of potentially pathogenic bacterial strains.
There is a rapid development of gut microbiota composition in the first year of life, thereafter a more childhood-type microbiota is established (38), which takes approximately 2-3 years (39). Recent studies indicate that there is ongoing development of the gut microbiota beyond that age (40).

*The environment influences the intestinal colonization pattern*

The gut microbiota establishment is dependent on genetic and epigenetic factors, mode of delivery (caesarean section vs. vaginal delivery), maternal microbiota (gastrointestinal, vaginal and skin microbiota), geographic origin and cultural traditions (especially diet related) (37), medical practices (including antibiotics) (38) and infant feeding practices (41).

Children delivered by caesarean section have a lower gut microbial diversity compared to vaginally delivered children (42). Facultative anaerobic species such as *Escherichia coli*, *Staphylococcus* and *Streptococcus* colonize the infant gut during vaginal delivery, producing an environment that allows strict anaerobes to thrive, e.g. *Bacteroides* and *Bifidobacterium* ssp. (43). There are marked differences between vaginally born infants and infants delivered by caesarean section. The latter group harbours no vaginal microbes, instead they are colonized by skin bacteria, i.e. *Staphylococcus*, *Corynebacterium*, *Propionibacterium* ssp. with a delay in colonization by *Bacteroides* and *Bifidobacterium* ssp.

Decreased bacterial diversity in the neonate’s stool as well as lower abundance of lactobacilli and bifidobacteria in the neonatal gut have been associated with intrapartum antibiotics (43). In one study, clustering analysis on gut microbiota composition showed two distinct clusters; one infant cluster and one childhood cluster, and repeated treatment with antibiotics during the first 6 months of life appeared to delay the development of a childhood-type microbiota (38) ([Figure 2](#figure2)).

*Classification of gut microbiota*

The gut microbiota can be classified as either endogenous (autochthonous), i.e. residing in the gut or passenger (allochthonous), i.e. passing through the gut (44). The autochthonous bacteria are considered permanent members of the microbiota while the allochthonous bacteria may be related to diet and are of a more transient nature. The adult
microbiota is dominated by four phyla; Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria (45) and the adult gut microbiota is dominated by genera from Firmicutes and Bacteroidetes phyla (Figure 2). The early life microbial composition is less diverse and complex compared to that of the adult (39, 46).

Figure 2. Overview of the relative abundance of key phyla of the human microbiota at different stages in life. Image published with permission from Ottman et al., Front Cell Infect Microbiol 2012 (Copyright © 2012 Ottman, Smidt, de Vos and Belzer).

**Diet influences gut microbiota composition and functions**

An important environmental factor affecting the gut microbial composition is infant feeding regimes, i.e. breastfeeding or formula feeding. Breast milk is rich in human milk oligosaccharides (HMOs) which act like “natural prebiotics”, thereby promoting intestinal colonisation with bifidobacteria and lactobacilli (37, 47, 48). Bifidobacteria and lactobacilli are considered important for inhibiting the growth of pathogens, in the modulation of mucosal barrier function and immunological and inflammatory responses (43). With breast milk constituting an example, our modern infant formulas are constantly being improved in order to stimulate similar intestinal colonisation patterns as in breastfed infants (48). Formula-fed infants have higher gut microbial diversity and species richness compared to breastfed children (37, 42).
newborn’s gut microbiome is enriched in genes for carbohydrate uptake and degradation of sugars from breast milk whereas at 12-months of age genes involved in the breakdown of complex sugars and starch are enriched with an associated increase in the abundance of Bacteroides thetaiotaomicron (49). The increase of B. thetaiotaomicron and pectinesterase seen at 1 year of age is probably due to an increased intake of solids or semisolid foods. The use of new advanced techniques such as metagenomic shot-gun sequencing will allow a deeper exploration of the influence feeding practice has on gut microbial establishment.

The gut microbiota and its potential role in the obesity “epidemic”

The relationship between gut microbes and host metabolism, energy utilization and storage has been disclosed in both animal and human studies. Differences in the gut microbiota composition have been shown to precede the development of obesity thus providing a potential possibility for preventive treatment (14). Increased nutrient and energy harvest from the diet, prolonged intestinal transit time, altered fatty acid metabolism and composition in adipose tissue, chronic low-grade inflammation triggered by the endotoxin toll-like receptor–4-axis and alteration of intestinal barrier function have been proposed as potential underlying mechanisms for this relationship (50).

Lipopolysaccharides (LPS) constitute a major lipid component of the outer membranes in cell walls of Gram-negative bacteria. They are an important signalling molecule for the innate immune system inducing strong inflammatory responses in the host to counteract infection (51). Further, LPS is a known endotoxin and responsible for the protective barrier function that the outer membrane forms to protect the bacteria from environmental compounds such as antibiotics.

The finding of a high percentage of Gram-positive Firmicutes and low percentage of Gram-negative Bacteriodetes first in genetically obese leptin-deficient ob/ob mice by Ley and colleagues (15) and later also in adult humans (12) suggested that the gut microbiota composition is associated with obesity. This composition also seems to be affected by weight changes (52). In children results are contradictory, with some finding an association between the Firmicutes/Bacteroidetes ratio and obesity (53) whereas others do not (54). Recently, the B. fragilis group was associated with lower BMI z-score in a sub-group of children whose
mothers had an alternative lifestyle, e.g. vegetarian and/or organic diets (55). Even though the underlying mechanisms are not fully understood there are strong indications that an altered gut microbial diversity and composition is associated with the metabolic dysregulation seen in many non-communicable diseases (11). Further understanding of factors modulating the gut microbial composition may lead to new preventive or therapeutic strategies for metabolic disorders.

**Systemic low-grade inflammation**

The connection between gut microbial composition and immunological pathways is influenced by diet. A Western diet is often considered “obesogenic” and is generally energy-dense, low in nutrient-rich components and fibre. A high-fibre diet increases the production of diet-induced metabolites including short-chain fatty acids (SCFAs) e.g. acetate, propionate and butyrate with known anti-inflammatory effects (56). SCFAs also reinforce gut epithelial integrity. Hence, a low-fibre diet has been suggested to contribute to the development of inflammatory disorders (57).

Inflammation is a normal defence mechanism protecting the host from infections. In healthy individuals homeostasis is maintained through finely regulated inflammatory responses. A loss of tolerance and/or regulatory processes induces pathological inflammation, which can lead to irreparable damage to host tissue and ultimately onset of disease (58). Obesity is accompanied by a state of chronic systemic low-grade inflammation as a consequence of a dysfunction in the adipose tissue (59). Although the diagnostic criteria for a low-grade systemic inflammation has as of yet not been clearly defined, the phenotype, characterised by an increased concentration of inflammatory markers in the systemic circulation, is not disputed (60). The adipose tissue releases many pro- and anti-inflammatory mediators, but which mediator that best represents chronic low-grade inflammation is yet to be decided. Increased levels of high sensitivity C-reactive protein (hsCRP) have been associated with childhood overweight and obesity (61). Systemic inflammation is most pronounced in visceral obesity and is connected to an increased risk for developing cardiovascular diseases, insulin resistance and type 2 diabetes (60, 62).
Probiotics

The year 2016 marks the centenary of the death of Élie Metchnikoff, the “father” of the concept that the addition of lactic acid bacteria via fermented foods could restore a disrupted colonic flora to promote health (63). In 1960 the word probiotic was coined but it was not until the early 1990s that the concept of ‘probiotics’ was introduced. In 2014 the definition of probiotics was revised by the International Scientific Association for Probiotics and Prebiotics (ISAPP) (64), leading only to a minor grammatical alteration from the definition initiated by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) in 2001 (65).

![Probiotics WHO](image)

Even though the definitions of probiotics have been slightly modified over the years, the key points are that probiotic products should contain live microorganisms in adequate amounts.

The most commonly used probiotics belong to the genera *Lactobacillus* and *Bifidobacteria*, which are Generally Recognised as Safe (GRAS) (66). This is supported by their natural occurrence in the mammalian microbiota. However, the yeast *Saccharomyces boulardii* has also been used as a probiotic (67). To pass as probiotic, a microbe should not only survive the passage through the gastrointestinal (GI) tract but also proliferate in the gut (66). Probiotics can be provided through a variety of ways, i.e. in food, drops or capsules. Even though they are generally regarded as safe there are some theoretical risks that need to be addressed, i.e. some probiotics might cause infections due to bacterial translocation, transfer of genes of resistance to antibiotics and production of potentially toxic metabolites (67). However, cases are rare and have been reported in immunocompromised patients, patients with short-bowel or a central venous catheter (67).
**Probiotic effects**

In the 2001 WHO report on properties, functionality, benefits, safety and nutritional features of probiotic products the emphasis was on bacteria viability and effectiveness at the target site (65). Probiotics have been proposed as one tool for gut microbiota modulation (68) and several probiotic bacteria have been identified as potential candidates to favourably improve host metabolism by influencing the gut microbiota (69). Analysis of fecal samples has been conducted before and after probiotic ingestion in order to evaluate survival during intestinal transit in humans. In several studies the administered probiotic has been isolated in stool samples following supplementation (2, 70-72). Possible beneficial mechanisms of action for probiotics for intestinal health were proposed by Gareau et al (27) including increased integrity of tight junctions and thereby maintaining/strengthening the intestinal barrier leading to reduced permeability (Figure 3). Current evidence infers that the effects of probiotics are strain specific (27, 69) and therefore consideration should be taken when selecting the strain depending on the desired outcome.

The metabolic products of probiotics are referred to as postbiotic activity (73) and for *L. paracasei* these activities have shown luminal effects, i.e. competition with pathogens and modulation of inflammatory responses (74).
Figure 3. Potential mechanisms of action for probiotics include: a) Colonization resistance, i.e. blockage of pathogen entry into epithelial cells; b) Creation of a mucus barrier; c) Maintenance of intestinal permeability; d) Production of antimicrobial factors; e-f) Stimulation/regulation of the innate immune system. Reprinted by permission from Macmillan Publishers Ltd: [Nat rev Gastroenterol Hepatol] (27) copyright (2010).

**Lactobacillus**

Lactobacilli are facultative anaerobic Gram-positive bacteria. They are members of the lactic acid bacteria, named after their ability to produce lactic acid following fermentation of lactose and other sugars (74). They are present in the gastrointestinal tract in humans and constitute a small part of the human microbiota. The taxonomy of the probiotic used in the present study, *Lactobacillus paracasei* ssp. *paracasei* F19 (LF19) is depicted in Figure 4. In brief, LF19 belongs to the Firmicutes phylum, class *Bacilli*, order *Lactobacillales* and family *Lactobacillaceae* (www.ncbi.nlm.nih.gov taxonomy).
Figure 4. The taxonomy of *Lactobacillus paracasei* ssp. *paracasei* F19.

*Lactobacillus paracasei* ssp *paracasei* F19

To be classified as a probiotic the selected strain must endure the tough environment with low pH, digestive enzymes and bile in the GI tract. LF19 (dep. nr LMG P-17806) was first isolated from deep colonic mucus layer of individuals with no GI disorders and no prior (<3 months) antibiotic treatment (75). It is considered to be part of the commensal microbiota in a small percentage in the Nordic population (70). LF19 successfully survived gastric transit in healthy infants when consumed in foods, transiently colonized both the colonic lumen and the mucosa (70, 75) and was well tolerated in individuals 1-85 years of age (76). It might be undesirable for a probiotic bacterium to disturb the commensal bacteria within the intestinal tract. However, overall gut microbiota composition as assessed by polymerase chain reaction-DGGE did not correlate with consumption of LF19, indicating that major population groups in the gut microbiota are not affected following supplementation with this probiotic bacterium (70).

Experimental animal studies have shown that supplementation with LF19, had protective effects of oxidative and hepatic injury in a rat model (77). In murine models LF19 decreased the risk of developing overweight and decreased total body fat (78), affected the expression of several genes influencing metabolism via up-regulation of adiponectin and adipsin insulin-sensitizing hormone and down-regulation of resistin like β known
to induce insulin resistance (79). In one of these studies, the lipoprotein profile was influenced following LF19 supplementation through increased triacylglycerol (TAG) load in very low density lipoproteins (VLDL) via increased circulation of Angiopoietin-like 4 protein (ANGPTL4), a circulating lipoprotein lipase inhibitor with importance for the regulation of deposition of TAG in adipocytes (78). Taken together, these studies suggest that LF19 can affect both body composition as well as metabolism and inflammation.

**Probiotic studies in childhood**

Probiotics have been assessed for treatment and prevention purposes in many clinical conditions affecting infants and children. To date, the most evident effect is seen in treatment of acute gastroenteritis, prevention of antibiotic-associated diarrhoea and nosocomial diarrhoea (80). Hence, one of the first recommendations for the use of probiotics was for treatment of acute gastroenteritis, with a shortened duration of ~1 day and a decreased number of stools (81, 82). The level of evidence is strong for efficacy of treatment with *L. rhamnosus* GG (LGG) and *Saccharomyces boulardii* (83) and also for *L. reuteri* ATCC 55730 and *L. casei* DN-114 001 (84). For several other indications the level of evidence is still limited or absent (29, 80, 82).

**Long-term effects following early probiotic supplementation**

During recent years there has been extensive research done in the probiotic field, however few studies have examined the long-term effects on body composition, growth and metabolic health outcomes following early supplementation with probiotics (Table 1). The majority of studies reported in Table 1 found no effect on growth, i.e. weight, height and/or BMI at the time of the follow-up (85-91). In children who remained normal weight at 7 years of age, Kalliomäki et al (14) found higher bifidobacterial numbers and lower numbers of *Staphylococcus aureus* in faecal samples in a subgroup of children during the first year of life.
<table>
<thead>
<tr>
<th>Author, year</th>
<th>Strain</th>
<th>Design, duration</th>
<th>Dose</th>
<th>Age, N</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laitinen et al, 2005 (85)</td>
<td><em>L. rhamnosus</em> GG ATCC 53103</td>
<td>Subjects selected from a prospective follow-up. Perinatal suppl (2–4 w), postnatal suppl (6 mo)</td>
<td>10∧10 CFU/d</td>
<td>4 yr, 159</td>
<td>No effect on growth</td>
</tr>
<tr>
<td>Luoto et al, 2010 (92)</td>
<td></td>
<td></td>
<td></td>
<td>10 yr, 113</td>
<td>Moderation of initial phase of weight gain at 4 yr, but no effect on growth, BMI at 10 yr</td>
</tr>
<tr>
<td>Kuitunen et al, 2009 (86)</td>
<td>Mix of <em>L. rhamnosus GG</em> and LC705 (both 5 x 10⁹ CFU) and <em>B. breve</em> Bb99 and <em>Propionibacterium freudenreichii</em> ssp. <em>shermani</em> JS (both 2 x 10⁹ CFU) plus prebiotic GOS</td>
<td>RCT, perinatal suppl twice daily to mothers 2–4 w before delivery, then to infants for 6 months</td>
<td>5 x 10⁹ CFU and 2 x 10⁹ CFU, twice daily</td>
<td>5 yr, 891</td>
<td>No effect on growth</td>
</tr>
<tr>
<td>Luoto et al, 2010 (93)</td>
<td><em>L. rhamnosus</em> GG + <em>B. lactis</em> Bb12</td>
<td>Prospective RCT, perinatal suppl from 1st trimester to end of exclusive BF, intervention group received probiotics and dietary advice</td>
<td>10∧10 CFU/d</td>
<td>24 mo, 191</td>
<td>No effect on fetal and infant growth</td>
</tr>
<tr>
<td>Abrahamsson et al, 2013 (87)</td>
<td><em>L. reuteri</em> ATCC 55730</td>
<td>RCT, perinatal suppl from last 4 w of gestation through the first year.</td>
<td>10⁸ CFU/d</td>
<td>7 yr, 184</td>
<td>No effect on growth</td>
</tr>
<tr>
<td>Wickens et al, 2012 (88)</td>
<td><em>L. rhamnosus</em> HN001 (6 x 10⁹ CFU/d) or <em>B. lactis</em> HN019 (9 x 10⁹ CFU/d)</td>
<td>RCT, perinatal suppl from 2–5 w before delivery and the to the infant for 2 yr</td>
<td>6 x 10⁹ CFU/d or 9 x 10⁹ CFU/d</td>
<td>4 yr, 425</td>
<td>No effect on growth, BMI</td>
</tr>
<tr>
<td>Wickens et al, 2013 (89)</td>
<td></td>
<td></td>
<td>6 x 10⁹ CFU/d or 9 x 10⁹ CFU/d</td>
<td>6 yr, 422</td>
<td>No effect on growth, BMI</td>
</tr>
<tr>
<td>Taylor et al, 2012 (90)</td>
<td><em>L. acidophilus</em> (LAVRI-A1)</td>
<td>RCT, postnatal suppl from birth to 6 mo of age</td>
<td>3 x 10⁸ CFU/d</td>
<td>5 yr, 123</td>
<td>No effect on weight</td>
</tr>
<tr>
<td>Loo et al, 2014 (91)</td>
<td><em>L. rhamnosus</em> LPR (1 x 10⁹ CFU/d) and <em>B. longum</em> (BL999) (6 x 10⁹ CFU/d)</td>
<td>RCT, postnatal suppl (in infant formula) from birth to 6 mo of age</td>
<td>1 x 10³ CFU/d and 6 x 10⁸ CFU/d</td>
<td>5 yr, 220</td>
<td>No effect on growth, BMI</td>
</tr>
</tbody>
</table>

BF, breastfeeding; RCT, randomised controlled trial; GOS, galacto-oligosaccharides
In the same study population, Luoto et al found that administration of LGG (92) altered the growth pattern in children by restraining excessive weight gain during the first year of life compared to placebo. There was however no difference in BMI at 10 years of age between the probiotic and the placebo group. Perinatal supplementation with the combination of LGG and \textit{B. lactis} Bb12 together with dietary counselling reduced the risk of gestational diabetes mellitus in mothers, however there was no impact on children’s growth at 24 months of age (93). It should be noted that the primary aim of the majority of the studies reported in Table 1 has been allergy and eczema prevention and that growth, body composition and metabolic markers have been included as secondary outcomes.

**Growth and body composition**

Body composition can be measured in numerous ways. By measuring weight and length/height, body mass index (BMI) can easily be calculated using the equation; body mass (kg)/height (m)$^2$ and be used as a screening tool for identification of overweight and obese children. Growth in children is however a dynamic continuum, i.e. both weight and height is part of the normal growth until final height is obtained, thus adult cut-offs for overweight and obesity cannot be used. Based on data from six large cross-sectional growth studies, Cole et al (94) developed a definition based on age- and sex-specific cut-offs between the ages 2 and 18 years called iso-BMI. The cut-offs are constructed to correspond to adult BMIs of 25 and 30. The distribution of adipose tissue within the body is of clinical relevance and BMI as a substitute for adiposity is problematic in the pediatric population because the contribution of fat mass and lean mass varies by age, sex, pubertal status and ethnicity (95). Accumulation of adipose tissue in the thorax or abdomen is often called visceral or android obesity and has been associated with increased risk of diabetes and atherosclerosis. On the other hand, adipose tissue located in the lower part of the body, i.e. gynoid adipose tissue, does not seem to be associated with similar risks (60). BMI gives an indication of body mass but does not differentiate between fat mass and lean mass, therefore more sophisticated measures are needed to evaluate body composition. Commonly used body composition assessment methods include the non-invasive skinfold thickness, DXA and sagittal abdominal diameter (SAD). Skinfold thickness is a cheap method suitable for use in the community and outside fixed facilities, for evaluation of body fatness in infants and children. However it might be subject to great interobserver variability and the accuracy is
dependent of the pediatric predictive equation used (96). DXA is a robust standard measurement for evaluating body composition, ideal for pediatric use due to its rapid scan time and low radiation exposure equivalent to ~1 % of a chest X-ray. DXA provides estimates of three main components, i.e. fat and lean soft tissue and bone mineral. SAD is defined as the shortest distance between the anterior and posterior trunk at the level of the iliac crest or lowest trunk at the level of the iliac crest or lowest trunk site (96), measured in a supine position and thus offers a prediction of visceral fat (97).

**Dietary assessment**

Dietary intakes are difficult to assess and can be measured in numerous ways, both from a prospective and a retrospective point of view with all methods having their individual strengths and limitations.

Food records is a prospective open-ended assessment method that allows the subject in question to self-record all food items and beverages consumed over a specific time period decided beforehand (98). Detailed information is given on brands, portion sizes, fat content and cooking methods. The number of days needed to represent information on habitual food consumption are being discussed, however 3 days is considered a minimum. Although 7 days often are referred to as “gold standard” it has been shown that responders are prone to underreport after day four due to fatigue as well as a tendency to start filling out the report in retrospect rather than the concurrent intake (98). Both weekdays and weekend days are however required to be included but it remains undecided whether or not the days reported need to be consecutive. The advantage with food records is that they collect both qualitative and quantitative information on individual foods. Since the focus is on current intakes inferences on long-term dietary exposure cannot be made.

Food frequency questionnaires (FFQs) are more commonly used for evaluation of dietary intake in larger epidemiological studies and answer questions in retrospect on how often and how much food of a specified character that is consumed over a reference period (99). The questions asked in the FFQs reflect the interest of the researcher and should mirror food habits of the study population at the time of data collection. FFQs consist of three main components; the food list, frequency of consumption and portion sizes. To capture the overall energy and nutrient intake the
FFQs need to be comprehensive including ~100 to 150 food items, however the list can range from 20 to 200 items. One advantage with FFQs is the low response burden in comparison to other dietary assessment methods and the relatively low cost, however accurate reporting depends on the respondent’s memory. In addition, the accuracy of the quantitative intake in FFQs is lower compared with food records.

**Metabolomics**

The advancement of chemical analysis instrumentation for characterizing biological samples together with more sophisticated data analysis and bioinformatics strategies for handling large complex data provide possibilities for an unprecedented detailed description of the human metabolome (100). The metabolome consists of the entire set low molecular weight metabolites in a biological sample (101). Metabolomics is one of the ‘omics’ methods used for studying biochemical processes in tissues and biofluids (100). The method is focused on detecting concentration changes of metabolites, which are the end products of a cellular process and closest to the phenotype (100) with the main objective to identify differences in metabolite profile between samples linked to phenotypic variation.

Metabolomics uses a combination of sensitive analytical methods to detect and quantify metabolites followed by bioinformatics commonly based on multivariate projection methods for data analysis and evaluation (Figure 5). The method has proven effective in screening for biomarkers or biomarker patterns as well as for providing insight into underlying mechanisms of different biochemical processes (102, 103). Due to the close relationship between nutrient intake and metabolism the interest for metabolomics as a tool in nutritional research has rapidly increased. As gut microbial composition also influences the metabolome (25), there is also emerging interest in employing metabolomics within this field. However, because of the relative novelty of the metabolomics field a complete catalogue of the human metabolome does not yet exist (104) and the availability of reference spectral libraries for known metabolites is limited, making the identification of detected metabolites incomplete. The aim of the metabolomics approach is to distinguish the unique systemic features that define the system from a large, complex background. Biological systems are commonly affected by environmental factors such as age, sex, diet and growth phase. Furthermore, unavoidable variation in the spectral
data caused by instrument instability and variability in sample conditions need to be taken in account. Thus, the generation, processing and analysis of metabolomics data require a robust methodology in order be able to extract the relevant information to obtain a correct interpretation of the system under study.

Figure 5. Multivariate metabolomics study strategy. Published with permission from Prof Henrik Antti, Umeå University.

Gas chromatography (GC) coupled to mass spectrometry (MS) is a chemical analysis technique that combines the separating power of gas chromatography with the detection power of mass spectrometry. It is a sensitive technique allowing a relatively straightforward detection of identified metabolites when combined with efficient data processing, e.g. curve resolution and comprehensive spectral libraries (105). Prior to GC analysis the samples require derivatization to become volatile. Samples are then injected into the gas-chromatograph where they are vaporized and carried through the column by a carrier gas. The time it takes for the molecule to travel through the column (i.e. retention time) depends on the properties of the molecule, mainly boiling point and polarity (105). The
difference in retention time is what separates one molecule from another. The MS then separates fragments of the molecules according to their mass-to-charge ratio. Thus, unique “fingerprints” known as mass spectra are created and together with the retention time molecules can be identified by data base searches in mass spectral libraries. The 2 dimensional (2D) GC uses two columns, which results in higher separation capacity together with enhanced detection limit (106, 107).

Following both 1D and 2D GC not all compounds in a complex sample are completely separated, thus many peaks overlap. In order to resolve signals from each compound, i.e. obtaining pure chromatographic peaks and mass spectra for every individual metabolite, the hierarchical multivariate curve resolution (HMCR) method can to be applied (108, 109). HMCR is used to mathematically resolve complex GC-MS data for calculation of relative levels of individual metabolite peaks prior to multivariate or other statistical analysis.

**Multivariate data analysis**

A challenge in the metabolomics analysis flow is the data analysis with large sample sets usually consisting of a large number of variables (metabolites) that by far exceeds the number of observations (samples). In addition, the variables are also often highly co-varying. This makes classical statistical methods such as linear regression methods sub-optimal due to multicollinearity issues. Thus multivariate analysis methods which are designed to handle the collinearity in the data by means of projections to latent variables are required. To model and visualize the systemic variation, the generated data matrix can be decomposed using unsupervised multivariate dimensionality reduction methods such as principal component analysis (PCA). For regression or discrimination purposes supervised multivariate methods such as orthogonal projections to latent structures (OPLS) (110) and OPLS-discriminant analysis (OPLS-DA) (104) can be used. The data and scientifical hypotheses will direct the choice of method used. PCA offers an overview of the variation in a lower dimensionality output. However, PCA only provides group structure when the between-group variation is sufficiently larger than the within-group variation and is not specifically aimed for classification analysis. OPLS-DA in the other hand relies on that class membership of each observation is known to the model and thus searches the data for systemic variation associated with the class difference. The OPLS methods also have an integrated filter separating the variation orthogonal to the response of
interest (e.g. class in a DA model) from the variation correlated to (predictive) the response, which greatly facilitates interpretation of the results.
Objectives

The general objective of this thesis was to investigate if supplementation with the probiotic LF19 during weaning exerts effects on the global metabolome, body composition, the metabolic and inflammatory profile and the risk of developing overweight and obesity.

The specific objectives are as follows (respective paper in parenthesis):

- To examine the effects of supplementation with LF19 during weaning on growth, body composition, serum lipid profile and global plasma metabolome (I).

- To evaluate long-term effects on body composition, growth and metabolic markers following early probiotic supplementation: a follow-up at school age (II).

- To assess long-term impact of LF19 on the metabolic and inflammatory profile at age 8-9 years and its association with previously reported body composition measures (III).

- To explore possible long-term effects of LF19 on the global metabolic profile at school age as assessed by a metabolomics approach (IV).
Materials and methods

The methods are described in the material and methods section of each paper (I – IV). A brief summary is presented below.

Study population and study design

The intervention study (Paper I)

The intervention study was a double-blind, placebo-controlled allergy prevention trial conducted from 2000 to 2003 (1, 2), comprising 179 infants randomized to daily feeds of cereal with \( n=89 \) or without \( n=90 \) \( 10^8 \) colony-forming units (CFU)/serving of the probiotic bacterium \textit{Lactobacillus paracasei} ssp. \textit{paracasei} F19 (LF19) (dep.nr. LMG P-17806) from 4 to 13 months of age (Figure 6). The cereals contained milk protein and were rice-based between 4-6 months and wheat-based between 6-13 months of age (111). Parents were asked not to give any other products containing probiotics during the intervention period; no other dietary advice was given. LF19 was chosen since it fulfilled the criteria for a probiotic bacterium (66). At the time of the intervention LF19 was not available in any commercial products. In the baseline study, extensive demographic characteristics were collected (1).

Inclusion criteria were healthy infants, born at term, vaginally delivered with a birth weight of >2500 g. Further, the infants should have no atopic manifestation or medication that could have affected the gut microbiota, i.e. antibiotics or prior intake of pre- or probiotics. The general hypothesis was that supplementation with probiotics during the time when solid foods were introduced would maintain the beneficial gut microbial milieu that breastfeeding generates with effects on gut microbial function, adaptive immunity and allergy development (1, 2).
Anthropometrics; body composition.

Figure 6. Study design of the intervention and follow-up. Adapted from West C.E et al. 2008.

The follow-up (Paper II-IV)

One-hundred and seventy-one children completed the baseline study, 84 children in the LF19 group and 87 children in the placebo group. These were contacted and invited to participate in the follow-up study when the children were 8-9 years of age. The follow-up was initiated in 2009 and completed in 2011. A total of 120 children and their parents participated in the follow-up. Sixteen children had moved away from the area, and 35 declined for other reasons. The drop-out rate did not differ between the LF19 and the placebo group ($p>0.05$).

Data collection

Anthropometric measurements

During the intervention phase (paper I) repeated measurements on anthropometrics, i.e. weight, length, head circumference and knee-heel length were collected (Figure 6). For evaluation of body composition triceps, biceps, suprailiac and subscapular skinfold thickness was measured using a skinfold caliper.

At the time of the follow-up the childrens’ and accompanying parents’ weight, height and SAD were measured by one research nurse at the clinical visit (papers II-IV). BMI was calculated (body mass (kg)/height
(m)²) according to the International Obesity Task Force (IOTF) criteria (94). Overweight and obesity were defined by age and sex specific cut-off points, iso-BMI 25 and 30 kg/m², respectively. SAD was measured with the child and accompanying parent/s in supine position on a firm examination table, after normal expiration, the highest point between the abdomen and examination table was measured using a square rule (paper II). Body composition of the children was assessed using DXA scan (Lunar prodiy whole-body scanner GE Medical Systems, Madison, WI, USA) in a three-compartment model (i.e. bone, lean and fat mass). Manufacturer-supplied software was used, versions 8.70 and 13.31, with no changes made regarding reference population (NHANES/USA whole body reference population [v101]).

![One of the study participants going through a DXA-scan.](image)

Further, a retrospective collection of data (Figure 7) on weight and height from the regular check-ups at 4 years of age at the health care centres was done (paper II).
Age 4-13 mo
Paper I
Intervention
n=171
Metabolome
GC-TOF/MS
n=49
LF19
n=20
Placebo
n=20
Growth n=167
Body composition n=142
Blood lipids n=135 (at 5.5 mo)
 n=146 (at 13 mo)
Weight & length
Skinfold caliper
Roche/Hitachi

Age 4 yr
Paper II
Growth n=120
Weight & height

Age 8-9 yr
Paper II-IV
Follow-up
n=120
Food record n=113
Physical activity n=110
Growth n=120
Body composition n=117
Metabolic markers/
Inflammatory markers n=114
Metabolome n=112
Dietist XP
Pedometer
Weight & height
DXA
ELISA/
Luminex
GC-GC/TOFMS

**Figure 7.** Overview of data collection and methods used in the papers presented in this thesis. In paper **II** growth data from the intervention (paper **I**) is included.

**Biochemical measurements**

Serum samples were drawn at 5.5 and 13 months (presented in paper **I**) and 8-9 years (presented in paper **II-III**) of age for analysis of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), apolipoprotein A-1 (apo A-1), apolipoprotein B (apo B), triacylglycerol (TAG), low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula (i.e. LDL = TC – HDL – 0.45 x TG) (112). Further, analyses at 8-9 years also included glucose, aspartate transaminase (AST) and alanine transaminase (ALT) according to accredited methods at the Department of Clinical Chemistry, University Hospital of Umeå. Homeostatic Model Assessment (HOMA) index was calculated according to the equation (S-insulin x P-glucose)/22.5 (113). At 8-9 years of age the blood samples were drawn after overnight fasting by one research nurse. The samples these were centrifuged within an hour and serum was frozen at -20°C and then stored at -70°C until analysis (presented in paper **II-IV**).

Further, using high multiplex immunoassay technology (i.e. Luminex); C-peptide, ghrelin, gastric inhibitory polypeptide (GIP), glucagon-like peptide 1 (GLP-1), glucagon, insulin, leptin, plasminogen activator inhibitor-1 (PAI-1), resistin and visfatin were analysed in plasma. High
molecular weight (HMW) adiponectin and high-sensitivity C-reactive protein (hsCRP) were analysed in serum using a commercial ELISA kit (described in detail in paper III).

Blood sampling and anthropometric measurements performed by the research nurse.

**Metabolomic analysis**

In paper I and IV a global metabolomic approach was used. From the intervention a subgroup ($n=40$) was randomly chosen for characterisation of the global plasma metabolome using a GC-time-of-flight (TOF)/MS approach (paper I). At the time of follow-up plasma samples from 112 children were analysed using 2D GCxGC/TOFMS analysis (paper IV). The plasma samples were extracted and further processed according to the Swedish metabolomics centre (SMC) protocol for plasma metabolomics analysis (114).

**Dietary registration and nutritional calculation**

The participating families were asked to fill in a food frequency questionnaire (FFQ) and a four-day food record, of which two were weekend days. Type and quantity of each food item consumed by the child were recorded. When the child attended school, parents informed school personnel to be present at meal times and aid the child to record food intake, parents then transferred the information to the food records. The daily dietary intakes of energy (EI) were calculated for each child in a dietary analysis program Dietist XP version 3.2 2011-03-10 (Kost och Näringsdata AB, Stockholm). Dietist XP was based on the Swedish Food
composition database version 2011-02-14. If food items or meals were not included in the database, these were substituted with similar food items or meals. If no equal replacement could be found, information of energy and nutrient content was manually entered into the database. Weight tables from National Food Administration were used for estimating the weight of the food. Parents were asked to fill in a questionnaire regarding chronic diseases, medication and consumption of nutritional supplements and probiotics. These data were used in papers II and III.

The research nurse instructing one participating family how to fill in the food records.

**Physical activity**

To monitor physical activity the participating children wore a pedometer during seven days. This was calculated and is presented as mean number of steps in paper II.
Statistical methods

All statistical analyses with the exception of the multivariate analysis were performed using SPSS for Windows version 18.0 (Paper I) and 21.0 (Paper II-IV) (SPSS Inc. Chicago, IL, USA). The level of significance was set at $p<0.05$. All variables were assessed for normality and non-normal distributed variables were either log$_{10}$-transformed or analysed with non-parametric tests. Log variables are presented as geometric means.

Multivariate statistical analysis was performed in SIMCA version SIMCA-P+ 12.0.1 (Paper I) and 13.0 (Paper IV) (Umetrics, Umeå, Sweden).

Statistical methods used in this thesis are presented in Table 2.

Table 2. Overview of the statistical methods used in papers (I-IV) included in the thesis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parametric tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Student’s t test</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeated measures ANOVA</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>General linear mixed model</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple linear regression</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>(standard)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Non-parametric tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chi square test</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Mann-Whitney U test</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Spearman rank-order correlation</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><strong>Multivariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCA</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

ANCOVA, analysis of covariance; ANOVA, analysis of variance; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis.
Results

The main results from each paper (I-IV) are presented below. More detailed information is given in the individual papers.

Baseline characteristics

A summary of the baseline characteristics of the study population is presented below.

The baseline characteristics did not differ between the LF19 and the placebo group (data not shown). This was consistent also when the statistical analyses were restricted to the subgroup of infants included in the analysis of the plasma metabolome (n=40) (paper I).

Analysis on demographic characteristics from children participating in the follow-up did not differ between the children with the exception of higher gestational age in the LF19 group and heavier fathers in the placebo group (p<0.05). Ninety-three % (n=54/58) and 92 % (n=57/62) of the children in the LF19 and placebo groups, respectively, had a birth weight >3000 g.

At the time of the follow-up approximately one-third of the children were diagnosed with allergic disease, i.e. eczema, food allergy, and/or respiratory allergy (115). Other reported chronic diseases were: celiac disease (n=1), lactose intolerance (n=1), congenital heart disease (n=1) and enuresis (n=1); some children had more than one diagnosis (116).

As 7 families, 4 in the LF19 group and 3 in the placebo group, did not complete the 4-day food records, a total of 113 records were analysed for energy content, reported as mean intake. The LF19 and placebo groups did not differ in EI. For evaluation of physical activity mean number of steps/day was measured during seven days. The mean number of steps/day in the whole study population (n=110) was 8155 (SD 3028) with a maximum number of steps being 14528 and a minimum of 1915. There was no difference between the LF19 and the placebo group (p>0.05).

When combining the two intervention groups (n=120), 16% were overweight and 4% obese according to iso-BMI, 25 and 30 kg/m², respectively. Overweight and obese children were combined into one group for statistical analysis. Demographic characteristics and physical activity...
did not differ between the overweight/obese and the normal weight children with the exception of higher proportion of paternal smoking ($p=0.03$) and higher paternal BMI ($p=0.03$) among overweight/obese children compared to normal weight children (Table 3).

**Table 3.** Demographic characteristics in children classified as normal weight or overweight/obese.

<table>
<thead>
<tr>
<th></th>
<th>Normal weight $n=96$</th>
<th>Overweight/obese $n=24$</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>43</td>
<td>46</td>
<td>0.78</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3693 (0.5)</td>
<td>3769 (0.5)</td>
<td>0.51</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>51 (2.1)</td>
<td>51 (2.5)</td>
<td>0.60</td>
</tr>
<tr>
<td>Gestational age (w)</td>
<td>40.2 (1.3)</td>
<td>40.3 (1.4)</td>
<td>0.94</td>
</tr>
<tr>
<td>Exclusively BF (mo)</td>
<td>3.8 (1.2)</td>
<td>3.7 (1.3)</td>
<td>0.73</td>
</tr>
<tr>
<td>Tot. duration of BF (mo)$^a$</td>
<td>7.7 (3.3)</td>
<td>9.1 (6.1) ($n=23$)</td>
<td>0.26</td>
</tr>
<tr>
<td>Received LF$^b$</td>
<td>49</td>
<td>46</td>
<td>0.78</td>
</tr>
<tr>
<td>Maternal smoking (%)$^b$</td>
<td>8</td>
<td>8</td>
<td>1.00</td>
</tr>
<tr>
<td>Paternal smoking (%)$^b$</td>
<td>3</td>
<td>17</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>Maternal univ. edu (%)</td>
<td>66</td>
<td>75</td>
<td>0.38</td>
</tr>
<tr>
<td>Paternal univ. edu (%)</td>
<td>48</td>
<td>50</td>
<td>0.89</td>
</tr>
<tr>
<td>Maternal BMI$^a$</td>
<td>24.0 (3.4) ($n=95$)</td>
<td>25.5 (4.0) ($n=22$)</td>
<td>0.07</td>
</tr>
<tr>
<td>Paternal BMI$^a$</td>
<td>25.7 (3.4) ($n=91$)</td>
<td>27.6 (3.8) ($n=21$)</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>Maternal SAD$^a$</td>
<td>19.4 (2.3) ($n=75$)</td>
<td>20.5 (2.9) ($n=14$)</td>
<td>0.10</td>
</tr>
<tr>
<td>Paternal SAD$^a$</td>
<td>21.3 (3.2) ($n=23$)</td>
<td>21.4 (1.7) ($n=8$)</td>
<td>0.91</td>
</tr>
</tbody>
</table>

BF, breast fed; univ. edu, university education; BMI, body mass index; SAD, sagittal abdominal diameter.

Data presented as mean (SD). Continuous variables analysed using Student’s $t$-test, categorical variables analysed with Chi square test.

$^a$Data not available for all participants, numbers included in italic and brackets. $^b$Calculated using Fischer’s Exact test. Bold font indicates statistically significance.
Growth, body composition and metabolic markers during weaning (Paper I)

Our aim was to evaluate growth, body composition and lipid status in the infants participating in the intervention study ($n=171$). Further, in order to explore the global plasma metabolome, a subgroup of 20 infants in each study group was randomly selected.

Between 4 and 13 months of age anthropometrics measurements, i.e. weight, length, knee-heel length and head circumference were collected. Analysis showed no statistical difference between the LF19 group and the placebo group ($p>0.05$).

As probiotic intake has been suggested to affect fat disposition in animal models (78), body composition was assessed using biceps, triceps, suprailiac and subscapular skinfold thickness measurements on six occasions during the intervention, i.e. 5.5, 6.5, 9, 12 and 13 months. Statistical analysis adjusted for measurements at entry (i.e. 4 months) showed no difference between the LF19 and the placebo groups.

At 5.5 months of age serum samples from 68 and 67 infants in the LF19 and the placebo group, respectively, were available. At 13 months of age samples were available from 73 infants from each group. TC, LDL-C, HDL-C, ApoA1, ApoB and TAG concentrations were analysed. There were no statistical differences between the two groups in any of these measures ($p>0.05$), there was however a trend towards lower levels of TC, LDL-C and ApoA1 at 13 months of age in the LF19 group ($p>0.05$).

For characterisation of the metabolome, 20 infants from each group were randomly selected and a total of 37 plasma samples at 5.5 and 13 months of age were analysed by GC/time-of-flight-MS (Figure 8).

![Figure 8](image)

Figure 8. Thirty-seven plasma samples were eligible from 5.5 and 13 months of age from the LF19 ($n=19$) and placebo ($n=18$) groups.
The overall plasma metabolite profile at 5.5 and 13 months of age did not differ between the LF19 and the placebo group, but there were differences in some unique metabolites (Table 4). There were lower levels of Methyl-β-D-glucopyranoside ($p=0.02$), palmitic acid and palmitoleic acid (both $p<0.04$) and higher levels of putrescine and tryptophan (both $p<0.01$) found in the LF19 group compared to the placebo group. In both groups age related differences were seen, with a statistically significant change in the amino acid profile during the second half of infancy.

Table 4. Identified and classified metabolites that differed between the LF19 and the placebo groups

<table>
<thead>
<tr>
<th>Compound class</th>
<th>Identification</th>
<th>LF19 vs placebo at 13 months (response in LF19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amine</td>
<td>Putrescine</td>
<td>↑</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>Palmitic acid (16:0)</td>
<td>↓</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>Palmitoleic acid (16:1)</td>
<td>↓</td>
</tr>
<tr>
<td>Amino acid</td>
<td>Tryptophan</td>
<td>↑</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Methyl-β-D-glucopyranoside</td>
<td>↓</td>
</tr>
</tbody>
</table>

**Main results**

Supplementation with LF19 during weaning influenced the metabolic profile at 13 months of age with lower levels of the saturated fatty acid palmitic acid and the monounsaturated fatty acid palmitoleic acid and higher concentrations of putrescine.

LF19 had no effect on anthropometry, body composition or the lipid profile.
Growth, body composition and metabolic markers at school age (Paper II)

In this study we aimed to evaluate possible long-term effects on growth, body composition and metabolic markers at 8-9 years of age.

As presented in paper II, 120 of the initial 171 children that completed the intervention were re-recruited in the follow-up at 8-9 years of age (Figure 7). Drop-out analysis revealed no differences regarding baseline characteristics between the two groups. However, families with allergies were more likely to participate (115).

There were no differences in growth, i.e. weight, length/height or body composition assessed by DXA and SAD between the LF19 and the placebo groups. As depicted in Figure 9 the prevalence of normal weight, overweight and obesity based on iso-BMI 25 and 30 kg/m² as cut-offs did not differ between the probiotic and the placebo group (p=0.78).

![Figure 9. Prevalence of normal weight, overweight and obese in the LF19 (n=58) and placebo groups (n=62) at 8-9 years of age.](image)

The analysed metabolic markers, i.e. TC, TG, HDL-C, LDL-C, Apo A1, Apo B, AST, ALT, insulin, glucagon and HOMA index did not differ between the probiotic and the placebo groups (p>0.05). Adjusting for sex, total duration of breastfeeding and gestational age did not affect the outcome. Analysis between the association of selected measurements from the DXA-
scan, SAD, BMI and BMI z-score showed strong correlation \( p<0.01 \) as depicted in selected scatterplots (Figure 10).

![Figure 10. Scatterplots displaying the correlations between BMI z-score, SAD (top) and total body fat (%) (bottom) based on measurements obtained from the DXA-scans.](image)

Tracking of growth was measured on eight occasions from baseline to follow-up, regarding weight and length/height. There was no difference between the overweight/obese or normal weight children regarding birth characteristics \( p>0.05 \) (data not shown). Mean weight, length/height and BMI z-score were higher \( p<0.05 \) at each measurement in overweight/obese children compared to normal weight children (Figure 11).
Follow-up analyses demonstrated that differences existed between overweight/obese and normal weight children at every measurement \((p<0.05)\) with the exception of length at 4 and 5.5 months of age (data not shown). Birth weight and length as a covariate did not affect the outcome.

Figure 11. Mean BMI z-score (vertical bars indicating SEM) between the overweight/obese and normal weight children, measurements at eight occasions from baseline (4-13 months) to follow-up (8-9 years). *\(p < 0.05\) Student’s t-test, adjusting for birth measurements using a mixed model approach did not affect the outcome.

We also observed that overweight/obese children had higher s-insulin levels and HOMA-index and lower HDL levels compared with normal weight children (Table 5). When excluding obese children from the statistical analyses, overweight children still had higher insulin concentrations \((p<0.05)\), but HOMA-index was comparable \((p=0.06)\) (data not shown).
Table 5. Fasting values of metabolic markers in the whole study population and according to BMI classification (overweight/obese and normal weight)

<table>
<thead>
<tr>
<th></th>
<th>All n=114</th>
<th>Overweight /Obese n=22</th>
<th>Normal weight n=92</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.60 (0.30)</td>
<td>1.46 (0.27)</td>
<td>1.62 (0.30)</td>
<td>0.02</td>
</tr>
<tr>
<td>Insulin^a (mU/L)</td>
<td>4.60 (2.56)</td>
<td>6.17 (3.54)</td>
<td>4.29 (2.09)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HOMA index</td>
<td>0.99 (0.54)</td>
<td>1.33 (0.75)</td>
<td>0.90 (0.44)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*P-value for differences between overweight/obese and normal weight children from one-way between-groups ANCOVA adjusting for sex, total duration of breastfeeding and gestational age. ^Insulin was log-transformed before the analysis. Data are presented as mean (SD) or as geometric mean (SD) if log-transformed prior to analysis.

**Main results**

There were no long-term effects on body composition, growth or metabolic markers following early probiotic supplementation. This supports that supplementation with the probiotic LF19 is safe to administer with regard to body composition and metabolic programming.

Metabolic differences with lower HDL-C and higher levels of insulin and HOMA index were observed together with an overall higher BMI z-score at every measurement occasion in the overweight children emphasizing the need for early prevention programs.
Extended panel of metabolic and inflammatory markers (Paper III)

In this study we examined long-term effects on the metabolic and inflammatory profile between the LF19 and placebo groups using multiplex immunoassay technology. One-hundred and fourteen blood samples (serum or plasma) collected following overnight fasting, were available for analysis of metabolic and inflammatory markers.

The LF19 and placebo groups did not differ in macro-nutrient intake or current probiotic intake (Figure 12). Nor did micronutrient intake (sodium, iron, calcium and vitamin D), differ between the two groups ($p>0.05$).

Biomarkers included in the analysis were; plasma C-peptide, ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1, resistin and visfatin. HMW adiponectin and hsCRP were analysed in serum. Leptin was removed from the multivariate analyses due to collinearity. In the correlation and regression analysis hsCRP levels below ~1 ng/ml (i.e. detection limit) were excluded. There were no statistically significant differences between the
children in the LF19 and the placebo group for any of these variables. However, differences between the overweight/obese (n=22) and normal weight children (n=92) were observed. Overweight/obese children had higher levels of C-peptide (p=0.01), leptin (p<0.01), PAI-1 (p=0.02) and hsCRP (p<0.01) compared with normal weight children. When obese children were excluded from the analysis, leptin (p<0.01) and hsCRP (p<0.01) were still higher among overweight children compared with normal weight children.

Several biomarkers correlated with measures of body composition as assessed by DXA-scan, SAD and anthropometric measurements. The variable making the strongest unique contribution was PAI-1 explaining all dependent variables, i.e. total body fat (%) (beta =0.47, p<0.01), truncal fat % (beta=0.38, p<0.01), SAD (beta=0.33, p<0.01), BMI z-score (beta=0.33, p=0.01) and FFM % (beta=0.47, p<0.01).

Main results

There was no difference in an extended panel of metabolic and inflammatory markers between the LF19 and the placebo group.

BMI z-score, SAD, truncal and total fat mass were associated with several of the metabolic and inflammatory markers.

Overweight/obese children had higher levels of C-peptide, leptin, PAI-1 and hsCRP compared with normal weight children – emphasising the need for early prevention and treatment strategies in pre-pubertal children with overweight and obesity.
Long-term effects on the metabolome at 8-9 years of age (Paper IV)

Our primary aim in paper IV was to investigate the metabolome at follow-up. In particular, we aimed at examining whether the lower levels of the saturated fatty acid palmitic acid and the monounsaturated fatty acid palmitoleic acid as well as the higher levels of putrescine seen in the LF19 group during the intervention remained at school age.

Here, we included all children in the follow-up and plasma samples were available from 112 children. Calculation for separation was performed using OPLS-DA between; the LF19 and placebo groups, overweight/obese and normal weight children as well as boys and girls.

A model was calculated between LF19 and placebo, describing 21.5% of the variation in the metabolite data (R2X), 47% of the between class variation (R2Y) and predicting 14.6% of the between class variation according to cross-validation (Q2Y). In total, 8 metabolites were identified that were statistically significant (p≤0.05) between the LF19 and the placebo group. Levels of xylose, tryptamine and methyl-β-d-glucopyranoside were lower and levels of 4-methoxyphenylpropionic acid, myo-inositol, threonic acid, hydrocinnamic acid and proline were higher in the LF19 group compared with the placebo group. Palmitic acid, palmitoleic acid and putrescine were identified, however they were not statistically significantly different between the two groups.

Stronger models were found between overweight/obese and normal weight children (R2Y=0.697, Q2Y=0.35), with 17 metabolites identified of which 11 were statistically significantly different between these two groups (Table 6).
Table 6. Pathway table of statistically ($p \leq 0.05$) different metabolites between the overweight/obese compared to the normal weight children.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Identification</th>
<th>Response in overweight/obesity</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid cycle</td>
<td>Citric acid</td>
<td>↓</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fructose and Mannose</td>
<td>Mannose</td>
<td>↑</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>degradation</td>
<td>Fructose</td>
<td>↓</td>
<td>0.01</td>
</tr>
<tr>
<td>Glutamate metabolism</td>
<td>Glutamine</td>
<td>↓</td>
<td>0.03</td>
</tr>
<tr>
<td>Glycerol kinase pathway</td>
<td>Glycerol</td>
<td>↑</td>
<td>0.04</td>
</tr>
<tr>
<td>Nucleotide sugars metabolism</td>
<td>Xylulose</td>
<td>↑</td>
<td>0.01</td>
</tr>
<tr>
<td>Purine metabolism</td>
<td>Uric acid</td>
<td>↑</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tyrosine metabolism</td>
<td>Tyrosine</td>
<td>↑</td>
<td>0.01</td>
</tr>
<tr>
<td>Other</td>
<td>Fucose</td>
<td>↑</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Glutaric acid</td>
<td>↓</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Monooleoylglycerol</td>
<td>↑</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The strongest OPLS-DA model obtained was the one between boys and girls ($R^2_Y=0.729$, $Q^2_Y=0.487$). Boys and girls did not differ in prevalence of overweight/obesity and normal weight (Figure 13).

![Prevalence of overweight/obesity and normal weight among boys and girls at 8-9 years of age.](image)

**Figure 13.** Prevalence of overweight/obesity and normal weight among boys and girls at 8-9 years of age.
Twenty-two metabolites were identified that differed between the boys and the girls \((p \leq 0.05)\). Of these, the concentration of several metabolites associated with fatty acid biosynthesis was lower among boys compared to girls. Boys reported a higher protein intake compared to girls \((p = 0.02)\), this was however not reflected in the metabolite profile since identified amino acids were lower among boys.

**Main results**

*The previously reported results with lower levels of palmitic acid and palmitoleic acid and higher levels of putrescine in the LF19 group during the intervention did not remain at the time of the follow-up.*

*The OPLS-DA analysis revealed stronger models between overweight/obese versus normal weight children and between boys and girls, as compared to analysis between the LF19 and the placebo group.*

*The disparities seen in metabolic profile between overweight/obese and normal weight children using an untargeted metabolomic approach underscore the need for effective treatment strategies to prevent the development of later comorbidities in overweight/obese children.*
Discussion

This thesis describes the results from an intervention trial with infants randomised to early supplementation with the probiotic bacterium LF19 or placebo and its follow-up 8 years later. This part of the project included analysis on growth, body composition, metabolic and inflammatory markers as well as exploring the plasma metabolome of the children participating in the study.

Main findings

Blood lipids and metabolomics

Animal studies have implicated that the addition of LF19 to the diet could decrease fat storage in the abdominal and visceral regions of mice (78). The proposed mechanism was that inhibition of lipoprotein lipase via increased levels of ANGPTL4 following administration with LF19 decreased fat storage. Our intervention did not result in any difference between the LF19 and the placebo groups in HDL-C, LDL-C, ApoA1, ApoB and TAG. There was a trend towards lower TC concentrations in the LF19 group during the intervention. This could potentially be a controversial issue since human milk contains a fair amount of cholesterol, approximately 90-150 mg/L (117) and breastfeeding compared to formula-feeding results in higher TC (118). Still, this difference does not exist after 1-2 years and later breastfed infants have slightly lower TC. It has also been suggested that exclusive breast-feeding can lead to a reduction of the population prevalence of cardiovascular disease by 5 % (118). As probiotics have been suggested to improve the lipid profile mainly through reduction of TC and LDL-C mostly in adults (119), more infant studies are needed. However, as no major decrease in cholesterol reduction was seen in the LF19 group, it appears as if it is safe to administer LF19 in that aspect.

During the intervention an untargeted metabolomic analysis was performed in a randomised subgroup of infants (n=40). Unique metabolites were identified that differed between the groups, although there was no difference in the global metabolome between infants fed LF19 or placebo. The 1D GC-TOFMS analysis at 13 months of age revealed lower levels of the saturated fatty acid (SFA) palmitic acid (16:0) and the monounsaturated fatty acid (MUFA) palmitoleic acid (16:1) and higher levels of putrescine in infants in the LF19 group. Palmitic acid is a major
component of human milk but also in palm oil and bovine milk (117). Palmitoleic acid is also present in breast milk (117), which has been associated with visceral obesity in children (120), with body composition indices (121) and triglyceridemia (122) in adults. Since duration of breast-feeding did not differ between the LF19 and the placebo group this could not explain the observed differences seen in the levels of palmitic and palmitoleic acid. Palm oil, one of the most widely used vegetable oils in the world, constitutes a high proportion of palmitic acid and has been suggested in the development of obesity and cardiovascular diseases although results are conflicting (123). On the other hand, one study reported that infants randomised to high palmitic acid formula had higher fecal lactobacilli and bifidobacteria counts compared to infants randomised to low palmitate formula (124). The counts of these beneficial bacteria in the high palmitic acid group were equally high as in a breast fed reference group, indicating a beneficial effect of palmitic acid on gut microbiota.

Since palmitic acid has been associated with adverse health effects, although with conflicting results (123) and palmitoleic acid strongly correlates with visceral obesity (120, 121) we wanted to examine if the observed differences between the two groups persisted at the follow-up. If the effects remained this would indicate that LF19 could exert programming effects in the human setting. All available samples were analysed at the time of the follow-up using a 2D GCxGC/MS approach. Although the multivariate model obtained was not as strong between the LF19 and the placebo group as between overweight/obese and normal weight children, unique metabolites that differed were identified. Even though palmitic acid, palmitoleic acid and putrescine were among the identified metabolites, the differences seen between the LF19 and the placebo groups during the intervention did not remain at the time of follow-up, indicating that early probiotic supplementation did not exert long-term programming effects. Since obesity has been associated with increased intestinal permeability (125) the effect of LF19 on putrescine levels during the intervention could still be of significance as this polyamine appears important for gut integrity (126).

We found age-dependent differences during the intervention, which might be linked to a more diversified gut microbiota. The amino acid profile increased with age in both the LF19 and the placebo groups, possibly
reflecting diet induced changes with higher protein content as solid foods were introduced during the intervention period.

**Growth and body composition**

Growth was assessed on several occasions during the intervention and at follow-up. In accordance with others, we found neither short- (127) nor long-term (**Table 1**) effects on weight or length/height according to probiotic intake.

Body composition was evaluated both during the intervention using skinfold caliper measurements and at the time of follow-up using DXA-scans and SAD. There were no differences regarding body composition between the two groups. It is unlikely that more sophisticated measurements of body composition during the intervention (96, 128) could have detected differences, since the weight, length and BMI of the children were almost identical in the two groups. BMI has been questioned as a reliable marker of body composition since it doesn’t distinguish between lean or fat mass and interpretation of results are age dependent in children (129, 130). We found strong correlations between the measurements from the DXA-scan and assessed BMI and SAD, indicating that in this age group both BMI and SAD are useful indicators of obesity. The inter- and intraobserver precision of SAD has been evaluated with small variations between observers (131, 132). A detailed study protocol for measuring weight, length and SAD and the same trained nurse performing all measurements are factors that are likely to explain the strong correlations obtained in this thesis.

**Adipose tissue and metabolic markers**

The adipose tissue is an important endocrine organ with the adipocytes playing a critical role in the inflammatory response by releasing several inflammatory mediators and signalling molecules (60). A wide array of biomarkers can be used to characterise a deviant metabolic state (**Figure 14**). Leptin is preferably expressed and secreted by subcutaneous adipose tissue while the expression and secretion of PAI-1, resistin, visfatin, adiponectin and others is higher in abdominal fat (60).
Figure 14. Inflammatory and metabolic biomarkers used to characterize an aberrant metabolic state.

The lipid profile, and the metabolic and inflammatory markers were comparable between the groups at the follow-up. During the intervention, there was a trend towards lower TC, HDL-C and ApoA1 in the LF19 group, but this did not remain at follow-up. Brahe et al. (133) found no effect on the metabolic markers glucose, insulin, C-peptide, HOMA index, TAG, HDL-C and LDL-C following a six-week intervention with LF19 in obese, postmenopausal women. This could be consistent with our results although they included a group of patients for treatment purposes and we studied the preventive effect of LF19 in children. The authors speculated that an effect might have been detectable if LF19 had been administered together with a fermentable milk product that could potentially enhance the probiotic efficacy (133).

Conclusion

Collectively, feeding LF19 in infancy caused neither benefit nor harm on growth, body composition, metabolic or inflammatory markers or the metabolome in a long-term perspective, i.e. at 8-9 years of age. The effects seen on the global metabolome at 13 months of age with lower levels of
palmitic and palmitoleic acid and higher levels of putrescine in the LF19 group were not confirmed at follow-up. During the intervention period LF19 was an excellent transient colonizer with the probiotic isolated in 90% at 6.5 months and in 64% of the children throughout the intervention in the LF19 group (1). At the follow-up however, LF19 was not detected in any of the fecal samples (115) or saliva samples (134). Taken together this indicates that LF19 is a temporal colonizer that exerts transient beneficial effects (1, 2).

**Effects of probiotics**

*Modulation of the gut microbiota*

Members from Jeff Gordon’s group (15, 26) presented results in the beginning of the 21st century that conventionally raised mice had more body fat compared to germ-free mice, despite that the energy intake was higher in the latter. Together with the observation that the ratio of Bacteroidetes/Firmicutes in the fecal microbiota differed between lean and obese individuals and that this ratio changed with weight loss (12, 15), this suggested that the microbial composition could affect the development of obesity and thus offer a novel approach in obesity treatment and prevention (12). As *Lactobacillus* strains belong to the Firmicutes phylum, which in some studies has been associated with obesity (12) this caused some concerns (135). However, in observational human studies *L. paracasei* was associated with normal weight, contrary to for example *L. reuteri*, which was associated with obesity (136). Then again, Abrahamsson et al. (87) used *L. reuteri* in an RCT and reported no differences in growth indices between children in the intervention and the control group. As mentioned, here we found neither benefit nor harm of supplementation with LF19 on growth and body composition.

Probiotic studies, both in animals (78, 79, 137) and humans (138) have indeed shown promising results in the modulation of the gut microbiota with a subsequent change in body composition, reduced weight gain and improved metabolic markers. It has been demonstrated that the gut microbiota in conventionally raised mice down-regulated ANGPTL4 with consequently increased adiposity (26). Of note, administration of LF19 in a murine model increased serum TAG plausibly through increased circulating levels of ANGPTL4 (78). In another study in mice, LF19 induced up-and down-regulation of genes involved in insulin sensitivity and insulin
resistance, respectively (79). Mekkes et al. (138) published a review on probiotic treatment of obesity and possible mechanisms of action of the microbiota on metabolism. Different strains (single or in combination) of Lactobacillus showed short-term effects with reduced body weight, adiposity, central adiposity and fat mass in obese adults. Further, two studies included in the review found effects on the lipid profile with decreased levels of TAG and LDL-C (138). The included studies varied however, in sample size, strains used, dosage, range of study duration, heterogeneity in study participants, mode of administration and controlling for confounding factors e.g. diet. Thus, although promising effects have been reported in a short-term perspective, inferences are difficult and more homogenous studies are needed before recommendations of probiotics in the treatment or prevention of overweight/obesity and its related comorbidities can be given.

The aetiology of obesity is complex (9), involving energy intake and expenditure together with environmental factors such as the gut microbiota composition where microbes can play a major role in thriftiness of energy harvest from the diet (13). A likely explanation that the effects seen in our study population during the intervention did not persist at the time of the follow-up is that several external factors, such as diet (type, composition, timing) (37, 41, 139), antibiotics (38), stress and age (28) influence the gut microbial composition. It is reasonable to believe that effects seen during probiotic supplementation are diluted with time as the abovementioned factors have potential to influence gut microbiota composition and functions.

A healthy microbiome

In 2011, the concept that the gut microbiota of humans could fall into three distinct enterotypes based on the composition of the gut microbiota was proposed by Arumugam et al. (140). The authors speculated that this could allow classification of groups who responded differently to dietary intake or drugs. This theory has been questioned and it appears that the distribution of enterotypes is not as discrete as first thought and should be considered more as enterogradients (141). A dysbiotic gut microbiota has been associated with several intestinal and systemic diseases both in adults and children (11, 28, 29). However, the cause-effect relationship remains to be established, i.e. we do not know whether the dysbiosis is causing the disease or if it is a consequence of the disease. One limiting factor and an important first step to unravel this relationship is to determine what
constitutes a healthy human gut microbiome. Reduced bacterial diversity as seen in many NCDs e.g. obesity, inflammatory bowel disease and allergic diseases may represent a suboptimal microbiome (11, 28, 29). The gut microbiota’s ability to remain stable during times of continuous and potentially disruptive perturbations such as when exposed to antibiotics appears important (28).

Antibiotic treatment is known to drastically alter the gut microbiota (135), promote growth and increase fat mass in animals (142). Further, repeated treatment with broad-spectrum antibiotics from birth to 2 years of age was associated with early childhood obesity (143). A Finnish study reported long-lasting shifts in gut microbiota composition following early exposure to macrolide and a predisposition to antibiotic-associated weight gain (144). Cox et al. (31) found lasting metabolic alterations in mice following early low-dose penicillin treatment whereas there was only a transient effect on the gut microbiome. Penicillin is frequently given to children worldwide. In infants <6 months of age early antibiotic usage increased body mass from 10 to 38 months of age, but not if given later in infancy (145). This might indicate that antibiotic treatment during a critical window of gut microbiota establishment has long-lasting effects. During the intervention, the infants in the LF19 group had fewer days with antibiotics compared to placebo (1). This could indicate that LF19 protected against infections with less antibiotic usage, which could in turn lead to changes in gut microbiota composition that could have effects during a prolonged period of time. However, as mentioned, we found no effect, neither short- nor long-term, on growth or body composition between the two groups.

Long-term follow-ups of probiotic intervention trials are warranted because of the increased marketing and use of these products. Current evidence points at strain-specificity of probiotics (80). Safety of one product should therefore not be extrapolated to other probiotics or products and for assessment of clinical effects each strain should be evaluated separately (80). As the underlying mechanisms mediating the clinical effect of different probiotics are yet not fully elucidated the European Food Safety Authority (EFSA) has refused health claims of marketed probiotics (67).

We can only speculate whether a different study approach would have resulted in different results. In the future a more clear-cut message of choice of probiotic strain(s) depending on the indication, optimal timing
and duration of administration is needed. An essential part of using probiotics for health effects is the dosage, which should ultimately be ‘administered in adequate amounts’. The optimal dose has not yet been established but probably varies in different clinical conditions and for different probiotic strains (80, 127). Until a dose – response consensus has been stated it is reasonable to use the protocols shown effective in well-designed RCTs for the intended indication.

**Methodological discussion**

*Comparison of animal and human studies*

Most studies associating gut microbiota and host metabolism have been performed in rodents. Even though experimental animal models provide important information on causality of the complex host-microbiota interactions, there are some important discrepancies between animal models and humans that need to be taken under consideration before making inferences. Despite the obvious prevailing conditions, i.e. animals can be bred to provide a homogenous genetic background and identical diets and housing conditions are generally controlled for thereby limiting “noise” from the surrounding environment. In 2014, Nguyen et al, published a comprehensive overview of differences, advantages and limitations of mouse models used in gut microbiota research (146). Important limitations are the genetic, anatomical and physiological differences. The cross-talk between the host and the gut microbiota is host-specific, thus interpretations from mouse models might not apply to humans. The genetic homogeneity in mice might fail to capture the genetic variability of humans. Finally, all factors participating in shaping the ‘real-life’ gut microbiota of the human, e.g. mode of delivery, feeding practices and medical history, the absence of these factors in mice implies that the gut microbiota of the mouse cannot reflect a ‘real-life’ microbiota. Recently, as the gut microbiota of pigs and their gastrointestinal physiology, microbiology, genetics and diets appear more comparable to humans these models have been suggested for use in future studies (35). Despite these limitations, the advantages are numerous and mouse models have rendered a clearer picture on underlying mechanisms behind the pathophysiology of obesity (146). Although it still remains to be clarified if the proposed mechanisms seen in rodents can be extrapolated to humans, animal studies still provide valuable information from experiments that cannot be performed in humans.
Metabolomics

Here we used untargeted metabolomics as an explorative approach to detect known and unknown metabolites. One advantage with this approach is that, in a fairly novel area of research such as metabolomics in probiotic studies, this method can act as hypothesis generating. The use of metabolomics in studies in infants and young children is suitable since it requires low sample volumes \((147)\). Analyses can be conducted in numerous ways, e.g. in blood serum or plasma, urine, or fecal samples. As for other methods, standardised procedures are important in order to obtain representative samples for metabolomics analyses. During the intervention we used a 1D GC-TOFMS and a 2D GC-TOFMS at the follow-up. The main advantage with the 2D GC is higher separation capacity together with enhanced detection limit \((106)\). Even though the analysis time is longer the information generated from each sample is higher, which makes it worthwhile.

Few studies have been performed in the age group of the current study population. In primates the metabolome has been associated to age, with changes seen in different metabolic pathways that are also sex specific \((148)\). This is a novel field of research making inferences from our results based on other studies difficult. However, our results could in the long run serve as a useful base for future research.

Physical activity and energy/nutritional intake

In this follow-up study information on physical activity and dietary intake was collected mainly to enable adjustments in the statistical analyses on growth, body composition and metabolic markers. There was no difference between the LF19 and the placebo group or between the normal weight and the overweight/obese children regarding physical activity. Boys were however more physically active compared to the girls. It appears that the minimal recommendation of 60 minutes moderate-to-vigorous physical activity/day in primary/elementary schoolchildren is associated with 13-15,000 steps/day in boys and 11-12,000 steps/day in girls \((149)\). Compared to these numbers the corresponding mean number of steps in the present study was considerably lower, i.e. 9210 in boys and 7275 in girls. In adults, <5000 steps/day is used as a step-defined sedentary lifestyle index, but there is inadequate evidence to advocate a corresponding number for children and adolescents \((150)\). This could indicate a more sedentary lifestyle among the participating children, but could also reflect a
limitation with the used method for measuring physical activity. Accelerometers offer a great potential to study complex patterns of physical activity and sedentary behaviours (149). Even so, due to its “ simplicity”, low cost and interpretability the use of pedometers are widely accepted by researchers, practitioners and the general public. Non-ambulatory activities such as cross-country or alpine skiing, ice hockey, bicycling or swimming, i.e. sports commonly performed among Swedish children are also valuable.

We found no differences in the reported energy- and nutrient intake based on the four-day food record or the FFQs; although not unexpected when comparing the LF19 and the placebo group, a difference, albeit small was anticipated between the overweight/obese and normal weight children. A known problem in dietary assessment of children is the difficulty of getting accurate dietary reports that measure the true intake during the study period (151). Over- and underreports are common depending on the subject’s weight, with low-energy reports and increased body fatness showing positive correlations (151). Age also seems to be an important factor where younger children are dependent on the care-givers’ enthusiasm (151). Another factor that makes evaluation of EI difficult is that the participants may change their eating behaviour by eating healthier or eating less during the days of reporting. One technique for validating dietary intake is doubly labelled water (DLW) which however is a costly and technical complex method and therefore not used as a routine for detecting bias in EI data (151). Despite this, a validation study of the accuracy of weighted food records and FFQs by reports from children, age 8-11, and their parents using DLW showed that the child-reported FFQ was the most accurate compared to reports from their parents (152). Although the weighted food records tended to under-report EI and the FFQs to over-report by approximately 13% of daily kcal intake, the methods were almost comparable. Even so, until the nature of misreporting has been identified and to what extent this occurs, dietary data on children should be interpreted with caution.

Strengths and limitations

All studies have its strengths and weaknesses. This study was powered to assess cumulative eczema incidence, growth and immune markers during the intervention. To evaluate long-term effects of nutritional interventions or as in this case a supplementation with a probiotic bacterium, follow-up studies are necessary (153). A major challenge with follow-ups is cohort
attrition or “loss to follow-up” which reduces the power of the study and individuals staying in the study at follow-up rarely represent the original cohort. One main concern for RCTs is if randomised groups present differential bias. This can however be investigated by comparison of characteristics in subjects kept or lost to follow-up in the study groups (154). The “80% rule” is a concept that in order for a follow-up study to ensure statistical validity a minimum of 80% follow-up rate must be ensured. However, as discussed by Fewtrell et al. (154) a 20% attrition does not necessarily introduce bias if the attrition is equally spread between groups and if the subjects that remain in the study are representative to those lost. Reassessment of approximately 70% of children at the follow-up might pose a risk for introducing bias. Drop-out analysis revealed no differences between the LF19 and the placebo groups, although overall families with allergies were more likely to participate (115). Extensive demographic characteristics were collected and with the exception of gestational age and paternal overweight/obesity, there were no differences between the LF19 and the placebo group at the time of the follow-up.

Many probiotic studies initiated in the beginning of the 21st century were originally designed with the aim of evaluating the preventive effects of probiotics on early onset allergic manifestations (namely eczema) (155). Variables on growth and metabolism were included as secondary outcomes. In fact, most probiotic studies evaluating long-term effects were not originally powered to detect differences between the intervention and placebo groups at a follow-up. As discussed by Grüber et al. (156) when the occurrence of an event is low, such as obesity in some of these trials including our own, the number needed to treat to prevent one case becomes extensive. For a number of reasons, including ethical and economical reasons, there should be a balance between efforts (e.g. early probiotic supplementation) and transient gains.

This study has several strengths such as a prospective design, analyses based on repeated blood samplings and that trained research nurse/-s performed all measurements. In addition, DXA is a powerful method suitable for pediatric use, which allowed us to assess body composition not solely based on BMI. Another strength is the use of high sensitivity multiplex immunoassay technology for analysis of the biomarkers in paper III and that the metabolomic approach and the multivariate data analyses in paper I and IV allowed us to handle large data sets.
**Future directions**

There is consensus that the microbiota interacts with human health (11, 28) and that gut microbiota modulation strategies might offer a way to prevent the onset of obesity and related comorbidities. In the pediatric setting, the heterogeneity and relative small study populations as well as the diversity of used probiotic strains of previously conducted probiotic studies makes inferences difficult. Future studies in this field should strive towards greater homogeneity including dose, use of strain, timing of supplementation, i.e. pre-, post- or perinatal supplementation, duration, administration and so forth (80, 82). Larger scale studies are needed to account for inter-patient variability as well as markers of diseases related to the microbiota in order to adequately power clinical trials. The best approach in this area of research is yet to be decided, do we need “better” probiotics? In combinations? Fecal microbial transplantation? An interesting emerging field is targeted prebiotics, i.e. prebiotics designed to enhance the effect of a certain probiotic strain.

The interest for the gut microbiome in health and disease is immense and new highly advanced "omics” methods allow not only for globally mapping the microbiome but also offer an increased understanding on its function, and a deeper knowledge of the interaction/connection with later disease onset.
Personal reflection

In all papers presented in this thesis with the exception of paper I, we explored growth, body composition, inflammatory and metabolic markers in children classified as overweight/obese and normal weight. Even though only 5% of the children were obese we still found deviating metabolic and inflammatory profiles in pre-pubertal children at 8-9 years of age. This deviating pattern was also evident when including only overweight children in the analyses. The aetiology of overweight and obesity is complex and involves genetics, diet, physical activity, social aspects and as more recently proposed the gut microbiota. The role of probiotics in these conditions still remains to be elucidated and although it may not be the solution for the obesity “epidemic”, administration might aid in the treatment of individuals undergoing weight loss. Not all children with overweight/obesity display an aberrant metabolic profile. Hence, efforts should be made to investigate underlying mechanisms between metabolically healthy obese and those who are not. Timing of intervention to prevent obesity is crucial, since obesity tends to track from childhood to adulthood.

“To make an end is to make a beginning. The end is where we start from.”

T.S. Eliot
Acknowledgements

Participating children and families in the ELEFANT study – Thank you for taking the time to contribute to the study. Without you this thesis would not have been possible to compile.

Christina West (main supervisor). Thank you for inviting me to be part of the ELEFANT study. During these years you have truly been a fantastic mentor in this research field. Thank you for teaching me the thoroughness of research, for your encouragement and enthusiasm and for your never ending patience in correcting my incorrectly written “were” and “was”.

Olle Hernell (co-supervisor). Your knowledge and never ending curiosity when it comes to pediatric nutrition is tremendously inspiring. Thank you for continuously offering great advice along the way.

Inger Öhlund (co-supervisor). Thank you for suggesting me as a PhD student to this inspiring project and for being part of my introduction to the world of research as well as for your invaluable knowledge regarding pediatric clinical nutrition.

Hans Stenlund (co-author paper II, III). Thank you for your vast knowledge in the statistical field and for doing your best trying to pass some of it on to me.

Henrik Antti (co-author paper I and IV). Thank you for taking the time to share your great advice, expertise and invaluable assistance in the world of metabolomics and multivariate analysis, again and again and again...

Yvonne Andersson (co-author paper III). Thank you for the guidance in the world of ELISA and Luminex and for always taking the time to answer my questions.

Elin Chorell (co-author paper I). Thank you for your great contribution in paper I.

Research nurse Åsa Sundström for your invaluable contact with the children and families. Thank you for keeping them in the study during the time of the follow-up, as well as for running all the blood samplings, taking measurements, giving information and answering questions from the
participants (and from me). Research nurse Lena Uddståhl – responsible for running the DXA scans. Thank you for taking such good care of our participants.

Catarina Lundell, Carina Lagerqvist and Yvonne Andersson – Thank you for outstanding work on the laboratory analysis.

Thanks to Michelle for helping out with entering data from the food frequency questionnaires.

Karin Moström – Thank you for taking care of us PhD-students with reminders of applications, documents to fill out as well as great help during all the administrative work along the way and especially before the dissertation.

Carina, Elisabeth and Marlene Engström – Thank you for keeping an eye on the finances.

Lena Hansson and Tove Mårs – You have the ability to always make me feel better when I leave your rooms at the department than I did when I first entered and for that I thank you. Tove Grip – Thank you for shared experiences in the baffling world of metabolomics and for always offering a coffee break exactly when I needed it the most.

Staffan Berglund and Torbjörn Lind – Thank you for your invaluable discussions regarding pretty much everything in the research world and for always taking time off from your busy schedules to answer my “it’ll just take 2 minutes” questions.

My PhD fellows at the department of Pediatrics during this time, with a special thanks to: Cornelia, Ulrika, Tove G, Lena, Emilia, Elena, Josefine, Björn-Markus, Jenny and Itay. And the ones with me at the beginning: Elisabeth, Niklas and Staffan.

Ulla Norman – What would I have done without you? Besides being fantastic at your “real” job, you are also a “handyman” in every other sense. Thank you for always taking the time to help a technical illiterate.
Ett stort tack till alla kollegor (tidigare och nuvarande) vid Enheten för klinisk vetenskap Pediatrik och vid Barn- och Ungdomscentrum, Norrlands universitetssjukhus för delade samtal och skratt under det obligatoriska 9.30 och 14.30 fikat.

To my friends: Anders and Karin, Frída and Fredrik, Vickan and Anders, Stina and Johan, Lina, Åsa...thanks for shared baby walks, family dinners, “pulkaåkning”, BBQs, game nights, workouts... It's a pleasure to know you all. To Emmeli – Thank you for being a friend, for lending me a horse every now and then (the best therapy ever) and for letting me take out my frustrations by cleaning the stables. I guess it’s a win-win situation for us both.

To my oldest and closest friends: Monica, Jennie and Karolina with families. Thank you for being just that and for always allowing us to pick up where we left off.

Thanks to my family and relatives, especially my parents Kärstin and Nicke, my sister Elin and her family and my brother Oskar. Thanks for all the family dinners, laughter’s, discussions and for always being there for me and my family. Thank you for questioning and supporting all the things I just somehow “happen” to get involved in. I feel truly blessed to have you all nearby.

My boys. To my husband Kristofer and my two sons, Vidar and Ebbot the most important people in my life. Kristofer - Thank you for being my exact opposite and through that making us the great team that we are. What would I be without you? Vidar och Ebbot – mina modiga, starka och fantastiska pojkar. Tänk vilken tur jag har att just jag får vara just er mamma – tack för att ni varje dag delar med er av era klokheter. “Always remember that you are braver than you believe, stronger than you seem and smarter than you think” - A.A Milne
**Funding**

Financial support for this thesis was gratefully supported with grants from:

- Semper AB, Sweden
- Arla Foods AB, Denmark
- Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS)
- Swedish Agency for Innovation Systems (VINNOVA)
- Ronald McDonald Fund
- Swedish Nutrition Foundation (SNF)
- Ekhaga foundation
- Oskar-foundations
- European Union’s Seventh Framework Programme under grant agreement no 222720
- Regional agreement between Umeå University and Västerbotten County Council on cooperation in the field of Medicine
- The Swedish Society of Medical Research
- Stiftelsen Samariten
- Insamlingsstiftelsen at Umeå University
- Kronprinsessan Lovisas Förening För Barnasjukvård/ Stiftelsen Axel Tielmans Minnesfond
References


