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The Gut Bacterial Flora
Focus on Early Life and Physiological Traits

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Contents

Abstract

The gastrointestinal tract of the foetus is considered sterile but during vaginal birth the neonate comes into contact with bacteria from the maternal vaginal and intestinal microbiota.

The main focus of this doctoral thesis was to elucidate the initial bacterial ecosystem in newborns and to relate microbial perturbations to physiological traits. The bacterial flora was mainly studied with molecular-genetic methods.

When the microbiota was assessed in one-week-old infants, reduced faecal bacterial diversity was found in infants developing atopic eczema at the age of 18 months compared to those infants not developing eczema.

To further elucidate the pioneer microbiota, stool samples from healthy full-term vaginally-born neonates was studied. *Lactobacillus* was found in all newborns within 48 hours after birth. Species commonly found in the vaginal microbiota were to some extent detected among the babies. Other bacterial groups were found in varying prevalence. Interestingly, a subgroup of neonates born large for gestational age had significantly more *Proteobacteria* compared to neonates born appropriate for gestational age.

Maternal microbiota and dietary habits are known to impact offspring physiology. Results presented in this thesis show impaired physiology in suckling rat pups to dams of the outbred Sprague-Dawley stock when these were treated with high-energy dense diet during the gestation and lactation period. Offspring body weight, adiposity, gut permeability and systemic inflammation were further accentuated if the Gram-negative *Escherichia coli* was given to the dams in combination with the high-energy dense feed. In a similar experimental design, the pups were monitored in a longitudinal study. *E. coli* exposure from foetal life until six months of age decreased the diversity of the caecal microbiota along with enhanced adiposity. In contrast, when the Gram-positive *Lactobacillus plantarum* was given instead of *E. coli,* body weight gain and fat accumulation were lower in addition to a more favourable gut microbiota, implying the prospect of effect on health homeostasis by bacterial consumption. This thesis suggests that a high load of *E. coli* should be avoided in pregnant mothers and in children, and treatment with *L. plantarum* may be a therapeutic option. However, more extensive research is needed to establish the relationship between inflammation, obesity and the bacterial flora of the gut. A general conclusion concerning the microbiota is that the intestinal ecosystem should be studied at least at the hierarchical level of genus or family, but preferably on the species level since looking at the phylum level can give superficial information.

Summary in Swedish

Populärvetenskaplig sammanfattning

Fetma, övervikt och allergi är ett problem i många delar av världen och förändringar i tarmens bakterieflora kan vara en av flera orsaker. Människan har tio gånger fler mikroorganismer än mänskliga celler. Flest mikroorganismer finns i tarmen och de brukar gemensamt kallas för tarmfloran. Många bakterier är helt oskadliga och en del är dessutom nödvändiga för att vi till exempel ska kunna motstå sjukdomar och tillgodogöra oss näringen i maten vi äter. En del bakterier som finns naturligt i tarmfloran har dock förmågan att skapa en mild inflammation i hela kroppen, utan att vi för den saken känner oss sjuka.

Det övergripande målet med det här avhandlingsarbetet har varit att klargöra sammansättningen av spädbarns första tarmflora, samt att undersöka om man med bakterietillskott kan påverka fetma och inflammationsnivå. För att studera det sistnämnda gav vi råttor antingen inflammationsskapande bakterier eller probiotika tillsammans med en energirik kost. Probiotika kallas sådana bakterier som ger hälsomässiga fördelar när man äter dem.

Till skillnad från barn och vuxna så befinner sig ett foster i en steril miljö. I samband med en vaginal förlossning kommer barnet däremot i kontakt med de första bakterierna som kommer från mammans vagina och tarmkanal. Spädbarn har ett outvecklat immunförsvar som behöver stimuleras för att kroppen ska lära sig vilka ämnen som är ofarliga respektive farliga. Forskning tyder på att det är viktigt att nyfödda barn utsätts för rätt sorts bakterier för att programmeringen av immunförsvaret ska bli bra.

För att ta reda på vilka bakteriesorter som finns i tarmfloran så kan prov från tarmslemhinnan eller avföring analyseras. Tidigare odlades bakterier från exempelvis avföring fram på laboratoriet, men nya genbaserade tekniker gör att det numera är möjligt att studera även svårodlade och döda bakterier. Det är framförallt sådana genbaserade metoder som har använts i studierna som presenteras i denna avhandling.

Resultat som presenteras i denna avhandling visar att barn med allergiskt eksem vid ett och ett halvt års ålder hade en lägre mångfald i sin tarmflora när de bara var en vecka gamla, jämfört med de barn som inte hade allergiskt eksem. Det tyder på att en tidig stimulering av immunförsvaret med fler sorters bakterier minskar risken för allergiutveckling.

Eftersom sammansättningen av tarmfloran vid en veckas ålder är viktig för den framtida hälsan så antog vi att bakteriesammansättningen är betydelsefull även innan barnet är en vecka. Vi valde därför att undersöka tarmfloran hos spädbarn som föddes genom vaginal förlossning eftersom de barnen då kom i nära kontakt med sin mammas tarm- och vaginalbakterier. Laktobaciller är bakterier som är vanliga i kvinnors vagina och resultat från vår studie visar att alla barn hade laktobaciller inom de två första dagarna i livet. Samma laktobacillarter som normalt finns i kvinnors vagina kunde hittas hos de nyfödda barnen. Det tyder således på en överföring av bakterier från mamman till barnet

i samband med förlossningen. Spädbarnen hade även andra typer av bakterier men dessa förekom i varierande omfattning.

Hos nyfödda barn brukar det inte talas om övervikt och fetma. Däremot kan de nyfödda vara stora, små eller ha normal vikt i förhållande till i vilken graviditetsvecka de har fötts, med andra ord sin graviditetsålder. När vi jämförde tarmfloran hos barn som var stora för sin graviditetsålder med de som hade normal vikt så visade det sig att barnen som var stora för sin graviditetsålder hade mer av inflammationsskapande bakterier, som exempelvis *Escherichia coli,* i sin tarmkanal. Barnen med normal vikt hade däremot större variation i sin tarmflora och de hade oftare bakterier som inte skapar inflammation, utan istället exempelvis laktobaciller som kan ha motsatt effekt.

Den ökade kroppsmassan hos feta och överviktiga individer är sammankopplad med mild inflammation vilket i sin tur kan leda till andra sjukdomstillstånd såsom exempelvis diabetes. Sambandet mellan fetma och tarmens bakterieflora är inte helt klarlagt men troligen beror det på att tarmflorans komposition är störd och det kan bidra till den milda inflammation som finns hos feta individer. Mycket forskning återstår dock för att förstå det komplexa samspelet mellan tarmflora, fetma och inflammation.

Det är sedan tidigare känt att mammans levnadsvanor påverkar fostret. För att närmare studera hur mammans kost och bakterieflora påverkar barnet och dess hälsa gav vi råtthonor mat med högt energiinnehåll under dräktigheten och perioden då de diade sina ungar. Det visade sig att ungarna vid två veckors ålder hade högre kroppsvikt och mer fett i kroppen samt högre inflammationsnivå om deras mamma fick energirik föda jämfört med ungar vars mamma fick en mer balanserad kost. Om råttmammorna förutom energirik mat dessutom fick *E. coli*-bakterier i dricksvattnet så gick ungarna upp ännu mer i vikt och mängden kroppsfett ökade ytterligare samtidigt som inflammationsnivån och tarmens genomsläpplighet också ökade. Det är alltså inte hälsosamt att utsättas för en alltför energirik kost och inflammationsskapande bakterier som *E. coli* under fostertiden och tidigt i livet.

I ett annat experiment följde vi råttungarna tills de blev vuxna. Precis som i den tidigare studien så fick deras mammor en kost med högt energiinnehåll under dräktigheten och diperioden. En grupp råttor fick dessutom *E. coli* i dricksvattnet medan en annan grupp fick en probiotisk stam av *Lactobacillus plantarum* i sitt dricksvatten. Samma behandling fortsatte för ungarna efter att de skiljts från sina mammor och den pågick tills de var sex månader. Råttor kan då anses vara medelålders. Resultatet blev att de djur som fick *L. plantarum* gick upp mindre i vikt och hade mindre fettmängd samtidigt som tarmfloran var annorlunda. Till exempel hade djuren som fått *L. plantarum* en större mångfald av bakterier i sin tarmflora.

Sammanfattningsvis visar resultaten som presenteras i denna avhandling att den tidiga tarmfloran har betydelse för såväl allergiutveckling som fetma. En ogynnsam tarmflora kan till exempel vara en orsak till uppkomst av fetma. Resultaten visar också att det är möjligt att påverka hälsotillståndet beroende på vilken typ av bakterier man konsumerar. Intag av den använda probiotiska stammen av *L. plantarum* kan vara ett sätt att uppnå en bättre hälsa.

List of papers

This thesis is based on the following papers, which will be referred to by their Roman numerals in the text.

I   Wang M., **Karlsson C.**, Olsson C., Adlerberth I., Wold A., Strachan D. P., Matricardi P. M., Åberg N., Perkin M. R., Tripodi S., Coates A., Hesselmar B., Saalman R., Molin G., Ahrné S.
Reduced diversity in the early fecal microbiota of infants with atopic eczema
Journal of Allergy and Clinical Immunology 2008;121:129-134

II   Fåk F., **Karlsson C. L. J.**, Ahrné S., Molin G., Weström B.
Effects of a high fat diet during pregnancy and lactation is modulated by
E. coli in rat offspring
Submitted

III   **Karlsson C. L. J.**, Molin G., Fåk F., Johansson Hagslätt M.-L., Jakesevic M., Håkansson Å., Jeppsson B., Weström B., Ahrné S.
Effects on weight gain and gut microbiota in rats given bacterial supplement and a high-energy-dense diet from foetal life through to 6 months of age
British Journal of Nutrition, 2011; doi:10.1017/S0007114511001036

IV   **Karlsson C. L. J.**, Molin G., Cilio C. M., Ahrné S.
The pioneer gut microbiota in human neonates vaginally born at term – a pilot study
Pediatric Research (*in press*)

Published peer-reviewed paper not included in this thesis

Karlsson C., Ahrné S., Molin G., Berggren A., Palmquist I., Nordin Fredrikson G., Jeppsson B.
Probiotic therapy to men with incipient arteriosclerosis initiates increased bacterial diversity in colon: A randomized controlled trial
Atherosclerosis 2010;208:228-233

The author's contribution to the papers

Paper **I**   The author, C Karlsson, performed the analysis of the intestinal microbiota in collaboration with M Wang. CK took part in the evaluation of the results and in preparation of the manuscript.

Paper **II**   The author, C Karlsson, designed the study in collaboration with the co-authors. CK performed the animal experiment in collaboration with F Fåk, was responsible for microbiological analysis, and participated in evaluating the data and manuscript preparation.

Paper **III** The author, C Karlsson, designed the study together with the co-authors. CK coordinated the study, was responsible for the animal care and performed the experimental work, except for histological evaluation. CK evaluated the results and was responsible for writing the manuscript.

Paper **IV**   The author, C Karlsson, designed the study together with the co-authors. CK coordinated the study, including sample collection, and performed all the analytical experiments. CK evaluated the results and was responsible of writing the manuscript.

Abbreviations

A	Adenine
AGA	Appropriate for gestational age
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
BMI	Body mass index
bp	base pair
BSA	Bovine serum albumin
BV	Bacterial vaginosis
C	Cytosine
CCUG	Culture Collection, University of Göteborg
CFU	Colony-forming unit
c-section	caesarean section
DGGE	Denaturating gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dsDNA	double-stranded DNA
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E%	Energy percent
FISH	Fluorescence in situ hybridisation
G	Guanine
GF	Germ-free
GI	Gastrointestinal
HEDD	High-energy dense diet
HF	High-fat
I	Inosin
IFN	Interferon
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
LF	Low-fat
LGA	Large for gestational age
LPS	Lipopolysaccharide
MRP	Migration inhibitory factor-related protein
NAFLD	Non-alcoholic fatty liver disease

NF-κB	Nuclear factor kappa B
OPLS-DA	Orthogonal partial least-squares to latent structures discriminant analysis
PCR	Polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RDP	Ribosomal database project
rRNA	ribosomal ribonucleic acid
SCFA	Short-chain fatty acids
SI	Small intestine
T	Thymine
T2D	Type 2 diabetes
TE	Trizma® base ethylenediaminetetraacetic acid
TGGE	Temperature gradient gel electrophoresis
TJ	Tight junction
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
TTGE	Temporal temperature gradient gel electrophoresis
VRBD	Violet red bile dextrose
WHO	World Health Organization

Introduction

Although invisible to the human eye, microorganisms encompass all kinds of ecological niches. Microbes that collectively make up an ecosystem are termed microbiota or microflora. The mammalian gastrointestinal (GI) tract harbours a fascinating microbial ecosystem that is essential for host maintenance. For example, the gut microbiota have immune-regulatory functions, metabolic functions including fermentation of otherwise indigestible carbohydrates and synthesis of bioactive compounds and protective functions including competitive actions and maintaining gut barrier integrity. However, perturbation in the microbial ecology may affect host resilience, gut permeability and alter the susceptibility of metabolic disorders (Cani *et al.*, 2008, Miele *et al.*, 2009). In human beings the prokaryotic cells outnumber the eukaryotic cells by an order of magnitude (Bäckhed *et al.*, 2005) and given the fact that the highest bacterial density associated with the mammalian body is found in the gut, the gut microbiota deserves attention and exploration for further understanding of its complex relationship with the host.

It can be proposed that we are born as humans but die as a microbial horde. That statement is based on the general knowledge of a sterile foetal environment but during the birth process and thereafter microbial colonisation proceeds. The microbial composition early in life is influenced by numerous factors, for instance delivery mode, hygiene circumstances, maternal microbiota, and infant nutritional intake, all of which impact the shaping of the microbial ecosystem (revie wed in Mackie *et al.*, 1999). The gut microbiota is often dominated by bacteria belonging to the *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria* and *Verrucomicrobia* phyla (Eckburg *et al.*, 2005, Tap *et al.*, 2009). However, at the lower phylogenetic levels the microbial composition is highly individual (Tap *et al.*, 2009, Walker *et al.*, 2011) and additionally, the infant gut microbiota is different to that of adults. Once established after the weaning period, the gut microbial composition was previously considered stable throughout life. However, technological advances clearly show variation in the gut microbiota depending on extrinsic factors. Development of the microbial ecosystem is for example influenced by heredity, lifestyle factors and health homeostasis (Koenig *et al.*, 2010, Roger and McCartney, 2010). There is convincing data showing connections between gut microbial composition, low-grade inflammation, obesity and related disorders. Furthermore the microbiota early in life seems to be important for later health outcomes (Penders *et al.*, 2007, Kalliomäki *et al.*, 2008).

The work presented in this doctoral thesis focuses on the structure and diversity of the gut microbial community, and how it influences the host. The human studies reported in this thesis are of observational character. In the animal studies the system was defied with an energy-rich diet and supplementation of either the non-pathogenic but Gram-negative (with pro-inflammatory lipopolysaccharides (LPS) in its cell wall) *Escherichia coli* Culture Collection University of Göteborg (CCUG) 29300[T], or by the Gram-positive *Lactobacillus plantarum* Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) 15313. In the papers that make up this thesis, particular focus was placed on the microbiota early in life and its implication in development of atopic eczema,

body weight gain and adiposity in combination with biochemical markers of metabolic interest. Molecular methods relying on analysis of the 16S ribosomal ribonucleic acid (rRNA) gene sequence are suitable for bacterial analysis and were subsequently used to investigate the gut microbial population. In addition, culture-dependent methods were applied to some extent. An outline of the studies, major methods and investigated parameters that are included in this thesis is given in Table 1.

Table 1. Outline of the studies, major methods and investigated parameters that are included in this thesis.

Paper	Type of study	Samples for analyses	Methods used	Investigated parameters	Comments
I	Observational, human	Faeces	T-RFLP TTGE	Bacterial diversity	Faecal microbiota was studied in infants with and without atopic eczema.
II	Intervention, rat pups	Plasma Caecal content	Colorimetric assay Rocket immunoelectrophoresis Cultivation	Body weight Adiposity Organ weights Enzymology Haptoglobin Intestinal permeability *Enterobacteriaceae* count	Maternal HEDD feeding with or without addition of *E. coli* during pregnancy and lactation.
III	Intervention, rat	Caecal content Liver tissue Plasma Rectal content	T-RFLP Cultivation Cloning and sequencing Histology Colorimetric assay Enzyme-linked immunosorbent assay	Body weight Adiposity Organ weights Bacterial diversity *Enterobacteriaceae* counts *Lactobacillus* counts Phylogenetic affiliation Clone libraries differences Steatosis Liver enzymes Endotoxin Haptoglobin Leptin Calprotectin	Maternal HEDD feeding with addition of *E. coli* or *L. plantarum* during pregnancy and lactation. Offspring continued with the same treatment until six months of age.
IV	Observational, human	Faeces/meconium Rectal content	qPCR Cloning and sequencing	LGA/AGA *Lactobacillus* *Enterococcus* *Bifidobacterium* *Enterobacteriaceae* *Bacteroides fragilis* group Phylogenetic affiliation Clone libraries differences	The pioneer microbiota of healthy vaginally-born neonates were investigated, including subgroups of those born LGA and AGA.

For explanation of abbreviations, see list of abbreviations.

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Aim

The general aim of this thesis was to clarify the gut microbial composition and diversity in young individuals and relate it to physiological traits. In order to explore this, the following questions were addressed:

- What bacterial groups can be found in the pioneer gut microbiota of human neonates vaginally born at term without complications?

- Are there any differences in the gut microbiota of neonates born large for gestational age compared to neonates with birth weight appropriate for gestational age?

- Is there any difference in the gut microbiota of one-week-old infants who later developed atopic eczema compared to those that did not?

- What effects does the maternal diet have on offspring physiology in terms of systemic inflammation and body fat accumulation?

- Can gut microbiota and adiposity be affected by supplementation of either the Gram-negative *E. coli* CCUG29300$^{\mathrm{T}}$ or the Gram-positive *L. plantarum* DSM15313 in combination with a high-energy dense diet to rats from foetal life to adulthood?

The gut microbiota early in life

The environment *in utero* and the foetal GI tract is considered sterile but at birth the neonate is exposed to various bacteria from for example the birth canal, the hospital environment and from handling by the parents and nursing staff (Bettelheim *et al.*, 1974, Brook *et al.*, 1979, Mackie *et al.*, 1999, Lindberg *et al.*, 2004, Adlerberth and Wold, 2009). The pioneer microbiota is considered to be of significant importance for instance for the development of the neonatal immune system (Levy, 2007). Already in the first day of life, the number of 16S rRNA gene copies per gram faeces has been reported to reach 10^6 (range 10^4-10^{10}) (Palmer *et al.*, 2007). Historically, microbiological investigations have been performed by cultivation, but in recent years the majority of studies rely on molecular methods. A few years ago, Palmer *et al.* (2007) reported the succession of the intestinal microbiota in 14 infants up to 17 months of age. Future studies should further address these kinds of detailed succession studies in order to extend knowledge about fluctuations in the infant intestinal microbiota and preferably relate it to health and disease. The microbiota changes in response to lifestyle changes, such as dietary changes, fever and antibiotic treatment (Koenig *et al.*, 2010, Roger and McCartney, 2010), which may be a reason for the inconclusive results that have been presented on the infant gut microbial composition. In this chapter the gut microbial composition early in life and factors important for shaping the gut ecosystem will be discussed.

The bacterial flora

The composition of the initial bacterial colonisation is known to be highly individual and to cluster apart from the microbiota later in infancy (Wang *et al.*, 2004, Palmer *et al.*, 2007, Roger and McCartney, 2010). The traditional view of the gut microbial ecosystem reveals a less complex infantile gut microbiota and higher proportion of facultative anaerobic bacteria compared to the adult microbiota (Adlerberth and Wold, 2009).

In human infants

In a culture-based study from rural Guatemala in the 1970s, *Streptococcus* and *E. coli* were detected in many of the newborns within the first hours after birth (Mata *et al.*, 1971). During the first few days of life several studies have shown that only limited types of bacteria are present (Favier *et al.*, 2002, Songjinda *et al.*, 2005, Palmer *et al.*, 2007).

Lactobacillus is known to dominate a healthy human vagina (Yamamoto *et al.*, 2009) so this bacterial group is assumed to be found in vaginally delivered newborns. In paper **IV** *Lactobacillus* was found in every neonate. However, other studies using both culture-dependent and culture-independent methods have only found this bacterial group to be present in up to 20% of the newborns (Grönlund *et al.*, 2000, Mitsou *et al.*, 2008, Tanaka *et al.*, 2009). Different methodology and other confounding factors may be a reason for the observed discrepancy. *Enterococcus* has been found among one-third of

vaginally born neonates in their first days of life (Tanaka *et al.*, 2009). *Streptococcus* has been detected in about one-third and *Staphylococcus* in 50-100% of the neonates at this early age (Songjinda *et al.*, 2005, Adlerberth *et al.*, 2007, Tanaka *et al.*, 2009).

In 30-100% of neonates, the facultative anaerobic *Enterobacteriaceae* was detected during the first few days of life (Mata *et al.*, 1971, Favier *et al.*, 2003, Adlerberth *et al.*, 2007, Palmer *et al.*, 2007, Tanaka *et al.*, 2009, paper **IV**). The wide range may be a consequence of the studies being performed in geographically and hygienically different locations and at different points in time. Adlerberth *et al.* (2007) discussed the possibility that improved hygienic circumstances may have reduced the circulation of faecal bacteria. The anaerobic *Bacteroides* has been found in 6-50% of the neonates during the first days of life and in those harbouring this bacterial group, the concentration was fairly high (Mata *et al.*, 1971, Adlerberth *et al.*, 2007, Palmer *et al.*, 2007, Tanaka *et al.*, 2009, paper **IV**).

Various studies have found *Bifidobacterium* prevalence among 10-30% of neonates their first days of life (Mata *et al.*, 1971, Mitsou *et al.*, 2008, Tanaka *et al.*, 2009, paper **IV**), suggesting that bifidobacteria colonises slightly later in most neonates (Stark and Lee, 1982, Adlerberth *et al.*, 2007).

At one week of age, *Streptococcus* became less prevalent (Park *et al.*, 2005, Solís *et al.*, 2010) whereas *Staphylococcus* has been demonstrated to colonise all infants in a culture-based study with approximately 300 infants from Sweden, UK and Italy (Adlerberth *et al.*, 2007). *Lactobacillus* has been found in 10-24% of the infants (Grönlund *et al.*, 2000, Adlerberth *et al.*, 2007) and *Enterococcus* colonise more than 80% of the infants at one week of age (Stark and Lee, 1982, Adlerberth *et al.*, 2007, Adlerberth and Wold, 2009). Furthermore, *Proteobacteria* seemed abundant in considerably many infants (Stark and Lee, 1982, Wang *et al.*, 2004, Palmer *et al.*, 2007, Adlerberth and Wold, 2009). However, other studies have found this phylum at lower prevalence at the age of one week (Park *et al.*, 2005, Adlerberth *et al.*, 2007, Koenig *et al.*, 2010). The anaerobic *Bacteroides* has been detected in every fourth to second infant and *Bifidobacterium* was found in about 70-80% (Stark and Lee, 1982, Grönlund *et al.*, 2000, Adlerberth *et al.*, 2007). Clostridia is an anaerobic group that has been found in up to half of the neonates during the first week of life (Adlerberth *et al.*, 2007). However, this frequency has not been confirmed by others (Park *et al.*, 2005, Palmer *et al.*, 2007).

At the age of six months, about 10^{10} 16S rRNA genes/g faeces have been observed, with higher viable abundance of anaerobic bacteria compared to aerobic ones and additionally the microbiota seem to be more diverse (Mata *et al.*, 1971, Palmer *et al.*, 2007). During the first year of life the anaerobic bacteria outnumber the aerobic bacteria by a factor of 100-1,000 (Mata *et al.*, 1971). Clostridia have been reported in most children at six months of age (Adlerberth *et al.*, 2007) and *Bacteroides* seems to be more prevalent at this age compared to the age of one week (Grönlund *et al.*, 2000, Adlerberth *et al.*, 2007). *Enterococcus* has been detected in all infants and *Lactobacillus* has been reported in about half of the infants (Grönlund *et al.*, 2000, Adlerberth *et al.*, 2007). According to the study by Palmer *et al.* (2007), all infants harboured considerable amounts of γ-*Proteobacteria* but otherwise no clear colonisation pattern could be distinguished. Furthermore, Adlerberth *et al.* (2007) reported γ-*Proteobacteria* to be present in all infants. However, the microbial succession in the child reported by Koenig *et al.* (2010) showed less *Proteobacteria*. Several studies have reported *Bifidobacterium* to be present in all infants, usually at a level of about 10^{10} colony-forming units (CFU)/g faeces (Mata *et al.*, 1971, Stark and Lee, 1982,

Grönlund *et al.*, 2000, Adlerberth *et al.*, 2007), but conflicting results have been reported and the succession differs between infants (Palmer *et al.*, 2007, Koenig *et al.*, 2010). Table 2 indicates the viable count of common bacterial groups in the infant gut microbiota. Contradictory to abundant bacterial colonisation, the abundance of *Archaea* and fungi are reported to be low throughout the first year of life (Palmer *et al.*, 2007).

The nutritional habits change dramatically at the time of weaning, generating perturbation of the intestinal microbiota and diarrhoeas are not uncommon (Magne *et al.*, 2006). When solid food is introduced, the inter-variability between subjects seems to be reduced (Magne *et al.*, 2006, Roger and McCartney, 2010). Furthermore, several studies reveal increased diversity and the microbiota seems to become more adult-like, for example by increased levels of clostridia at weaning (Wang *et al.*, 2004, Magne *et al.*, 2006, Roger and McCartney, 2010).

Table 2. Viable counts of bacterial groups found in the infant gut microbiota[1].

	Age			
	1 week	2 months	6 months	12 months
Bacterial group	$\log_{10}$ CFU/g faeces	$\log_{10}$ CFU/g faeces	$\log_{10}$ CFU/g faeces	$\log_{10}$ CFU/g faeces
Lactobacillus	6	8	8	6
Enterococcus	8	8	8	7
Staphylococcus	7-8	6	5	4-5
Bifidobacterium	9-10	10	10	9-10
Clostridium	6	6	6	6
Enterobacteriaceae	8-9	8-9	8-9	7-8
Bacteroides	9	9-10	9	9

[1]Data taken from Adlerberth *et al.* (2007) in which faecal samples from 300 European infants were cultivated using standard procedure. The mean $\log_{10}$ count for colonised infants is presented. According to Adlerberth *et al.* (2007) colonisation range was 10-100% depending on bacterial group.

In childhood

During childhood the *Bifidobacterium* prevalence remains controversial since some investigators report detection in every child while others could hardly detect any bifidobacteria at all (Collado *et al.*, 2009b, Schwiertz *et al.*, 2010a). Also the concentration has been reported to differ between $\log_{10}$ 7.5-10 CFU/g faeces (Salminen *et al.*, 2004, Collado *et al.*, 2009b, Schwiertz *et al.*, 2010a) or to be even higher (unpublished results). *Lactobacillus* has been found in similar prevalence among infants and older children and the concentration is reported to be similar (Collado *et al.*, 2009b, Schwiertz *et al.*, 2010a, paper **IV**) whereas the prevalence and concentration of *Staphylococcus* are known to be lower in older children compared to the early microbiota (Adlerberth *et al.*, 2007, Collado *et al.*, 2009b).

The Gram-negative *E. coli* has been detected in half of the children (Collado *et al.*, 2009b) and a concentration of around $\log_{10}$ 6 CFU/g faeces has been reported. However, among children with diseases the prevalence and concentration of *E. coli* and other *Enterobacteriaceae* species have been shown to be increased (Collado *et al.*, 2009b, Schwiertz *et al.*, 2010a, unpublished results).

Clostridia has been detected in all children at a level of $\log_{10}$ 9-10 CFU/g faeces (Salminen *et al.*, 2004, Collado *et al.*, 2009b, Schwiertz *et al.*, 2010a) and makes up two-thirds of the microbiota (Schwiertz *et al.*, 2010a). The prevalence and concentration was higher compared to the first six months of life (Hopkins *et al.*, 2005, Adlerberth *et al.*, 2007). *Bacteroides* makes up one-quarter of the intestinal ecosystem in healthy children and the concentration seems to be in accordance with clostridia (Salminen *et al.*, 2004, Collado *et al.*, 2009b, Schwiertz *et al.*, 2010a), although higher concentration has been observed (unpublished results).

In rat pups

The microbial succession in rat pups is less explored compared to the ecosystem in human infants. However, in a study by Wenzl *et al.* (2001) Gram-positive cocci, for example *Staphylococcus* spp.*,* enterococci and micrococci, are considered the first colonisers of the neonatal rat small intestine (SI) and the Gram-positive cocci persisted in the same concentration at least until the study ended at postnatal day 42. Beyond the first days, Gram-negative bacteria were found in similar prevalence and concentration whereas *Lactobacillus* was present in higher numbers than Gram-positive cocci in almost all animals. At ten days of age, the aerobic microbiota was more dominant in rat pups and significantly more *Enterobacteriaceae, Enterococcus* and *Streptococcus* were found in the pups' faecal microbiota compared to the microbiota of rat dams. The latter had higher numbers of anaerobes, *Lactobacillus* and *Bacteroidaceae*. At 14 days postpartum, the pups' faecal microbiota was dominated by *Enterobacteriaceae, Lactobacillus* and *Enterococcus* (Yajima *et al.*, 2001).

Similar to the human situation, the microbiota of neonatal rats were less complex compared to the bacterial composition of their mothers (Yajima *et al.*, 2001). Assessed by Temperature Gradient Gel Electrophoresis (TGGE), the diversity was low before weaning but increased with age and in accordance with the human situation the microbiota is considered individual also in rats (Inoue and Ushida, 2003).

Factors influencing the microbial ecosystem of the gut

The effect of maternal microbiota and diet

Maternal vaginal microbes are transferred to infants during vaginal delivery (Brook *et al.*, 1979, Dominguez-Bello *et al.*, 2010). Thus, the vaginal microbiota is considered crucial since it is the initial inoculum to the previously sterile neonatal GI tract. The vagina of healthy women is mostly colonised by *Lactobacillus* species. These produce hydrogen peroxide that acts as an antimicrobial substance and lactic acid to create an acidic environment, which in combination with antimicrobial peptides such as calprotectin inhibits invading pathogenic bacteria (Valore *et al.*, 2002). The vaginal lactobacilli microbiota of pregnant women is not clarified but a healthy vagina is mainly dominated by *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus gasseri* and *Lactobacillus jensenii* (Vásquez *et al.*, 2002, Martin *et al.*, 2007a, Thies *et al.*, 2007, Yamamoto *et al.*, 2009).

Interestingly, *L. iners* dominated the cloned faecal sample from one of the neonates in paper **IV**. When Multiplex polymerase chain reaction (PCR) was performed according

to Song *et al.* (2000, with minor technical modifications), *L. crispatus* and *L. gasseri* were identified among neonates in paper **IV**. Because of the transfer of vaginal microbes to the neonatal child, it appears that lactobacilli should be the first microbes the newborn should meet. In paper **IV** lactobacilli was observed in all vaginally born neonates. The finding of *Lactobacillus* presence in every neonate at this early age is, to the best of our knowledge, novel. In some neonates, species of vaginal origin were detected with quantitative polymerase chain reaction (qPCR) (paper **IV**). Methodological issues concerning the qPCR assays may be a reason why vaginal lactobacilli could not be detected at greater frequency.

Temporal variability of the human vaginal bacteria is observed upon hormonal variability during the menstrual cycle (Srinivasan *et al.*, 2010). In the case of bacterial vaginosis (BV), excessive ecological alternation is detected, *Lactobacillus* becomes less prevalent, and instead a mixed flora dominated by for example *Gardnerella, Prevotella, Atopobium* and *Megasphaera* is present (Fredricks *et al.*, 2007, Thies *et al.*, 2007). One-third of American women have BV but most of them are asymptomatic (Allsworth and Peipert, 2007). BV occurrence varies with lifestyle factors and is also connected with inflammatory disorders (Haggerty *et al.*, 2004, Ness *et al.*, 2005, Allsworth and Peipert, 2007, Neggers *et al.*, 2007). Notably, increased dietary fat intake has been associated with enhanced risk of BV in apparently healthy women of reproductive age (Neggers *et al.*, 2007). Presence of BV in mothers to neonates in paper **I** and **IV** could not be determined, but BV may be a reason for discrepancy in the pioneer microbiota.

Hardly surprisingly, the diet affects body weight and other physiological parameters. Furthermore, maternal diet affects offspring development and physiology, for instance maternal vitamin and mineral intake during pregnancy is positively associated with reduced risk of asthma in children (Devereux *et al.*, 2006). Another example of strikingly epigenetic outcomes was found in individuals who were prenatally exposed to famine during the Dutch Hunger Winter at the end of World War II in the winter of 1944-45. It was reported that maternal malnutrition early in gestation was associated with increased body mass index (BMI) of the offspring in adulthood (Ravelli *et al.*, 1999).

Maternal obesity and excessive weight gain during pregnancy increase the risk of giving birth to neonates with high birth weight (Collado *et al.*, 2008, Bodnar *et al.*, 2010, Santacruz *et al.*, 2010), which impacts the risk of metabolic disturbance such as type 1 diabetes and type 2 diabetes (T2D), increased BMI and fat mass, as well as attention deficit hyperactivity disorder later in life (McCarne *et al.*, 1994, Gunnarsdottir *et al.*, 2004, Boney *et al.*, 2005, Rodriguez *et al.*, 2008, Harder *et al.*, 2009, Crozier *et al.*, 2010). The gut microbial composition during pregnancy has been demonstrated to differ among overweight and normal-weight women. Higher birth weight of neonates to overweight women correlated with *Enterobacteriaceae* and *E. coli* loads in these women and it was further associated with excessive weight gain during pregnancy. On the other hand, *Bifidobacterium* loads were higher in normal-weight women and in those with normal weight gain (Collado *et al.*, 2008, Santacruz *et al.*, 2010). Interestingly, infants of obese mothers and those with excessive weight gain had higher concentrations of *Bacteroides* and *Staphylococcus*, but lower amounts of *Bifidobacterium* at six months of age (Collado *et al.*, 2010). Moreover, lower *Bifidobacterium* numbers but higher *Staphylococcus aureus* numbers were found in infants who became overweight at the age of seven (Kalliomäki *et al.*, 2008).

Mothers of infants in papers **I** and **IV** were not weighed in a standardised fashion and no vaginal or faecal sample could be obtained. Consequently, in these studies it was not feasible to assess the effect of maternal obesity and pregnancy weight gain on birth weight or microbiota of their baby.

The Nordic Nutrition Recommendations suggest that about 30 percent of the total energy intake (E%) in the human nutrition should come from fat, 50-60E% should come from carbohydrates and 15E% from protein (Becker *et al.*, 2004). Obviously these guidelines are not always followed and in the experimental studies we aimed to use a diet with macronutritional composition in accordance with previous energy-rich diets evoking metabolic disorders in experimental animals (Bayol *et al.*, 2005, Bäckhed *et al.*, 2007) in order to mimic the nutritional intake by people who are at elevated risk of metabolic disorders.

The animal studies presented in this thesis attempted to more extensively evaluate effects of the maternal diet on offspring physiology. Experimental data suggests that intrauterine nutritional disturbance may evoke metabolic disorders. Historically, focus has been on protein deprivation. Interestingly, obesity in rodents can be programmed by prenatal exposure to diets either with low or high protein content (Langley-Evans *et al.*, 2005, Mortensen *et al.*, 2010). However, the increasing incidence of obesity and related disorders has shed light upon the effect of excessive nutritional abundance *in utero* on offspring development.

Numerous animal studies have reported various effects of maternal diet on offspring development. For instance, rat dams fed cafeteria diet (*ad libitum* choice of palatable processed foods with high fat and/or sugar content) gave birth to pups with markedly enhanced adiposity, characterised by increased fat pad weight and intramuscular lipid deposition but decreased functionality at weaning (Bayol *et al.*, 2005). Long-term consumption of a high-fat (HF) diet by rats induced foetal mal-programming predisposing to metabolic disorders regardless of whether offspring were weaned onto a low-calorie diet or a high-sucrose diet, although the latter induced more pronounced effects (Srinivasan *et al.*, 2006). When dams were fed a HF diet four weeks before mating and throughout gestation, pups had increased total body fat, abdominal fat and liver triglyceride content at three months of age, despite offspring being weaned to regular chow diet (Buckley *et al.*, 2005). Zambrano *et al.* (2010) suggest an ability to partly reverse metabolic programming, assessed by fat deposition, serum leptin, insulin and triglycerides, in offspring of obese dams if these were placed on a regular chow diet one month prior to breeding. The effects were more enhanced at weaning compared to four and five months of age, a phenomenon that partly resembles the situation in papers **II** and **III**. A proposed explanation may be higher energy and fat content but lower protein content in milk from dams fed palatable food (Rolls *et al.*, 1986).

Rat dams in papers **II** and **III** were maintained on standard chow before gestation and during the first third of pregnancy. The same energy-rich HF diet (Table S1, paper **III**) was given to the dams in papers **II** and **III** during the last two-thirds of pregnancy and during the lactation period and weaned pups in paper **III** continued on the high-energy dense diet (HEDD). The diet was custom modified from the OpenSource diet D12451 produced by Research Diets Inc. (New Brunswick, NJ, USA) and similar to the Western diet used by Bäckhed *et al.* (2007). Other studies of diet-induced obesity have used dietary regimes with 60-72E% from fat (Srinivasan *et al.*, 2006, Cani *et al.*, 2007a, Cani *et al.*, 2007b, Cani *et al.*, 2008). The low-fat (LF) fed rats in paper **II** were

maintained on the standard chow (RM3, SDS, Essex, UK) throughout the gestation and lactation periods. Our experiments confirmed that the type of diet clearly affected offspring development through for example increased body, liver and fat pad weights and enhanced inflammatory tone but decreased GI function in offspring to dams fed HEDD (papers **II** and **III**).

Microbial effect of delivery mode in human infants

As already mentioned, infants born vaginally are in contact with their mother's vaginal and intestinal microbiota during the passage through the birth canal and consequently, microbes are transferred from mother to child (Brook *et al.*, 1979, Dominguez-Bello *et al.*, 2010). For example, the same *E. coli* serotypes were found both in the baby immediately after birth and in the mother's faeces (Bettelheim *et al.*, 1974). Furthermore, the probiotic strain *Lactobacillus rhamnosus* GG (American Type Culture Collection (ATCC) 53103) given to pregnant women was found in their babies' faeces at one and six months of age, despite no probiotic administration to the infants (Schultz *et al.*, 2004). In contrast, infants born by caesarean section (c-section) acquire their initial microbes from the surrounding environment, for example from air, equipment and nursing staff since they are deprived contact with the vaginal microbiota (Morelli, 2008). Infants delivered by c-section have less diverse microbiota in terms of richness according to denaturing gradient gel electrophoresis (DGGE) and TGGE analysis (Biasucci *et al.*, 2008). Dominguez-Bello *et al.* (2010) found c-section infants to harbour bacterial communities resembling those found on skin surface, dominated by *Staphylococcus, Corynebacterium* and *Propionibacterium*, while vaginally delivered infants had more lactobacilli and *Prevotella* (Dominguez-Bello *et al.*, 2010). Additionally, c-section infants less often harboured *E. coli* on their third day of life and also during their first six months of life (Adlerberth *et al.*, 2006, Biasucci *et al.*, 2008). On the other hand, *Klebsiella, Enterobacter* spp. and *Clostridium* were more frequently early colonisers in c-section infants while *Bacteroides* were found less frequently (Adlerberth *et al.*, 2006, Adlerberth *et al.*, 2007). A recent study indicated *Proteobacteria* to be dominant in c-section infants. Although all infants had members of this phylum, the composition varied between subjects, with *Citrobacter, Klebsiella, Shigella,* and *Enterobacter* common in infants with eczema. *Bifidobacterium* was present at significantly higher abundance in non-eczema infants (Hong *et al.*, 2010). When the influence of delivery mode on gut microbiota composition was assessed in 60 seven-year-old children, the faecal concentration of clostridia was significantly lower in children born by c-section as determined by fluorescence in situ hybridisation (FISH), but other bacterial groups did not differ between the study groups. However, only one analysis method was used and information about diet, sickness history and other confounding factors were lacking (Salminen *et al.*, 2004).

All participants in paper **IV** were born vaginally. In contrast, the delivery mode was not a criterion for inclusion or exclusion of subjects in paper **I**, so a minority of the infants were delivered by c-section. However, the mode of delivery did not seem to affect the level of diversity in our study.

Effects of human infant feeding regimen

Breast milk is the ultimate feed that fulfils all nutritional requirements for the newborn and rapidly growing neonate. The milk contains oligosaccharides, immunoglobulins (Ig), lactoferrin and other glycoproteins and antimicrobial peptides and fatty acids, which protect from infectious diseases (Morrow and Rangel, 2004, Morrow *et al.*, 2005, Newburg, 2005). The most frequently encountered bacterial groups in human breast milk are staphylococci, streptococci, lactobacilli but for example propionibacteria, bifidobacteria, *Pseudomonas* and *E. coli* can also be found. The microbes probably originate from the nipple and the surrounding skin and from the milk ducts (West *et al.*, 1979, Martin *et al.*, 2007b, Collado *et al.*, 2009a). Women are believed to have their own specific concentration of constituents and bacterial pattern in their breast milk and that is suggested to affect the microbial colonisation of her neonate (Morrow *et al.*, 2005, Martin *et al.*, 2007a, Martin *et al.*, 2007b). Lactic acid bacteria has frequently been found in human breast milk, and this has been proposed as a source of microbes to the neonate since the same species have been found in the milk and its corresponding infant faeces (Martin *et al.*, 2007b, Collado *et al.*, 2009a, Solís *et al.*, 2010).

In contrast to breast milk, formula feed is not a natural source of bacteria. However, infant formulas have been improved and now resemble the composition of human milk. Addition of prebiotic fructo-oligosaccharides and galacto-oligosaccharides to infant formula has been described as stimulating the growth of intestinal *Bifidobacterium* and *Lactobacillus* species in a similar manner to breast milk. Also the species distribution resembles the one in breast-fed infants. In contrast, infants fed standard formula had significantly lower *Bifidobacterium* and *Lactobacillus* amounts (Haarman and Knol, 2006). A synbiotic formulation, *L. plantarum* ATCC20195 and fructo-oligosaccharides, of infant formula has been shown to increase the bacterial diversity and especially the number of Gram-positive species, while Gram-negatives were lower (Panigrahi *et al.*, 2008).

The extent to which the type of infant feeding affects the gut microbiota acquisition is controversial and not fully understood, probably because of other factors such as delivery mode, age at sampling, methodologies used and improved nutritional formulation. These influence the observed bacterial composition, making it a challenge to determine the effect of feeding regimen (Adlerberth and Wold, 2009). However, Swedish infants receiving breast milk at six months of age were more often colonised by lactobacilli, in particular *L. rhamnosus* and *L. gasseri*, compared to weaned infants (Ahrné *et al.*, 2005). Rinne *et al.* (2005) demonstrated both *Bifidobacterium* and *Lactobacillus/Enterococcus* counts to be higher in breast-fed compared to formula-fed infants at six months of age. Infants exclusively breast-fed for at least three months had higher *Bifidobacterium* counts. All infants in paper **I** were breast-fed and in paper **IV** the mothers were strictly advised to breastfeed, so it was not feasible to assess the effect of different feeding regimen upon microbial composition in the studies included in this thesis.

Physiological effects of microbial manipulation

Antibiotics can have long-lasting effects on the intestinal microbiota (Jernberg *et al.*, 2007, Jakobsson *et al.*, 2010) and antibiotic treatment to the pregnant mother or the infant associates with delayed establishment of anaerobic dominance in the gut

microbiota (Adlerberth *et al.*, 2007). In papers **I** and **IV**, there were no differences in maternal antibiotic treatment between the groups of infants with or without eczema or in the neonates born large for gestational age (LGA) compared to those appropriate for gestational age (AGA) respectively, implying the result was not affected by this parameter.

Infant birth weight was reported to be lower although not statistically significant when the probiotics *L. rhamnosus* GG and *Bifidobacterium lactis* Bb12 were given to pregnant women, who showed improved blood glucose concentrations and insulin sensitivity. However, neither extensive evaluations of metabolic parameters in the infants nor microbial investigations were performed (Laitinen *et al.*, 2009). In a study originating from the Finnish cohort described by Kalliomäki *et al.* (2001b), maternal overweight did not seem to increase the risk of children being overweight at the age of ten. But administration of the probiotic *L. rhamnosus* GG for four weeks before expected delivery and six months postnatal was correlated to lower birth weight and there was a trend for lower BMI at the age of four (Luoto *et al.*, 2010).

Broad-spectrum antibiotic treatment to rat dams for a few days before expected parturition reduced the faecal lactobacilli in the dams. Furthermore, it could be speculated whether the antibiotic treatment initiated a microbial disturbance that contributed to the fact that pups, at postpartum day 14, had higher caecal counts of *Enterobacteriaceae* compared to control pups. Moreover, intestinal permeability and inflammation, assessed by plasma bovine immunoglobulin G (BIgG) and haptoglobin, was enhanced in accordance with liver weight (Fåk *et al.*, 2008b). It was also shown that maternal supplement of *E. coli* resulted in higher *Enterobacteriaceae* counts but no difference in lactobacilli counts in pups at day 14 postpartum. Similar to the antibiotic treatment, plasma haptoglobin and liver weight were increased in pups whose mothers consumed *E. coli* pre-parturition. Furthermore, the development and function of the GI tract was affected (Fåk *et al.*, 2008b). The same strain of *E. coli* was used in combination with HEDD feeding in paper **II**. The elevated haptoglobin levels, intestinal permeability and liver weight were confirmed in the rat pups, in addition to increased fat deposition when HEDD feeding was associated with bacterial administration to the dams. However, exposure of *E. coli* until six months of age had slightly other effects, for instance lower haptoglobin and reduced diversity of the intestinal microbiota but still increased adiposity (paper **III**). In contrast, when dams were exposed to the probiotic *L. plantarum* DSM9843 from one week before expected day of parturition and during the lactation period, the bacterial supplement improved gut barrier function and stimulated GI growth and development without involvement of systemic inflammation (Fåk *et al.*, 2008c). Supplementation of *L. plantarum* DSM15313 from foetal life until six months of age revealed improved microbial diversity in combination with reduced body weight gain (paper **III**).

Bacterial composition along the gastrointestinal tract in adults

The majority of the intestinal bacteria are commensals (one partner benefits and the other is apparently unaffected), while others are potential pathogens. Some are resident colonisers while others appear just transient when following the body fluids through the system. Up to 13 different bacterial phyla have been found, but *Firmicutes* and *Bacteroidetes* are by far the numerically dominant (Dethlefsen *et al.*, 2007). Despite striking conservation at a higher phylogenetic level, the abundance of bacteria at species or strain level varies extensively between individuals (Eckburg *et al.*, 2005, Lay *et al.*, 2005, Tap *et al.*, 2009). Demographical reasons and geographical regions may affect the microbial composition and differences in local environment, heredity, diets or other lifestyle factors can be potential reasons for the discrepancy in intestinal ecology (Lay *et al.*, 2005, Mueller *et al.*, 2006, Biagi *et al.*, 2010, De Filippo *et al.*, 2010). Nevertheless, a core gut microbiome that is shared between different individuals ensures conservation of metabolic functions provided by the microbiota (Turnbaugh *et al.*, 2006, Tap *et al.*, 2009). This chapter gives an overview of the microbial composition at different gut locations in adult humans. Examples of bacterial taxa found in the intestinal tract are given in Table 3.

Table 3. Example of bacteria found in the adult human intestinal microbiota[1].

Phylum	Class	Family	Genus
Gram-positive taxa			
Actinobacteria	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*
Firmicutes	*Bacilli*	*Streptococcaceae*	*Streptococcus*
Firmicutes	*Bacilli*	*Lactobacillaceae*	*Lactobacillus*
Firmicutes	*Bacilli*	*Enterococcaceae*	*Enterococcus*
Firmicutes	*Negativicutes*	*Veillonellaceae*	*Veillonella*
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Roseburia*
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Ruminococcus*
Firmicutes	*Clostridia*	*Ruminococcaceae*	*Faecalibacterium*
Firmicutes	*Clostridia*	*Clostridiaceae*	*Clostridium*
Firmicutes	*Clostridia*	*Eubacteriaceae*	*Eubacterium*
Gram-negative taxa			
Bacteroidetes	*Bacteroidia*	*Bacteroidaceae*	*Bacteroides*
Bacteroidetes	*Bacteroidia*	*Porphyromonadaceae*	*Parabacteroides*
Bacteroidetes	*Bacteroidia*	*Prevotellaceae*	*Prevotella*
Proteobacteria	*δ-Proteobacteria*	*Desulfovibrionaceae*	*Desulfovibrio*
Proteobacteria	*γ-Proteobacteria*	*Enterobacteriaceae*	*Escherichia*
Proteobacteria	*γ-Proteobacteria*	*Enterobacteriaceae*	*Klebsiella*
Proteobacteria	*γ-Proteobacteria*	*Enterobacteriaceae*	*Enterobacter*
Fusobacteria	*Fusobacteria*	*Fusobacteriaceae*	*Fusobacterium*
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiaceae*	*Akkermansia*

[1]Data taken from Pettersson *et al.* (2003), Hayashi *et al.* (2005), Wang *et al.* (2005) and Tap *et al.* (2009). Taxonomic identification relied on identification of the 16S rRNA gene by cloning and sequencing.

The microbiota of the upper digestive tract

The mouth comprises many different types of surfaces. Recently, the microbiota of several oral niches were analysed with the 454 pyrosequencing technique. The individual human oral cavity was showed to harbour over 500 species and about 100 higher taxa (genus level or above) (Zaura *et al.*, 2009), which is considerably more than figures previously reported using conventional cloning and sequencing (Aas *et al.*, 2005). The number of taxa and diversity varied according to sampling location. *Firmicutes* (*Streptococcus*, *Veillonellaceae*, *Granulicatella*), *Proteobacteria* (*Neisseria*, *Haemophilus*), *Actinobacteria* (*Corynebacterium*, *Rothia*, *Actinomyces*), *Bacteroidetes* (*Prevotella*, *Capnocytophaga*, *Porphymonas*) and *Fusobacteria* (*Fusobacterium*) seem to dominate the oral microbiota in both adults and children (Aas *et al.*, 2005, Zaura *et al.*, 2009, Ling *et al.*, 2010).

Previously, the oesophagus and the stomach were thought to be sterile due to short retention time and acidic environment, and the latter is considered an effective barrier killing microbes encountering the GI tract. However, Pei *et al.* (2004) identified phylotypes belonging to six different phyla, mainly *Firmicutes* and *Bacteroidetes*, in oesophagus and the microbial composition was similar to the oral microbiota. In 1984 Marshall and Warren discovered *Helicobacter pylori* colonisation of the gastric mucosa. *H. pylori* has long been blamed for the aetiology of gastritis, but recent data suggests the presence of *H. pylori* even when there was no sign of disease (Bik *et al.*, 2006). Even if the number of bacteria is substantially lower due to the acidic environment, a more diverse microbiota than previously hypothesised can be assumed since phylotypes of five major bacterial phyla were found in the gastric mucosa. At the high taxonomic level it resembles the microbiota of oral cavity and oesophagus (Bik *et al.*, 2006, Dicksved *et al.*, 2009) and in the absence of *H. pylori* the diversity was enhanced (Andersson *et al.*, 2008).

The small intestinal microbiota

The SI, comprising duodenum, jejunum and ileum, is the location of enzymatic digestion and absorption of nutrients and a major part of the immune system. In the proximal part of the SI, bile, pancreatic secretions, low pH and the high transit time, impedes bacterial density (Guarner and Malagelada, 2003) whereas a gradient to the distal part allows bacterial concentration and diversity to increase and the composition to be different (Hayashi *et al.*, 2005, Wang *et al.*, 2005). Because of technical difficulties associated with sampling, the microbiota of SI is poorly investigated in humans. However, Wang *et al.* (2005) revealed *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia* and *Actinobacteria* to be present in the mucosal SI microbiota of a healthy middle-aged woman. The jejunum microbiota, dominated by *Streptococcus*, was significantly different to the ecosystem observed from distal ileum to rectum since that was dominated by *Bacteroidetes* and *Clostridium* cluster XIVa and IV (Collins *et al.*, 1994, Wang *et al.*, 2005). Analysis of SI luminal content revealed lactobacilli, streptococci, enterococci and γ-*Proteobacteria* to be prevalent in the jejunum of three elderly humans. Similarly, facultative anaerobes were detected in the ileum (Hayashi *et al.*, 2005).

The large intestinal microbiota

In humans, the colon is the predominant site for fermentation and, due to slower transit rate and rich nutritional environment, the bacterial density is higher, about 10^{12} cells/g luminal content (Guarner and Malagelada, 2003). On the taxonomic level of phylum, the large intestinal ecosystem is dominated by *Firmicutes* (mainly *Clostridium* cluster XIV) and *Bacteroidetes* (Eckburg *et al.*, 2005, Wang *et al.*, 2005, Walker *et al.*, 2011). In healthy humans *Proteobacteria* seems to make a minor contribution to the colonic ecosystem (Eckburg *et al.*, 2005, Wang *et al.*, 2005). However, in diseases such as ulcerative colitis, *Proteobacteria* has been shown to dominate the colonic microbiota (Wang *et al.*, 2007a).

Despite physiological differences along the large intestine, bacteria are uniformly distributed, at least according to the higher phylogenetic orders. It has been suggested that the human intestinal microbiota is rather stable over time (Costello *et al.*, 2009). However, recent data indicates that the dominating gut microbiota became more diverse by consumption of the probiotic bacteria *L. plantarum* DSM9843 (Karlsson *et al.*, 2010), while antibiotic treatment reduces diversity and induces changes persisting for several years (Jernberg *et al.*, 2007, Jakobsson *et al.*, 2010).

The faecal microbiota

Most human studies exploring the intestinal microbiota have examined the faecal microbiota due to its easy access and collection. However, it has been postulated that the faecal microbiota represents a mixture of shed mucosal bacteria and a separate non-adherent luminal microbiota (Eckburg *et al.*, 2005). The luminal and the surface-adherent bacterial populations may be distinct and have different properties. Zoetendal *et al.* (2002) demonstrated that the colonic mucosa-associated microbial populations were significantly different in composition compared to the faecal population. This has also been shown in mice where increased diversity was seen in intestinal tissue of those administered *Lactabacillus casei* and *L. plantarum* while no compositional changes were found in the faecal microbiota (Fuentes *et al.*, 2008). However, since stool samples are most accessible, the majority of studies referred to in this thesis have explored the faecal microbiota. Although it is not exactly known, the faecal microbiota is believed to mirror the microbiota inside the alimentary canal since the same dominant phyla are present in faeces as well as in SI mucosa. More than 90% of sequences obtained from human faecal microbiota belonged to the *Firmicutes* and *Bacteroidetes* phyla (Ley *et al.*, 2008, Larsen *et al.*, 2010). However, it seems warranted that the microbial ecosystem needs to be identified and dealt with at the lower taxonomic levels when the composition and microbial constituent are considered in health and disease.

Tools for studying gut microbiota

Cultivation and molecular approaches

Cultivation has been the traditional way to analyse microbial habitats, including the GI tract. Serial dilutions of the sample are prepared and appropriate dilutions are plated on general or selective media, for instance brain heart infusion and violet red bile dextrose (VRBD), respectively. In this thesis, viable count was addressed in paper **III** as live bacteria translocating to liver tissue were cultivated on brain heart infusion agar, and caecal content of lactobacilli and *Enterobacteriaceae* were selectively grown on Rogosa and VRBD, respectively. Also, in paper **II**, cultivation on VRBD was performed to assess *Enterobacteriaceae* concentrations. Caecum is the primary location for microbial fermentation in rodents and the bacterial flora was therefore studied at this location.

Molecular approaches are widely used to characterise microbial communities and their constituents, and in this thesis molecular analysis is the methodological backbone. By these methods the studied ecosystem can be characterised and monitored without prior knowledge of its structure or composition. Analysis and microbial identification usually rely on the 16S rRNA gene, which is the gene coding for the small subunit in the prokaryotic ribosome. The protein-synthesising machinery is essential since it translates the cell's genetic information. Because no big changes in the ribosomal gene have occurred during evolution, the 16S rRNA gene (approximately 1,500 nucleotides), with both conserved and variable regions, is suitable for comparison between bacteria and is also the most accepted gene for bacterial classification and identification. The sequence variability of 16S rRNA genes is useful for analysis of community dynamics, both within the same subjects (Wang *et al.*, 2004, Jernberg *et al.*, 2007, Karlsson *et al.*, 2010) and between groups of individuals (Dicksved *et al.*, 2007, Wang *et al.*, 2009, papers **I** and **III**).

Polymerase Chain Reaction

The PCR technique was described by Mullis and co-workers in 1986 (Mullis *et al.*, 1986) and is now one of the most widely used techniques in molecular biology. In the reaction, target deoxyribonucleic acid (DNA) is amplified to sufficient numbers to enable further analysis. Primers are synthetic oligonucleotides complementary to a part of the target DNA. Universal regions of the 16S rRNA gene are suitable for primer binding in PCR amplification and DNA sequencing while variable regions make phylogenetic studies possible. Degenerative primers can be used to increase the possibility of the primers to bind target DNA, for example inosin (I) in the primer sequence ensure any of adenine (A), thymine (T), cytosine (C) or guanine (G) on the complementary sequence. Primer ENV-1 (5´-AGA GTT TGA TII TGG CTC AG-3´) and ENV-2 (5´-CGG ITA CCT TGT TAC GAC TT-3´) (Pettersson *et al.*, 2003), annealing with 8-27 base pair (bp) and 1511-1492 bp respectively in *E. coli* (Brosius *et al.*, 1978), have I incorporated in order to facilitate diverse binding to 16S rRNA genes (papers **I**, **III** and **IV**). In end-point PCR,

the amplification product is determined after the end of the last PCR cycle, while in qPCR the concentration is measured during the run.

Quantitative PCR

In microbial ecology, qPCR is widely applied and is commonly used to quantify bacteria at the family and genus levels. In contrast to conventional end-point PCR, qPCR enables quantification in real-time of the abundance of taxonomic genes within the studied samples. A reporter-quencher system (*Taq*Man technology) or fluorescence chemistry (for example SYBR Green I) is used to determine the kinetics of PCR product accumulation. The qPCR instrument detects amplicons during the exponential phase when reaction components are in abundance and the target gene can therefore be accurately quantified. The signal intensity is proportional to the concentration of target DNA in the sample, enabling the use of the product accumulation rate to calculate the concentration of target molecules in a sample. The quantities of a battery of bacterial groups were estimated in paper **IV**. SYBR Green chemistry was used and, when intercalated to double-stranded DNA (dsDNA), a fluorescent signal is emitted following light excitation. As amplicon numbers accumulate in each PCR cycle, a corresponding increase in fluorescence is obtained (Smith and Osborn, 2009). In paper **IV**, bacterial DNA was extracted from pure cultures of *Lactobacillus plantarum* DSM9843, *Lactobacillus iners* CCUG28746[T], *Enterococcus faecalis* CCUG19916[T], *Bifidobacterium infantis* DSM15159, *Escherichia coli* CCUG29300[T] and *Bacteroides fragilis* CCUG4856[T]. Amplicons of 16S rRNA from the corresponding PCR reactions (qPCR primers according to Table 1 in paper **IV**) were subjected to cloning into high competent *E. coli* cells. Clones were cultured overnight in Luria-Bertani broth and plasmid purification was made with QIAprep Spin MiniPrep kit (Qiagen, Hilden, Germany). Pure DNA was then ten-fold diluted in Trizma® base ethylenediaminetetraacetic acid (TE)-buffer supplemented with herring sperm DNA and used as standard curves in the PCR reactions.

After completion of the numbers of PCR cycles, a melting curve analysis should be performed to confirm specific target amplification. When dsDNA is heated, it denatures and a corresponding decline in fluorescence is obtained since SYBR Green dissociates from the DNA. Sharp curves at the same position suggest that only one specific DNA sequence was generated during the amplification (Smith and Osborn, 2009). Several qPCR instruments are available on the market. The principle of detection is the same but the Rotor-Gene Q (Qiagen), used in paper **IV**, differs slightly from others since a centrifugal force rotates samples in a 72-tube rotor instead of using a 96-well plate systems (Gibson, 2006). The reproducibility with the Rotor-Gene Q was observed to be very high in triplicate runs for both samples and standards.

Gradient electrophoresis

Temporal Temperature Gel Electrophoresis (TTGE), TGGE and DGGE are related methods that have been widely used to investigate complex microbial communities such as the GI microbiota (for example Zoetendal *et al.*, 1998, McCracken *et al.*, 2001, Favier *et al.*, 2003, Licht *et al.*, 2006, Magne *et al.*, 2006, paper **I**). These methods separate sequences according to GC content, which causes melting behaviour of the dsDNA to

differ. In DGGE a chemical gradient (urea and formamide) will cause the separating environment, while TGGE and TTGE use a temperature gradient (Huys *et al.*, 2008). The gradient causes conformational changes of the dsDNA, which decrease electrophoretic mobility of the partially dissociated dsDNA. On TGGE and TTGE gels, fragments with lower melting temperature will migrate shorter distances compared to fragments with higher melting temperature. To prevent complete denaturation of the DNA molecule, a GC-clamp is attached to the 5´-end to one of the primers. It is noteworthy that amplicons with different base pair sequence may migrate to the same position due to similar GC content (Macfarlane and Macfarlane, 2004). Importantly, these methods are not truly quantitative since band density does not necessarily relate to target abundance (Wu *et al.*, 2010). Bands of interest can be excised and sequenced to distinguish their identity (Favier *et al.*, 2003, Magne *et al.*, 2006). In this thesis TTGE was used in paper **I**. In a study by Biasucci *et al.* (2008), TGGE was reported to have greater discriminatory power than DGGE when the effect of delivery mode on the neonatal microbiota was evaluated.

Terminal Restriction Fragment Length Polymorphism

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is a powerful method for analysing the numerically dominant populations in the microbial community (Hayashi *et al.*, 2002, Hayashi *et al.*, 2005, Dicksved *et al.*, 2007, Jernberg *et al.*, 2007, Karlsson *et al.*, 2010). The method can be used for semi-quantification and for identification if accompanied by clone libraries. In this thesis T-RFLP was used in papers **I** and **III**.

For T-RFLP, the universal forward PCR primer is fluorescently labelled on the 5´-end. PCR products are purified and digested with restriction endonuclease, usually one with tetranucleotide recognition sequence (Kitts, 2001, Schütte *et al.*, 2008), which enables only terminal restriction fragment (T-RF) to be detected. Different species have restriction sites at different locations in their 16S rRNA genes, which results in different organisms having T-RFs of different sizes. Digested PCR products are mixed with a fluorescently labelled DNA size standard and the mixture can be separated either by polyacrylamide gel (paper **I**) or by capillary gel electrophoresis (paper **III**) to visualise the fluorescently labelled T-RFs (Figure 1). Capillary electrophoresis needs less material and is more reproducible because injection procedure is automatic and it has high separation efficiency and better resolution compared to gel electrophoresis (Behr *et al.*, 1999). Furthermore, it is more precise since an internal standard with higher specificity is used.
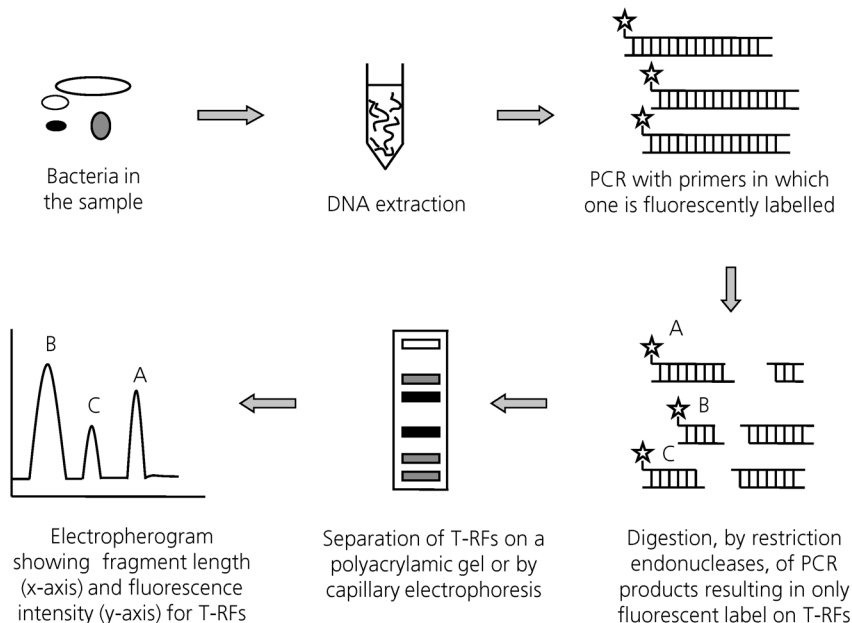
Figure 1. Schematic view of the T-RFLP technique.

The output T-RFLP raw data is visualised in an electropherogram. The lengths, in base pairs, and the amount (peak height and area) of the fluorescent T-RFs can be calculated with fragment analysis software where T-RF peak retention time is compared to a DNA size standard. Before calculation, raw data should be normalised and thresholds for standards and sample DNA should be applied in the analytical software used. T-RFLP estimates community richness and evenness but should not be considered as a quantitative method. Instead, relative abundance of individual T-RFs can be calculated as percentage of the total peak area in each specific profile. By this procedure, the percentage of specific T-RFs can be compared between profiles. Identification of unknown T-RFs can be made by comparison with T-RFs from clone library with sequenced clones.

Cloning and sequencing

Cloning combined with sequencing is another method for exploring microbial communities. The method cannot be used for quantification but it gives a relatively precise identification of the bacteria that dominates the population of the sample. The accuracy of the method is highly dependent on a sufficient amount of clones being picked from a sample and length of the nucleotide sequence to be identified. Amplicons of the 16S rRNA gene can be ligated into a sequencing vector and recombinant DNA molecules are then transformed into living cells such as *E. coli* high competent cells to yield clone libraries. These inserted fragments are sequenced and then aligned with sequences in public databases, for example GeneBank (http://www.ncbi.nlm.nih.gov/genbank/) and

Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) in order to ascertain their closest relatives. The longer the fragments, the more reliable the identification.

With the aim of identifying T-RFs in T-RFLP profiles, cloning and sequencing was applied in paper **III** for samples representing the most diverse T-RFLP profiles. In paper **IV** cloning and sequencing were applied for subgroups of neonates born LGA and AGA. These sequences are deposited to GeneBank, accession number HQ53018-HQ536167, and compared in RDP for closest phylogenetic affiliation. Sequences of two clone libraries can be compared in LIBSHUFF for overall community structure analysis in the mothur software package (Singleton *et al.*, 2001, Schloss *et al.*, 2009) and in the RDP feature LibCompare to reveal differences between the two microbial ecosystems.

454 pyrosequencing

In the relatively new 454 pyrosequencing technique, parallel sequencing of a large number of templates can be performed without need for cloning. In short, DNA fragments are bound to beads under conditions favouring binding of one DNA fragment per bead. The DNA fragments are amplified on the beads in separate reagent droplets (Margulies *et al.*, 2005). The amplicons can be sequenced using pyrosequencing where nucleotides are sequentially added to the reaction and the incorporation is detected by an enzymatic reaction in real-time, generating light emission (Ronaghi *et al.*, 1998). Even if enormously large amounts of amplicons can be sequenced, their lengths are so far relatively short (Andersson *et al.*, 2008, Zhang *et al.*, 2009) and biases introduced by PCR are not overcome, although cloning is avoided.

Fluorescence In Situ Hybridisation

Microscopy and fluorescently labelled oligonucleotides are used in an attempt to detect and quantify the number of bacteria, using the FISH method. During the laboratory procedure the probes penetrate the cell membrane and bind to the complementary 16S rRNA gene. Consequently, sequence information is required but no amplification is performed and so the method is not subjected to biases introduced by PCR. However, the counting of fluorescently units can be challenging and the method is laborious if quantification at lower taxonomic levels is desirable (Zoetendal *et al.*, 2004). Nonetheless, several studies have used this approach to quantify specific bacterial groups (Collado *et al.*, 2008, Duncan *et al.*, 2008, Nadal *et al.*, 2009, Collado *et al.*, 2010).

Microbial diversity

Studying and measuring diversity is a way to explain ecological patterns in various bacterial communities. High biological diversity normally provides higher resilience to ecological disturbances (Yachi and Loreau, 1999). In contrast, low bacterial diversity in the GI tract has been observed in patients with Crohn's disease, ulcerative colitis, obesity and in infants developing atopic eczema (Wang *et al.*, 2007a, Dicksved *et al.*, 2008, Turnbaugh *et al.*, 2009, papers **I** and **III**). Diversity can be described as the number of

different species or bacterial groups, defined as richness, in a given ecosystem. In paper **I**, atopic infants had reduced bacterial richness compared to their non-atopic counterparts as assessed by both the number of T-RFs and TTGE-bands.

Diversity can further be defined as species evenness, which reflects the distributional composition of species in an ecosystem by including their relative abundances (Magurran, 2004). The degree of diversity can be explored by a variety of indices. Microbial diversity in papers **I** and **III** were assessed by calculation of Shannon (H´ = - $\sum p_i \ln p_i$) and Simpson (D= $\sum p_i^2$) indices of diversity. These indices are commonly used and take into account both richness and evenness. Simpson index increases when diversity decreases and so this index is usually expressed as 1-D (Magurran, 2004). Calculation of diversity indices for subjects in both papers **I** and **III** suggests that higher diversity correlates with better health status. For instance, rats exposed to *E. coli* from foetal life until six months of age had reduced bacterial diversity but increased viable count of *Enterobacteriaceae*, suggesting *E. coli* consumption is associated with overgrowth of unfavourable bacterial groups. Although the cause and effect of diversity could not be determined, exposure of *E. coli* resulted in metabolic disturbances exemplified by enhanced adiposity (paper **III**), which corroborates the observation that obese humans have decreased microbial diversity (Turnbaugh *et al.*, 2009). Decreased intestinal diversity has been further associated with an accentuated inflammatory tone in centenarians compared to younger people (Biagi *et al.*, 2010). This further highlights the beneficial effect of high diversity.

It should be remembered that microbial diversity assessment based on T-RFLP and gradient electrophoresis techniques is not a measure of true diversity since only the most dominant constituents of the community are assessed due to limitations such as PCR bias. However, diversity indices can serve as an indicator of diversity and provide a relative diversity measure for comparisons between samples and for determining how the community changes according to different treatments (Karlsson *et al.*, 2010).

Statistical evaluation of data

Univariate statistical analysis is the traditional way to process data. Data not normally distributed were tested non-parametrically with the Mann-Whitney Rank Sum test to reveal differences between two study groups, for instance difference in diversity indices between infants with and without atopic eczema (paper **I**). For evaluation of three groups with non-parametric data distribution, the Kruskal-Wallis one-way analysis of variance by ranks followed by the Nemenyi-Damico-Wolfe-Dunn test (Hollander and Wolfe, 1999) was used (paper **III**). Parametric methods were applied if data was normally distributed (papers **II** and **IV**). One-way ANOVA with Dunnet's post hoc test was used in paper **II**.

Fisher's exact test was used to reveal difference in incidence among specific T-RFs and translocation of viable bacteria to the liver (paper **III**). Spearman Rank order correlation was used to identify correlations between parameters (paper **III**). For all univariate statistical evaluations, a *p*-value of ≤0.05 was considered statistically significant.

Nature is multivariate, i.e. one particular phenomenon most often depends on several factors. Multivariate data management is an attempt to simultaneously investigate the relationships between several variables and its main purpose is to reduce the complexity of the dataset to identify patterns that unify the samples. For example, animals that respond similarly to a treatment will group tightly together whereas samples with dissimilar

response will tear apart. For projection-based models, for example principal component analysis and partial least-squares to latent structures, the relationships between samples are preferably displayed in plots. For multivariate analysis of the data from paper **III**, orthogonal partial least-squares to latent structures discriminate analysis (OPLS-DA) was performed with unit variance scaling and no transformation. The OPLS-DA method was chosen since it simultaneously performs good prediction and interpretation of complex multivariate datasets (Trygg and Wold, 2002, Trygg, 2004, Wiklund *et al.*, 2008). Interpretations from multivariate analysis should be confirmed by other methods, such as univariate statistics, since different approaches sometimes give inconsistent results because of incorrect usage.

The LIBSHUFF software is designed to compare two libraries of 16S rRNA gene sequences and to determine whether they are significantly different, and libraries with relatively few sequences can also be analysed. Accordingly, the sequences from neonates born LGA and AGA were compared with LIBSHUFF using the mothur software package (Singleton *et al.*, 2001, Schloss *et al.*, 2009, paper **IV**). To reveal differences in the taxonomic context of microbial community comparison based on 16S rRNA gene sequence libraries, the LibCompare feature with Naïve Bayesian rRNA Classifier in RDP was used (papers **III** and **IV**). The latter software combines RDP Classifier, which proposes phylogenetic affiliation of the sequence, with a statistical test to identify taxa differentially represented between samples (Wang *et al.*, 2007b).

Methodological considerations concerning gut microbiota

As previously mentioned, most data regarding human gut microbiota has been generated using stool samples due to practical and ethical issues. However, collection and storage of faecal samples can be challenging and diverse conditions are known to generate different results (Monteiro *et al.*, 2001, Ott *et al.*, 2004). The bacterial diversity of the faecal microbiota and the total number of bacteria has been shown to be significantly reduced after 8 and 24 hours at both room temperature and 4°C (Ott *et al.*, 2004). Another study, assessing microbial content of meconium, suggested significantly increased bacterial counts in samples stored at 4-8°C for four days compared to immediate processing (Jiménez *et al.*, 2008). Interpretation and comparisons should therefore be made with caution if storage conditions differ between samples and studies. In all papers of this thesis, samples were immediately frozen and so the results are believed to reflect the actual microbial ecosystem in that subject.

Cultivation versus molecular approaches

The great advantage of cultivation is that isolates can be further studied, for instance in terms of their ability to utilise different substrates. *Enterobacteriaceae* and *Lactobacillus* are examples of organisms that are relatively suitable for cultivation (papers **II** and **III**). However, the majority of intestinal microbes are difficult or impossible to culture under laboratory conditions due to, for example, difficulties associated with sampling, their unknown growth conditions and requirement of a strict anaerobic environment throughout the procedure. The exact proportion of cultivable intestinal microbes is not known, although approximately 20% has been suggested (Eckburg *et al.*, 2005). However, in neonates the percentage may be higher according to the general concept that aerobes and facultative anaerobes are the initial colonisers (Adlerberth and Wold, 2009, Hong *et al.*, 2010). While only live bacteria can be cultivated, a more complete picture of the microbial intestinal ecosystem can be obtained by use of molecular-genetic methods. As already mentioned, the 16S rRNA gene is the predominant source for phylogenetic determination. However, as discussed below, several considerations should be taken into account when these molecular methods are used.

DNA extraction

Numerous protocols can be applied and various kits are available for extracting DNA from faecal and biopsy samples. Extraction conditions that are too harsh may shear the DNA in organisms that are easy to lyse, whereas insufficient lysing of, for example, Gram-positive bacteria with rigid cell walls will lead to misinterpretation of the community

analysed. Bead beating is considered to be most suitable for efficient lysis of Gram-positive bacteria (Zoetendal *et al.*, 2001). Furthermore, McOrist *et al.* (2002) compared five different DNA extraction methods, including the QIAamp DNA Stool Mini Kit which they found to be most effective. In addition to bead beating, the QIAamp DNA Stool Mini Kit has been shown to be successful in producing high quality DNA that could be further used in techniques investigating community structure (McOrist *et al.*, 2002, Li *et al.*, 2003a, Li *et al.*, 2007, Ariefdjohan *et al.*, 2010). Consequently, in papers **I**, **III** and **IV**, the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used in combination with bead beating to facilitate DNA extraction. The procedure is further described in paper **I**.

For standardisation and effectiveness, the last extraction step was performed in BioRobot EZ1 (tissue kit and card; Qiagen). This minimised the risk of contamination since the DNA was extracted in a system with automated handling. The Qiagen Tissue kit has now been developed so that it is suitable for DNA extraction from faecal samples. In a separate project, faecal samples were extracted with both the QIAamp DNA Stool Mini Kit (in combination with 30 min bead beating) and the Qiagen Tissue kit (Qiagen, Hilden, Germany). Analysis with qPCR revealed higher concentrations of *Lactobacillus*, *Enterobacteriaceae* and *Desulfovibrio*, after extraction with the Tissue kit, reaching statistical significance for the *Enterobacteriaceae* and *Desulfovibrio* concentrations. The concentrations from the Stool and Tissue kit were positively and significantly correlated (Table 4). Moreover, analysis with the Tissue kit is less time consuming, making it an attractive choice for future procedure.

Table 4. Quantification of bacteria, extracted with QIAamp DNA Stool Mini Kit and Qiagen Tissue Kit, in human faecal samples revealed by qPCR[1].

Bacterial taxa	Stool kit $\log_{10}$ 16S rRNA gene copies/g faeces	Tissue kit $\log_{10}$ 16S rRNA gene copies/g faeces	*p*-value Stool vs. Tissue kit	Correlation coefficient	*p*-value correlation coefficient
Lactobacillus (n=47)	7.09 (6.67-7.69)	7.21 (6.85-7.88)	0.092	0.652	<0.001
Enterobacteriaceae (n=45)	8.33 (SD 0.96)	9.58 (SD 0.95)	<0.001	0.781	<0.001
Desulfovibrio (n=37)	7.20 (6.67-8.76)	8.38 (7.73-9.49)	0.001	0.561	<0.001

Statistical analyses were performed using SigmaStat 3.1, Systat Software, Point Richmond, USA.
[1]Analysed with the Rotor-Gene Q (Qiagen) instrument.

Bias associated with PCR

There are several biases and limitations associated with PCR reactions. The first critical step is the choice of primers. Universal primers should by definition be complementary to DNA from all kinds of bacteria. However, a vast majority of bacteria remain unknown and need to be characterised in order to know their 16S rRNA sequence. Furthermore,

new sequences are constantly being identified and databases with 16S rRNA genes are constantly expanding. Primers considered as universal may therefore exclude sequences that recently have been identified or are still to be explored. However, the ENV-primers used in papers **I**, **III** and **IV** have previously been reported to amplify a variety of bacterial groups common in the GI tract (Pettersson *et al.*, 2003, Wang *et al.*, 2004, Wang *et al.*, 2005). Importantly, in PCR reactions only numerically abundant populations will be amplified and PCR-based methods do not reveal information about the ratio of live and dead bacteria.

Primer specificity should be carefully addressed. For example, primers Lact-F and Lact-R were reported to be specific for the genus *Lactobacillus* (Rinttilä *et al.*, 2004, Wise and Siragusa, 2007) but the primers were shown to also amplify *Enterococcus*, although at a considerably lower extent (paper **IV**). The annealing temperature should be considered for each primer pair. Too low annealing temperatures will probably lead to unspecific binding since it allows mismatches, and too high annealing temperature will result in unamplified sequences in a mixed template mixture. Obviously, for comparison within a project it is essential that reagents and conditions are constant.

To assess diversity, the number of PCR cycles should be as low as possible (Bonnet *et al.*, 2002). Papers in this thesis have used 25-30 cycles in end-point PCR reactions. Pooling multiple PCR reactions is a way of limiting biases (Kitts, 2001). This was applied for the T-RFLP method and in cloning and sequencing (papers **I**, **III**, **IV**).

Furthermore, the genome size and 16S rRNA gene copy number can affect the diversity obtained from the PCR reaction (Farrelly *et al.*, 1995). It is worth mentioning that faecal samples can contain PCR inhibitors, for example polysaccharides from plant material (Monteiro *et al.*, 1997, Monteiro *et al.*, 2001). However, in the QIAamp DNA Stool Mini Kit the InhibitEX matrix absorbs DNA-damaging substances and PCR inhibitors in the stool samples.

Concerns about qPCR

The main advantage of qPCR is that it allows quantification of a specific bacterial group. However, the studied group has to be well defined by a relatively short sequence in order to facilitate accurate amplification. It is of utmost importance that the selected sequence is shared by all members of the target taxa and by no others.

The number of copies of the rRNA operons in a bacterial genome can vary from 1-15 (Klappenbach *et al.*, 2000). Therefore, quantification should be interpreted with some caution when analysing the 16S rRNA gene since this can occur in different numbers across various bacterial groups and copy numbers cannot directly be converted into cell numbers (Farrelly *et al.*, 1995). However, the abundance of specific groups can be compared between samples and within the cohort (paper **IV**). The neonate bacterial groups were quantified using standard curves obtained from cloned PCR fragments. Intercalating dyes detect all dsDNA including primer-dimers and nonspecific reaction products. Optimisation of reaction conditions can limit these potential problems and melt-curve analysis should always be performed to detect false positive fluorescence (Carey *et al.*, 2007). Different apparatus for detection is another source of variation among samples. Bacterial quantification in human neonates was performed using the Rotor-Gene Q system (paper **IV**). However, for comparison, lactobacilli content was

also tested in a 96-well system (RealPlex^2, Eppendorf, Germany), although quantification with Rotor-Gene Q was more reproducible.

Fragment analysis

T-RFLP has higher sensitivity than TTGE since bacterial groups present as 0.1% of the sample are detected with T-RFLP, but for methods relying on sequence-specific melting behaviour the amplicon abundance should be 1% in order to be detected (Dunbar *et al.*, 2000, Zoetendal *et al.*, 2004). In contrast, TTGE has a more fine-tuned discriminatory power, for instance different species of *Enterobacteriaceae* give rise to different band position in TTGE (Olsson *et al.*, 2004) while most of the genera of this family end up in the same T-RF (Wang *et al.*, 2004). The T-RFLP and TTGE methods consider the numerically dominant groups in the ecosystem. Since smaller fractions of the total community DNA will not be amplified, the diversity can be underestimated. In contrast, if digestion in the T-RFLP procedure is incomplete, additional T-RFs may appear which can overestimate diversity (Osborn *et al.*, 2000). Furthermore, T-RFLP is not a measure of the total diversity of the sample. Instead it should be regarded as a measure of the relative diversity for comparison between samples. It has been found that initial DNA concentration, number of cycles and other PCR conditions affect T-RFLP profile composition (Osborn *et al.*, 2000). Some critical steps associated with the T-RFLP method will be discussed below.

Typically, restriction enzymes with four base-pair recognition sites are used due to high frequency of these recognition sites (Schütte *et al.*, 2008). Before project set-up, enzymes and digestion conditions should be carefully tested for specificity. Initial testing revealed *Msp*I and *Alu*I (Fermentas, St. Leon-Rot, Germany) to be the most specific enzymes and so were used in the papers in this thesis. The two enzymes were also chosen because of their generation of dissimilar T-RFLP profiles of the same sample. More than one restriction enzyme, separately used, is preferred as some organisms can have T-RFs of the same length when digested with one enzyme (Liu *et al.*, 1997).

As mentioned earlier, T-RFLP and other sequence-dependent electrophoresis methods are suitable for community analysis rather than direct identification. For the same reason these methods are not suitable for detection of administered, for instance probiotic, strains because of the relatively poor detection limit and lack of resolution to discriminate administered strains from other closely related strains of the same species. If identification is desirable, clones from clone libraries should be run under the same conditions as the T-RFLP profiles. However, T-RFs can be identified *in silico* in databases where existing 16S rRNA genes sequences can be digested. Several web tools have been reported but although fragment analysis by capillary electrophoresis is very precise (±1 bp), it is not necessarily accurate (Schütte *et al.*, 2008). A range of parameters affects the T-RF size, making identification according to general databases problematic. For example, variation between sequence-determined or true T-RF length and observed T-RF length, known as T-RF drift, has been observed and T-RF drift differs depending on analysis systems such as ALFwin Express and GeneMapper (Kaplan and Kitts, 2003). For instance it was observed that clones run on the polyacrylamide gel (and analysed with ALFwin software) and capillary electrophoresis (analysed with GeneMapper) generated T-RFs of different lengths. The analytical software GeneMapper version 4.0 (Applied

Biosystems, Foster city, CA, USA) offers five size calling methods. Different size calling methods produce different sizing, for example local southern uses size standards closer to the unknown T-RF while global southern do sizing over the whole range covered by the standard (Elder and Southern, 1983), thereby creating differences in observed T-RF length (Kaplan and Kitts, 2003) and identification according to web tools could obviously be a problem. To assess the effect of T-RF drift, the size calling methods *i)* 2nd order least square, *ii)* 3rd order least square, *iii)* local southern, *iv)* global southern and *v)* cubic spline were tested on T-RFLP profiles obtained from rat caecum. The local southern method assumes linear migration time with T-RF size. This is not always true and sizing of large fragments can be erroneous (Schütte *et al.*, 2008). However, in the test of T-RFLP profiles from rat caecum, the local southern method appeared to have least T-RF drift, which was in accordance with other studies (Osborn *et al.*, 2000, Kaplan and Kitts, 2003). Consequently, the local southern method was used in paper **III**. A reason for drift can be different migration of standard and sample DNA as they are labelled differentially, for instance LIZ and 6-FAM respectively. The molecular weight of the fluorescent dye affects migration time, and this is more pronounced for shorter DNA fragments (Pandey *et al.*, 2007). Furthermore, purine content discrepancy can be a reason for T-RF drift as related organisms seem to have similar T-RF drift (Kaplan and Kitts, 2003).

The methodological considerations mentioned above highlight the need to standardise the procedure, sample treatments and reagents within the same project and between projects if comparisons are to be reliable. By doing this, the T-RFLP method is suitable for analysis and comparison of the dominating microbiota. For identification purposes, T-RFLP should be accompanied with clone libraries.

Study designs

Human studies

The early intestinal microbiota and the possibility of predicting later health outcome was of interest in paper **I**. Infants included in this study were selected from the ALLERGYFLORA project that included three cohorts of approximately 100 infants each from Sweden, UK and Italy (Adlerberth *et al.*, 2007). Because of well characterised study subjects it was feasible to select those that clearly had atopic eczema and those without eczema. Consequently, differences could be found in the relatively limited cohort described in paper **I**. Faecal samples were obtained one week after birth.

To further investigate the early intestinal colonisation in human babies, the pioneer microbiota was studied in newborns (paper **IV**). Parents collected the meconium from the diaper of their vaginally born baby. Meconium is the first stool passed by the neonate and it is composed of mucin, amniotic fluid, exfoliated epithelial cells and has historically been considered sterile (Sunderarajan and Kelkar, 1979) but microbes are now known to be present (Jiménez *et al.*, 2008).

When designing a study, the time point for sampling should be carefully addressed since especially the early gut microbiota is known to differ. Furthermore, knowledge about host-related or external factors that may affect the gut microbial composition is valuable. When possible, this kind of information should be taken into account when designing studies, in order to reduce the number of confounding factors. Moreover, this should be born in mind when evaluating the data.

The parents of the babies in papers **I** and **IV** gave written informed consent and the studies were performed in compliance with the Helsinki Declaration and approved by the regional ethical committee in the south of Sweden. Because of ethical concerns about biopsies to obtain mucosal samples from children without apparently intestinal disorders, we investigated the faecal microbiota of subjects in papers **I** and **IV**.

Animal studies

Experimental studies can generally be well controlled and are ideal for mechanistic investigations where *in vitro* studies are not enough and human studies are not practical or acceptable. Rats (*Rattus norvegicus*) of the outbred Sprague-Dawley stock (Taconic, Ry, Denmark) were used for the intervention studies described in papers **II** and **III**. Previous studies have reported significant effects in offspring to rat dams whose microbiota was manipulated (Fåk *et al.*, 2008c, Fåk *et al.*, 2008b). Obesity is an increasing health problem and the maternal diet is known to affect offspring physiology; for example, energy-rich diet to dams makes offspring more prone to metabolic disorders (Bayol *et al.*, 2005, Buckley *et al.*, 2005, Srinivasan *et al.*, 2006, Bayol *et al.*, 2007, Zambrano *et al.*, 2010). Additionally, it has been proved that the maternal microbiota of obese mice is transferred to the offspring (Ley *et al.*, 2005). However, energy-rich diet in combination

with microbial manipulation of rat dams during pregnancy and lactation has been poorly investigated.

The study design in paper **II** aimed to investigate the GI function, systemic inflammation, fat deposition and body weight in the suckling rat pups after maternal HEDD feeding combined with modulation of the gut microbiota by administration of *E. coli*. Refrigerated foods such as milk, meat and vegetables in an early phase of deterioration, before loss of apparent edibility, expose the consumer to different types of bacteria, typically Gram-negative *Proteobacteria* such as *Pseudomonas* and *Enterobacteriaceae* (Gram *et al.*, 2002). *E. coli* CCUG29300[T], used in papers **II** and **III**, has previously been reported to evoke an inflammatory tone (Fåk *et al.*, 2008b).

The intervention study described in paper **III** aimed to investigate the longitudinal effect of an energy-dense diet, combined with two fundamentally different bacteria, on body weight development, fat deposition and gut microbiota along with various biochemical markers. One group of animals were exposed to *E. coli* CCUG29300[T] and another group received *L. plantarum* DSM15313. *L. plantarum* frequently occurs spontaneously and in high numbers in most lactic acid fermented foods, especially those based on plant material (Molin, 2008). *L. plantarum* is also present on human intestinal mucosa (Molin *et al.*, 1993) and the particular strain used in paper **III** has been isolated from healthy human intestinal mucosa (Molin *et al.*, 2004) and used in several studies to investigate the probiotic potential (Osman *et al.*, 2007, Bränning *et al.*, 2009, Andersson *et al.*, 2010, Lavasani *et al.*, 2010).

Animals of an outbred stock were chosen since the purpose was to simulate, to some extent, the general human population. The animals were kept under specific pathogen-free conditions and a controlled environment (21±1ºC, 50 ± 10% relative humidity, 12:12-hours light-dark cycle). Dams were randomly mated with males selected to maximise the genetic variation within the treatment groups but to minimise variations between the groups. One week after mating, dams were separated into individual cages and assigned dietary regimes. In paper **II** pups were sacrificed at postnatal day 14. By then they have undergone major postnatal development but milk is the sole source of nutrients and they have not yet started to eat solid food (Rolls *et al.*, 1986). Animals in paper **III** were weaned at about four weeks of age and nine female offspring from each study group were randomly mixed into cages with three animals per cage and assigned the same diet as their mothers. The animals continued in the experiment for six months. Along with other investigators (Cani *et al.*, 2007a, Cani *et al.*, 2008) we studied the gut microbiota in caecum since most of the fermentation occurs at this site (papers **II** and **III**). The animal studies described in this thesis were approved by the regional ethical committee in the south of Sweden.

Physiological evaluations

General evaluation of study subjects

In paper **I**, 15 infants were diagnosed with atopic eczema and increased total and specific IgE levels, and 20 infants served as controls. Newborn babies recruited to the study described in paper **IV** were all healthy and vaginally born at term without complications.

Rats in papers **II** and **III** appeared healthy throughout the studies. The only exception was one rat receiving *E. coli* that was sacrificed after 12 weeks after veterinarian consultation and recommendation, due to the finding of a subcutaneous tumour. Another rat receiving *E. coli* was perceptually leaner than the others, as confirmed at dissection after six months in the study. However, no signs of disease could be detected (paper **III**).

Atopic eczema

Atopic eczema is often the first manifestation of atopic disease in infants (Eichenfield *et al.*, 2003) and is characterised by itching skin rashes. The toddlers in paper **I** were diagnosed with atopic eczema if they fulfilled William's UK diagnostic criteria for atopic eczema (Williams, 1996) or the International Study of Asthma and Allergy in Childhood criteria of an itching rash that has come and gone for at least six months and has affected typical locations (Asher *et al.*, 1995). Toddlers not fulfilling these criteria were included as control cases. Skilled paediatricians made the diagnosis of atopic eczema at the age of 18 months. A SCORAD value was obtained by use of the ScoradCard software (Tripodi *et al.*, 2004).

Body weight and fat deposition

Body weight can be regarded as an indicator of the overall health status. The rat pups' body weights were evaluated the first day after parturition (birth weight) and at the day of killing (paper **II**). There was no difference in birth weight depending on maternal diet (papers **II** and **III**), which was in accordance with other studies (Bayol *et al.*, 2005, Buckley *et al.*, 2005, Bayol *et al.*, 2007, Zambrano *et al.*, 2010). However, at postnatal day 14 the body weight was significantly higher in pups of dams fed HEDD compared to LF diet and the offspring body weight was further increased if dams were supplemented with *E. coli*. At this age, adiposity (epididymal and retroperitoneal fat pad weight as percentage of body weight) was increased (paper **II**). Possibly the enhanced permeability and inflammation observed in the pups of paper **II** is a reason for their increased adiposity.

Rats in paper **III** were weighed weekly from weaning until sacrifice. Interestingly, rats supplemented with *L. plantarum* had lower body weight throughout the study compared to control animals or those fed *E. coli* (*p*=0.086 at six months of age). Significantly less retroperitoneal fat was observed in the group consuming *L. plantarum* compared to animals consuming *E. coli* (*p*=0.03) and the retroperitoneal fat weight correlated with body weight after six months (*r* 0.454, *p*=0.02). Animals administered *E. coli* had a

higher median weight of the periovarial fat deposit compared to the other groups but the difference was not statistically significant (paper **III**). Figure 2 illustrates the difference in body weight depending on bacterial supplementation from foetal life through six months of age.
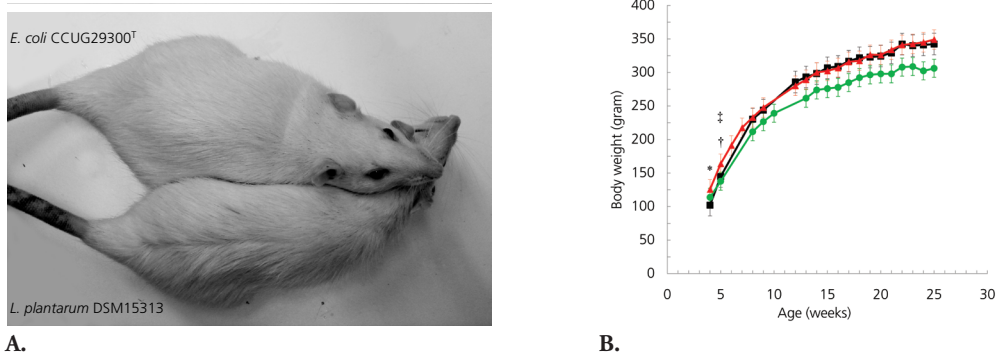

A.

B.

Figure 2. Bacterial supplement from foetal life through six months of age affected body weight gain. **A.** The rat at the top of this photograph was exposed to *E. coli* CCUG29300[T] from foetal life through six months of age and the rat at the bottom was exposed to *L. plantarum* DSM15313 for the same time period. **B.** Body weight was monitored weekly from weaning to sacrifice. During the entire study period, the group exposed to *L. plantarum* DSM15313 exhibited lower body weight. ■, control; ●, *L. plantarum* DSM15313; ▲, *E. coli* CCUG29300[T].

Newborn babies were weighed and classified as AGA or LGA (paper **IV**). According to the guidelines from the Swedish National Board of Health and Welfare, AGA was determined as the mean birth weight ±2SD and LGA was assigned babies with birth weight above 2SD of the mean for that specific gestational age (Marsál *et al.*, 1996). Ten of the 79 neonates in paper **IV** were classified LGA. Maternal diet and body weight could not be determined for mothers to babies in paper **IV**. However, as previously discussed, maternal faecal microbiota is known to differ depending on body weight (Collado *et al.*, 2008).

Biochemical analyses

Gut maturation in suckling rat pups

Rodents who are born relatively immature in terms of gut maturation undergo postnatal structural and functional development of the GI tract (Pacha, 2000). In the suckling rat pups of paper **II**, organ weights and digestive enzyme activities were investigated to determine the GI growth and function. The intestinal protein content (relative to the body weight) increases with age and so can be used as a marker for intestinal growth. In paper **II** the SI was homogenised and thereafter analysed with the Lowry method to colorimetrically compare the protein content of the samples with a bovine serum albumin

(BSA) standard (Lowry *et al.*, 1951). HEDD to rat dams resulted in lower protein concentration in pups' SI although the SI length was increased.

The fact that lactase activity in the SI decreases with age, while sucrase and maltase increases, can be utilised to investigate the degree of intestinal functional maturation in the suckling animals. In paper **II** the intestinal disaccharidase activities were measured using the Dahlqvist method (Dahlqvist, 1984) where lactose, maltose or sucrose are incubated with the samples to allow any lactase, maltase or sucrase respectively to degrade the sugar to glucose. The glucose concentrations were determined colorimetrically and compared to a standard (Dahlqvist, 1984). For example the HF group and the HF-EC group had lower maltase activities compared to the LF group. Possibly the dietary regimes induced stress that affected the developing GI system, so the functional adaptation of the GI tract depending on dietary regimes needs further investigation. The study design in paper **III** can partly be regarded as a follow up study to the experiment reported in paper **II**. However, the purpose of paper **III** was different and the GI growth and development parameters were not evaluated in the adult animals.

Biochemical markers evaluated in the adult rats

Leptin is a hormone that regulates the balance between food intake and energy expenditure by signalling to the brain. The protein is mainly secreted from the adipose tissue and obese individuals have increased leptin levels (La Cava *et al.*, 2004). Furthermore, leptin increased proportionally to the increase in body fat of germ-free (GF) mice subjected to colonisation (Bäckhed *et al.*, 2004). It is well known that leptin has immune stimulative features such as stimulation of T_H1 cells to secrete pro-inflammatory cytokines (Lord *et al.*, 1998, Batra *et al.*, 2010). In addition, patients with inflammatory disorders like, for example, pelvic endometriosis and rats suffering from colitis have enhanced plasma leptin concentrations (Barbier *et al.*, 2001, La Cava *et al.*, 2004). In paper **III** we found significantly higher leptin concentrations in the group that was administered *E. coli*. This was in accordance with the higher body weight and weight of fat pads in these animals. The immunological properties were to some extent in alliance with the higher plasma haptoglobin concentrations in animals administered *E. coli* (paper **II**).

The liver is a vital organ involved in a range of biological processes, for example the enterohepatic circulation, metabolic processes, production of acute-phase proteins and the liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). AST is also known to be produced by heart and skeletal muscles, kidney and pancreas while ALT is more specifically produced by hepatocytes (Giboney, 2005). In paper **III**, the AST, ALT and ALP activity was measured enzymatically. It was shown that animals fed HEDD without any supplemented bacteria had the highest AST concentrations, although it did not attain statistical significance (*p*=0.087) (paper **III**).

The acute-phase protein haptoglobin is mainly produced by the liver (Taes *et al.*, 2005). The protein is known to increase in response to inflammation and it has been implicated as a useful inflammatory marker in rodents (Giffen *et al.*, 2003). However, contradictory results have been reported. For instance, data did not support the hypothesis that haptoglobin would positively correlate with increased adiposity in rats (Due *et al.*, 2005, Perez-Echarri *et al.*, 2008). In paper **II**, HEDD to rat dams increased haptoglobin concentration in the suckling pups and HEDD in combination with *E. coli*

further increased the plasma haptoglobin. A previous study showed similar increased haptoglobin in rat pups when dams were exposed to either antibiotics or *E. coli* (Fåk *et al.*, 2008b). However, when pups were monitored to the age of six months, the haptoglobin was lower in these animals compared to rats fed HEDD without bacteria, despite more body fat (paper **III**). The primary function of haptoglobin is to restrict iron availability to pathogenic bacteria by formation of an irreversible complex with haemoglobin that is released from damaged erythrocytes (Eaton *et al.*, 1982, Wejman *et al.*, 1984). The decreased haptoglobin concentration found in the six-month-old rats on HEDD and *E. coli* supplementation might be a result of the fact that even non-pathogenic *E. coli* strains are equipped with efficient means (siderophores) to absorb iron (Smarda *et al.*, 2006). In the developing pups this feature might not be manifested to the same degree.

The LPS in the outer cell membrane of Gram-negative bacteria are composed of lipid A (fatty acids), a carbohydrate core and a polysaccharide O antigen. The hydrophobic fatty acids in lipid A anchor the LPS into the bacterial membrane, and upon lysis the cells release their LPS to the surroundings. LPS triggers for example macrophages and monocytes to release cytokines that further accentuate the immune reaction. LPS is sometimes used interchangeably with the term endotoxin. However, the former term refers to the chemical structure and composition of the molecule while the latter emphasises its biological activity (Hurley, 1995). Importantly, the chemical composition of the LPS molecules differs and so its ability to promote inflammation and metabolic disturbances might be different because of the various endotoxemic capacities (Lindberg *et al.*, 1990, Gangloff *et al.*, 1999) and, furthermore, synergistic effects of LPS from different bacteria can be obtained (Namavar *et al.*, 1987). Intestinal alkaline phosphatase is an enzyme found in duodenum and known to detoxify LPS. Obese rats have shown decreased concentration of this enzyme activity but increased intestinal inflammation and permeability (de La Serre *et al.*, 2010). Cani *et al.* (2007a) observed increased endotoxemia and altered microbiota, for example reduced number of bifidobacteria and other Gram-positives, after high-fat feeding. Plasma LPS has been shown to positively correlate with fat and total energy intake (Amar *et al.*, 2008) and significantly higher LPS has been reported in humans after a HF meal (Erridge *et al.*, 2007). According to previously published data and the fact that *E. coli* has LPS in its cell wall, we assumed increased plasma LPS in rats on HEDD in combination with the Gram-negative *E. coli*. In paper **III**, LPS was measured using an accredited chromogenic end-point method based on limulus amebocyte extract (Charles River Laboratories, Charleston, SC, USA). Plasma samples were withdrawn using endotoxin-free syringe, tips and tubes. In our study there were no significant differences in LPS content between the rat groups. Interestingly, the levels in our animals were considerably lower compared to other experimental studies (Cani *et al.*, 2007a, Cani *et al.*, 2007b, Amar *et al.*, 2008, Thuy *et al.*, 2008) and human studies (Erridge *et al.*, 2007, Amar *et al.*, 2008). Methodological properties can be a reason and possible contamination should not be excluded since LPS is heat-stable and found everywhere (Hurley, 1995). Furthermore, plasma LPS is known to increase after a meal (Cani *et al.*, 2007a, Erridge *et al.*, 2007) but eating behaviour was not assessed prior to blood sampling at sacrifice in paper **III**. Nevertheless, an external laboratory with experience in LPS quantification performed the LPS measurement. To ensure lack of inhibitors in plasma and to verify reliability, each sample was spiked with LPS and at least 50% recovery was guaranteed (paper **III**).

Calprotectin is a complexed form of two calcium-binding proteins of the S100 family called migration inhibitory factor-related protein (MRP)-8 and MRP-14 or S100A8/S100A9. The protein is predominantly secreted by neutrophils and monocytes to neutralise the activity of pro-inflammatory cytokines (Ikemoto *et al.*, 2007). The marker is widely used in the diagnosis of patients with inflammatory bowel diseases and colorectal carcinoma (Damms and Bischoff, 2008). In paper **III**, rectal content of calprotectin in obese rats was analysed with enzyme-linked immunosorbent assay according to the manufacturer's instruction (Immundiagnostic AG, Bensheim, Germany). The type of diet and bacteria used in this study did not influence the concentration of rectal calprotectin, which indicates the diet regimen did not cause extensive intestinal inflammation.

Histological analyses

Ectopic fat accumulation can impair tissue function and the degree of hepatic fat accumulation can be assessed by a steatosis scoring system. In paper **III**, histological evaluation of the livers was performed in order to assess the level of steatosis. Liver tissue was fixed in 4% buffered formaldehyde (Histolab, Göteborg, Sweden) for 24 hours, rinsed in water and dehydrated in graded ethanol concentrations, followed by xylen and finally embedded in paraffin. Sections of 5 µm were mounted on slides, deparaffinised, and stained with haematoxylin and eosin. A blinded scientist, with special interest in pathology, scored the steatosis prevalence using the following scoring system: 0 = no visible steatosis, 1 = steatosis in <1/3 of the hepatocytes; 2 = steatosis in 1/3 to 2/3 of the hepatocytes; 3 = steatosis in >2/3 of the hepatocytes (Brunt *et al.*, 1999).

Functions of the gut microbiota

The gut microbiota has numerous functions. These can roughly be divided into three compartments: *i)* protective, *ii)* immune-regulatory and *iii)* metabolic functions.

Protective functions

The microbiota occupying intestinal surfaces prevent potential pathogens from colonising and interacting with epithelial and immunological cells. Furthermore, competition for substrate and mucosal adhesion sites contribute to exclusion of exogenous bacterial colonisation. Microbes can adhere to the intestinal mucosa in various ways (Adlerberth *et al.*, 1996, Roos and Jonsson, 2002, von Ossowski *et al.*, 2010). Antimicrobial substances can also be released, for example hydrogen peroxide, nitrogen oxide and different antimicrobial peptides (Valore *et al.*, 2002, Stecher and Hardt, 2008).

A single layer of epithelial cells lines the GI tract to separate the luminal content from the body. Tight junctions (TJ) are the dynamic protein complex forming apical seals between enterocytes. This forms an effective barrier preventing paracellular diffusion of macromolecules and microorganisms from the luminal content to come into direct contact with the basolatheral compartments (Pacha, 2000). However, various stimuli including pro-inflammatory cytokines can induce alteration in expression and assemblage of for example zona occludence-1 and occludin, which are parts of the TJ structure, leading to changed permeability (Schneeberger and Lynch, 2004).

HF feeding to rodents induced lower *Lactobacillus* and *Bacteroides/Prevotella* viable counts, and DGGE analysis showed that the intestinal microbiota was altered in combination with increased intestinal permeability and decreased expression of genes coding for TJ proteins (Cani *et al.*, 2008). Brun *et al.* (2007) observed decreased levels and redistribution of TJ proteins in the SI of genetically obese mice and that was in accordance with increased circulating pro-inflammatory cytokines and endotoxemia, suggesting enhanced intestinal permeability leading to increased endotoxemia.

Gut permeability can be assessed through oral administration of a lactulose, mannitol, sucralose solution and measurement of corresponding urinary excretion. Lactulose/mannitol ratio is the marker of SI barrier function and the lactulose/sucralose ratio is the marker of distal gut barrier function (Brignardello *et al.*, 2010). FITC-dextran can also be used for gut permeability measurement by plasmatic determination after oral administration (Cani *et al.*, 2008, de La Serre *et al.*, 2010). The Ussing chamber is a possibility in experimental conditions and in a study by Mangell *et al.* (2002) *E. coli*-induced permeability was inhibited in rats pre-treated with *L. plantarum* DSM9843. *L. plantarum* DSM9843 are known to compete with *E. coli* for binding and colonisation in the intestine and both systemic and intestinal immunity are affected, for instance by enhanced IgA concentration when the lactobacilli was given in combination with *E. coli* compared to *E. coli* alone. Translocation also seemed to be positively affected (Herias *et al.*, 1999).

Permeability is extensive across the gut epithelium in newborn rat pups and translocation occurs, preferentially to mesenteric lymph nodes but also to the liver and spleen to some extent (Wenzl *et al.*, 2001, Yajima *et al.*, 2001). In suckling rat pups, gut permeability and the capacity of macromolecular absorption gradually decreases with age. Gut closure occurs when foetal-type enterocytes are replaced with adult-type enterocytes that lack the neonatal Fc receptors to bind milk IgG in proximal SI and large absorptive vacuoles to transport BSA in distal SI (Pacha, 2000). In paper **II**, permeability of BIgG and BSA was assessed as an indicator of SI maturation and to examine whether bacterial supplementation could affect permeability. Four hours prior to sacrifice, the pups were separated from the dam. After one hour separation pups were gavaged with the marker molecules BIgG and BSA. At sacrifice, blood was withdrawn in order to analyse the levels of the administered molecules in plasma. BSA and BIgG absorption was significantly higher in the HF group supplemented with *E. coli.* In contrast, plasma BIgG in the HF group was significantly lower compared to the other groups (paper **II**). In an attempt to connect intestinal permeability and the microbial ecosystem, caecal *Enterobacteriaceae* was determined but no difference between the groups was observed. However, it cannot be excluded that the concentration and prevalence of other bacterial groups were altered since that was the case in paper **III**. Permeability of BIgG has previously been shown to be lower in pups when the dam was treated with *L. plantarum* DSM9843 (Fåk *et al.*, 2008c), but previous studies have not been totally conclusive (Fåk *et al.*, 2008a, Fåk *et al.*, 2008b, Fåk *et al.*, 2008c). Although maternal feeding with HEDD and microbial manipulation increased gut permeability in our experiment, further investigations regarding neonatal permeability depending on maternal diet are needed.

The gut microbiota is known to be altered in obesity (Bäckhed *et al.*, 2004, Ley *et al.*, 2006, Duncan *et al.*, 2008, Turnbaugh *et al.*, 2009) and a recent study showed that obese humans have altered gut microbiota and increased levels of the inflammatory marker C-reactive protein. This was in accordance with changed gut permeability (Brignardello *et al.*, 2010). Furthermore, it has also been shown that systemic inflammation, indicated by for example increased interleukin (IL)-6, tumour necrosis factor (TNF)-α and interferon (IFN)-γ plasma concentrations, correlates with increased intestinal permeability in human volunteers subjected to endotoxemia since urinary excretion of the marker molecule polyethylene glycol was significantly increased after LPS injection. The permeability was explained by an inflammation-induced increase in paracellular permeability rather than ischemia of enterocytes (Hietbrink *et al.*, 2009). Probiotics are suggested to ameliorate the barrier function (Ohland and Macnaughton, 2010) and recently, Karczewski *et al.* (2010) showed enhanced barrier function both *in vitro* and *in vivo* in duodenum of healthy humans after probiotic administration of *L. plantarum* WCFS1. *L. plantarum* (strain not specified) has further been implicated in improving intestinal barrier function and clinical outcomes in patients with acute pancreatitis (Qin *et al.*, 2008). In addition, probiotics prevent bacterial translocation after chronic psychological stress (Zareie *et al.*, 2006).

Immune-regulatory functions

At birth, the neonate leaves the germ-free intrauterine environment for entrance to the extrauterine antigen-rich environment. It is fundamentally important for the body to distinguish between potential pathogens and commensal bacteria and to elicit an appropriate response and thereby prevent diseases. The intestinal bacteria are mandatory for this process. The foetal immune system is skewed towards T_H2 phenotype but upon correct stimulation a balance between T_H1 and T_H2 response is obtained. Allergy is manifested by an immune system polarised towards T_H2 response since these cells stimulates IgE production (Levy, 2007).

The main interface between the host immune system and the external environment is the intestinal mucosa where the cells are in constant interchange (Guarner and Malagelada, 2003). The gut-associated lymphoid tissue is the body's largest collection of organised lymphoid tissues such as mesenteric lymph nodes and Peyer's patches, and more diffusely located lymphocytes in the intestinal lamina propria and epithelium, including IgA secreting cells (Forchielli and Walker, 2005). Intestinal bacteria are supposed to enter by M (microfold) cells at the Peyer's patches but also to be phagocytised by dendritic cells in the lamina propria since these cells are able to express TJ proteins and can by that extend their dendrites to the luminal compartment without compromising the barrier function (Rescigno *et al.*, 2001).

Intestinal microbes can signal through transmembrane receptors on epithelial cells such as Toll-like receptors (TLRs), resulting in activation of complex intracellular signalling cascades and leading to recruitment of immune cells and altered expression of genes coding for mediators involved in pro- and anti-inflammatory processes (Round and Mazmanian, 2009). A range of TLRs can be expressed at the epithelial cell surface, each having its preferred ligands. LPS signal through TLR4 (Hedlund *et al.*, 2001) while lipoteichonic acid from Gram-positive bacteria acts through TLR2 (Grangette *et al.*, 2005).

Secretory IgA is one of the key components of the intestinal humoral immune system as it binds to microbial components, preventing these from inducing excessive immune stimulation. It has been shown that secretory IgA levels during the first year of life are positively correlated with diversity of *Bifidobacterium* during the first month of life and reduced risk of developing allergy (Sjögren *et al.*, 2009). Although we did not measure secretory IgA or the diversity of specific microbial genera in paper **I**, higher diversity in the dominating microbiota was associated with lower risk of atopic eczema.

Intestinal microbes stimulate immune cells differently. For example, irrespective of phylogenetic position, Gram-positive bacteria induced more IL-12 and IFN-γ while Gram-negative bacteria induced more IL-6, IL-8 and IL-10 (Skovbjerg *et al.*, 2010), which indicates a different immune response depending on stimuli and so the types of antigens are believed to be important. The cross-talk between host immune system and the intestinal microbiota is highly complex. For example, *B. fragilis* is a Gram-negative bacteria with LPS, but it also induces anti-inflammatory cytokine response by regulatory T cells through its polysaccharide A (Round and Mazmanian, 2009).

Metabolic functions

Vitamin K_2 is a lipophilic vitamin synthesised by gut microbes, for instance *B. fragilis* and *E. coli* (Parker *et al.*, 2003) but not by *Bifidobacterium* (Shearer, 1995). This vitamin is an essential co-factor for enzymes involved in bone and vascular health, and vitamin K deficiency has been reported to associate with hemorrhagic disorders in newborn infants. Neonates do not have endogenic vitamin K and human breast milk has limited amounts of this compound (Shearer, 1995, Greer, 2010). Vitamin B_{12} is another vitamin produced by intestinal microbes, but it is absorbed in the SI and not in colon. Hence humans must rely on B_{12} in their diet. During foetal life and infancy, vitamin B_{12} is required for normal growth and the development of the nervous system (Dror and Allen, 2008). Intestinal microbes can synthesise amino acids from urea and ammonia (Metges *et al.*, 1999) and commensal microbes like lactobacilli, lactococci, bifidobacteria, pediococci, enterococci, streptococci and propionibacteria can produce conjugated isomers of the essential fatty acid linoleic acid (Lee *et al.*, 2007, Wall *et al.*, 2009, Ross *et al.*, 2010). A potential therapeutic opportunity, raised for conjugated linoleic acid, is prevention of necrotising enterocolitis in preterm infants (Rosberg-Cody *et al.*, 2004).

Non-digestible carbohydrates escape enzymatic digestion in the SI. Instead, the intestinal microbiota ferments these compounds to short-chain fatty acids (SCFA), mainly acetic, propionic and butyric acids (Blaut and Clavel, 2007). Dietary proteins, pancreatic enzymes, mucus, sloughed epithelial cells and bacterial debris are other substrates for intestinal microbes to convert into SCFA (Guarner and Malagelada, 2003, Blaut and Clavel, 2007). Colonocytes use butyric acid as its main energy source, but SCFA can also enter the circulation and be metabolised in other tissues (Blaut and Clavel, 2007, Samuel *et al.*, 2008). Furthermore, it has been suggested that its activation of the G-protein-coupled receptors Gpr41and Gpr43, expressed by enteroendocrine cells in the gut epithelium and immune cells, enhance caloric utilisation from a polysacharide-rich diet (Samuel *et al.*, 2008, Tazoe *et al.*, 2008). Oligosaccharides in human breast milk are fermented by for instance bifidobacteria and *Bacteroides* spp. (Marcobal *et al.*, 2010).

Disorders related to the gut microbiota

Microorganisms, especially lactic acid bacteria, have historically been of utmost importance in food preservation but during the last centuries more focus has been on pathogens. However, as already extensively discussed, the gut microbiota is now considered a vital organ in close interaction with the host. Nevertheless, various microbial disturbances can be a cause or consequence of host disorders. Differences in the gut microbial composition have been proposed to be implicated in for example, allergy, obesity, T2D, insulin resistance, cardiovascular diseases and non-alcoholic fatty liver disease (NAFLD) (Naruszewicz *et al.*, 2002, Cani *et al.*, 2007a, Miele *et al.*, 2009, Karlsson *et al.*, 2010, Larsen *et al.*, 2010, Schwiertz *et al.*, 2010b, paper **I**). The next sections focus on allergy and obesity, and the role of the gut microbiota in that context will be discussed.

Allergy

The World Allergy Organization defines allergy as "a hypersensitivity reaction initiated by specific immunologic mechanisms". Dermatitis is the general term for local skin inflammation while atopic eczema refers to the dermatitis in subjects with a hereditary predisposition to allergic manifestations by an underlying inflammation dominated by an IgE-antibody associated reaction. Atopic eczema has replaced the former terms atopic dermatitis and atopic eczema/dermatitis syndrome (Johansson *et al.*, 2004) and is often the first manifestation of atopic disease in infants (Eichenfield *et al.*, 2003). However, even if predisposed, not all infants develop atopic eczema, suggesting external factors to be important. Lack of microbial exposure during infancy, and subsequent failure of adequate tuning of the immature immune system, has been suggested as one reason for the allergy epidemic in westernised populations (Strachan, 1989, Bach, 2002).

The results of paper **I** corroborate this, as reduced diversity in the dominating microbiota was observed in those infants developing atopic eczema (Figure 3). Similar results were confirmed by others, who also showed that the reduced diversity persisted for the first four months of life (Forno *et al.*, 2008). The disturbed microbiota preceding atopic eczema in infants can be exemplified by significantly higher *E. coli* prevalence (Penders *et al.*, 2006a). Newborns with excessive birth weight are at greater risk of later disease (Boney *et al.*, 2005, Harder *et al.*, 2009) and interestingly, neonates born LGA also had higher prevalence of *E. coli* (paper **IV**) and rats exposed to *E. coli* early in life developed obesity (papers **II** and **III**).
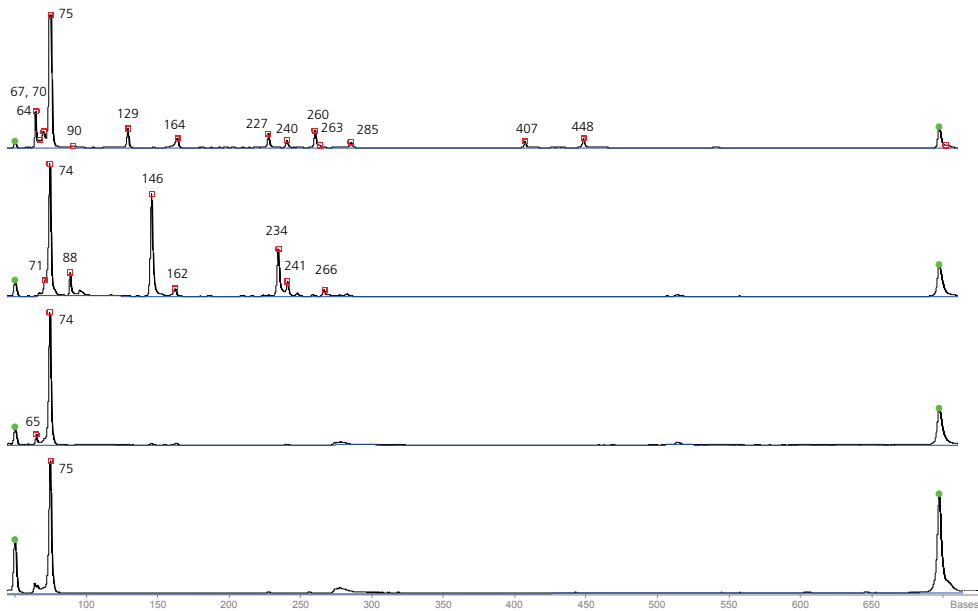
Figure 3. The diversity of the faecal microbiota was reduced in infants developing atopic eczema. The two electropherograms at the bottom of the figure show the dominating faecal microbiota in infants who developed atopic eczema during their first 18 months of life. The two electropherograms at the top represent the dominating faecal microbiota in those who did not develop atopic eczema. Faecal specimens were analysed when the infants were one week old, digestion with restriction endonuclease *Alu*I. Bacterial diversity was assessed by calculating bacterial richness (number of T-RF) and by using the relative abundance (T-RF peak area) for calculation of diversity indices.

The hygiene hypothesis

In 1989 Strachan coined the "hygiene hypothesis", by proposing that the increased allergy prevalence observed in developed countries could be related to the reduced microbial exposure to children born in these parts of the world compared to children born and raised in areas where microbial exposure and infections are more extensive (Strachan, 1989). The hygiene hypothesis has been well accepted. For instance, Illi *et al.* (2001) monitored children from birth to the age of seven and found repeated viral infections during the first year of life that directed the immature immune system towards the T_H1 phenotype. This reduced the likelihood of developing atopy and asthma at the age of five and seven years, respectively. Björkstén *et al.* (1999) observed allergic infants to be colonised less often by lactobacilli and bifidobacteria while the number of aerobes, coliforms, and *S. aureus* were higher in allergic infants compared to non-allergic control subjects. In addition, one-year-old Estonian infants were more often colonised by lactobacilli, and at higher counts. *Clostridium difficile* was more common in Swedish infants (Sepp *et al.*, 1997). Dicksved *et al.* (2007) found differences in intestinal diversity depending on lifestyle factors, for instance consumption of organic food products, and no antibiotic usage or vaccination were associated with increased diversity.

Infants in paper **I** were all born in western societies and therefore reduced microbial exposure could be assumed. However, the exact initial microbial exposure was not unified or controlled. No difference in diversity was observed related to the study centres. Number of siblings, antibiotic use during pregnancy or mode of delivery did not affect the result that infants not developing atopic eczema had higher microbial diversity. In line with the hygiene hypothesis, the results in paper **I** demonstrated that different microbial composition preceded development of atopic eczema, which has also been observed by others (Björkstén *et al.*, 2001, Kalliomäki *et al.*, 2001a, Penders *et al.*, 2006b, Penders *et al.*, 2007, Forno *et al.*, 2008, Hong *et al.*, 2010).

Probiotic interventions to prevent allergy

The World Health Organization (WHO) and American Food and Agricultural Organization define probiotic bacteria as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. Numerous studies report that probiotic bacteria have effects on the immune system. However, further research is needed since the interaction between probiotics, other intestinal bacteria and the host immune system is highly complex and affected by numerous confounders.

Nevertheless, there have been several attempts to reduce allergic manifestations by prophylactic administration of probiotic bacteria to mothers and infants with high risk of allergy. In a Finnish randomised placebo-controlled study, capsules with placebo or *L. rhamnosus* GG (10^{10} CFU/day) were given to pregnant woman two to four weeks before expected delivery and during the six months after delivery. At the age of two, children were diagnosed for atopic eczema, asthma and allergic rhinitis. In total, 35% of the 135 children were diagnosed with atopic eczema and the frequency in the probiotic group was half of that of the placebo group, 23% and 46% respectively (Kalliomäki *et al.*, 2001b). Similar prevalence was observed at the follow-up control when children were four years and also the follow-up at seven years of age confirmed protection against eczema, suggesting probiotic beneficial effect beyond infancy (Kalliomäki *et al.*, 2003, Kalliomäki *et al.*, 2007). However, the results of the Finnish study could not be reproduced by others, despite similar study design and use of the same probiotic strain (Kopp *et al.*, 2008).

A study with similar study design was carried out by Abrahamsson *et al.* (2007) but with administration of *Lactobacillus reuteri* ATCC55730 (10^{10} CFU/day) instead of *L. rhamnosus*. *L. reuteri* was given to pregnant women from gestational week 36 until delivery. Then the babies were given the same product during their first year of life in this prospective double-blind, randomised, placebo-controlled trial. The intervention with *L. reuteri* failed to prevent eczema in children with a family history of allergic disease. However, IgE-associated eczema during the second year of life was less common in children receiving *L. reuteri* indicating some protective effect of the probiotic administration.

Probiotic introduction during weaning can be an attractive approach since the microbial ecosystem undergoes remarkable alteration at that time. The cumulative risk of eczema at 13 months was significantly lower in infants subjected to *Lactobacillus paracasei* F19 from weaning until 13 months of age and immune modulation was observed. Infants in the placebo group were more often prescribed antibiotics (West *et al.*, 2009). Atopic eczema has been associated with decreased mucosal barrier function, and a mixture of *L. rhamnosus* 19070-2 and *L. reuteri* DSM12246 has been shown to stabilise barrier function and gastrointestinal symptoms in the children (Rosenfeldt *et al.*, 2004).

Administration of the single probiotic bacterium *L. plantarum* DSM9843 was recently shown to increase diversity of the dominating colonic microbiota in adults (Karlsson *et al.*, 2010). Along with the finding in paper **I** that infants remained non-atopic if bacterial diversity was higher, it can be hypothesised that administration of this probiotic to pregnant women and/or newborn infants can reduce the risk of developing allergic manifestations.

Obesity

WHO defines overweight and obesity as an abnormal fat accumulation that may impair health. Obesity is known to be an important risk factor for T2D, cardiovascular disease, NAFLD and other metabolic disturbances (Lakka and Bouchard, 2005, Cani *et al.*, 2007a, Creely *et al.*, 2007, Krawczyk *et al.*, 2010). BMI is defined as the weight in kilograms divided by the square of the height in meter (kg/m^2). WHO defines overweight in adults as BMI ≥25 and obesity as BMI ≥30. According to estimates by WHO, almost half of European adults are overweight and 8-23% (depending on country) are obese. Overweight and obesity of children are defined according to the International Obesity Task Force BMI criteria (Cole *et al.*, 2000), which differs from the ranges for adults and takes into account child age and gender. It is not considered appropriate to discuss obesity in newborns. However, neonates can be classified as LGA compared to AGA or small for gestational age (Marsál *et al.*, 1996).

Adipocytes and stromal cells make up the adipose tissue. There are two types of adipose tissue, namely brown and white adipose tissue. The latter is by far the dominating type and is consequently referred to as adipose tissue in the text of this thesis. In obesity, the adipose tissue is expanded by increase of the average adipocyte volume and/or the number of adipocytes. During the last two decades it has become clear that adipose tissue, except for its fat storage function, has endocrine as well as immunological features (Hotamisligil *et al.*, 1993, Weisberg *et al.*, 2003, Fantuzzi, 2005, Tilg and Moschen, 2006). Macrophages in adipose tissue secrete for example pro-inflammatory cytokines like TNF-α and IL-6 (Weisberg *et al.*, 2003). Furthermore, obese children are reported to have increased total leukocytes counts, neotrophils, monocytes and T lymphocytes compared to their counterparts of normal weight, and obesity was positively correlated to the amount of blood T lympocytes (Zaldivar *et al.*, 2006). As indicated, obesity is characterised by a chronic low-grade systemic inflammation.

Virus-induced obesity

In 1982 a publication by Lyons *et al.* reported for the first time obesity to be induced by a virus. Body weight and both the number and volume of adipocytes were shown to increase significantly in mice upon viral infection. In spite of this observation, not much attention was paid to this. However, approximately a decade ago, Dhurandhar reviewed the presence of several different viruses that have been correlated with obesity prevalence and the term "infectobesity" was coined (Dhurandhar, 2001). Adenovirus-36 was the first human virus to be implicated in obesity. BMI has been shown to positively correlate with antibodies against Adenovirus-36 and obese subjects more often have Adenovirus-36 antibodies compared to non-obese individuals. But other human adenovirus does not seem to have the same effect (Atkinson *et al.*, 2005).

The relationship between gut microbiota and obesity

Although "infectobesity" was coined and discussed earlier, it was not until 2004 that the hypothesis received an impetus. The group comprising Jeffrey Gordon and colleagues drew attention to the relationship between gut microbiota and obesity, by publishing the first paper in this area (Bäckhed *et al.*, 2004). Since then the field has exploded in new reports about the subject.

Animal studies to reveal relationship between obesity and gut microbiota

The pioneer evidence of obesity and gut microbiota relationship originated from GF mice that were inoculated with caecal microbiota from conventionally raised mice. Mice that were raised under conventional circumstances had 42% more total body fat compared to mice reared in a GF environment. After two weeks of conventionalisation, GF mice increased their body fat by 60% and hepatic triglycerides increased 2.3-fold. Furthermore the expression of mRNA coding for enzymes involved in *de novo* fatty acid biosynthesis was increased but insulin sensitivity was decreased (Bäckhed *et al.*, 2004). In addition, conventionalised mice fed a diet rich in fat and sugar (similar to HEDD in paper **III**), had greater adiposity whereas energy intake and faecal energy excretion was similar to the one observed in GF mice on the same diet (Bäckhed *et al.*, 2007). A mechanistic explanation suggests that conventionalisation increased the density of SI villi capillaries and enhanced monosaccharide uptake from the gut, leading to fat accumulation in the liver and adipose tissue. There seems to be a microbial suppression of intestinal fasting-induced adipose factor, a protein that is produced by for example intestinal cells and adipose tissue. The suppression enhances lipoprotein lipase activity that leads to increased triglyceride storage in adipocytes while increased fasting-induced adipose factor expression and/or activity may promote leanness (Bäckhed *et al.*, 2004). The HF-fed C57BL/6J mouse is a commonly used model in metabolic studies due to its propensity to develop pre-diabetic features (Cani *et al.*, 2007a, Cani *et al.*, 2008, Andersson *et al.*, 2010, Aronsson *et al.*, 2010). Recently, *L. paracasei* F19 and HF administration to C57B/6J mice resulted in less body fat which was explained by higher expression of fasting-induced adipose factor in these mice (Aronsson *et al.*, 2010), suggesting a possibility of probiotic modulation to reduce obesity prevalence.

One reason for the increased adiposity can be the microbes' energy-harvesting capacity. Turnbaugh *et al.* (2006) showed that colonisation of GF mice with microbiota from obese individuals resulted in significantly greater body fat accumulation in contrast to colonisation of GF mice with a microbiota from lean individuals. The authors suggested that an obese microbiome has an increased capacity to harvest energy from the diet while lean mice excreted significantly more energy through the faecal route. In the studies discussed above, the obese phenotype has been associated with increased abundance of *Firmicutes* and decreased *Bacteroidetes* compared to lean individuals (Ley *et al.*, 2005, Turnbaugh *et al.*, 2006).

In an alternative hypothesis, Cani and co-workers proposed a hypothesis of increased intestinal permeability and metabolic endotoxemia-induced inflammation to be the reason for the connection between gut microbiota and obesity (Figure 4) (Cani *et al.*, 2007a, Cani *et al.*, 2007b, Cani *et al.*, 2008). Cani and co-workers showed that HF feeding for four weeks significantly altered the metabolic state of the host and they found

increased plasma LPS to be the triggering factor for expression of pro-inflammatory cytokines, liver dysfunction, and adipose tissue expansion. In addition, the gut microbiota was changed by for example decreased levels of *Bifidobacterium, Lactobacillus* and the *Eubacterium rectale-Clostridium coccoide* group during HF feeding (Cani *et al.*, 2007a, Cani *et al.*, 2007b, Cani *et al.*, 2008). In agreement with a previous investigation (Ley *et al.*, 2005), a group of *Bacteroidetes* decreased upon HF feeding (Cani *et al.*, 2007a, Cani *et al.*, 2008). Although *Bacteroidetes* are Gram-negatives with LPS in its cell wall, its endotoxic potential is lower compared to, for example, *E. coli* (Lindberg *et al.*, 1990, Gangloff *et al.*, 1999). Normally, plasma LPS has a diurnal rhythm in which the highest level is reached at the end of the dark feeding period. However, HF feeding has been shown to disrupt this cycle, meaning endotoxemia remains high throughout the day but at a level ten to fifty times lower than during sepsis, therefore the phenomenon was termed metabolic endotoxemia (Cani *et al.*, 2007a). Fat facilitates LPS absorption and increased intestinal permeability (Brun *et al.*, 2007, Cani *et al.*, 2007a, Cani *et al.*, 2008) and a newly described mechanism involving chylomicron-facilitated transport of LPS from the intestinal lumen via mesenteric lymph nodes to target tissues (Ghoshal *et al.*, 2009), are mechanisms proposed to increase plasma LPS. Once in the circulation, LPS is a potent stimulator of inflammation by binding to, for example, TLR4 on adipocytes inducing NF-κB-signalling and expression of TNF-α and IL-6 provoking for example insulin resistance (Song *et al.*, 2006). Adipose TNF-α and IL-6, along with other pro-inflammatory mediators and insulin resistance, were induced systemically upon LPS challenge to healthy human volunteers (Mehta *et al.*, 2010).
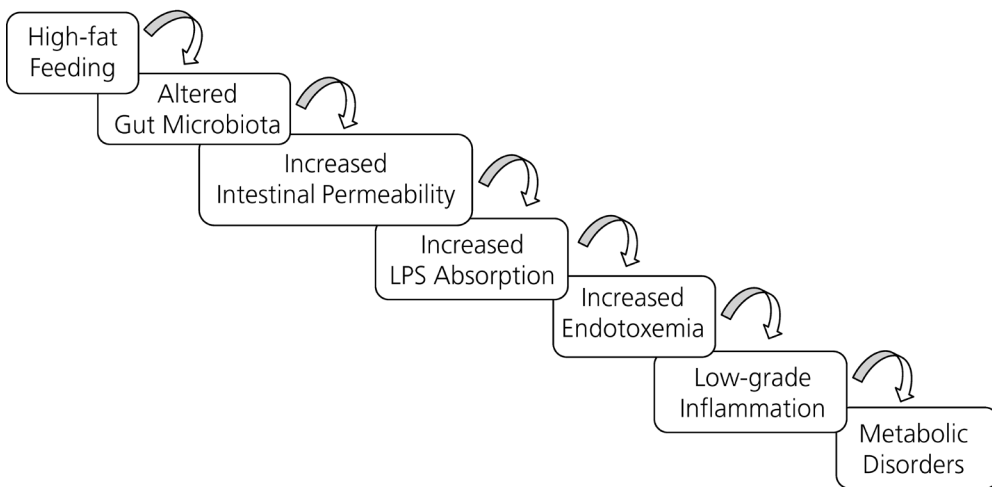
Figure 4. Hypothesis for how an altered gut microbiota induces metabolic disorders (modified from Cani *et al.*, 2008). The hypothesis grew from the observation that a HF diet increased plasma LPS (referred to as metabolic endotoxemia) and that the gut microbial composition was changed for example by reduced amount of bifidobacteria (Cani *et al.*, 2007a, Cani *et al.*, 2007b). Furthermore, HF feeding and obesity increased gut permeability (Cani *et al.*, 2008).

When HF diet was given in combination with prebiotic oligofructose, the *Bifidobacterium* concentration was restored and correlated positively to normalised concentration of pro-inflammatory cytokines, improved glucose tolerance, decreased body weight gain and visceral adiposity along with decreased endotoxemia (Cani *et al.*, 2007b). Using the FISH method, *Enterobacteriaceae* counts were decreased in HF plus prebiotic fed animals compared to HF alone, but surprisingly *Enterobacteriaceae* concentration did not correlate with LPS concentration (Cani *et al.*, 2007b). This phenomenon was analogous to the observation in paper **III**. In the study by Cani *et al.* (2007b) *Bifidobacterium* was the only tested microbial group that significantly was inversely correlated to LPS. Furthermore, prebiotic treatment reduced the intestinal permeability and TJ integrity increased along with reduction of plasma LPS and hepatic expression of inflammatory and oxidative stress markers (Cani *et al.*, 2009).

In a study by Membrez *et al.* (2008), antibiotic modulation of the mice gut microbiota resulted in decreased *Enterobacteriaceae* and LPS load along with ameliorated insulin sensitivity, glucose tolerance and liver function and decreased adiposity. In addition, genetically obese mice fed HF diet for ten weeks developed hyperglycemia but two weeks of antibiotic treatment improved the oral glucose tolerance. A changed microbiota after antibiotic treatment was in accordance with alleviated triglyceride levels and steatosis in the liver along with reduced ALT concentration, suggesting that gut microbiota has a significant role for liver function. In accordance with augmented obesity, offspring liver weight was increased upon maternal HF feeding and even more pronounced if HF feeding was combined with *E. coli* (paper **II**). Furthermore, macroscopic assessment of livers from adult rats in paper **III** suggested enhanced hepatic fat accumulation.

NAFLD encompasses a histological spectrum ranging from hepatocyte steatosis without concomitant inflammation or fibrosis to hepatic steatosis with inflammatory components and is characterised by mildly elevated transaminases (Krawczyk *et al.*, 2010). The role of the gut microbiota in NAFLD was recently reviewed by Abu-Shanab and Quigley (2010). Alterations of TJ and increased intestinal permeability in addition to bacterial overgrowth have been observed in humans with NAFLD (Miele *et al.*, 2009). Probiotic (VSL#3) treatment to obese mice improved liver histology while reducing serum transaminases and inflammatory markers including NF-κB activity (Li *et al.*, 2003b, Esposito *et al.*, 2009). The gut microbiota of animals in paper **III** was altered but liver enzymes were not significantly different among the treatment groups. However, liver TNF-α and IL-1β, bilirubin, myeloperoxidase activity and bacterial translocation to the liver and mesenteric lymph nodes were significantly decreased when *L. plantarum* DSM15313, the same strain as used in paper **III**, was administered to rats with endotoxin- and D-galactosamine-induced liver injury. In addition ALT was significantly reduced if *L. plantarum* DSM51313 was given in combination with blueberry (Osman *et al.*, 2007). In paper **III**, liver steatosis was significantly higher in the control group, in accordance with higher AST and haptoglobin concentration. The reason for enhanced steatosis in the control rats is not known but it was associated with higher proportion of *Bacteroidetes* in the gut.

It should be remembered that most of the HF feeding experiments described above used diets where 72E% came from fat and only 1E% from carbohydrates. In contrast, 41E% came from fat in the diet used in papers **III** and **II**. Even though all treatment groups in paper **III** were fed the same HEDD, differences in body weight and adiposity were found between groups of rats exposed to bacterial supplements in addition to the

HEDD feeding. A proposed mechanism involves reduced adipocyte size, which has been observed in mice treated with another strain of *L. plantarum* (Takemura *et al.*, 2010).

In accordance with the above studies we found significant changes in the gut microbiota (paper **III**). Rats supplemented with *E. coli* had lower diversity in the caecal microbiota compared to the control rats and those supplemented with *L. plantarum*. Incidence of specific T-RFs varied among the groups, which further supports a changed intestinal ecosystem according to treatment. Additionally, *Enterobacteriaecae* viable count positively correlated to body weight at six months of age (*r* 0.428, *p*=0.029) and was significantly higher in those animals supplemented with *E. coli* compared to *L. plantarum,* which confirm the observation that the caecal proportion of *Enterobacteriaceae* is by far more abundant in obese rats (de La Serre *et al.*, 2010).

In paper **III** *Firmicutes, Bacteroidetes* and *Verrucomicrobia* phyla were present in the clone libraries from all groups regardless of treatment. Five families were observed in the control group and in those exposed to *E. coli.* However, nine families were found in the *L. plantarum* group. *Bacteroidetes* were less prevalent in the control library compared to libraries from those consuming *E. coli* or *L. plantarum*, although differences in body weight and adiposity were observed, which did not correspond to the results of others (Ley *et al.*, 2005, Turnbaugh *et al.*, 2009). Independent of treatment group, all *Verrucomicrobia* sequences corresponded to *Akkermansia muciniphila*, which is similar to results of other studies in obese and non-obese individuals (Collado *et al.*, 2008, Santacruz *et al.*, 2010). In contrast to some previous studies (Ley *et al.*, 2005, Ley *et al.*, 2006, Turnbaugh *et al.*, 2006), no significance in proportions of sequences belonging to *Firmicutes* was observed among the treatment groups in paper **III**, suggesting microbial differences need to be dealt with on lower hierarchy levels to reveal differences according to obesity and inflammation.

The gut microbiota in obese humans

Differences in gut microbiota of obese versus lean humans have been reported, and it was shown that lean individuals had a higher proportion of *Bacteroidetes* at the expense of *Firmicutes* (Ley *et al.*, 2006). However, this relationship is controversial since later reports demonstrate conflicting results (Duncan *et al.*, 2008, Zhang *et al.*, 2009, Schwiertz *et al.*, 2010b). Higher proportion of *Bacteroidetes* has been shown in overweight and obese subjects whereas the proportion of *Firmicutes* was markedly higher in normal-weight individuals (Schwiertz *et al.*, 2010b). The increased *Firmicutes* abundance in lean human was mainly explained by increased abundance of *Clostridium leptum* group and *Ruminococcus flavefaciens* subgroup while there were no differences in counts of the *Clostridium coccoides* group or *Lactobacillus/Enterococcus.* Furthermore *Bifidobacterium* counts were lower in obese subjects (Schwiertz *et al.*, 2010b). Obese individuals have also been shown to have a less diverse intestinal microbiota compared to lean ones (Turnbaugh *et al.*, 2009).

Obese women have been shown to harbour more *Bacteroides* and *Staphylococcus*, when assessed by FISH and qPCR, in their gut microbiota during pregnancy compared to normal-weight women. Body weight and BMI before pregnancy positively correlated with concentrations of *Bacteroides, Clostridium,* and *Staphylococcus.* Moreover, high *Bacteroides* concentrations were associated with excessive weight gain during pregnancy. It was further suggested that *Bifidobacterium* counts were higher in women who gained less weight

during pregnancy (Collado *et al.*, 2008). When the gut ecosystems were investigated in the middle of the pregnancy period, overweight women had reduced amounts of *Bifidobacterium* and *Bacteroides* whereas the opposite was observed for *Staphylococcus* and *Enterobacteriaceae* (*E. coli* included). Moreover, *E. coli* numbers were higher in those with excessive weight gain, while *Bifidobacterium* and *Akkermansia muciniphila* showed the opposite trend (Santacruz *et al.*, 2010). Although we did not assess the maternal weight factors or maternal gut microbiota in paper **IV**, it should be noted that neonates born LGA had higher prevalence of *Enterobacteriaceae* and *E. coli*, assessed by clone library comparison and qPCR. Because of the higher prevalence of *Proteobacteria* in LGA and since maternal microbiota is known to be transferred to the newborn (Bettelheim *et al.*, 1974, Brook *et al.*, 1979), it is tempting to hypothesise that the mothers of LGA babies had a disturbed microbiota. This hypothesis can be supported by the knowledge that one woman in three is asymptomatic for BV (Allsworth and Peipert, 2007) and by the observation that babies to obese mothers had a changed microbiota compared to those of normal-weight mothers (Collado *et al.*, 2010), also in terms of *Proteobacteria* (Santacruz *et al.*, 2010).

The above findings together with the fact that obese women more often give birth to babies with higher birth weight (Collado *et al.*, 2008, Collado *et al.*, 2010, Santacruz *et al.*, 2010) should be acknowledged in the preventive care of pregnant women since the mother's characteristics seem to be transferred to the newborn. Since high birth weight increases the risk of future metabolic disturbances (Boney *et al.*, 2005, Harder *et al.*, 2009), improving the gut microbiota of pregnant women is attractive. One such intervention study involved dietary consultation and consumption of *L. rhamnosus* GG and *B. lactis* Bb12, and found glucose tolerance and insulin sensitivity to be improved during and after pregnancy although the gut microbial ecosystem was not investigated (Laitinen *et al.*, 2009).

The microbiota of diabetic humans

Along with microbial differences associated with obesity, patients with T2D are claimed to have an altered intestinal microbiota, although the overall bacterial diversity did not seem to differ compared to non-diabetic subjects with similar BMI (Larsen *et al.*, 2010, Wu *et al.*, 2010). Anyhow, the relative proportion of *Firmicutes* was lower while *Bacteroidetes* and *Proteobacteria* were higher in patient with T2D compared to non-diabetic counterparts. In addition, *Prevotella* and *Proteobacteria* positively correlated with plasma glucose whereas the proportion of *Roseburia* was negatively correlated (Larsen *et al.*, 2010). Patients with T2D were also shown to have reduced *Bifidobacterium* concentration whereas the enhanced abundance of *Bacteroides* and *Parabacteroides* compared to healthy individuals was confirmed in another study (Wu *et al.*, 2010). This can be reflected in the increased endotoxemia found among T2D patients (Creely *et al.*, 2007).

Dietary interventions in obese humans

In 2006, Ley *et al.* published the first study investigating the microbial composition upon weight loss in obese human subjects. When monitoring the faecal microbiota during one year, the relative proportion of *Bacteroidetes* decreased in obese compared to lean individuals and the *Bacteroidetes* proportion increased in correlation to increased weight

loss in people subjected to either a fat-restricted or a carbohydrate-restricted low-calorie diet. The opposite was shown for *Firmicutes*.

The finding that *Bacteroidetes* was associated with weight loss (Ley *et al.*, 2006, Nadal *et al.*, 2009, Santacruz *et al.*, 2009) could not be supported in a study where two different weight-loss diets were tested on obese humans, and there were no differences in *Bacteroidetes* prevalence between obese and non-obese subjects when using the FISH method (Duncan *et al.*, 2008). Nonetheless, the faecal microbiota of obese volunteers contained reduced amounts of the butyrate-producing *Roseburia* and *E. rectale* group (belonging to *Clostridium* cluster XIVa and the phylum *Firmicutes*) when the dietary amount of fermentable carbohydrates was reduced (Duncan *et al.*, 2008). Obese humans maintained on a diet high in carbohydrates harboured more of the butyrate-producing subgroup *Roseburia* spp. and *E. rectale,* which was accompanied by higher faecal butyrate concentration. Moreover, reduced amount of *Bifidobacterium* was observed when subjected to a diet with limited amount of carbohydrates. No changes in the relative proportion of *Bacteroidetes* or several of the clostridial clusters could be observed according to the dietary interventions (Duncan *et al.*, 2007).

Recently, Walker *et al.* (2011) showed that dietary changes strongly influence the microbial phylotypes present in the gut microbiota of obese volunteers. In accordance with other studies investigating lean as well as obese subjects, the same dominant phyla were present, namely *Firmicutes* and *Bacteroidetes*. The study by Walker and co-worker is the first to combine precise control of the human dietary intake with detailed investigation of changes in the gut microbiota at the level of bacterial phylotypes. By use of qPCR, DGGE and 16S rRNA clone libraries and careful data evaluation, they did not detect any significant differences in the proportion of *Bacteroidetes, Firmicutes, Actinobacteria* or *Proteobacteria* upon dietary regimen. No significant changes were observed when calculating Shannon and Simpson diversity indices. However, when looking into the lower taxonomic levels, they found rapid changes in the gut microbiota when the main type of ingested fermentable carbohydrates was shifted. The proportion of phylotypes related to *E. rectale* and *Ruminococcus bromii* (both belonging to *Firmicutes)* were increased upon higher dietary intake of resistant starch that could be converted to SCFA. It was also shown that the proportion of *Collinsella aerofaciens* (belonging to *Actinobacteria*) was decreased when subjects consumed a diet high in protein. The response was highly specific to the individual and the authors suggest that it largely depends on the initial composition of an individual's microbiota (Walker *et al.*, 2011).

In the farming industry, antibiotics modulating the microbial ecosystem have been used for growth promotion (Dibner and Richards, 2005). It has previously been mentioned that antibiotic consumption has long-lasting effects on the gut microbiota (Jernberg *et al.*, 2007, Jakobsson *et al.*, 2010). However, the longitudinal effects of short-term antibiotic treatment on body weight and metabolic parameters are poorly studied in humans. Probiotics that also modulate the microbiota have been blamed for contributing to increased body weight and the worldwide human obesity epidemic (Raoult, 2009). However, proof of concept is essentially lacking, and in fact the opposite has been shown. Recently, probiotic intervention with *L. gasseri* SBT2055 administration to overweight adults resulted in significantly decreased BMI and adiposity (abdominal, visceral and subcutaneous) and increased serum adiponectin (Kadooka *et al.*, 2010). At least in rats this was explained by reduced adipocyte size accompanied by inhibition of dietary fat absorption (Hamad *et al.*, 2009).

Summary of present investigations

Results presented and discussed in this thesis clearly indicate that the pioneer microbiota is important for health homeostasis later in life. For instance, reduced diversity of the intestinal microbiota at the age of one week was a precursor to the development of atopic eczema at the age of 18 months.

The microbiota of healthy neonates vaginally born was studied with the aim of investigating their pioneer microbiota. Few types or bacterial groups were shown to dominate the faecal microbiota of the neonates within 48 hours after birth. Mainly one type of bacteria dominated each neonate. There was a profound microbial difference between neonates born AGA and LGA. Neonates born AGA seemed to have a more diverse microbiota dominated by Gram-positive *Firmicutes* such as *Lactobacillus, Staphylococcus* and *Clostridium* whereas neonates born LGA were dominated by Gram-negatives, most often *E. coli* belonging to the phylum of *Proteobacteria*. All neonates had detectable levels of *Lactobacillus*, a bacterial group that is believed to be beneficial for the neonate since a healthy vagina is dominated by this genus. Although we did not evaluate the maternal microbiota, we assume a microbial transfer from the mother to the newborn, as indicated by the presence of lactobacilli in all neonates. Also typically vaginal *Lactobacillus* species were found. *Enterococcus, Bifidobacterium, Enterobacteriaceae* and *B. fragilis* group were found at various prevalences.

Obesity is associated with low-grade systemic inflammation (Fantuzzi, 2005) and since Gram-negative bacteria can exert pro-inflammatory activity through its LPS (Cani *et al.*, 2007a, Cani *et al.*, 2008) it can be assumed that Gram-negative bacteria such as *E. coli* can be a factor in the connection of obesity and inflammation. Discussion of obesity in newborns is inappropriate. Nevertheless, neonates born LGA were predominantly inoculated with Gram-negative bacteria. The intestinal microbiota is known to be influenced by the diet (De Filippo *et al.*, 2010, Walker *et al.*, 2011) and because of the microbial transfer from mother to child it was appealing to investigate the above hypothesis in an experimental situation.

In order to study the effect of maternal diet on offspring physiology, rat dams were given HEDD during the gestation and lactation period. Compared to standard chow diet, the suckling rat pups of dams consuming HEDD had increased adiposity along with increased systemic inflammation and decreased functional maturation of the gut. The effects were even more profound when rat dams were supplemented with *E. coli* along with HEDD, and the intestinal permeability was also increased.

In order to further explore the long-term impact of maternal microbial manipulation, a similar study design was applied, but in addition the offspring were challenged with the same bacteria until the age of six months. Exposure to *E. coli* from foetal life through adulthood was shown to decrease the diversity of the intestinal microbiota along with accentuated fat accumulation. In contrast, when rat dams were administered *L. plantarum* the body weight gain and fat accumulation were lower compared to animals consuming *E. coli*. Furthermore, viable count of caecal *Enterobacteriaceae* was lower and bacterial diversity was higher in the *L. plantarum* group, indicating a more favourable microbiota

and health homeostasis in animals consuming this particular Gram-positive bacteria. Results obtained with T-RFLP revealed significant differences in gut microbiota according to bacterial supplementation. The caecal microbiota of control rats and those consuming *E. coli* was characterised by the same dominant families while the *L. plantarum* group had additional bacterial families. An association between obesity and gut microbiota is now well established. However, the cause and effect and the details about bacterial groups connected to obesity remains controversial. Even if conflicting results have been published (Duncan *et al.*, 2008, Zhang *et al.*, 2009, Schwiertz *et al.*, 2010b), the original hypothesis postulates that the proportion of *Bacteroides* is decreased while *Firmicutes* is increased in obese subjects (Ley *et al.*, 2005, Ley *et al.*, 2006). However, this hypothesis could not be acknowledged in the present investigations since the proportion of *Bacteroidetes* was lower in both the rats consuming *E. coli* and in those consuming *L. plantarum* compared to control rats. The proportion of *Firmicutes* was not significantly different between the study groups. These results highlight the need to discuss the microbiota of lower hierarchy levels, preferably at genus and species level but at least on the level of family. Nevertheless, it can be concluded that microbial supplementation from foetal life through adulthood has longitudinal physiological effects in the outbred rat strain used in the present studies, and colonisation by the right kind of microbes early in life seems to be important in preventing diseases later in life.

Concluding remarks

The major conclusions from the findings of the studies presented in this thesis are:

- A microbial transfer from the mother to the newborn can be assumed since *Lactobacillus* species commonly found in the vagina were detected among the neonates. Bacteria of the *Lactobacillus* genus were found within 48 hours after birth in all healthy full-term vaginally-born neonates.

- The gut microbiota differed significantly depending on birth weight since a subgroup of neonates born LGA more often harboured *Proteobacteria*, preferentially *E. coli*, during their first 48 hours of life. The subgroup of neonates born AGA were more often colonised by species belonging to *Firmicutes*.

- Perturbation of the gut microbial ecosystem procursed later health homeostasis since reduced faecal bacterial diversity at one week of age was related to atopic eczema at the age of 18 months.

- Maternal diet and microbial manipulation affected offspring physiology. This was exemplified by enhanced body weight, fat accumulation and systemic inflammation in offspring of dams consuming HEDD during the gestation and lactation period. The effects were further accentuated if dams were exposed to *E. coli* CCUG29300^T in addition to the HEDD. Impaired gut barrier function in pups was related to the observed effects.

- The negative effects of HEDD feeding and *E. coli* CCUG29300^T consumption persisted when the treatment lasted from foetal life until six months of age, exemplified by for instance enhanced body weight gain, adiposity and higher caecal *Enterobacteriaceae* counts. In contrast, exposure to the probiotic *L. plantarum* DSM15313 from foetal life until adulthood showed lower body weight gain and adiposity along with a more diverse caecal bacterial flora.

- Molecular-genetic techniques are suitable for characterisation of the gut microbiota. The T-RFLP method should preferably be used for an overview of the dominating microbial community while qPCR is suitable for quantification of specific bacterial groups. Notably, the qPCR method assumes that the bacterial groups can be well ascertained and that relatively short sequences can define the bacterial groups. Cloning and sequencing makes it possible to reveal identities of the dominating bacterial taxa.

Taken together, results presented in this thesis suggest that a disturbed microbiota is a cause rather than a consequence of atopic eczema and obesity development.

Future perspectives

Since the gut microbiota plays an essential role in the interaction with the host, both immunologically and metabolically, it is of utmost importance to further explore this area. The relationship between the microbial ecology and its stimulation of the immune system, along with the connection to low-grade systemic inflammation and associated disorders, deserves further attention. Allergic manifestations and gut microbial dynamics needs further elucidation and although it has become clear that the gut microbiota is involved in obesity, far more work is needed before we can draw conclusions about the interrelationship between the microbial ecosystem and adiposity development. In particular, the microbiota should be considered in more detail, preferably at genus and species level but at least at the level of family. The difference in microbial ecosystem among various animal models and categories of humans should be elucidated and the maternal microbiota should be monitored more extensively. Future advances in both existing and new technologies should facilitate screening of large numbers of samples that can provide insights into the microbial composition and dynamics among populations.

Since the pioneer microbiota is important for future health homeostasis, the outcome of papers **I** and **IV** should be considered in the care of pregnant women and newborns. An interesting approach would be an intervention study where the microbiota of pregnant women is manipulated by probiotic consumption and physiological traits are measured in both women and babies to elucidate microbial transfer and effects on the newborn and as it grows older.

Furthermore, future studies should investigate the microbial succession from the initial microbiota to adulthood and connect it with metabolic features. It is of special interest to examine whether babies born LGA and with a microbiota dominated by specific Gram-negative bacteria, predict overweight or related metabolic and immunological disturbances later in life.

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