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PROBIOTIC INTERVENTIONS IN INFANCY:

Benefit and Safety Assessment of Extended Applications

by

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ABSTRACT

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PROBIOTIC INTERVENTIONS IN INFANCY: Benefit and Safety Assessment of Extended Applications

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Immaturity of the gut barrier system in the newborn has been seen to underlie a number of chronic diseases originating in infancy and manifesting later in life. The gut microbiota and breast milk provide the most important maturing signals for the gut-related immune system and reinforcement of the gut mucosal barrier function. Recently, the composition of the gut microbiota has been proposed to be instrumental in control of host body weight and metabolism as well as the inflammatory state characterizing overweight and obesity. On this basis, inflammatory Western lifestyle diseases, including overweight development, may represent a potential target for probiotic interventions beyond the well documented clinical applications.

The purpose of the present undertaking was to study the efficacy and safety of perinatal probiotic intervention. The material comprised two ongoing, prospective, double-blind NAMI (Nutrition, Allergy, Mucosal immunology and Intestinal microbiota) probiotic interventions. In the mother-infant nutrition and probiotic study altogether 256 women were randomized at their first trimester of pregnancy into a dietary intervention and a control group. The intervention group received intensive dietary counselling provided by a nutritionist, and were further randomized at baseline, double-blind, to receive probiotics (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis*) or placebo. The intervention period extended from the first trimester of pregnancy to the end of exclusive breastfeeding. In the allergy prevention study altogether 159 women were randomized, double-blind, to receive probiotics (*Lactobacillus rhamnosus* GG) or placebo 4 weeks before expected delivery, the intervention extending for 6 months postnatally. Additionally, patient data on all premature infants with very low birth weight (VLBW) treated in the Department of Paediatrics, Turku University Hospital, during the years 1997 - 2008 were utilized.

The perinatal probiotic intervention reduced the risk of gestational diabetes mellitus (GDM) in the mothers and perinatal dietary counselling reduced that of fetal overgrowth in GDM-affected pregnancies. Early gut microbiota modulation with probiotics modified the growth pattern of the child by restraining excessive weight gain during the first years of life. The colostrum adiponectin concentration was demonstrated to be dependent on maternal diet and nutritional status during pregnancy. It was also higher in the colostrum received by normal-weight compared to overweight children at the age of 10 years. The early perinatal probiotic intervention and the postnatal probiotic intervention in VLBW infants were shown to be safe.

To conclude, the findings in this study provided clinical evidence supporting the involvement of the initial microbial and nutritional environment in metabolic programming of the child. The manipulation of early gut microbial communities with probiotics might offer an applicable strategy to impact individual energy homeostasis and thus to prevent excessive body-weight gain. The results add weight to the hypothesis that interventions aiming to prevent obesity and its metabolic consequences later in life should be initiated as early as during the perinatal period.

Keywords: adiponectin; bifidobacteria; breast milk; colostrum; gut microbiota; probiotics; obesity; overweight

TIIVISTELMÄ

Raakel Luoto

Varhaislapsuuden probiootti-interventiot: uudenlaisten käyttöaiheiden hyödyt ja turvallisuus

Lastentautioppi, Turun Yliopisto
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Vastasyntyneen suolistoesteen kypsymättömyys voi altistaa sairauksille, jotka ilmenevät vasta myöhemmällä iällä. Suoliston mikrobisto ja rintamaito ovat tärkeimmät vastasyntyneen suoliston immuniteettia ja suolistoesteen kypsymistä tukevat tekijät. Suoliston mikrobikoostumuksen on osoitettu vaikuttavan sekä isäntänsä energiatasapainoon että ylipainoon ja lihavuuteen liittyvään matala-asteiseen tulehdustilaan. Tämän perusteella tulehdukselliset länsimaiset taudit, mukaan lukien lihavuus, voisivat olla uudenlaisten probiootti-interventioiden kohde jo tunnettujen kliinisten käyttötarkoitusten lisäksi.

Tämän väitöskirjatyön tarkoituksena oli tutkia perinataalisen probiootti-intervention vaikutusta ja turvallisuutta. Väitöskirjan aineisto koostui kahdesta Turun Yliopistollisen Keskussairaalan Lastenklinikan NAMI-projektin prospektiivisesta, kaksoissokkoutetusta probiootti-interventiosta. Äiti-lapsi-ravitsemus- ja probioottitutkimuksessa 256 raskaana olevaa naista satunnaistettiin ensimmäisen raskauskolmanneksen aikana ravitsemus- ja kontrolliryhmiin. Ravitsemusryhmälle annettiin intensiivistä tehostettua ravitsemusneuvontaa, ja ryhmä satunnaistettiin alkuvaiheessa kaksoissokkoutetusti probiootti- (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis*) ja lumeryhmiin. Interventio jatkui täysimetyksen ajan, ei kuitenkaan yli kuutta kuukautta lapsen syntymästä. Allergian ehkäisytutkimuksessa 159 raskaana olevaa naista satunnaistettiin probiootti- (*Lactobacillus rhamnosus* GG) ja lumeryhmiin 4 viikkoa ennen laskettua aikaa. Interventio jatkui 6 kuukauden ajan. Lisäksi tutkittiin Turun Yliopistollisen Keskussairaalan Lastenklinikassa vuosien 1997-2008 aikana hoidettujen pikkukeskosten potilastiedot probioottiprofylaksin turvallisuuden selvittämiseksi.

Tämän väitöskirjan tulokset osoittivat, että perinataalinen probiootti-interventio vähensi raskausdiabeteksen esiintyvyyttä ja perinataalinen ravitsemusneuvonta hillitsi sikiön ylenmääräistä kasvua raskausdiabeteksen komplisoimissa raskauksissa. Varhaisvaiheen suolistomikrobiston muokkaus probiooteilla hillitsi lasten ylipainon kehittymistä ensimmäisten elinvuosien aikana. Äidin ternimaidon adiponektiinipitoisuuden osoitettiin riippuvan äidin raskaudenaikaisesta ravitsemuksesta ja ravitsemustilasta. Lisäksi ylipainoisten lasten äitien ternimaidon adiponektiinipitoisuuden havaittiin olleen pienempi kuin äitien, joiden lapset olivat normaalipainoisia 10-vuotiaina. Tulokset osoittivat myös probiootti-intervention olevan turvallinen sekä varhain perinataalisesti että pikkukeskosille postnataalisesti annettuna.

Tämän väitöskirjan tulokset tuottivat uutta kliinistä tietoa varhaisvaiheen suolistomikrobiston ja ternimaidon adiponektiinipitoisuuden yhteydestä lapsen metaboliseen ohjelmoitumiseen. Varhaisen suolistomikrobiston muokkaus probiooteilla voisi tarjota uudenlaisen mahdollisuuden vaikuttaa yksilön energiatasapainoon ja siten ehkäistä ylipainon kehittymistä. Tulokset tukevat hypoteesia, jonka mukaan ylipainon ehkäisemiseen pyrkivät toimenpiteet tulisi aloittaa jo perinataaliaikana.

Avainsanat: adiponektiini; bifidobakteerit; lihavuus; probiootit; rintamaito; suolistomikrobisto; ternimaito; ylipaino

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ABBREVIATIONS

AMPK	5’ Adenosine monophosphate-activated protein kinase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Antigen-presenting cell
BMI	Body mass index
CD14	Innate microbial receptor CD14
CFU	Colony-forming unit
CI	Confidence interval
DC	Dentritic cell
FIAF	Fasting-induced adipose factor
FISH	Fluorescent in situ hybridization
GALT	Gut-associated lymphoid tissue
GDM	Gestational diabetes mellitus
GPR	G protein-coupled receptor
hsCRP	High sensitive C-reactive protein
IFN-γ	Interferon gamma
IL	Interleukin
LBP	Lipopolysaccharide-binding protein
LGG	*Lactobacillus Rhamnosus* GG
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAMP	Microbe-associated molecular pattern
M cell	Microfold cell
NAMI	Nutrition, Allergy, Mucosal immunology and Intestinal microbiota project
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
NF-κB	Nuclear factor kappa B
OGTT	Oral glucose tolerance test
OR	Odds ratio
PRR	Pattern recognition receptor

PUFA	Polyunsaturated fatty acids
SCFA	Short-chain fatty acids
SD	Standard deviation
SFA	Saturated fatty acids
sCD14	Soluble innate microbial receptor CD14
sIgA	Secretory IgA
TGF-β	Transforming growth factor beta
Th	Helper T cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alfa
T_{reg}	Regulatory T cell
VLBW	Very-low-birth-weight
VON	Vermont Oxford Network

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I – V. The original communications have been reproduced with permission from copyright holders.

I Raakel Luoto, Kirsi Laitinen, Merja Nermes, Erika Isolauri. Impact of maternal probiotic-supplemented dietary counselling on pregnancy outcome and prenatal and postnatal growth: a double-blind, placebo-controlled study. *British Journal of Nutrition 2010;103:1792-9.*

II Raakel Luoto, Kirsi Laitinen, Merja Nermes, Erika Isolauri. Impact of maternal probiotic-supplemented dietary counselling during pregnancy on colostrum adiponectin concentration: a prospective, randomized, placebo-controlled study. *(Submitted)*

III Raakel Luoto, Marko Kalliomäki, Kirsi Laitinen, Erika Isolauri. The impact of perinatal probiotic intervention on the development of overweight and obesity: follow-up study from birth to 10 years. *International Journal of Obesity (Lond) 2010 Mar 16. [Epub ahead of print]*

IV Raakel Luoto, Marko Kalliomäki, Kirsi Laitinen, Nathalie M. Delzenne, Patrice D. Cani, Seppo Salminen, Erika Isolauri. Initial dietary and microbiological environments deviate in normal-weight compared to overweight children at 10 years of age. *Journal of Pediatric Gastroenterology and Nutrition (in press)*

V Raakel Luoto, Erika Isolauri, Liisa Lehtonen. Safety of *Lactobacillus* GG probiotic in infants with very low birth weight: twelve years of experience. *Clinical Infectious Diseases 2010;50:1327-8*.

1. INTRODUCTION

The term ´probiotic´ was originally proposed in 1965 to denote any microbial organism or substance contributing to the intestinal microbial balance (Lilly and Stillwell 1965). According to the current definition adopted by FAO (2009) and WHO (2001, 2002), probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". The most commonly used probiotics to date are lactic acid bacteria, including lactobacilli and bifidobacteria, but other non-pathogenic bacterial strains (*Streptococcus, Eschericia Coli*) and non-bacterial organisms such as yeasts (*Saccharomyces boulardii*) have also been used (Guarner and Schaafsma 1998). The first scientific reports on the beneficial effects of lactobacilli and bifidobacteria on human health were, however, already published a century ago. The French scientist, Tissier, recommended large doses of bifidobacteria for the treatment of infantile diarrhea (Tissier 1906), and Metchnikoff, a Nobel laureate working at the Pasteur Institute, suggested that putrefactive bacteria contribute to various disease processes and that the regular consumption of lactic acid bacteria might help to improve health and increase longevity (Metchnikoff 1907).

The accumulating evidence suggests that deviations in the gastrointestinal microbiota may act as underlying prerequisites for several human diseases. At the same time, preventive and therapeutic manipulation of microbial communities with probiotics has been reported to have the potential to ameliorate such conditions. Nevertheless, the use of probiotics as a therapeutic approach has led to consensus statements only for the following entities: prevention and treatment of acute gastroenteritis and antibiotic-associated diarrhea has been demonstrated with *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus* GG (LGG), *Saccharomyces boulardii*, and a mixture of *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Streptococcus thermophilus* and *Bifidobacterium bifidum* (Wolwers et al. 2010). A specific probiotic strain combination (VSL#3) has been proved to be clinically effective in maintaining remission in patients with ulcerative colitis, and a multispecies probiotic mixture of 8 strains in the maintenance of remission in pouchitis (Haller et al. 2010). A recommendation has also been given for probiotics LGG and *Bifidobacterium lactis* in the treatment and prevention of atopic eczema associated with cow´s milk allergy (Floch et al. 2008, Kalliomäki et al. 2010).

Results from clinical trials, as further evidenced by a number of recent meta-analyses and systematic reviews, suggest that specific probiotics might also be useful in risk reduction in other inflammatory diseases related to the disruption of the intestinal barrier, loss of immune tolerance to the gut microbiota and the consequent inappropriate inflammatory responses. One of these new extended potential applications for probiotics

is in overweight development and obesity-related metabolic abnormalities (Isolauri et al. 2009). The rationale for this linkage is based on a recent hypothesis which envisages the gut microbiota as instrumental in the control of energy metabolism and consequently also body weight (Bäckhed et al. 2004), the ´hygiene hypothesis´, which conceives the growing epidemic of autoimmune and allergic diseases to be related to reduced exposure to microbes at an early age as a result of improved hygiene conditions in the industrialized world (Bach 2002), and the notion that similar environmental influences appear to underlie the increasing prevalence of obesity and allergic diseases, i.e. a low-grade systemic inflammation (Weiss 2005).

2. REVIEW OF THE LITERATURE

The two last decades have provided a vast array of evidence indicating that the gut microbiota provides the host with essential genetic and metabolic attributes (Bäckhed et al. 2005). In the gut itself this includes nutrient and drug metabolism, synthesis and bioavailability of several vitamins, epithelial cell proliferation and the immune system and gut barrier function against enteric pathogens (Jia et al. 2008, Neish 2009, Round and Mazmanian 2009).

2.1. The gut barrier – a target for probiotic action

2.1.1. Intestinal epithelial barrier

The intestine forms the most extensive mucosal surface area in the human body, the epithelial layer being as large as 400 m^2, comparable to the area of a tennis court (Artis 2008). The epithelial layer acts as a selectively permeable barrier, permitting the absorption of nutrients, electrolytes and water while maintaining an effective defense against intraluminal and some environmental toxins, antigens and enteric microbiota (Baumgart and Dignass 2002). Indeed, a single epithelium layer is all that separates the commensal micro-organisms, pathogens and food-derived antigens from the underlying immune cells, the epithelial barrier thus functioning as a key component in the human defence mechanisms required to control antigen transfer and consequent immune responses (Sanderson and Walker 1993). In order to protect itself from uncontrolled inflammatory responses, the epithelium has developed mechanisms to restrain bacterial growth, limit direct contact with bacteria and other foreign antigens, and prevent antigen dissemination into the underlying tissue (Brandtzaeg 2009).

The intestinal epithelial barrier defences consist of the epithelial junctional adhesion complex, the mucous layer, and antimicrobial factors (Figure 1). The intestinal epithelium is anatomically a single layer of densely packed enterocytes along the villous axis of the crypt, with tight intercellular junctions preventing leakage through this layer (Ruemmele et al. 2009). Fluxes through the intestinal epithelium proceed either *via* the transcellular route, with specific membrane pumps and channels, or *via* a paracellular route controlled by tight junctions (Baumgart and Dignass 2002). The epithelial cells, in addition to constituting a structural barrier, manufacture most components of the secreted barrier, sensor the external environment, and provide signals for underlying innate and adaptive immunity (McGuckin et al. 2009). The secreted mucus barrier provides both a physical and a chemical barrier against microbes, as well as keeps the mucosal surface

well hydrated and provides lubrication, allowing the continuous flow of luminal contents (McGuckin et al. 2009). The most important defence mechanism in the epithelial barrier is, however, polymeric (p)IgA, which is present at high concentrations in the intestinal mucus layer. IgA enables the capture of a large array of antigens in the intestinal lumen and inhibits mucosal invasion and penetration of pathogens (Brandtzaeg 2009). The array of defence factors is constituted additionally of endogenous antimicrobial molecules produced and secreted by epithelial cells, for example α- and β-defensins, lysozyme, cathelicidins and chemokines (Ruemmele et al. 2009).

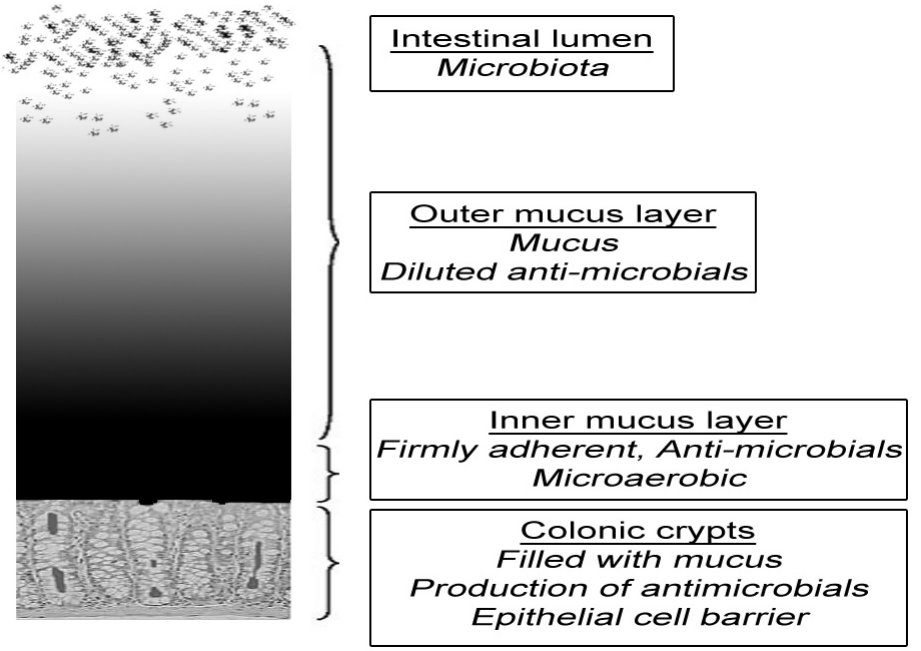

Figure 1. Diagrammatic representation of the intestinal mucosal barrier showing the thickness of the secreted mucus barrier and the relationship between the luminal microbiota and the epithelium. Modified from McGuckin et al. 2009.

2.1.2. Intestinal mucosal immune system

The importance of the intestine as an immune organ is mirrored in the fact that the intestinal immune system hosts more immune cells than all other immune-associated organs of the body together (Brandtzaeg et al. 1989). The intestinal immune system is in fact the sum of physical barriers, chemical barriers and reactive elements of local immune cells and cells recruited to a threatened site (van Elburg et al. 1992). This unique gut-associated immune system has probably evolved under selective pressure from dietary and commensal antigens, the aim being a tolerogenic and

symbiotic relationship with them. The anti-inflammatory strategies of the mucosal immune system are two-fold: immune exclusion effected by secretory (s)IgA to control epithelial colonization of microorganisms and to inhibit the penetration of potentially dangerous agents; and immunosuppression to avoid local and peripheral hypersensitivity to innocuous antigens which have trespassed across the epithelial barrier (Brandtzaeg 1996).

Intestinal immune cells are located in three compartments: in organized gut-associated lymphoid tissue (GALT), in the connective tissue layer (i.e. lamina propria), and in the surface epithelium. GALT, which comprises Peyer´s patches, the appendix, and numerous isolated lymphoid follicles, is the main inductive and regulatory site for intestinal immune responses (Neutra et al. 2001). The GALT structure resembles lymph nodes with inter-follicular T-cell zones and a variety of antigen-presenting cells (APCs) such as macrophages, microfold (M) cells and dentritic cells (DCs), but they are not encapsulated (Neutra et al. 2001). In response to microbial or other luminal antigen components, gut epithelial cells release chemokines which recruit immune cells, in particular DCs, into the gut mucosa (Sierro et al. 2001). The antigens, sampled directly from the mucosal surface (Figure 2), are presented to B and T cells in the adaptive immune system in mesenteric lymphoid follicles or alternatively within GALT after intracellular processing (Kraehenbuhl and Corbett 2004). Antigen presentation induces cytokine release from helper T (Th) cells. Th1-type cells produce interleukin (IL)-2 and interferon (IFN)-γ and promote cell-mediated immunity (Kidd 2003). Th2-type cells produce IL-4, IL-5, IL-6, and IL-13, thus activating the antibody-mediated immunity (Kidd 2003). Th3-type cells downregulate inflammatory responses through transforming growth factor (TGF)-β production (Izcue and Powrie 2008) and regulatory T (T_{reg}) cells alleviate the immune responses through IL-10 production (Wu et al. 2007). Especially the TGF-β response is of prime importance in inducing the switch of naïve plasma cells into IgA-producing B cells in GALT follicles (Macpherson and Uhr 2004). Alternatively, antigenic stimulation within GALT may induce the migration of IgA lymphoblasts to the lamina propria, where they differentiate into plasma cells and secrete IgA (Kraehenbuhl and Corbett 2004).

The production of appropriate amounts of specific cytokines is essential in response to antigen stimulus, since an inappropriate or overwhelming cytokine response may lead to a condition characterized by acute or chronic inflammation. It has also recently been demonstrated that the intestinal immune responses appear to pass beyond the intestine and have been shown to elicit systemic immunomodulatory effects, this substantiating the conception of a broader role for the intestinal immune system as a major modulator of systemic immunity (Clarke et al. 2010).

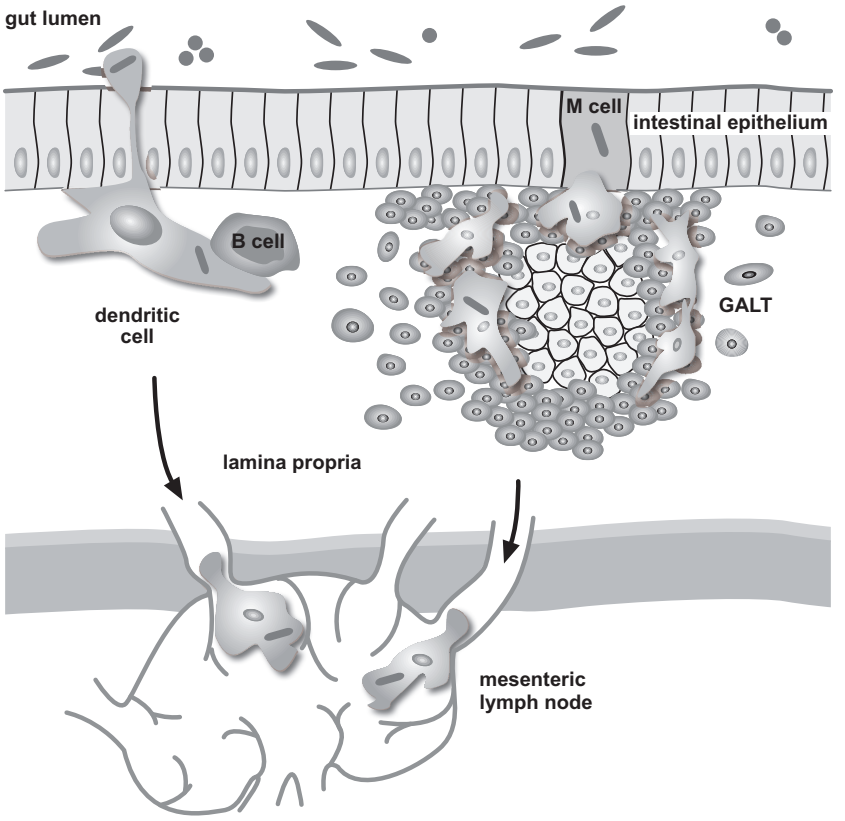

Figure 2. DC-mediated transport of antigens in the gut. Antigens are sampled by cells of the innate immune system, e.g. DCs and M cells. M cells in the gut epithelium may import antigens into the dome region of the GALT where DCs engulf them. Alternatively, DCs may sample antigens directly through the tight junctions between epithelial cells. DCs present antigens from captured microbes to B and T lymphocytes either locally in the GALT, or within the mesenteric lymph nodes. Presentation of microbial antigens to B cells triggers production of a commensal-specific IgA response. Modified from Kraehlenbuhl and Corbett 2004.

2.1.3. Immaturity of the gut barrier system

During a variable period after birth the gut barrier system is functionally immature, lacking many specific and nonspecific intestinal features necessary to protect it from environmental antigens (Walker 1988). The development of the gut barrier in the early extrauterine setting is dependent on external stimuli, the indigenous intestinal microbiota (Cebra 1999) and breast milk (Newburg 1999) being the most important sources of key regulatory signals. The immaturity of the barrier lays the host open to aberrant exposure to luminal antigens and bacteria. Further, the immaturity of mucosal barrier integrity and function and the intestinal immune system is implicated not only in acute illnesses such as bacterial translocation leading to life-threatening conditions such as sepsis, but also in

a number of chronic diseases having their origins during infancy and manifesting later. These include allergic diseases, inflammatory bowel diseases and celiac disease as well as presumably also overweight development (Vael and Desager 2009).

Both types of epithelial barrier mechanisms, non-immunologic and immunologic, are not completely functionally developed at birth (van Elburg et al. 1992). In the immature gut of the newborn, the sIgA is generally undetectable in the gut mucosa before 10 days of age and secretory (s)IgM antibodies are as a corollary of greater importance in the immune defence. Deficiency of sIgA predisposes the newborn to increased binding of antigens to the gut mucosal membrane, to aberrant mucosal leakiness and an increased uptake of commensal bacteria and dietary antigens (Johansen et al. 1999). The immature enterocytes may respond to these antigen challenges with an overactivation of the cellular signaling pathway and expression of proinflammatory cytokines, leading to a generalized hyperreactive state (Karlsson et al. 2010). Furthermore, the epithelial tightness and the immunoregulatory network remain fragile for a variable period after birth, possible remaining inadequate up to 2 years of age (Holt and Jones 2000). The mechanisms involved in this immaturity of epithelial barrier function remain poorly defined, but the development of secretory immunity is probably a decisive factor (van Elburg et al. 1992).

Although the structural and cellular components of the mucosal immune system are competent at least during the third trimester of pregnancy, some time elapses before they become activated (Holt and Jones 2000). The major feature of developmental immaturity in human intestinal immunity is a propensity to respond to antigen stimuli by mounting an overwhelming inflammatory response (Nanthakumar et al. 2000). The main reason is deficient APC function (Ridge et al. 1996), since the APCs need to be activated by microbial factors which enable them to provide appropriate co-stimulatory signals to naïve T cells to generate IgA-producing cells (Siegrist and Aspinall 2009). Given the fundamental role of APCs in determining whether mucosal contact with antigens results in a tolerogenic, local protective immune response or a systemic inflammatory reaction is to ensue, it is surprising that the immune responses during the early postnatal period are as controlled as they appear to be (Rautava and Walker 2007).

2.1.4. Impact of gut microbiota succession on gut barrier function

The gastrointestinal tract of the human adult harbors ~ 1 x 10^{14} (ten times more than the total number of cells in the human body) bacteria from more than 500 identified culturable and a large number of unculturable species, this antigen load thus vastly outnumbering the host cells (Ley et al. 2008). Recent reports suggest that 80-90% of the bacterial phylotypes are members of two *phyla*: the Bacteroidetes (e.g. *Bacteroides* and *Prevotella*) and the Firmicutes (e.g. *Clostridium*, *Enterococcus*, *Lactobacillus*, *Rumincoccus*), followed by the

Actinobacteria (e.g. *Bifidobacterium*) and the Proteobacteria (e.g. *Helicobacter, Escherichia*) (Eckburg et al. 2005). During the birth process and soon thereafter bacterial colonization of the hitherto germ-free infant intestinal tract takes place (Mackie et al. 1999). During the first few days of life the microbial colonization of the human intestine is a rapid and varying process which in physiological circumstances results in a symbiotic relationship with the host likely to maintain health and homeostasis (Hooper and Gordon 2001).

The initial colonization of the intestine takes place during delivery and within the first few hours from birth, when it comes in contact with the vaginal and intestinal microbiota of the mother (Mackie et al. 1999). Vaginally born infants and infants born by cesarean section evince differences in the composition of early intestinal microbiota, the method of delivery thus influencing as a corollary also the maturation of the humoral immune system (Grönlund et al. 2000). Thereafter, colonization is further modulated not only by environmental bacteria but also by early breast milk, colostrum, which contains live microbes, especially bifidobacteria and lactic acid bacteria, and a diverse spectrum of bioactive substances. Such substances participate actively in modulating the infant gut microbiota composition and activity. They also influence gut barrier and mucosal immune system priming and maturation. Intestinal colonization, a substantial antigen challenge for the newborn, is essential for the maturation of the gut-associated lymphoid tissue and for the developmental regulation of the intestinal physiology, especially of the epithelial barrier (Brandtzaeg 1998, Grönlund et al. 2000). Moreover, on molecular levels, members of the intestinal microbiota have been shown to possess a significant capacity to modulate the expression of a large number of host genes involved not only in mucosal barrier function but also in a variety of other intestinal functions, including nutrient absorption, metabolism, angiogenesis and intestinal maturation (Hooper and Gordon 2001). *Bacteroides* and *Escherichia coli* strains seem to be of particular importance in immune stimulation, but also lactic acid-producing bacteria contribute conspicuously to the maturation process (Moreau et al. 1978). The activation of sIgA-producing plasma cells in the human neonatal intestine is known to be dependent on the colonization of the gut by *Bifidobacteria* and *Lactobacilli,* stimulated by fermentation of nondigestible oligosaccharides in breast milk (Hyslop et al. 1974).

The bacterial interactions with the newborn during initial colonization are mediated largely *via* pattern recognition receptors (PRRs) expressed by the gut epithelium, particularly Toll-like receptors (TLRs) and their conserved microbial ligands, microbe-associated molecular patterns (MAMPs) (Artis 2008). The TLR most intensively studied is TLR-4, whose primary ligand is lipopolysaccharide (LPS), i.e. endotoxin, a surface molecule of gram-negative bacteria. TLR-4 interacts with LPS after an LPS – LPS-binding protein (LBP) complex is anchored to the cell surface by a surface molecule, the serum-soluble innate microbial receptor CD14 (Round and Mazmanian 2009). In general, the stimulation

of TLRs results in the activation of adaptor proteins, which event initiates a signalling cascade involving several kinases (Neish 2009). Eventually, the signals are transmitted via transcription factor nuclear factor κ B (NF-κB), which then initiates the transcription of genes leading to the synthesis of immunomodulatory molecules such as cytokines, chemokines and other inflammatory effector molecules, the aim being to generate immune responses against invading pathogens confined to the intestinal mucosa without systemic inflammatory reactions (Nenci et al. 2007). The activation of TLRs not only modulates the activation profiles of innate and adaptive immune cells but has also been demonstrated to participate directly in the epithelial barrier function (Lotz et al. 2006).

Taken together, the gut barrier-associated homeostasis during colonization depends on the ´cross-talk´ between the commensal bacteria, lamina propria cells including macrophages, DCs and T cells, and the epithelium *via* cytokines and other costimulatory molecules. The interaction of MAMPs with PRRs on enterocytes and intestinal lymphoid cells orchestrates the intestine´s capacity to mount an acute immunologic response to invading pathogens while at the same time maintaining intestinal homeostasis, thus averting chronic inflammatory responses (Walker 2009). Indeed, the significance of this host-microbiome interaction culminates at an early age, during the period when the gut barrier and intestinal immune system are still immature and the child´s immunological and metabolic phenotypes are still being consolidated (Abt and Artis 2009).

2.2. Dietary compounds interacting with the gut barrier function

Breast milk, specifically early breast-milk, colostrum, not only provides the infant with nutrients for growth and development, but also serves as an important source of factors which confer immune protection upon the infant and enhance the healthy maturation of both the non-immunologic and immunologic gut barrier system (Brandtzaeg 2010). The gut barrier-strengthening mechanisms provided by maternal breast milk can be grouped into three main categories exerting anti-microbial, anti-inflammatory and immune-modulatory effects.

The anti-microbial protection afforded by breast milk has been largely attributed to the presence of sIgA (Brandtzaeg 1998). In addition to the sIgA antibodies directed against microbes and antigens in the mother´s environment, the maternal sIgA provided by breast milk prevents attachment and invasion of pathogens in the infant´s intestine by competitively binding and neutralizing bacterial antigens (Corthésy 2007, Cruz et al. 1999). Furthermore, the shielding effect of maternal sIgA on the GALT of the breast-fed infant may contribute to systemic-type hyporesponsiveness to commensal bacteria and dietary antigens (Brandtzaeg 1998). Human breast milk also contains an array of other components evincing anti-microbial activity, including complex carbohydrates,

glycoproteins, glycolipids, glycosaminoglycans, mucins and oligosaccharides, these latter including sialylated galacto-oligosaccharides (Rautava and Walker 2009).

The indigestible oligosaccharides in breast milk serve as a passive protective function for the gut barrier and they also actively stimulate proliferation of the colonizing bacteria necessary to activate the infant´s intestinal defence mechanisms and maturation (Newburg 1999). Oligosaccharides can act as analogs to inhibit pathogen attachment to glycoconjugates on the intestinal surface (Newburg and Walker 2007). Moreover, they modulate the compositional development of the infant´s gut microbiota by promoting the growth of Bifidobacteria, which *per se* reduces the pathogenic potential of bacteria in the gut. Breast milk is in fact also a natural source of live Bifidobacteria (Gueimonde et al. 2006c), which explains the difference between the intestinal microbiota of breast-fed and formula-fed infants, Bifidobacteria comprising the predominant bacteria in full-term, breast-fed infants as early as 3-6 days of age (Favier et al. 2002). Additionally, breast milk contains a range of bacterial DNA signatures derived from maternal intestinal microbiota, these participating in immune system priming and regulation (Perez et al. 2007).

On this basis, modulation of infant formulas with prebiotic oligosaccharides has attracted scientific and commercial interest. The term prebiotic was first introduced in 1995 (Gibson and Roberfroid 1995, Gibson et al. 2004) and is now defined by the FAO (Pineiro et al. 2008) as "a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota".

Specific prebiotics, i.e. complex carbohydrates, may enhance the intestinal colonization, survival and function of probiotic organisms, the impact being limited, however, to specific classes of microbes already resident in the gastrointestinal tract (Preidis and Versalovic 2009). When fermented by gut bacteria to short-chain fatty acids (SCFAs), prebiotics lower luminal pH, thus creating an inhospitable environment for pathogens and stimulating mucin production. The SCFAs, among them butyrate, in addition to their anti-inflammatory properties (Tedelind et al. 2007), also contribute to the stimulation of GALT immune cells by direct interactions and induction of epithelial cell production of stimulatory cytokines and chemokines (Sakata 1987) and to gut barrier maturation in enhancing epithelial mucus expression and increasing the growth of intestinal epithelial cells (Willemsen et al. 2003). Recently, breast milk-derived polyunsaturated fatty acids (PUFA) have also been shown to support intestinal epithelial barrier integrity by improving resistance and reducing IL-4 mediated permeability (Willemsen et al. 2008). PUFAs may also influence probiotic surface structures and have an impact on adhesion and colonization (Kankaanpää et al. 2001). Hence, over and above the importance of both SCFA and PUFA in immune development, these components may support gut barrier closure, thus providing protection against infections and inflammatory diseases.

Moreover, breast milk-derived soluble receptors are also vital in linking dietary fatty acids with the innate immune system, further strengthening the host-microbe cross-talk beneficial for inflammation control (Laitinen, et al. 2006).

One particularly important property of breast milk is its capacity to down-regulate the immature, excessive inflammatory responses in newborns predisposed to a variety of stimuli until the infant can develop its own mature anti-inflammatory mechanisms. Lactoferrin has been demonstrated to inhibit LPS-induced proinflammatory cytokine release from human monocytic cells (Haversen et al. 2002). Erythropoietin, at concentrations found in breast milk, has been shown to reduce the excessive inflammatory response (IL-8) when incubated with a fetal human enterocyte cell line (Claud et al. 2003), a result which was repeated in another study made with breast milk hydrocortisone (Meng et al. 2007). The breast milk TGF-β and IL-10 might further contribute to immunological homeostasis, not only by merit of their immune-suppressive and anti-inflammatory effects, but also in promoting synergistically mucosal IgA induction (Field 2005). Additionally, a direct enhancing effect on the epithelial barrier has further been reported for TGF-β (Planchon et al. 1994).

Certain breast milk bioactive substances are considered to be mainly immune-modulatory, although some of them have been shown to exert multiple influences in immature enterocytes. One of these constituents is CD14, a soluble component of TLR-4 and a co-receptor for LPS, which regulates immune responses against Gram-negative bacteria. Soluble (s)CD14 is found in substantial quantities in breastmilk, especially in colostrum, and allows the immature intestine to respond if necessary to pathogen invasion (Labeta et al. 2002). Additionally, breast milk contains cytokines, cytokine receptors, chemokines, growth factors and functional maternal immune cells, these all participating in immune response modulation and intestinal maturation in the infant.

2.3. Breast milk adiponectin

One of the most recently discovered bioactive substances in breast milk is adiponectin, a most abundant circulating human cytokine secreted mainly by the adipose tissue (Scherer et al. 1995). The serum concentrations of adiponectin, unlike most of the other adipocytokines, are inversely correlated with the body mass index (BMI) and, most importantly, with visceral fat accumulation. Hence, circulating adiponectin concentrations are reduced in obesity and non-insulin-dependent diabetes (Arita et al. 1999). Adiponectin is an intriguing hormone, since it is known to modulate metabolic and inflammatory processes and to contribute to the pathophysiology of obesity-linked diseases. The health-ameliorating properties of adiponectin have been demonstrated

experimentally in mice lacking adiponectin, the animals subsequently developing insulin resistance, glucose intolerance, hyperglycemia and hypertension (Maeda et al. 2002).

Adiponectin has been shown to enhance insulin sensitivity by stimulating glucose utilization and fatty acid oxidation in muscle and liver (Berg et al. 2002) and to improve glucose tolerance by reducing gluconeogenesis in the liver (Combs et al. 2001). Furthermore, adiponectin exerts anti-inflammatory effects *via* its potential to counteract the expression of proinflammatory cytokines in adipocytes and macrophages (Wulster-Radcliffe et al. 2004). In macrophages and other monocyte-derived cells, adiponectin increases the expression of the anti-inflammatory cytokines (IL-10) and reduces that of pro-inflammatory cytokines (IL-6, Tumor necrosis factor (TNF)α and IFNγ) *via* inhibition of the NF-κB pathway (Ajuwon and Spurlock 2005). Adiponectin also acts as a negative regulator of CD14 / TLR-4 -mediated signalling (Yamaguchi et al. 2005), this being the main signalling channel for innate immunity which intestinal microbes (Rakoff-Nahoum et al. 2004) and dietary fatty acids (Laitinen et al. 2006) also engage. The anti-inflammatory action of adiponectin could, however, also be indirect; a tolerogenic response has been shown to accompany sustained exposure to LPS or adiponectin itself (Tsatsanis et al. 2005). Accumulating evidence would also suggest that adiponectin, apart from its peripheral actions, regulates energy balance centrally through its receptor in the hypothalamus (Kubota et al. 2007).

Groups under Martin (2006) and Bronsky (2006) were the first to report that adiponectin is also found in human breast milk. Its concentration was seen to decrease with the duration of lactation (Martin et al. 2006). Although adiponectin concentrations are significantly lower in breast milk than in maternal serum, Weyermann and associates (2006) showed that the adiponectin concentrations in breast milk at 6 weeks and 6 months after delivery correlated moderately with the concentration in maternal serum. This demonstration provided evidence that mammary epithelial cells are able to transfer adiponectin from the blood or to synthesize it. On the basis of the pleiotropic biological properties of adiponectin and its presence in breast milk, it has been hypothesized that human milk adiponectin may be biologically relevant and presumably involved in infant metabolic programming (Savino et al. 2008). Moreover, it is also possible that adiponectin confers its anti-inflammatory effects at a local level, in the intestine, where it might act to suppress inflammatory responses, thus participating in gut barrier maturation (Newburg et al. 2010).

2.4. Probiotic mechanisms and current clinical applications

The instrumental role of the compositional development of the gut microbiota in the healthy gut barrier and the immunological maturation of the newborn and in subsequent reduction of the risk of immune-mediated diseases has led to an interest in the development

of strategies aimed at manipulating intestinal bacterial colonization, including the admistration of probiotics (Madsen et al. 2001). The rationale for the use of probiotics is based on their capability to balance the intestinal microbiota, to reduce paracellular permeability, to enhance the physical impediment of the mucous layer, to provide innate defence against pathogens, and to alleviate abnormal immune responsiveness (Ohland and MacNaughton 2010). Interestingly, clinical studies have provided evidence that the consumption of probiotics has only temporary impacts on gut microbiota succession (Gueimonde et al. 2006a), even though the immunomodulatory effects achieved can confer long-lasting clinical effects (Kalliomäki et al. 2001).

As depicted in Figure 3, the levels of action of probiotics can be separated into three different levels. Probiotic bacteria can interfere with the growth or survival of pathogenic microorganisms in the gut lumen (level 1), probiotic bacteria can improve the mucosal barrier function and the mucosal immune system (level 2) and, beyond the gut, have an effect on systemic immunity as well as other cell and organ systems (level 3).

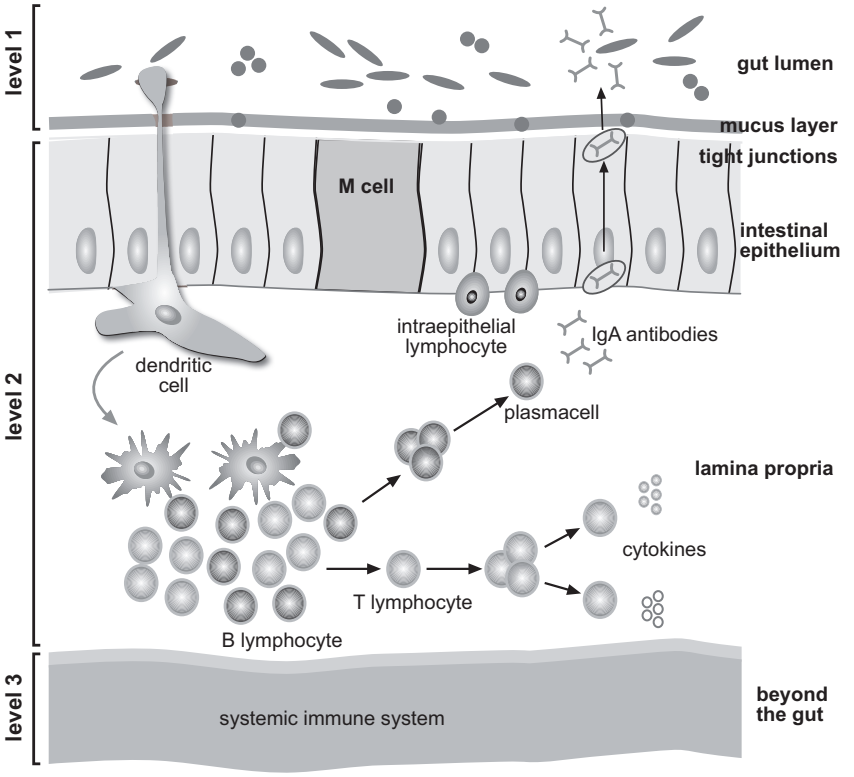

Figure 3. The levels of action of probiotics. Modified from Rijkers et al. 2010.

Exclusion of pathogens

Probiotics contribute to balancing of the gut microbiota by producing factors which inhibit pathogens and other commensal bacteria, enabling probiotics to compete effectively for nutrients in complex communities (Salminen et al. 2010, Collado et al. 2008b). In greater detail, probiotics have been shown to promote mucus secretion and mucin expression, contributing both to the exclusion of pathogens and to barrier function (e.g. Mack et al. 2003). Probiotics can also directly inhibit the growth of or kill pathogens by production of antimicrobial molecules including SCFAs, bacteriocins or microcins (e.g. Lievin et al. 2000, Corr et al. 2007). Moreover, probiotics stimulate host antimicrobial defense pathways, thus excluding pathogens indirectly by stimulating synthesis of defensin and by activating its propeptide form (e.g. Schlee et al. 2008). Probiotics may also alter the ability of pathogens to adhere to or invade colonic epithelial cells, sequester essential nutrients from invading pathogens, inhibit the expression of genes participating in the virulence functions of pathogens, as well as create an unfavorable environment for pathogen colonization (e.g. Johnson-Henry et al. 2007, Medellin-Peña et al. 2007).

The mechanisms whereby probiotics elicit pathogen exclusion capabilities also include stimulation of immune responses to pathogens *via* an increase of sIgA and anti-inflammatory cytokines and regulation of proinflammatory cytokines as well as stimulation of the epithelial barrier function (e.g. Johnson-Henry et al. 2008, Ruemmele et al. 2009). The effect of probiotic bacteria on B lymphocytes and antibody production has also been evaluated in clinical and vaccination trials; LGG has been demonstrated to enhance a specific IgA antibody response to rotavirus in infants with rotaviral gastroenteritis (Kaila et al. 1992) and *Lactobacillus casei* GG immunogenicity for oral rotavirus vaccination (Isolauri et al. 1995).

Gut barrier strengthening

The effects of probiotics in ameliorating intestinal barrier dysfunction have been evaluated in numerous studies in cell culture models exposed to enteropathogens or proinflammatory cytokines. Both in vitro and in vivo studies have shown that especially *Lactobacillus* and *Bifidobacterium* exert direct effects on intestinal epithelial barrier function, evidenced by decreased intestinal permeability and enhanced intestinal epithelial resistance (Madsen et al. 2001, Garcia-Lafuente et al. 2001, Parassol et al. 2005). Moreover, probiotic bacteria contribute to intestinal barrier function against invading pathogens in a strain-specific manner by competing for binding sites to epithelial cells and the overlying mucous layer (e.g. Zareie et al. 2006). Several studies have also demonstrated that pretreatment with probiotic bacteria can inhibit the decrease in resistance and epithelial cell tight junction alteration caused by stress, infection or pro-inflammatory cytokines (e.g. Seth et al. 2008) and additionally directly alter epithelial barrier function by influencing the structure of tight junctions (Ewaschuk et al. 2008, Anderson et al. 2010). Furthermore,

probiotics can reinforce the gut barrier by augmenting total and pathogen-specific sIgA levels upon infection, while typically not inducing production of probiotic-specific sIgA (e.g. Galdeano and Perdigon 2006, Qamar et al. 2001). Thus far no clinical trials have been conducted testing the effect of probiotics specifically on the intestinal barrier in infants. However, results of some experimental trials indirectly suggest that probiotics strengthen the intestinal barrier (Isolauri et al. 1993a and Isolauri et al. 1993b).

Immunomodulatory effects

Probiotics, like other intestinal bacteria, are recognized by TLRs in the gut epithelial cells and / or antigen-presenting cells and promote in consequence a cascade of immunological events aiming to maintain intestinal homeostasis. It is important to take into consideration that the netimate nature of a host cell response against pathogenic, commensal or probiotic microorganisms depends on the precise combination of MAMPs capable of interacting with the various PRRs and thus fine-tuning the immune response (Lebeer et al. 2010). Specific probiotics have been shown to confer many immunomodulatory effects in the human intestine, for example promoting tolerogenic dendritic cell and regulatory T cell phenotypes, inhibiting inflammatory cytokine production and enhancing natural killer cell activity (Chiba et al. 2010, Dong et al. 2010). Certain probiotic strains have also been found to elicit anti-inflammatory responses in intestinal epithelial cells in vitro, this also serving to reinforce the gut defence barrier (Ng et al. 2009, Donato et al. 2010).

Growing evidence also suggests that the functioning of the immune system at both systemic and mucosal level can be modulated by probiotics. More specifically, probiotics have been demonstrated to induce the production of protective cytokines on epithelial cells through TLR (2 and 4) sensing, this enhancing epithelial cell regeneration and inhibiting cell apoptosis (e.g. Rakoff-Nahoum et al. 2004). Probiotics may enhance the production of anti-inflammatory cytokines in epithelial cells as well as in monocytes and macrophages (e.g. Otte and Podolsky 2004). On the other hand, probiotics can also downregulate the production of proinflammatory cytokines in DCs and in the aforementioned immune cells (Ng et al. 2009). Furthermore, probiotic bacteria may exert beneficial effects by impacting on lymphocytes directly or secondarily *via* changes in stimulation induced by alterations in antigen-presenting cells. For example, certain probiotics have been found to elicit in interaction with mesenteric lymph nodes an up-regulation of sIgA production (Macpherson and Uhr 2004). In addition, T cell-mediated immune responses are modified by probiotics. Specific strains of probiotics have been shown to contribute to the differentiation of naïve T cells into Th1 and Th2 cells, thus controlling both cell-mediated and antibody-mediated immune responses, respectively (He et al. 2002). Additionally, probiotics modulate the differentiation of naïve T cells into Tr1 regulatory cells, this

contributing to a balanced immune function by inducing IL-10 and TGF-β production (von der Weid et al. 2001).

The mechanisms of action of probiotics are summarized in Figure 4.

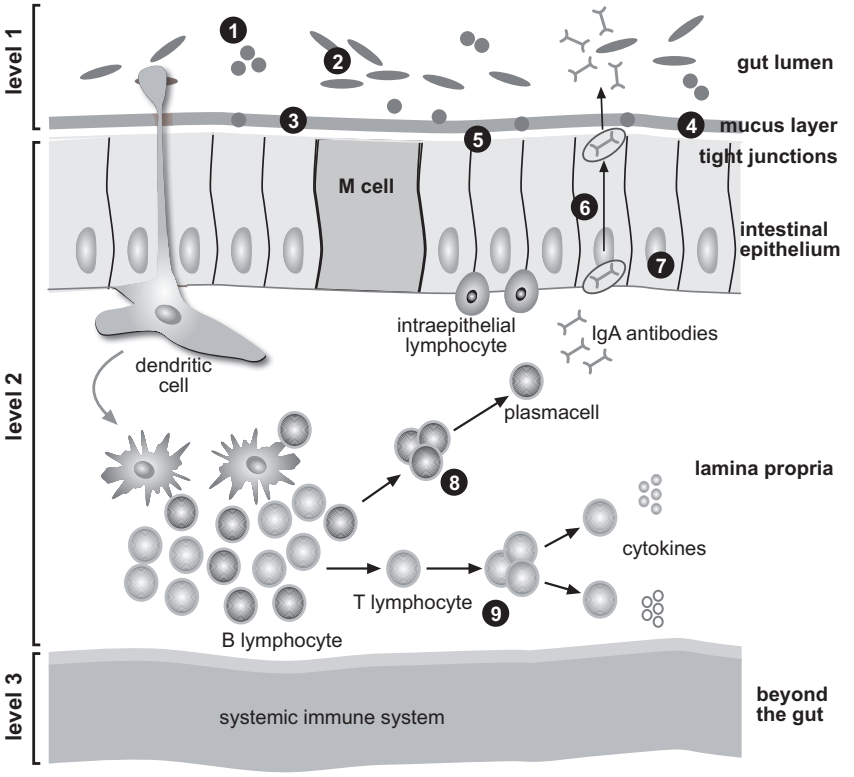

Figure 4. The mechanisms of action of probiotics. Modified from Rijkers et al. 2010.

1. Processing enteral antigens and balancing the gut microbiota.
2. Regulating energy balance.
3. Competitively excluding pathogens (inhibiting adhesion, competing for nutrients and receptors, producing antimicrobial molecules).
4. Inducing mucin production.
5. Strenghtening the gut barrier (regenerating epithelial cells, reinforcing tight junctions, restraining permeability).
6. Augmenting antigen-specific sIgA production.
7. Modulating the cytokine response of epithelial cells.
8. Controlling inflammatory responses (reducing proinflammatory cytokine production, promoting tolerogenic DCs and T cells, activating natural killer cells.
9. Modulating the differentiation of CD4+ naïve T cells.

Clinical applications of probiotics

The best documented clinical benefit of probiotic-induced pathogen exclusion is in acute gastroenteritis and antibiotic-associated diarrhea, as reviewed by Preidis and colleagues (2009). Probiotics may prevent the onset of disease when administered prophylactically or may suppress / diminish the severity or duration of disease. The rationale for the use of probiotics in inflammatory bowel and allergic diseases, on the other hand, lies mainly in their capability to maintain the gut barrier and to moderate overwhelming immune responses to intestinal bacteria or antigens induced by perturb gut microbiota composition as well as a disrupted and / or immature gut barrier system (Ng et al. 2009). Although no current recommendation has been given for the use of probiotics in the prevention of necrotizing enterocolitis (NEC) in premature infants (Szajewska 2010), results from clinical trials have been promising. The disease is considered to be caused by an inadequate, immature and excessive inflammatory response to both pathogenic and commensal bacteria (Nanthakumar et al. 2000). The mechanism of action for probiotics in the prevention of NEC would be mainly as an adjuvant to immune responses (Walker 2008). Also irritable bowel syndrome and radiation enteritis have been proposed as other potential target diseases for probiotics, but more clinical data should be forthcoming before any official recommendation can be issued (Floch et al. 2008).

Safety of probiotics

A number of probiotics have a long history of safe use and no health concerns have been recorded (Borriello et al. 2003, Salminen et al. 2002). Given the fact that probiotics are living micro-organisms possessing numerous immunological properties, uncertainty still prevails regarding the safety of their use among high-risk populations and under unphysiological conditions. This is especially true when administering them to immunocompromized individuals, since additional enteric bacteria, even such as are normally non-pathogenic, can have potentially harmful side-effects, including causing opportunistic infection. Most of the bacteremias described in the context of probiotic administration have been in individuals with immunosuppression, prior prolonged hospitalization and prior surgical interventions (Salminen et al. 2004a, Ohishi et al. 2010). On the other hand, specific strains of *Lactobacillus* probiotics have been administered to immunocompromized patients with HIV and no concerns have been reported (Salminen et al. 2004b).

The proneness to deviations in the stepwise compositional development of the gut microbiota in preterm infants originates in the delayed start of enteral feeding accompanied by the absence of the benefits of breast milk, frequent antibiotic exposure and the neonatal intensive care unit (NICU) environment (Sakata et al. 1985), extreme sequelae being NEC and septicemia induced by gastrointestinal bacteria (Lin and Stoll 2006, Claud and Walker 2001). In point of fact, NEC can be regarded as an immunoinflammatory

response of the immature and inexperienced gut barrier to sources of environmental antigens such as enteral nutrition and the presence of commensal bacteria. (Claud and Walker 2001) On this basis, intentional microbial manipulation of preterm infants with probiotics has attracted scientific and clinical interest (Caplan 2009), whereas the safety of this approach in this extremely fragile population remains elusive.

For the above reasons the indications for probiotic use in high-risk populations, i.e. preterm as well as term newborns, immunocompromized, critically ill patients etc, call for accurate safety demonstration, since the available data do not suffice to guarantee the safety of probiotic use in these patient populations (Allen et al. 2010, Szajewska 2010). Furthermore, the safety of early perinatal administration of probiotics must be separately and carefully evaluated, since specific strains of probiotics have been shown to contribute to the generation of Th1, Th3 and Tr1 regulatory cells (He et al. 2002, Christensen et al. 2002), with a counter-regulatory activity on the Th2-skewed immune responder type, this prevailing *in utero* (Piccini et al. 1998).

As probiotic bacteria constitute a tool with which to modify the gut barrier and microbiota, the strains used must further be obtained from acceptable sources with a proven safety record and efficacy to guarantee their future clinical applications. It should also be kept in mind that each probiotic bacteria and strain is itself a unique organism with specific properties which cannot be extrapolated from other, even closely related strains. The practice of combining data from studies of different genera, species, strains and doses of probiotics thus provides only limited information about specific therapeutic interventions (Preidis and Versalovic 2009).

3. AIMS OF THE STUDY

The main purpose of the present series was to assess the benefits and safety of perinatal probiotic intervention as a component in a balanced maternal nutritional environment.

The specific objectives in this thesis were:

1. To evaluate the influence of perinatal probiotic-supplemented dietary counselling on pregnancy outcome and on infants´ growth during 24 months´ follow-up (**I**).

2. To determine the impact of perinatal probiotic-supplemented dietary counselling on early postnatal nutritional environment (**II**).

3. To assess the impact of perinatal probiotic supplementation on childhood growth patterns and on the development of overweight during 10 years´s follow-up (**III**).

4. To establish whether early nutritional and microbiological environments deviate in children subsequently becoming normal-weight vs. overweight (**IV**).

5. To extend the safety aspect of probiotic administration to a high risk group, namely VLBW infants (**V**).

4. STUDY DESIGNS AND SUBJECTS

4.1. Study designs

The subjects in the present series comprised participants in two prospective, double-blind NAMI projects ongoing in the Department of Paediatrics, Turku University Hospital. Studies I and II in the present thesis were conducted as a part of a mother-infant nutrition and probiotic study and studies III and IV as a part of an allergy prevention study.

The patient material in study V consisted of all premature infants of birth weight ≤ 1500 g, i.e. VLBW infants, treated in the Turku University Hospital NICU during the years 1997 - 2008.

4.1.1. Mother-infant nutrition and probiotic study

The original study population comprised 256 mother-baby pairs participating in a randomized, double-blind mother infant nutrition and probiotic study (http://www.clinicaltrials.gov/ct/gui/show/NCT00167700, section 3). The women were recruited in early pregnancy during their first visit to maternal welfare clinics in Turku and neighbouring areas in South-West Finland. The inclusion criterion was that the subjects had no chronic metabolic diseases, allergic diseases (atopic eczema, allergic rhinitis or asthma) being however allowed.

At entry the women were randomized into a dietary intervention group or a control group. The dietary intervention group was further randomized at baseline in double-blind manner to receive either probiotics (diet/probiotics group), LGG (ATCC 53103, Valio Ltd., Helsinki, Finland) and *Bifidobacterium lactis* Bb12 (Chr. Hansen, Hoersholm, Denmark) at a dose of 1 x 10^{10} colony forming units (cfu) once daily each, or placebo (diet/placebo group), microcrystalline cellulose and dextrose anhydrate (Chr. Hansen, Hoersholm, Denmark) capsules. The control group received placebo capsules in single-blinded manner. The intervention period extended from the first trimester of pregnancy to the end of exclusive breastfeeding, however not beyond the infant age of 6 months.

All women received dietary counselling provided by maternal welfare clinics according to a national program. The intervention group received additionally intensive dietary counselling at every study visit, provided by a nutritionist and combined with conventional food products for use at home. Specifically, dietary counselling focused on the amount and the type of fat and the amount of fibre in the diet. Subjects were encouraged to increase their consumption of vegetables, fruits and wholegrain bread and

cereals, to consume leaner meat products, low-fat cheese and milk products, and to use vegetable oil or soft margarine as a spread and in food preparation. The recommended amounts of foods were planned to provide improvement in the consumption of fibre and unsaturated fatty acids and conversely reduction in the consumption of saturated fatty acids (SFAs). Total intake of fat was recommended to be 30 % energy, carbohydrates 55-60 % energy and protein 10-15 % energy, as was recommended during the study years 2002 - 2005 (Nordic Working Group on Diet and Nutrition 1996, Becker et al. 2004). The conventional food products provided were of favorable fat (e.g. oil-based spreads and salad dressing) and fibre content (e.g. fibre-enriched pasta, breakfast muesli and porridge cereals).

The women in all study groups visited the study clinic three times during pregnancy, in addition to their regular visits to maternal welfare clinics. Their clinical data were collected by interview at the first visit. Data on maternal heights and prepregnancy weights were collected by interview at the first study visit. The total gestational weight gain was calculated by subtracting self-reported prepregnancy weight from that recorded at a prenatal study visit or at the delivery hospital within 1 week before delivery. The duration of pregnancy was calculated from the date of the last menstruation. The results of a 75 g oral glucose tolerance test (OGTT) were recorded. This test is performed at 26 to 28 weeks of gestation in maternal welfare clinics in all risk pregnancies: pre-pregnancy BMI over 25 kg/m2; age over 40 years; GDM in a previous pregnancy; previous delivery of a child weighing more than 4500 g; detection of glucose in the urine or suspicion of a macrosomic fetus in the present pregnancy. The diagnosis of GDM was based on modified criteria of the Fourth International Workshop-Conference on GDM (Metzger and Coustan 1998), according to recommendations implemented in Finland during the study years 2002 - 2005. Specifically, OGTT was considered pathological when one value exceeded $\geq$ 4.8 mmol/L at baseline, $\geq$ 10.0 mmol/L at 1h or $\geq$ 8.7 mmol/L at 2 h.

Maternal dietary intake was assessed at each trimester using 3-day food diaries. Energy and nutrient intakes were calculated with a Micro-Nutricaw computerized program (version 2.5; Research Centre of the Social Insurance Institution, Turku, Finland). After delivery, study visits were scheduled at the infants´ages of 1, 6, 12, 24 and 48 months. The duration of exclusive and total breastfeeding was recorded from the mother.

4.1.2. Allergy prevention study

The original study population here comprised 159 mother-baby pairs participating in a randomized, double-blind prospective follow-up study of probiotics in allergic diseases (http://www.clinicaltrials.gov/ct/gui/show/NCT00167700, section1). The inclusion

criterion was that the fetus had at least one close relative (mother, father, sibling) with atopic dermatitis, allergic rhinitis or asthma. Mothers were recruited in antenatal clinics in Turku, Finland, and were randomized in double-blind, placebo-controlled manner to receive 1 x 10^{10} cfu of LGG (ATCC 53103, Valio Ltd., Helsinki, Finland) or placebo (microcrystalline cellulose, Chr. Hansen, Hoersholm, Denmark) in capsules once a day for 4 weeks before expected delivery. Data on maternal height and prepregnancy weight were collected from the hospital patient charts, these data being self-reported at the first prenatal visit to maternal welfare clinics. After delivery, the capsule contents were given either to the mothers if they were breastfeeding or otherwise to the infants mixed in water for 6 months. Postnatal study visits were scheduled at the childrens´ ages of 3 weeks, 3, 6, 12 and 24 months and at 4 and 7 years.

4.1.3. Premature infants

The rationale for this report was a 12 years´ history with prophylactic use of probiotic bacteria, LGG (ATCC 53103, Valio Ltd., Helsinki, Finland) in VLBW infants treated in the Turku University Hospital NICU. Since the year 1997 every enterally fed VLBW infant has been given LGG 6 x 10^9 cfu once daily until discharge. A total of 644 VLBW infants were treated in the Turku University Hospital NICU during these years, this representing 54 infants annually. The incidence of LGG septicemia and NEC (stage II or III) among all VLBW infants treated in the NICU during the years 1997 – 2008 was estimated using hospital patient charts and databases collected for the national database held by the National Institute for Health and Welfare and for Vermont Oxford Network (VON) reporting. VON comprises a voluntary collaboration of NICUs to improve quality and safety in the care of the newborn infant (Horbar 1999). The Network prospectively collects data on all newborn infants of birth weight ≤ 1500 g or gestational age less than 30 pregnancy weeks who are either born at a hospital with a participating NICU or are transferred to one within 28 days after birth. The annual Network Database Manual of Operations defines the diagnostic criteria for NEC, which is identified using clinical or radiographic criteria or findings in surgery or at post-mortem examination. Specifically, the clinical and radiographic features of the condition are defined by Bell staging (Bell et al. 1978). Stage II is defined when at least one clinical finding (bilious gastric aspirate or emesis, abdominal distension, or occult or gross blood in the stool in the absence of anal fissures) coincides with at least one radiographic finding (pneumatosis intestinalis or hepatobiliary gas) and stage III when at least one of the aforementioned clinical findings parallels a radiographic finding of pneumoperitoneum (Bell et al. 1978). LGG septicemia was defined as a bloodculture-positive disease.

4.2. Study subjects

The profiles of studies **I** and **II** are presented in Figure 5 and of studies **III** and **IV** in Figure 6.

4.2.1. Mother-infant nutrition and probiotic study

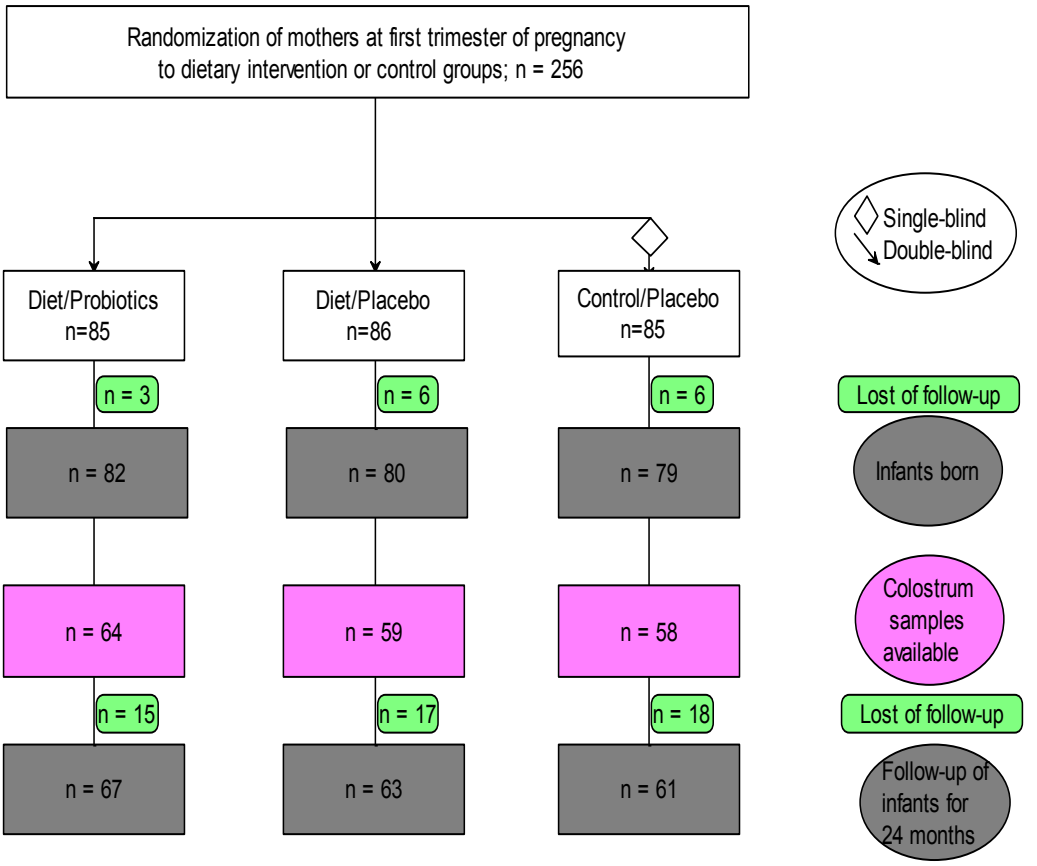

Figure 5. Trial flow of the subjects in studies I & II.

Of the 256 mothers recruited 238 continued to participate throughout pregnancy. Three of these women were carrying twins. The clinical characteristics of the mothers enrolled are set out in Table 1.

Table 1. Clinical characteristics of the women enrolled in the mother-infant nutrition and probiotic study (studies I & II).

Group	**Diet/probiotics** (n = 85)	**Diet/placebo** (n = 86)	**Control** (n = 85)
Age (years)	29.7 (4.1)	30.1 (5.2)	30.2 (5.0)
Higher degree education	68 (80 %)	59 (69 %)	67 (79 %)
Allergic diseases	66 (77.6 %)	69 (80.2 %)	65 (76.5 %)
Primipara	55 (65 %)	44 (51 %)	48 (56 %)
Prepregnancy BMI	22.9 (3.2)	24.3 (4.4)	23.7 (3.5)
Twin pregnancies	2 (2 %)	1 (1 %)	0 (0 %)

Results are given as mean (SD) or as numbers (%) of subjects.
None of the differences among the groups was significant.

Of the 241 children delivered, 191 completed the 24 months´ follow-up, these comprising the study subjects for study I in the present series. The aim of this study was to evaluate the impact of interventions on pregnancy outcome and children´s growth during 24 month´s follow-up. Of special interest was the safety of perinatal probiotic intervention.

Maternal colostrum samples for adiponectin concentration analyses were available from 181 mothers, these comprising the study subjects of study II. The aim of this study was to determine the impact of maternal nutritional status and diet on the adiponectin concentration in the colostrum.

4.2.2. Allergy prevention study

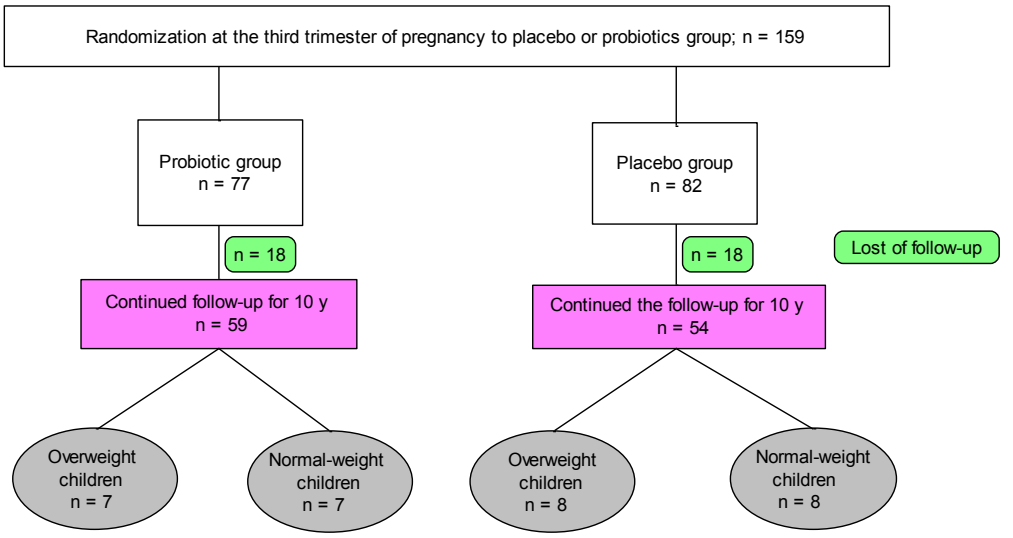

Figure 6. Trial flow of the subjects in the studies III & IV.

Of the 159 children enrolled, 113 completed the 10 years´ follow-up, these comprising the cohort in study III. The aim of this study was to evaluate the impact of perinatal probiotic intervention on childhood growth patterns and the development of overweight during a 10-year follow-up. The clinical characteristics of the patients are shown in Table 2.

Table 2. The clinical characteristics of the children in study III.

	Probiotic group (n = 54)	**Placebo group** (n = 59)
Sex (male)	34 (63 %)	34 (58 %)
Gestational age at birth (wk)	39.3 (38.9 - 39.6)	39.4 (39.0 - 39.6)
Cesarean delivery	7 (13 %)	12 (20 %)
Birth weight (g)	3556 (3423 - 3688)	3638 (3516 - 3759)
Birth length (cm)	51.0 (50.4 - 51.5)	50.6 (50.1 - 51.1)
Birth head circumference (cm)	35.1 (34.7 - 35.5)	35.1 (34.7 - 35.5)
Exclusively breast-fed (mo)	3.0 (2.6 - 3.4)	2.6 (2.1 - 3.1)
Total duration of breastfeeding (mo)	7.1 (6.2 - 8.0)	6.4 (5.3 - 7.5)

Results are given as mean (95 % CI) or as numbers (%) of subjects.
None of the differences among the groups was significant.

Among the study population used in study III 15 overweight or obese children at 10 years of age were identified. The overweight was defined according to international age- and gender-specific criteria (Cole et al. 2000). Normal-weight children (n=15) matched for gender, gestational age and BMI at birth, mode of delivery, probiotic intervention and duration of breastfeeding were identified from the same cohort as controls. These 30 children comprised the subjects in study IV in this series.

The adiponectin concentration in maternal colostrum was analyzed. On the basis of detected timing of deviation in growth patterns between children of normal weight and those overweight at the age of 10 years, children´s gut microbiota composition was analyzed by fluorescent in situ hybridization (FISH) at 3 months of age. Additionally, serum sCD14, LBP, LPS and high-sensitive C-reactive protein (hsCRP) concentrations were analyzed at the age of 3 months as putative early markers of low-grade inflammation.

The clinical characteristics of the children in study IV are depicted in Table 3.

Table 3. Clinical characteristics of the children in study IV.

	Normal-weight children (n = 15)	**Overweight children** (n = 15)
Probiotic supplementation	7 (47 %)	7 (47 %)
Probiotic supplementation directly to the child	3/7 (43%)	5/7 (71%)
Sex (male)	10 (67 %)	10 (67 %)
Cesarean delivery	2 (13 %)	2 (13 %)
Maternal peripartal antibiotic treatment	1 (7%)	1 (7%)
Gestational age at birth (wk)	39.8 (39.4 - 40.2)	39.1 (38.1 - 40.0)
Birth weight (g)	3702 (3494 - 3910)	3785 (3500 - 4070)
Birth length (cm)	51.0 (50.4 - 51.6)	50.9 (50.0 - 51.8)
Exclusively breast-fed (mo)	2.8 (1.8 - 3.7)	2.6 (1.6 - 3.6)
Total duration of breastfeeding (mo)	6.3 (4.1 - 8.5)	6.2 (3.7 - 8.7)

Results are given as mean (95 % CI) or as numbers (%) of subjects.
None of the differences among the groups was significant.

4.3. Anthropometric measurements

The birth weights and heights of infants were collected from the records of the delivery hospital and well-baby clinics. At every study visit weights and heights were measured. When a child was younger than two years, the weight was measured with a baby scale (Data Baby Scale model 930, Oriola, Espoo, Finland) and length on a length board (Pedi-Infantometer, Pedihealth, Oulu, Finland). From the age of two years onwards weights were measured with an electronic scale (Seca 709, Soehnle, Murrhardt, Germany) and standing heights were measured with a wallmounted stadiometer (Person Check, KaWe, Asperg, Germany). In the allergy prevention study the weight and height data at the age of 10 years were collected from the parents, the measurements having being made by school health nurses. All weights and lengths were recorded to the nearest 0.01 kg and 0.1 cm, respectively.

In the mother-infant nutrition and probiotic study all growth measurements of the children were estimated to precise daily ages (1 month, 6 months, 12 months and 24 months) using linear interpolation by reason of variable measuring ages. The growth rate was defined in grams and centimetres gained per month between two measurements. Three twin pairs were excluded from the growth follow-up.

The BMI was calculated as weight in kilograms divided by height in meters squared. A child was considered overweight if the BMI exceeded the international cut-off value (Cole et al. 2000) for overweight; 18.41 kg/m^2, 17.55 kg/m^2, 17.92 kg/m^2 and 19.84 kg/m^2 for boys and 18.02 kg/m^2, 17.28 kg/m^2, 17.75 kg/m^2 and 19.86 kg/m^2 for girls at the age of 2, 4, 7 and 10 years, respectively. Obesity at 2, 4, 7 and 10 years was defined according to international cut-off values 19.81 kg/m^2, 19.15 kg/m^2, 20.51 kg/m^2 and 24.11 kg/m^2 for girls and 20.09 kg/m^2, 19.29 kg/m^2, 20.63 kg/m^2 and 24.00 kg/m^2 for boys at the respective ages. These cut-off points have been developed on the basis of growth-curve data from large epidemiological studies in six countries and pass through the BMI of 25 and 30 at age 18, which is consistent with adult definitions for overweight and obesity. The BMI was chosen as an adequate and practical childhood obesity marker due to its correlation with other anthropometric indicators of obesity and central obesity (Corvalán et al. 2010). Additionally, the age- and gender-specific BMI z scores at birth and at the ages 3, 6, 12, 24, and 48 months and 7 and 10 years were calculated using the standardized BMI growth charts for children published by the WHO (2005) based on the lambda-mu-sigma (LMS) method described by Cole (1988) and Cole and Green (1992).

4.4. Biochemical analyses

Colostrum samples were collected from mothers by manual or mechanical expression as soon as lactation had commenced, however not later than during the third postpartum day. The samples were cooled to 6 to 8 °C and transported to the study clinic within 24 hours to be frozen at -75 °C.

Venous blood samples were drawn from the infants at the age of 3 months from an antecubital vein. Cutaneous analgesia (EMLA®; Astra, Södertälje, Sweden) was used if parents requested it. After clotting at room temperature for 30 to 60 minutes and centrifugation at 3400 x G for 12 minutes, sera were separated. The sera were stored at -25 ºC for up to one week and then at -70 ºC for later measurements.

4.4.1. Colostrum adiponectin

The NIHR Cambridge Biomedical Research Centre, Core Biochemical Assay Laboratory (Cambridge, UK) analyzed the samples. The adiponectin concentration was assayed manually using an in-house, two-step time-resolved fluorometric (DELFIA) assay (Semple et al. 2006). DELFIA reagents (multibuffer, wash buffer, enhancement solution, europium-labeled streptavidin and consumables) were purchased from PerkinElmer (Turku, Finland) and antibodies and calibration materials from R&D Systems Europe (Abingdon, Oxfordshire, UK). All samples were analyzed in duplicate. Samples in which the coefficient of variation of the duplicates was greater than 10 % were repeated. Quality control samples with concentrations spanning the working range of the assay were run at the beginning and end of each assay. According to the manufacturer, the assay measures all multimeric forms of adiponectin together, i.e. the total adiponectin concentration. The lower limit of detection was 0.8 ng/mL (in-house data) and the between-batch imprecision 5.4 % at 3.6 µg/mL, 5.2 % at 9.2 µg/mL and 5.8 % at 15.5 µg/mL (in-house data).

4.4.2. Serum sCD14, LBP and LPS

Serum sCD14, LBP and LPS analyses were performed in the laboratory of the Metabolism and Nutrition research group, Université catholique de Louvain, LDRI. Human sCD14 and LBP concentrations were assayed using a solid-phase enzyme-linked immunosorbent assay based on the sandwich principle (Hycult Biotechnology, Uden, the Netherlands) and adapted as follow: sera were diluted 1/10 with the appropriate buffer and subjected to an ultrasonic bath for 3 min and homogenized by vortex for 30 seconds prior to a high dilution rate: 1/500 (sCD14) and 1/1000 (LBP). The LPS concentration was measured using Endosafe-MCS (Charles River laboratories, Lyon, France) based on the Limulus amaebocyte Lysate (LAL) kinetic chromogenic methodology, which measures

the colour intensity directly related to the endotoxin concentration in a sample. Sera were diluted 1/10 with endotoxin-free buffer to minimize interference in the reaction (inhibition or enhancement) and heated for 15 min at 70 °C. Each sample was diluted 1/46 with endotoxin-free LAL reagent water (Charles River Laboratories) and treated in duplicate, and two spikes per sample were included in the determination. All samples were validated for recovery and coefficient of variation determination. The lower limit of detection was 0.01 EU/ml.

4.4.3. Serum hsCRP

The NIHR Cambridge Biomedical Research Centre, Core Biochemical Assay Laboratory (Cambridge, UK) analyzed the samples. Serum hsCRP levels were determined using an automated colorimetric immunoassay on the Dade Behring Dimension RXL autoanalyser (Siemens Healthcare, UK). The lower limit of detection was 0.1 mg/L. The imprecision between batches was 5.1 % at 1.8 mg/L and 2.4 % at 6.4 mg/L. Any hsCRP measurements over 8 mg/L were excluded from the analysis, since such a level is suggestive of active inflammation or infection. The final number of subjects in this analysis was 11 children of normal weight and 12 children overweight at the age of 10 years.

4.5. Gut microbiota analyses by FISH

Fecal specimens were collected from diapers after defecation. The specimens were immediately cooled to 6 to 8 °C and transported to the study clinic within 24 hours to be frozen at -75 °C for later analyses.

The main groups of fecal bacteria were analyzed. The samples were suspended in phosphate-buffered saline (PBS) and homogenized. Bacteria were fixed with 4 % paraformaldehyde, washed with PBS and stored in 50 % ethanol-PBS at -20 °C until analyzed. Probes included Bac303 (5'-CCAATGTGGGGGACCTT) for the *Bacteroides-Prevotella* group, Bif164 (5'-CATCCGGCATTACCACCC) for the *Bifidobacterium* genus, CHis150 (5'-TTATGCGGTATTAAT CT(C/T)CCTTT) for the C*lostridium histolyticum* group, and Lab158 (5'-GGTATTAGCA(T/C)GTGTTTCCA) for the *Lactobacillus-Lactococcus-Enterococcus* group. Total bacterial counts were determined by staining with 4',6-diamino-2-phenylindole. The bacteria were washed and filtered on 0.2 -μm polycarbonate filters. These were then mounted on slides and counted visually under an epifluorescence microscope (BX51; Olympus, Hamburg, Germany) using Cy3-labeled probes and 4',6-diamino-2-phenylindole–specific filters. At least 15 random fields were counted on each slide, and the average count was used for analysis.

4.6. Statistical analysis

All data were analyzed using SPSS version 14.0 or 15.0 (SPSS Inc, Chicago, IL). A *P* value of <0.05 was considered statistically significant. The clinical characteristics of the study subjects are reported as mean values with range, 95 % confidence intervals (CI) or standard deviations (SD) for continuous variables and as numbers with proportions for categorical variables. Differences among study groups in clinical characteristics and main outcome measures were compared using Chi-squared test for categorical variables, Student´s t-test or analysis of variance (ANOVA) for normally distributed continuous variables, and Mann-Whitney *U* or Kruskal-Wallis test for skewed continuous variables.

Mother-infant nutrition and probiotic study

Study I: Comparison of growth rates during the periods 0 - 6 months, 6 - 12 months and 12 - 24 months among the study groups was made by ANOVA for repeated measurements. Logistic regression analysis was used to compare the incidence of GDM among the groups. Results were given as odds ratios (OR) with 95 % CIs. ANOVA with two explaining factors, study group and GDM, was used to analyze the birth size variables. Due to significant interactions between dietary intervention and GDM, subgroup analyses were conducted to estimate the effect of GDM in the groups.

Study II: To improve the normality of the adiponectin concentration in the colostrum for use in statistical models, the data were log transformed and thereafter represented as geometric mean values with 95 % CIs. Since no differences in dietary intakes were detected between the diet/probiotics and diet/placebo groups, the impact of dietary intervention on the adiponectin concentration was analyzed using Student´s t-test also for the combined dietary intervention groups compared to the control group. Linear regression analysis, Student´s t-test or chi-square test was used to evaluate associations between maternal characteristics (pre-pregnancy BMI, weight gain during pregnancy, GDM) and the main outcome measure. The impact of dietary intervention on dietary intake was evaluated as change between first and third trimester of pregnancy. A general linear model was applied to analyze the association between the adiponectin concentration in the colostrum and dietary intake. In this model the independent variables were study group and change in dietary intake between the first and third trimester of pregnancy. The interaction between these two variables was also studied.

Allergy prevention study

Study III: The Breslow-Day interaction test was used to analyze possible interactions between gender and probiotic intervention in overweight. The genders were combined in further analyses, since no interaction was observed between gender and intervention in

anthropometric measurements. In view of a significant association between birth weight and overweight, the analysis of covariance (ANCOVA) with birth weight as a covariate was used to compare the BMI measurements between the study groups. In addition, ANCOVA for repeated measurements at the ages of 2, 4, 7 and 10 years, with birth weight as covariate, was used to compare the study groups. BMI results were given as birth weight-adjusted means with 95 % CIs. Logistic regression analysis, including birth weight as covariate, was used to test the effect of probiotics in overweight and obesity. The results were given as birth weight-adjusted ORs with 95 % CIs.

Study IV: ANOVA for repeated measurements was used to compare BMIs between the study groups at different age-points. The Breslow-Day interaction test was used to analyze possible interactions between fecal microbial counts and ages when fecal samples were taken. By reason of non-normal distribution, fecal microbial counts were expressed as means with 95 % CIs after log transformation. To examine the relationship between continuous variables linear regression models were constructed.

4.7. Ethics

The prospective studies were conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human participants were approved by the Ethical Committee of the Hospital District of Southwest Finland. Written informed consent was obtained from all participants before enrolment. The retrospective premature study design was approved by the Ethical Committee of the Hospital District of Southwest Finland and by the Ministry of Social Affairs and Health of Finland.

5. RESULTS

5.1. The impact of perinatal probiotic intervention on the frequency of GDM (I)

The mother-infant nutrition and probiotic study sought to assess the impact of probiotic-supplemented dietary counselling on pregnancy outcome. The frequency of GDM was significantly different among the study groups; 13% (diet/probiotics) vs. 36% (diet/placebo) and 34% (control), $P = 0.003$, logistic regression analysis (Figure 7). The subsequent subgroup analyses revealed that the probiotic supplementation independent of the dietary intervention, reduced the risk of GDM, since the risk was significantly smaller when compared to that in the control group; OR = 0.27 (95% CI 0.11 - 0.62); $P = 0.002$, whereas in the dietary intervention group without probiotics the risk was not significantly different when compared to the control group; OR = 1.08 (95% CI 0.55 - 2.12; $P = 0.823$.

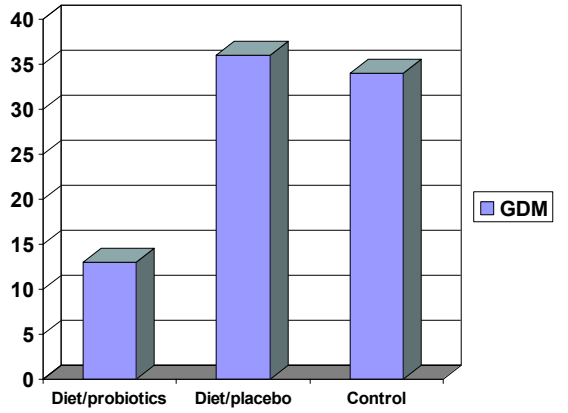

Figure 7. The frequency (%) of GDM in diet/probiotics, diet/placebo and control groups.

5.2. The impact of perinatal dietary counselling on prenatal growth in GDM-affected pregnancies

In evaluating fetal growth in the mother-infant nutrition and probiotic study, the perinatal dietary intervention, with or without probiotics, was observed to restrain excessive fetal growth in GDM-affected pregnancies. GDM increased the birth weight on the average 144 g (95% CI, -7-295) in the dietary intervention groups and 426 g (95% CI, 193-660)

in the control group, and birth length 0.4 cm (95% CI, -0.2-1.1) in the dietary intervention groups and 1.7 cm (95% CI, 0.7-2.7) cm in the control group (Figure 8). The significance of the difference between dietary intervention groups and control group was $P = 0.035$ for birth weight and 0.028 for birth length, 2-ANOVA. **(I)**

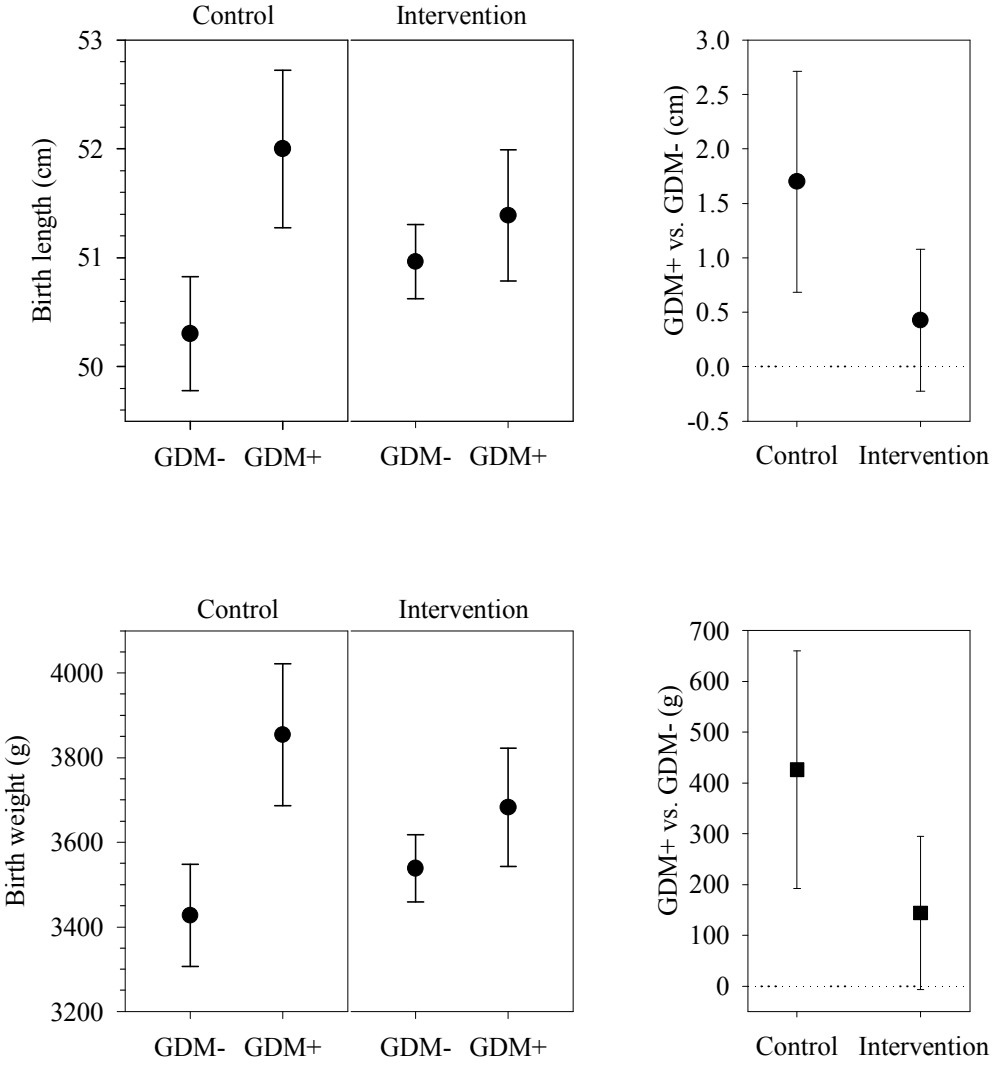

Figure 8. The mean (95% CI) birth length and birth weight of the infants in the control group (n = 73) and in the dietary intervention groups (n = 148) according to mothers´ GDM. On the right side is presented the difference between GDM+ (baseline) and GDM- in the control group and in the dietary intervention groups.

5.3. The influence of perinatal dietary counselling on adiponectin concentration in colostrum (II)

In order to assess the influence of perinatal dietary counselling on infants´ early dietary environment, the colostrum adiponectin concentration in 181 mothers participating in the mother-infant nutrition and probiotic study was analyzed. The results showed that the perinatal dietary intervention, with and without probiotics, increased the adiponectin concentration in the colostrum (Figure 9). The geometric mean adiponectin concentration in the colostrum was 12.7 [95% CI, 10.6-29.7] in the combined dietary intervention groups and 10.2 [95% CI, 9.9-13.2] in the control group; P = 0.024, Student´s t-test.

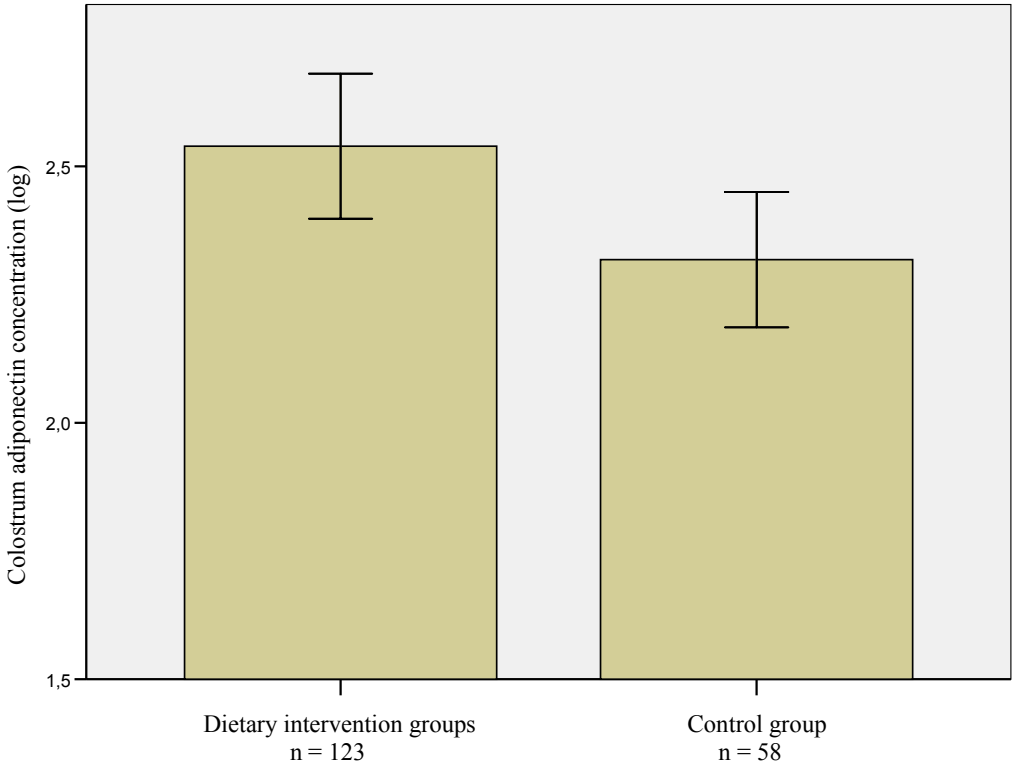

Figure 9. Maternal adiponectin concentration (log) in colostrum (mean, 95% CI) in dietary intervention and control groups.

5.4. The maternal determinants of colostrum adiponectin concentration (II)

An additional aim was to provide data regarding maternal determinants for the colostrum adiponectin concentration. In this study population, a significant linear inverse association was observed between the maternal weight gain (kg) during pregnancy and the adiponectin concentration in the colostrum. The higher the maternal weight gain was, the lower the adiponectin concentration in the colostrum; β (SE) = - 1.7 (0.1), $P = 0.020$ (Figure 10). Moreover, GDM, independent of gestational weight gain, was associated with the likelihood of the adiponectin concentration falling into the lowest quartile; OR 2.36, 95% CI 1.1-3.2, $P = 0.028$.

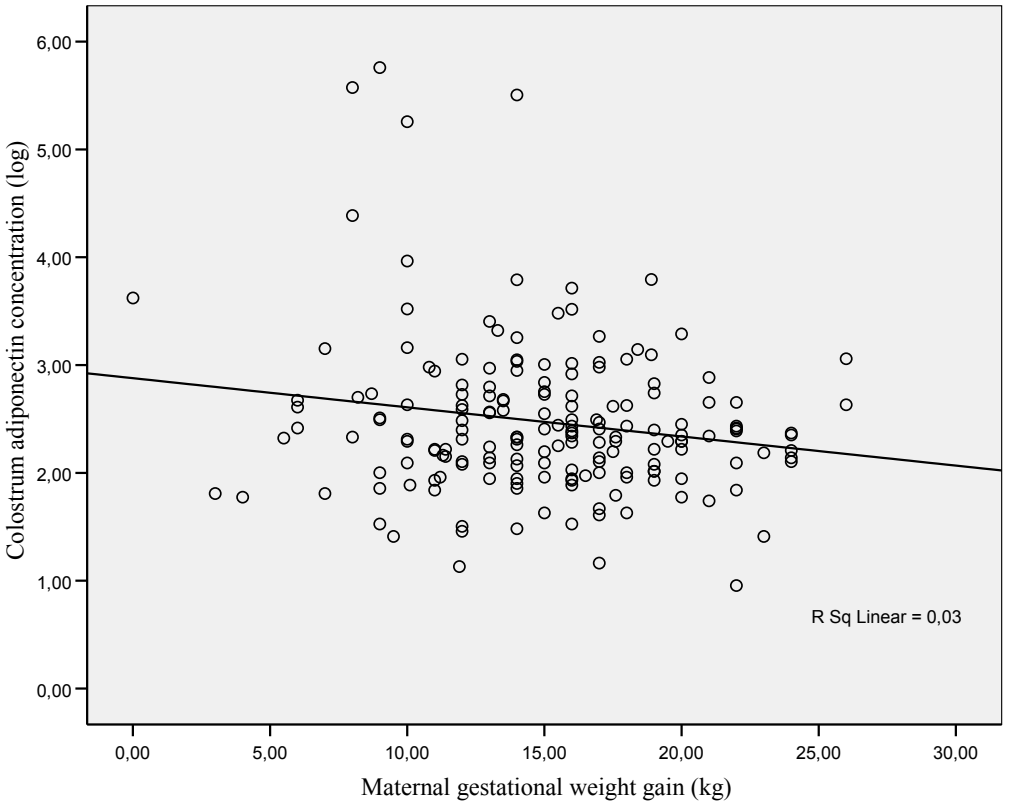

Figure 10. The linear association between maternal gestational weight gain (kg) and adiponectin concentration (log) in the colostrum.

5.5. The association between colostrum adiponectin concentration and weight status at 10 years of age (IV)

In study IV the aim was to compare the initial dietary environment in terms of colostrum adiponectin concentration received by 15 overweight and 15 normal-weight children at the age of 10 years. The results revealed that the mean (SD) adiponectin concentration in the colostrum was significantly higher in mothers whose children were normal-weight than in those whose children were overweight at the age of 10 years: 15.2 (23.4) vs. 5.9 (2.1) ng/mL respectively; $P = 0.001$, Mann-Whitney U test (Figure 11).

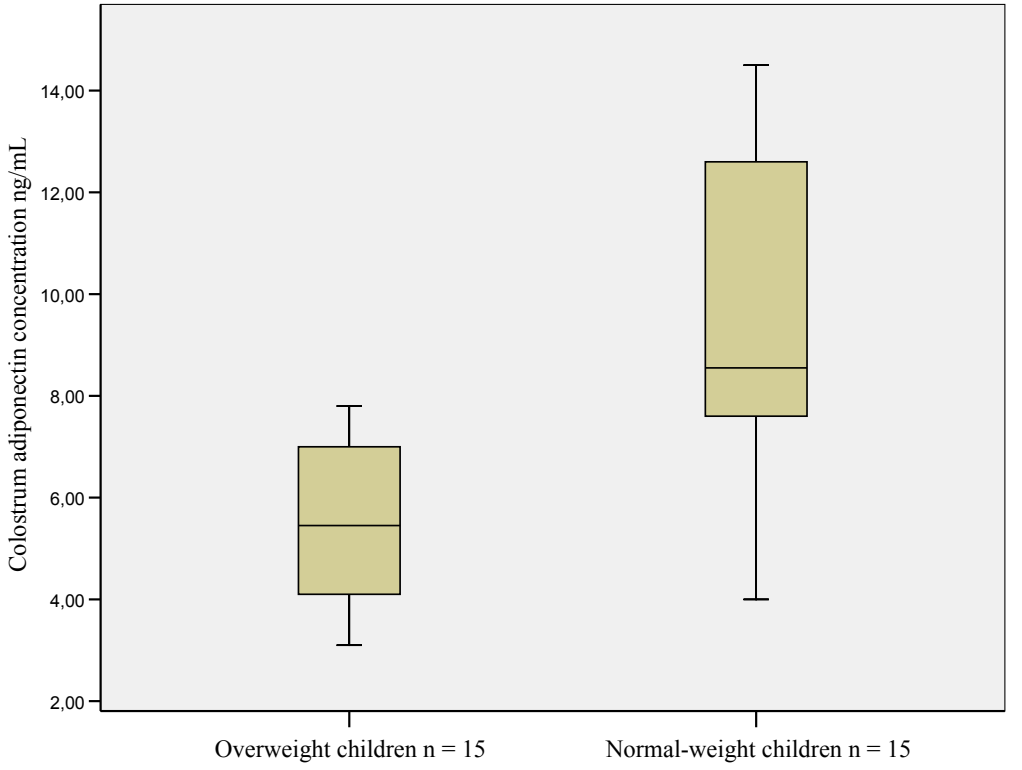

Figure 11. Colostrum adiponectin concentration (ng/mL) in mothers whose children were normal-weight and in those whose children were overweight at the age of 10 years. The box represents the interquartile range, the horizontal line the median, and the whiskers the 5th and 95th percentiles of distribution. (In: Luoto et al. J Pediatr Gastroenterol Nutr, in press).

5.6. The association between early gut microbiota composition and weight status at 10 years of age (IV)

In order to assess the potential relation of the early compositional development of the gut microbiota to overweight development, the gut microbiota composition at the age of 3 months was determined by FISH in these 15 overweight and 15 normal-weight children at the age of 10 years. The analysis showed a tendency towards higher Bifidobacterial numbers (mean x 10^9) in the stool at the age of 3 months in the normal-weight compared to overweight children; 10.0 (95% CI, 3.7 – 17.1) vs. 3.9 (95% CI, 2.4 – 5.3); $P = 0.087$, Student´s t-test (Figure 12).

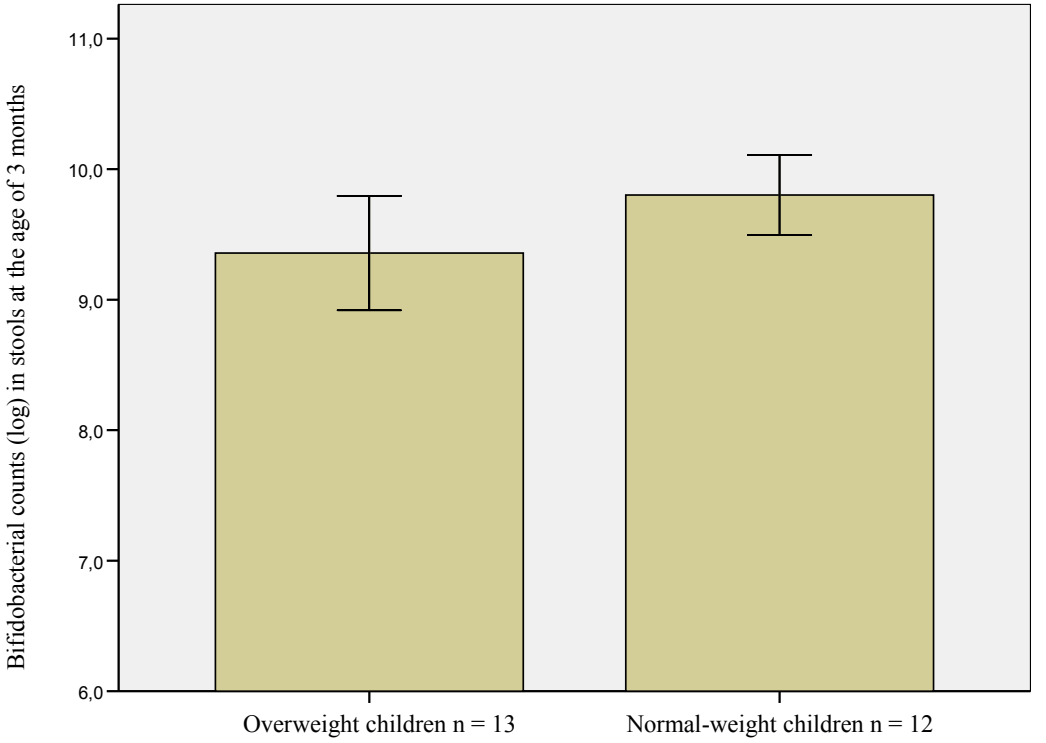

Figure 12. Mean Bifidobacterial count (log; 95% CI) in stools at the age of 3 months in children of normal-weight and children overweight at the age of 10 years.

5.7. Association between low-grade inflammation at the age of 3 months and weight status at 10 years of age (IV)

In assessing the putative early markers of low-grade inflammation in these 15 overweight and 15 normal-weight children at the age of 10 years, a statistically significantly higher mean (SD) sCD14 concentration in the serum at the age of 3 months was detected in children of normal weight compared to children who were overweight at the age of 10 years: 45.15 (5.82) vs. 38.71 (10.04) µg/ml; $P = 0.049$, Student´s t-test (Figure 13).

No statistically significant difference was found in LBP ($P = 0.712$), LPS ($P = 0.836$) or hsCRP ($P = 0.487$) concentrations measured at the age of 3 months of age between normal-weight and overweight children at the age of 10 years.

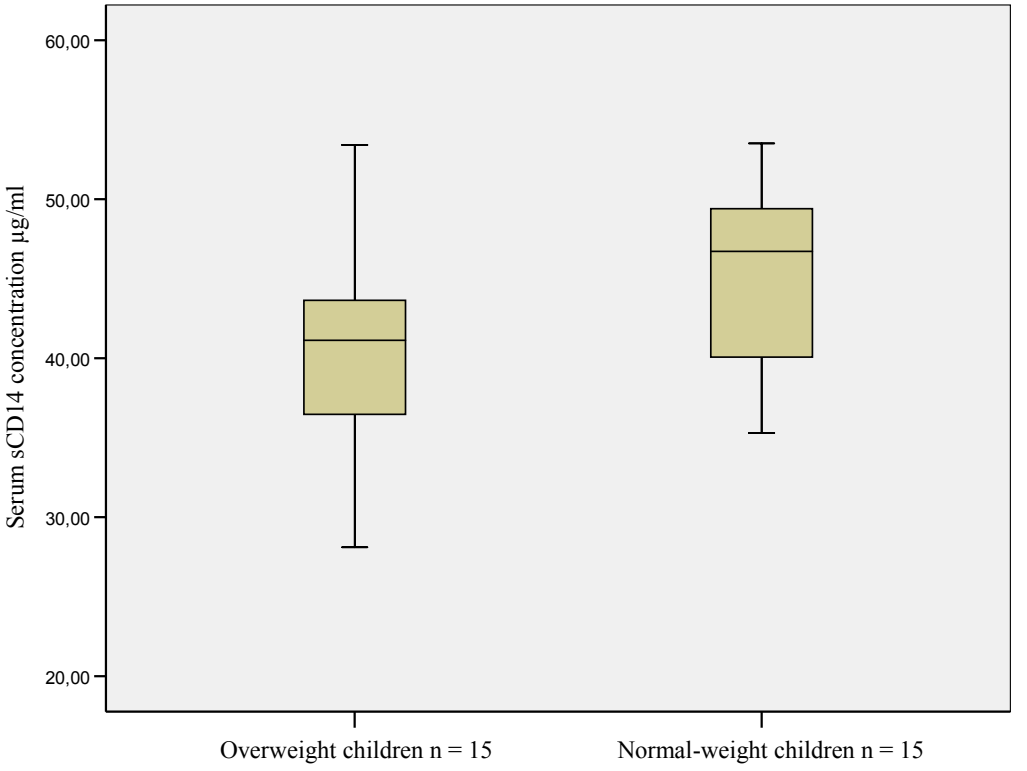

Figure 13. Serum sCD14 concentration at the age of 3 months (µg/mL) in children of normal weight and those overweight at the age of 10 years. The box represents the interquartile range, the horizontal line the median, and the whiskers the 5th and 95th percentiles of distribution. (In: Luoto et al. J Pediatr Gastroenterol Nutr, in press).

5.8. Characterization of the critical age for overweight development (III)

Assessment of the critical age for the development of overweight during the 10 years´ follow-up among the 113 children in the allergy prevention study revealed a statistically significant difference already in birth weight. The mean birth weight (g) of children subsequently becoming overweight (n = 25; 22%) was higher than in those remaining normal-weight (n = 88; 78%): 3838 (95% CI, 3659-4017) vs. 3532 (95% CI, 3420-3634); $P = 0.005$, Student´s t-test. In these children, correspondingly, a significant difference was also detected in the mean BMI (kg/m^2) at birth: 13.72 (95% CI, 13.47 – 13.98) vs. 14.54 (95% CI, 13.92 – 15.16); $P = 0.006$, Student´s t-test.

As represented in Figure 14, the mean BMI of children overweight at the age of 10 years was also constantly and increasingly higher at every measurement from birth to the age of 10 years compared to the normal-weight children, showing a statistically significant difference between these two groups at every measurement point; $P = <0.001$, ANOVA for repeated measures. This result was in line with those obtained using BMI z scores (Table 4).

Table 4. Mean (SD) BMI z scores at birth and at the ages of 3, 6, 12, 24, 48, 84 and 120 months in children overweight and those normal-weight at 10 years of age.

Age	Overweight children (n = 25)	Normal-weight children (n = 88)	Difference	P *
Birth	0.81 (1.08)	0.22 (0.94)	0.59	0.009
3 months	0.38 (1.15)	-0.21 (0.88)	0.59	0.007
6 months	0.76 (0.98)	-0.04 (0.96)	0.80	0.000
12 months	0.98 (1.00)	0.18 (0.93)	0.80	0.000
24 months	1.22 (0.94)	0.23 (0.87)	0.99	0.000
48 months	1.42 (1.11)	0.12 (0.86)	1.30	0.000
84 months	2.23 (0.32)	0.32 (0.60)	1.91	0.000
120 months	2.01 (0.41)	-0.01 (0.64)	2.02	0.000

* Student´s t-test

In those children who were normal-weight at the age of 10 years the mean BMI increased up to the age of 6 months, started to decline up to the age of 48 months and remained fairly steady thereafter until 10 years of age. In those children who were overweight at the age of 10 years the mean BMI increased up to the age of 6 months, started to

decline up to the age of 24 months and thereafter increased constantly. (Figure 14). The ´initial phase´ of excessive weight gain was thus observed to set in during the fetal period and to continue until 24 to 48 months of age, and a ´second phase´ of excessive weight gain set in after the age of 24 to 48 months (Figure 14).

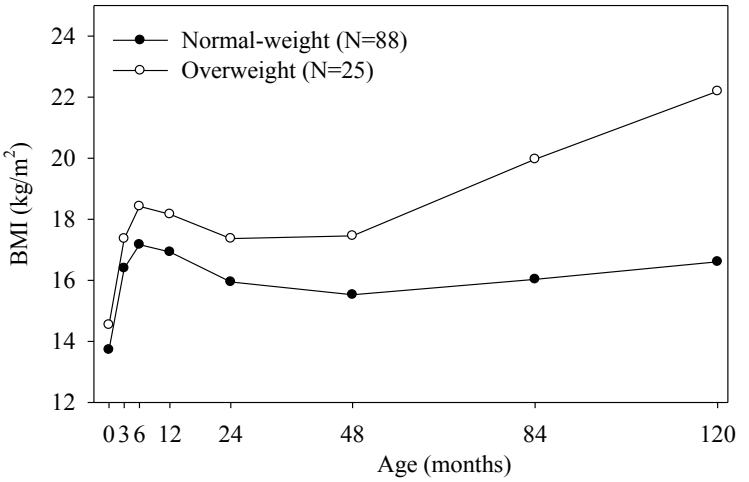

Figure 14. Mean BMI since birth in overweight children and in children of normal weight at the age of 10 years.

5.9. The effect of perinatal probiotic intervention on overweight development (III)

In the allergy prevention study the objective was to evaluate the impact of perinatal probiotic intervention on overweight development during the 10 years´ follow-up. The results demonstrated that probiotic intervention tended to reduce the birth weight-adjusted mean BMI at the age of 4 years; $P = 0.080$, ANCOVA. In the analysis using BMI z scores the impact was seen to be even more pronounced, the mean (SD) BMI z score at the age of 4 years being 0.17 (0.95) in the probiotic group and 0.62 (1.10) in the placebo group; $P = 0.038$, Student´s t-test.

The impact was observed to be more distinct among children who later became overweight. The probiotic intervention tended to moderate excessive weight gain among these children during the first years of life, the impact being most pronounced at the age of 4 years; $P = 0.063$, ANOVA for repeated measures (Figure 15).

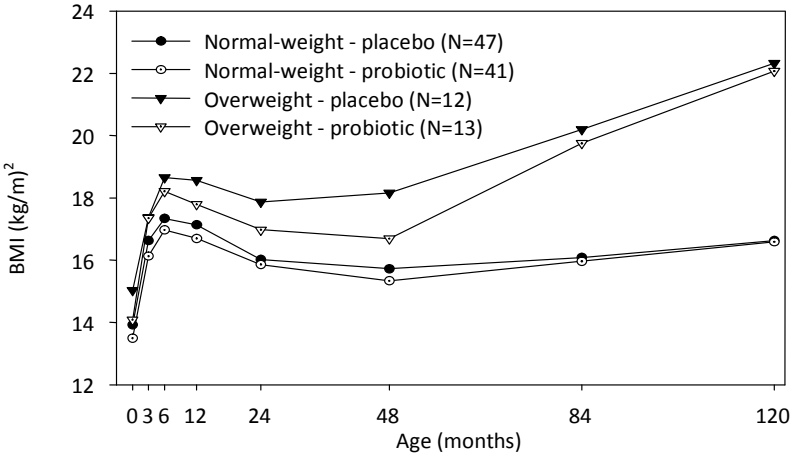

Figure 15. The mean BMI at birth and at the ages of 3, 6, 12 and 24 months and at the ages of 4, 7 and 10 years in children overweight and in those normal-weight at 10 years of age is given separately in the probiotic and in the placebo group. Significance for the mean difference between children overweight at 10 years of age is given separately in the probiotic and in the placebo group; P = 0.115, 0.979, 0.501, 0.228, 0.133, 0.063, 0.555 and 0.700 at respective ages, ANOVA for repeated measures.
(In: Luoto et al. Int J Obes, 2010 Mar 16. [Epub ahead of print])

5.10. The safety of the perinatal probiotic intervention (I & V)

The safety of the perinatal probiotic intervention in the mother-infant nutrition and probiotic study was attested by the normal duration of pregnancies and the absence of adverse events in mothers or children (Table 5). There were no significant differences among the study groups in the duration of gestation. The mean (range) birth weight of the neonates (n = 241) was 3551 (1610 - 4750) g and birth length 50.9 (44.0 – 57.0) cm, showing no significant differences among the study groups (Table 6). No perinatal deaths or serious adverse incidents such as sepsis occurred in mothers or newborns.

Table 5. Impact of perinatal probiotic-supplemented dietary counselling on pregnancies (studies I & II)

Group		**Diet/probiotics** (n = 85)	**Diet/placebo** (n = 86)	**Control** (n = 85)
Miscarriages <22 weeks		2 (2 %)	2 (2 %)	0 (0 %)
Duration of gestation		40.0 (36.9 - 42.8)	40.0 (30.5 - 42.4)	40.0 (34.9 - 43·3)
(weeks) †	< 32	0/80 (0 %)	1/79 (1 %)	0/79 (0 %)
	32 - 36	1/80 (1 %)	1/79 (1 %)	1/79 (1 %)
	37 - 41	73/80 (91 %)	72/79 (91 %)	74/79 (94 %)
	≥ 42	6/80 (8 %)	6/79 (8 %)	4/79 (5 %)
Cesarean delivery		12/75 (16 %)	12/77 (16 %)	11/76 (14 %)

Results are given as mean (95 % CI) or as numbers (%) of subjects.
None of the differences among the groups was significant.

Table 6. Impact of perinatal probiotic-supplemented dietary counselling on the neonates (studies I & II)

Group	**Diet/probiotics** (n = 82)	**Diet/placebo** (n = 80)	**Control** (n = 79)
Birth weight (g)	3467 (3370 - 3564)	3579 (3469 - 3688)	3611 (3494 -3727)
Birth length (cm)	50.7 (50.3 - 51.1)	51.2 (50.7 - 51.6)	51.0 (50.5 - 51.5)
Birth head circumference (cm)	34.8 (34.5 - 35.1)	35.0 (34.7 - 35.3)	35.2 (34.9 - 35.5)
5-minute Apgar score †	9 (6 - 10)	9 (3 - 10)	9 (4 - 10)

Results are given as mean (95 % CI) or as numbers (%) of subjects.
† median (range).
None of the differences among the groups was significant.

The prenatal and postnatal growth of the children in the mother-infant nutrition and probiotic study was chosen as one indicator of the safety of the perinatal probiotic intervention. No statistically significant differences were found among the study groups in prenatal weight gain ($P = 0.809$, ANOVA for repeated measurements, group effect, Figure 16), postnatal weight gain ($P = 0.983$ for group effect; $P < 0.001$ for time effect; $P = 0.520$ for group x time interaction; ANOVA for repeated measurements) or postnatal growth in length ($P = 0.872$ for group effect; $P < 0.001$ for time effect; $P = 0.325$ for group x time interaction; ANOVA for repeated measurements).

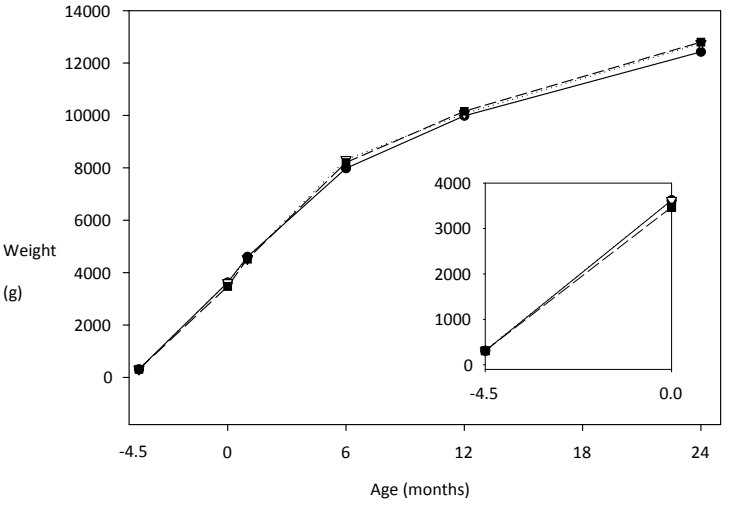

Figure 16. Impact of probiotic-supplemented dietary intervention on perinatal growth from 20th pregnancy week to delivery and over the follow-up period to 24 months´ age in the diet/probiotics group (n 28; ■), diet/placebo group (n 25; ▼) and control group (n 17; ○). Values are mean weights. (In: Luoto et al. Br J Nutr 2010;103:1792-9)

Study V:

In the assessment of the safety of postnatal prophylactic use of LGG as a part of enteral feeding in VLBW infants during the 12-year period 1997 - 2008 safety was attested with no cases of LGG septicemia. The incidence of NEC during the period in question was 4.6 % (range 0 – 11.3 %).

6. DISCUSSION

6.1. Overweight development

The maternal nutritional environment constitutes a decisive canvas in the *in utero* context impacting upon the health and well-being of mother and child also long-term. The importance of a balanced perinatal nutritional environment emerges in the perception that alterations in the intrauterine and early postnatal nutritional and metabolic environment have an impact on the long-term determination of fundamental processes of life and thus as a corollary may predispose to disorders and diseases throughout later life, both in the mother and in the child (Lucas 1998). The fetus is capable of responding to the mothers's diet by different mechanisms: altering gene expression, reducing / increasing cell numbers and selecting clones of cells, and thereby reducing / increasing body size and altering metabolism (Barker 2004).

In Western societies the fetus is often over-nourished due to the mother's excessive dietary intake of saturated fat and carbohydrates of high glycemic index, this leading to fetal macrosomia and consequently to a risk of overweight development in the child later in life (Parsons et al. 2001). Interestingly, the perinatal dietary intervention undertaken in the mother-infant nutrition and probiotic study was found to reduce the risk of fetal overgrowth associated with GDM-affected pregnancies (**I**). This finding is important, since maternal pre-pregnancy obesity, excessive weight gain during pregnancy and GDM are the major risks for fetal macrosomia (Lawror et al. 2010), creating the vicious circle of intergenerational overweight and metabolic disorders. In demonstrating that this risk is modiafiable, the present result add weight to the argument that the continuing burden of overweight may be reduced by interventions initiated already during the fetal period.

A growing body of evidence suggests that not only the fetal period but also early infancy is of major importance in the programming of adult health and disease (Barker et al. 2005). Epidemiological data point to high birth weight on the one hand (Eriksson et al. 2003, Hui et al. 2008) or faster postnatal growth on the other (Ekelund et al. 2006, Stettler et al. 2002, McCarthy et al. 2007, Braddon et al. 1986) as the initial requisite for later obesity. The results of this series of studies, in showing a significantly higher birth weight and constantly increasing BMI from birth onwards in children later becoming overweight (**III**), tend to confirm epidemiological data indicating that even prior to childhood, the perinatal period and early infancy may lay the foundation for the development of the child´s metabolic phenotypes. This notion was further supported in

the subgroup analyses from the allergy prevention study (**IV**), where the birth size did not differ between the children subsequently becoming overweight and normal-weight due to the matched BMI at birth. Among these children the excessive weight gain was observed to set in immediately after 3 weeks of age, the difference being already significant at the age of 3 months.

Furthermore, the findings in children followed for 10 years in the allergy prevention study (**III**) showed the excessive weight gain patterns leading to overweight to be two-dimensional. The initial phase set in already in the perinatal period and lasted to the age of 24 to 48 months, while a second phase started after 4 years of age. This finding conforms with the previously observed three-dimensional postnatal growth phases of the child; infancy, childhood and adolescence (Rosenbloom 2007). The initiation of each phase has been hypothesized to be the most sensitive period for overweight development prevention (Rolland-Cachera et al. 1984, Dietz 1994). On this basis, previous interventions targeting the long-term regulation of energy balance have focused primarily on childhood environmental factors such as dietary patterns and physical activity, especially during the periods when the child´s individual eating and activity patterns are developing (Summerbell et al. 2005). These interventions have thus far shown at best marginal efficacy.

6.2. The role of the initial microbial environment in overweight development

The key cause of overweight in humans is indubitably an imbalance between energy intake and expenditure. The early origins of overweight development however, challenge this orthodox point of view, since the diet during the first months of life, i.e. breast milk or formula with few solid foods, is markedly homogeneous worldwide and is thus unlikely to be *per se* the explanation for later overweight development. An innovative hypothesis has recently been proposed whereby deviations in gut microbiota composition could play an instrumental role in the control of host body weight and energy metabolism (Bäckhed et al. 2004, Claus et al. 2008), and furthermore favor the occurrence of the low-grade inflammatory state and metabolic diseases especially among individuals predisposed to obesity (Cani et al. 2007a, Membrez et al. 2008).

In a pioneer study conducted in germ-free mice, a group led by Jeffrey Gordon has provided evidence of gut microbiota metabolic activities facilitating the extraction of calories from ingested dietary substances and their storage in host adipose tissue for later use (Bäckhed et al. 2004, Bäckhed et al. 2007). Following observations of characteristic alterations in the gut microbiota composition of obese compared with

lean mice (Ley et al. 2005) the same group demonstrated the transferable nature of the obese phenotype by transplanting obese flora to germ-free mice (Turnbaugh et al. 2006). In a separate study design, the same researchers confirmed the presence and nature of alterations in the gut microbiota in obese individuals (Ley et al. 2006, Turnbaugh et al. 2009). Additionally, they demonstrated that the obese host microbiomes were found to be enriched in gene categories involved in carbohydrate and lipid metabolism (Turnbaugh et al. 2009).

In the aforementioned studies, several mechanisms were proposed to link the intestinal microbiota with obesity (Figure 17). Firstly, perturbations in the gut microbiota composition, i.e. dysbiosis, could promote intestinal monosaccharide absorption and energy extraction from nondigestible food components via SCFA production and hepatic de novo lipogenesis (Bäckhed et al. 2004). Furthermore, dysbiosis could increase fatty acid storage in adipocytes by suppressing the fasting-induced adipose factor (FIAF) in the gut which in turn increases enzyme lipoprotein lipase (LPL) activity. A second mechanism envisaged as linking balanced gut microbiota composition, i.e. eubiosis, to protection against diet-induced obesity could be inhibition of cellular energy-dependent protein kinase (AMPK) activation and thus fatty acid oxidation (Bäckhed et al. 2007). A third explanation is proposed to be the association between SCFA signaling molecules, G protein-coupled receptor (GPR41) activation and energy storage (Samuel et al. 2008). Whatever the actual mechanism, these studies elegantly provided innovative experimental and clinical evidence of a relation between gut microbiota composition and both energy harvest from nutrition and host gene and protein activity involved in the metabolism and storage of absorbed energy.

Interestingly, experimental findings to the contrary have also recently been reported. In a study conducted by Fleissner and associates (2010) the absence of intestinal microbiota did not protect mice from diet-induced obesity. Additionally, this study showed that the intestinal production of FIAF was not instrumental in gut microbiota-mediated effects on fat storage.

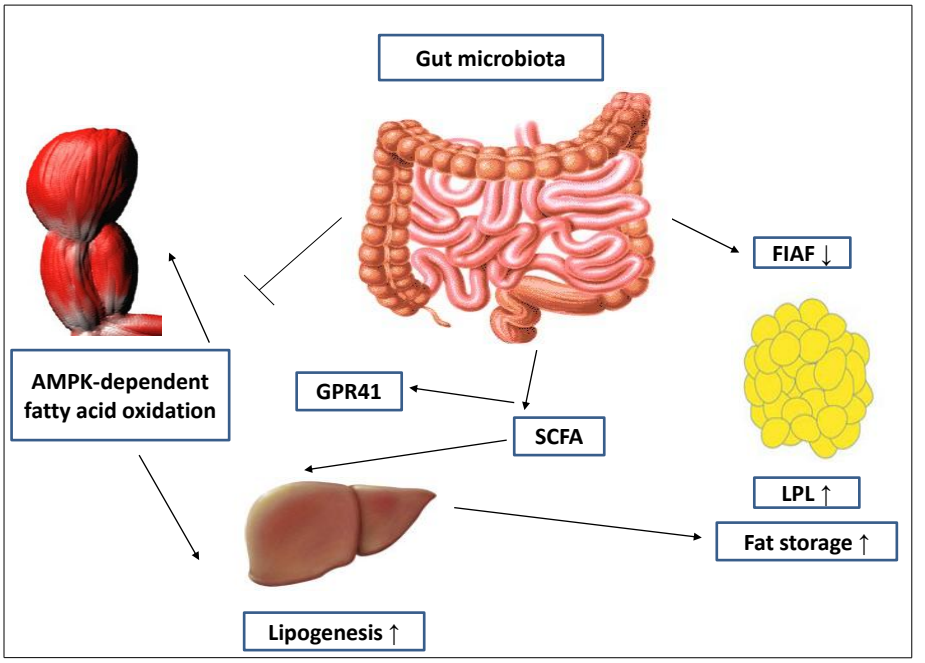

Figure 17. The gut microbiota may regulate energy storage by increasing fermentation of indigestible dietary polysaccharides, by increasing monosaccharide absorption, by producing lipogenic substrates (SCFAs), by increasing hepatic lipogenesis, by suppressing the FIAF in the gut, which in turn increases LPL activity, by inhibiting AMPK-dependent fatty acid oxidation, and by acting through the SCFA receptor GPR41. Modified from Cani and Delzenne 2009.

Recently, the relative abundance or paucity of various types of gut bacteria in obese and lean humans has also been demonstrated by other study groups (Duncan et al. 2008, Zhang et al. 2008, Armougom et al. 2009, Schwiertz et al. 2009, Tiihonen et al. 2009, Nadal et al. 2009, Santacruz et al. 2009). It is of note that in the aforementioned study by Santacruz (2009) the response of overweight adolescents to a diet and exercise weight-loss program was shown to be dependent on the initial microbiota prior to the treatment. In fact, a connection between a relative abundance of Bacteroidetes and obesity was first proposed, but in the light of the most recent findings, the possibility was envisaged that smaller changes in the gut microbiota community, rather than those obtaining at wide *phylum* levels, are involved in overweight development. Furthermore, administration of different strains of probiotics both exprimentally as well as in clinical trials have been shown to exert beneficial influence of metabolic disorders (Takemura et al. 2010, Kadooka et al. 2010)

Data on the relationship between early intestinal microbiota and subsequent overweight development in children are hitherto scant. Kalliomäki and colleagues (2008) have compared over time groups of children from the same allergy prevention study used in this thesis and observed that those who became overweight by 7 years of age had had lower levels of Bifidobacteria and higher levels of *Staphylococcus aureus* at 6 and 12

months of age compared to those remaining normal-weight. Interestingly, this type of result has also been shown in another studies conducted among pregnant women. The association between gut microbiota composition and maternal nutritional status during pregnancy supports the view that a gut microbiota profile favoring a higher number of Bifidobacteria and a lower number of *Staphylococcus aureus*, may provide protection against maternal overweight development (Collado et al. 2008a, Santacruz et al. 2010).

On this basis, the intriguing question can be proposed: could modulation of the initial gut microbiota contribute to a reduced risk of overweight development? The results of this series suggest such an effect for the first time. The perinatal probiotic intervention with LGG in the allergy prevention study was observed to moderate excessive weight gain during the 10 years´ follow-up (**III**). This effect was demonstrated as a tendency to restrain the initial phase of excessive weight gain, especially among the children who later became overweight, but not the second phase of excessive weight gain, the impact being most pronounced at the age of 4 years. It is conceivable that the possible protection against early excessive weight gain provided by the LGG probiotic may have been mediated *via* both energy harvest and immunomodulatory pathways, since probiotics may modify the intestinal *milieu* towards a nonobesitogenic environment by altering the fermentation of indigestible dietary polysaccharides and inhibiting fat storage through FIAF expression (Bäckhed et al. 2004).

6.3. The role of the gut microbiota in overweight-related low-grade inflammation

It is increasingly recognized that obesity is characterized by chronic activation of inflammatory pathways in adults (Wellen and Hotamisgil 2003, Hotamisgil 2006), but also in children (Skinner et al. 2010, Tam et al. 2010). These inflammatory signaling pathways are causally linked to insulin resistance (Kahn and Flier 2000), this being in turn a central prerequisite for a cluster of overweight-associated pathologies, including non-insulin-dependent diabetes, hypertension, hepatic steatosis and dyslipidemia (Saltiel and Kahn 2001).

Unequivocal experimental evidence of the mechanisms linking the gut microbiota to the development of obesity-related low-grade inflammation and metabolic disorders (Figure 18) has been published by a study group led by Nathalie M. Delzenne. First, the gut microbiota-derived LPS was shown to be a triggering factor for the early development of inflammation, insulin resistance and diabetes (Cani et al. 2007a). Excess dietary fat was shown to facilitate the absorption of LPS from the gut through an intestinal CD14 / TLR-4 -ligand, leading to a state defined as ´metabolic endotoxemia´ (Cani et al. 2007a, Amar et al. 2008). Next, high-fat feeding was found to bring about changes in the gut bacterial populations, especially to reduce the relative amount of Bifidobacteria (Cani

et al. 2007b) and to increase gut permeability (Cani et al. 2008). Thirdly, the association between changes in the gut microbiota toward a decreased number of Bifidobacteria and obesity-related metabolic disorders was further supported by the demonstration that prebiotics increase the number of intestinal Bifidobacteria and consequently reduce the impact of high-fat-diet-induced metabolic endotoxemia and insulin resistance (Cani et al. 2006, Cani et al. 2007b). Furthermore, gut microbiota modulation with prebiotics was shown to improve gut barrier function (Cani et al. 2009).

The association between obesity and elevated LPS levels, originating most probably from the gut microbial community, has also been demonstrated by Brun and associates (2007) and Creely and associates (2007). The linkage between relative shortage of intestinal Bifidobacteria and obesity has been further proved by Turnbaugh and associates (2008) and Waldram and associates (2009). These findings are indeed in line with the previously demonstrated capability of Bifidobacteria to reduce intestinal LPS levels and to improve mucosal barrier function (Griffiths et al. 2004, Commane et al. 2005, Wang et al. 2006, Ruan et al. 2007).

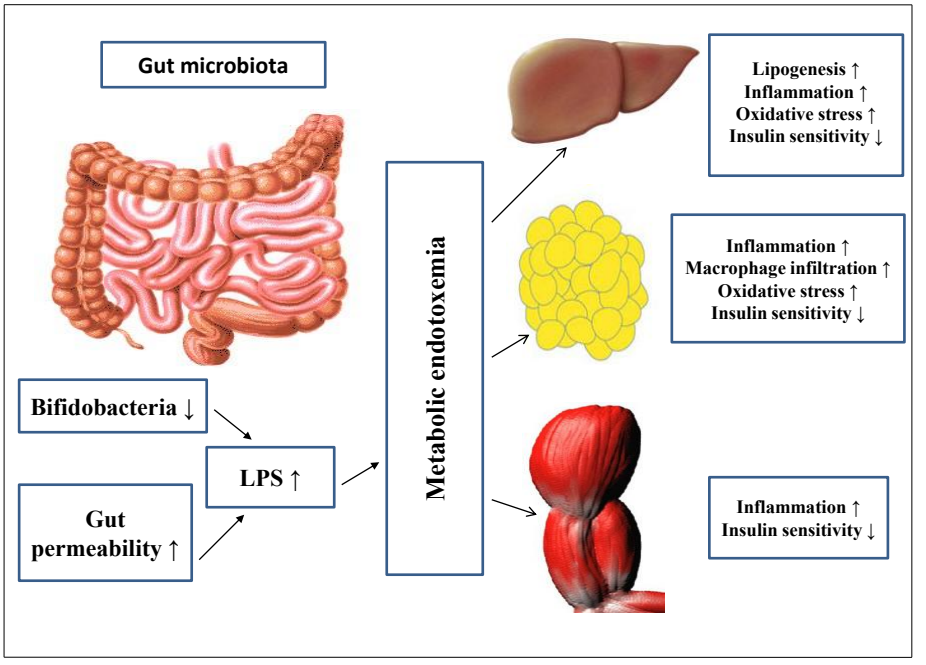

Figure 18. Changes in the gut microbiota following high-fat diet or obesity promote gut permeability, increase metabolic endotoxemia and trigger the development of metabolic disorders. High-fat diet feeding alters the gut microbiota composition in a complex way, with a specific decrease in Bifidobacterium spp. This phenomenon is associated with higher gut permeability, leading to a higher plasma LPS levels, e.g. metabolic endotoxemia, which promotes low-grade inflammation-induced metabolic disorders such as insulin resistance, diabetes, obesity, steatosis, oxidative stress and adipose tissue macrophage infiltration. Modified from Cani and Delzenne 2009.

On the basis of the above studies, the development of obesity-related metabolic disorders following high-fat diet-induced changes in the gut microbiota composition has been shown to be associated with the innate immune system *via* the mechanisms dependent on LPS-induced endotoxemia. Cytokines such as TNF-α, IL-1 and IL-6 are the major proinflammatory mediators produced in response to CD14 / TLR-4 -receptor activation by LPS (Parker et al. 2007). Due to the large number of LPS-containing gram-negative bacteria residing in the gut, chronic stimulation of intestinal TLR-4 may exacerbate the low-grade inflammatory state associated with obesity and insulin resistance (Reigstad et al. 2009). Such an assertion is further supported by the experimental demonstration that mice lacking TLR-4 are resistant to diet-induced obesity and insulin resistance (Tsukumo et al. 2007). In point of fact, the link between the LPS-TLR-4 pathway and the development of systemic low grade inflammation has been previously described (Stoll et al. 2006, Creely et al. 2007), the determinant role of the gut microbiota being the new extension in this line of research. Interestingly, the dietary fatty acids, whose circulating levels are often increased in obesity, have previously been shown to induce insulin resistance through TLR-4 signaling, this finding linking the innate immune system to insulin resistance also in response to changes in the nutritional environment (Shi et al. 2006). A novel putative link between gut microbiota composition, LPS, and low-grade inflammation has further been proposed to be the endocannabinoid system, which is *per se* modulated by the gut microbiota composition, and which further regulates gut permeability and as a consequency systemic LPS levels (Muccioli et al. 2010).

The results of these present studies suggest that the initial gut microbiota composition deviates in children later developing overweight from that in normal-weight children (**IV**). It is of note that despite the tendency to lower Bifidobacteria number in stools at the age of 3 months in children subsequently becoming overweight compared to those normal-weight, no signs of endotoxemia or low-grade inflammation were detectable at that same time point. It is feasible that despite the differences in fecal *Bifidobacterium* microbiota among the infants, the amount and composition of fat in the diet was still too similar during the first months of life to have elicited endotoxemia. Alternatively, the finding may be interpreted to indicate that a threshold for the development of low-grade inflammation was not passed. Moreover, the hsCRP synthesized solely by the hepatocytes is on the other hand regulated mainly by the pro-inflammatory cytokines produced by the adipocytes of visceral fat (Lemieux et al. 2001). The concentration of hsCRP does not thus rise until central obesity and age increase.

On the grounds of the data described a hypothesis may be formulated that the alleviation of bacterial-induced inflammatory responses play an instrumental role in the mechanisms whereby the perinatal probiotic intervention undertaken in the allergy prevention study

prevented overweight development during the first years of life (**III**). The protective role of LGG may be partly mediated by its own contribution to the inflammatory responses or by its impact on a more diverse *Bifidobacterium* microbiota establishment in a child (Gueimonde et al. 2006b). Bifidobacterial diversity may further enhance the maturation of the mucosal sIgA system, thus reinforcing the gut barrier (Sjögren et al. 2009). It is plausible that such an effect was achieved, since the intervention was carried out during the critical period of life, when the development of the child´s immunological and metabolic phenotypes are being consolidated (Cummings et al. 2004, Pesonen et al. 2009, Barker 1997). This finding (**III**) is in line with the previously demonstrated long-lasting clinical benefits of perinatal LGG administration in this same study population (Kalliomäki et al. 2001) irrespective of any long-term impact on gut microbiota composition (Rinne et al. 2006, Gueimonde et al. 2006a). The capacity to restrain dysregulated immune responses may originate not only from the amount or quality of antigens provided by balanced gut microbiota bacteria but also from the diminished number of signals from the damage and stress induced, the latter having presumably more responsibility in the size and longevity of an immune response (Noble 2009). It must nonetheless also be acknowledged that these responses evolving in the early extra-uterine setting may influence the risk of overweight development by interacting with socioenvironmental factors. One possible reason why the early but not the second phase of excessive weight gain was shown to be modifiable by perinatal probiotic intervention (**III**) is that the excessive weight gain starting after 4 years of age may be more strongly influenced by individual eating and activity patterns, this delineating the multifactorial requisites for overweight development during the later life span.

The observed pronounced effect of probiotic intervention, initiated during the first trimester of pregnancy in the mother-infant nutrition and probiotic study, on GDM prevention (**I**) was probably attributable to the immunoregulatory properties of probiotics LGG and *Bifidobacterium lactis* Bb12. The innate immune system has been demonstrated to participate, apart from microbial recognition, also in the regulation of glucose metabolism and insulin resistance (Shi et al. 2006). The reduction of endotoxemia and low-grade inflammation *via* intestinal microbiota modulation thus contributes to the systemic inflammatory tone control and as a corollary to insulin sensitivity improvement. This finding fully corroborates those of Laitinen and associates (2009), which provided the first clinical evidence of consistently improved plasma glucose concentrations and insulin sensitivity in these healthy women during pregnancy and 12 months *post partum* when advantageous dietary intake was combined with probiotics. It is however conceivable, that since this study population comprised metabolically healthy women, the results probably underestimate the beneficial impact of the probiotics used in the prevention of GDM. This result in a general normoglycemic population calls for further research in other, especially at-risk populations.

6.4. Prenatal microbial programming – hypothesis

During fetal life the development of the gastrointestinal tract is largely regulated by genetic programming, but external factors such as the amniotic fluid also play a fundamental role (Hirai et al. 2002). Given the importance of the gut microbiota in intestinal and gut barrier development as well as in the immunological programming of the neonate, it must be acknowledged that the general conception of a sterile intrauterine existence (Mackie et al. 1999) has recently been challenged. Commensal intestinal bacteria such as *Bifidobacterium* and *Lactobacillus* and their DNA have been found in placentas, including those derived from elective cesarean sections (Satokari et al. 2009) and in the meconium samples of healthy newborns (Jiménez et al. 2008, Mshvildadze et al. 2010). These findings suggest that horizontal bacterial transfer from mother to fetus may occur during pregnancy and thus programme the infant´s gut barrier and immune system development earlier than previously anticipated. In fetal murine models, gut epithelial cells have been shown to be sensitive to microbial factors such as LPS, as they express intracellularly a PRR for this ligand, namely TLR-4 (Lotz et al. 2006). Microbial exposure *in utero,* has also been shown affect gene expression with an impact on the clinical phenotype (Takahashi et al. 2009). It is thus possible that the nature of initial immunological responses to environmental agents is already determined *in utero* and the pattern of these responses may be critical in programming the expression of disease-specific responses later in life.

In this series of studies it was demonstrated that perinatal dietary counselling, equally with and without probiotics, moderated excessive fetal growth in GDM-affected pregnancies (**I**). It was previously reported in this particular mother-infant nutrition and probiotic study that intensive dietary counselling resulted in changes in dietary intake during pregnancy attributable to a higher intake of unsaturated and a lower intake of SFAs (Piirainen et al. 2006). On this basis it is conceivable that this present result may be explained by the change achieved in dietary fat composition, which also further modifies the gut microbiota composition and the development of innate immunity (Calder 2006). Additionally, the dietary fatty acids, whose circulating levels are often increased in obesity, have previously been shown to induce insulin resistance *via* TLR-4 signaling. This finding links the innate immune system to insulin resistance also in response to changes in the nutritional environment (Shi et al. 2006). Thus, the gut microbiota may regulate insulin sensitivity vicariously both by producing proinflammatory mediators and by regulating host lipid metabolism (Velagapudi et al. 2010).

6.5. The role of the initial nutritional environment in overweight development

Inasmuch as breastfeeding has a major influence on the composition of the infantile intestinal microbiota by providing both beneficial bacteria and prebiotic growth factors (Liepke et al. 2002), it may *per se* protect the infant from overweight development later in life (von Kries et al. 1999, Gillman et al. 2001, Owen et al. 2005). Breastfeeding has also been shown to reduce the risk of hypercholesterolemia (Owen et al. 2008) and non-insulin-dependent diabetes (Pettitt et al. 1997, Owen et al. 2006) later in life. The results of the studies mentioned demonstrate that the beneficial effects of breastfeeding may extend well beyond infancy and suggest that bioactive substances in breastmilk may prime the infant´s immune system and metabolism during infancy in a way that promotes health decades later. What is more, breast milk-derived soluble receptors are also vital in linking dietary fatty acids with the innate immune system, further strengthening the beneficial intestinal cross-talk for inflammation control (Laitinen et al. 2006).

Notwithstanding the well characterized beneficial effects of breast milk, breastfeeding does not prevent overweight development in all infants. The obscure bridge between early nutrition and weight development may thus lie in compositional differences in mother´s breast milk, this being dependent on the mother´s nutritional and immunological state and also on her gut microbiota composition. Adiponectin was chosen as a metabolic marker here, since it represents a key molecule in protection against metabolic syndrome (Li et al. 2009) with its anti-atherogenic and anti-inflammatory properties (Fantuzzi 2005) and its participation in glucose and lipid metabolism (Okamoto et al. 2006, Tsatsanis et al. 2006). Nevertheless, very little is known of the maternal factors determining the adiponectin concentration in breast milk. In a study by Martin and colleagues (2006) the postpregnancy BMI of mothers (n = 22) was found to correlate positively with the adiponectin concentration in their breast milk. Bronsky and associates (2006) demonstrated that the pre-pregnancy weight, but not the BMI, of the mothers (n = 59) correlated positively with the adiponectin concentration in the colostrum. Additionally, only scant evidence has thus far demonstrated an association between breast milk adiponectin concentration and childhood weight gain. A group under Weyermann (2007) found that higher levels of adiponectin in breast milk were associated with overweight in children at two years of age, particularly among those who were breast-fed for at least 6 months. The study in question was conducted in a cohort of 674 children and the breast milk samples were collected 6 months after delivery. In contrast to this finding Woo and associates (2009) demonstrated in two different mother-infant cohorts (n = 45 and 277), that higher milk adiponectin was associated with a lower infant weight-for-age Z score and weight-for-length Z score during a 6-month follow-up. The breast milk samples in that study were collected from the first cohort monthly up to 6 months

postpartum and from the other cohort at least twice between baseline (1 week) and 6 months postpartum.

The present results provide, firstly, evidence that the colostrum adiponectin concentration is markedly strongly dependent on maternal nutritional status and diet during pregnancy, even in a population of metabolically healthy women (**II**). In showing that dietary counselling was connected with increased adiponectin concentration in the colostrum, while in contrast excessive maternal weight gain and GDM during pregnancy were accompanied by lower adiponectin concentration in the colostrum, this result underscores the importance of the metabolic homeostasis of the mother for the child´s initial nutritional environment. Furthermore, this demonstration could furnish one explanation for the well characterized intergenerational vicious circle of overweight and obesity (Catalano et al. 2009, Whitaker et al. 2010). The mechanism of action remains elusive, but it is conceivable that the innate immune system, *via* the CD14 / TLR-4 pathway, might be involved.

Secondly, the results here provide novel evidence that the colostrum adiponectin concentration had been significantly higher in mothers whose children were normal-weight than in those whose children were overweight at the age of 10 years (**IV**). This finding reinforces the conception of the early nutritional environment as a potential initial requisite for later overweight development. The exact mechanism whereby adiponectin protects against subsequent overweight remains, however, to be deciphered. Breast milk adiponectin ingested by the infant could be partly absorbed and thus act systemically (Newburg et al. 2010). It is more likely, however, that part of it acts locally in the intestine, the impact being conveyed *via* its regulatory effects on the innate immune system (Yamaguchi et al. 2005). One interesting finding in this present series was that the children normal-weight at the age of 10 years had had significantly higher sCD14 concentrations in the serum at the age of 3 months than those who were overweight by the age of 10 years (**IV**). Since the sCD14 levels are strongly associated with endotoxin levels, current orthodoxy holds that sCD14 is a valuable marker of low-grade inflammation, whatever might be the reason for the state (McFarlin et al. 2007, Harte et al. 2010). An explanation for the result presented here might be that adiponectin, with its dual opposing activities in macrophages and adipocytes, is fundamental in establishing a systematically protective effect during the initiation of inflammation, yet locally can have an opposing, proinflammatory effect (Tsatsanis et al. 2005). As a tolerogenic response has been shown to accomplish sustained exposure to LPS or adiponectin itself, the higher concentrations of sCD14 in children of normal weight at the age of 10 years can be hypothesized to be a consequence of adiponectin exposure and to be further involved in the down-regulation of the deleterious immune responses mediated through TLR-4 (Kitchens and Thompson 2005), this preventing excessive weight gain later in life.

6.6. Safety documentation of perinatal probiotic intervention

This series demonstrated in two different study populations the safety of perinatal probiotic interventions. In the mother-infant nutrition and probiotic study the probiotic intervention with LGG and *Bifidobacterium lactis* Bb12 was proved to be safe, regardless of the fact that the intervention was initiated already during the first trimester of pregnancy (**I**). The normal duration of pregnancies and pregnancy outcome, as well as the complete absence of perinatal deaths or serious adverse incidents such as sepsis in mothers or newborns confirm that no interference with the physiological equilibrium and duration of the pregnancy was brought about by this approach. Moreover, the similar prenatal weight gain and postnatal weight gain and growth in length over the 24 months´ follow-up among the children in all three study groups indicates an adequate nutrition supply, absorption and exploitation of nutrients, and the long-term safety of the probiotic intervention (**I**).

The results here also provided evidence of the safety of LGG probiotic used as a prophylactic element in enteral feeding in a high-risk population, VLBW infants (**V**). The total absence of LGG septicemia in the population consisting of all VLBW infants treated in Turku University Hospital during the years 1997-2008 confirms that no interference occurs in the oral consumption of LGG, a viable bacteria, during the most sensitive period of premature life, when the child´s microbial and immunological phenotypes are being consolidated, this concomitantly assuring the safety aspect. Furthermore, the incidence of NEC was comparable with the level observed in such patient populations in other countries (Lin and Stoll 2006). These findings, together with the unchanged NEC-related mortality (one out of five died) among the same study population (Luoto et al. 2010), may justify further LGG intervention trials in the attempt to reduce the risk of NEC in populations with a higher incidence of this condition and putatively also in the many gastrointestinal complaints associated with prematurity.

6.7. The perinatal window of opportunity

Life in industrialized societies has introduced profound changes into the human environment (e.g. diet, antibiotics, hospital deliveries, improved hygiene etc.) which are markedly different from the conditions in which humans have evolved and which are likely to have occurred too abruptly for the human microbiome to adjust to (Bach 2002). Consequently, aberrations in the gut microbiota induced through lifestyle factors could be relevant to the etiology of a number of immune-mediated diseases whose occurrence has markedly increased in developed countries, for example allergic, auto-immune and inflammatory bowel diseases, a phenomenon defined by the ´hygiene hypothesis´ (Vuillermin et al. 2009). It is remarkable that the secular trends

in increasing immune-mediated diseases and obesity are so similar that they could almost be superimposed one over the other, and that according to current knowledge similar environmental influences underlie the increasing prevalence of obesity and allergic diseases, i.e. a low-grade systemic inflammation (Weiss 2005, Litonjua and Gold 2008).

The development of gut-associated immune homeostasis depends on a ´window of opportunity´ where innate and adaptive immunity are coordinated by APCs; their function is orchestrated not only by microbial products but also by breast milk constituents, both of which reinforce the gut barrier. Indeed, the gut barrier-related homeostasis depends on ´cross-talk´ between the epithelium (via cytokines and other factors) and lamina propria cells, including macrophages, DCs and T cells. When immune regulation is operating in a healthy manner the small amounts of antigens from commensal bacteria and diet reaching the lamina propria will be handled by DCs and macrophages in a homeostatic manner, with balanced cytokine secretion and induction of T_{reg} cells, a concept previously defined in the context of allergy as ´oral tolerance´ (Brandtzaeg 2010, Walker 2008). If the antigenic influx is excessive or immunoregulation defective, immune reactions may be driven into hypersensitivity and enter a vicious circle of proinflammatory cytokines and epithelial apoptosis (Groschwitz and Hogan 2009). Furthermore, it is possible that this excitability evolving during early postnatal life may determine life-long reactivity and responsiveness to medical and lifestyle interventions.

On the basis of the results reported in this thesis, it might be possible to extend the concepts of ´hygiene hypothesis´ and ´oral tolerance´ to the metabolic profiling of the newborn. In fact, newborn intestinal colonization appears to constitute a critical window for the interaction of the host with intestinal microbes, as a similar reconstitution of the gut microbiota at a later age has failed to have such a beneficial effect (Sudo et al. 1997). Hence, deviations in the initial intestinal colonization may be attributed to gut barrier failure, this predisposing the newborn to chronic inflammatory diseases such as overweight development. Likewise, the importance of the demonstration that colostrum adiponectin may protect from excessive weight gain during early extrauterine existence may culminate at the initial postnatal stage when the immunologically immature and inexperienced host encounters a myriad of environmental antigens via the mucosae in the gut (Round and Mazmanian 2009). The ´window of opportunity´ for preventive interventions appears to present itself during the perinatal period, since the infant´s intestinal microbiota already becomes established after 1 week of life (Palmer et al. 2007). It must however be acknowledged that the gut microbiota remains in a relative state of flux until it reaches equilibrium, an adult-like microbial population, by the end of infancy (Palmer et al. 2007).

The importance of the maternal nutritional status and diet during pregnancy may be extended to a completely novel line, a microbiological point of view. Considering that the maternal gut microbiota composition is dependent on diet, pre-pregnancy weight and weight gain during pregnancy (Collado et al. 2008a) and that the maternal microbiota is the first inoculum for the child's microbiota (Mackie et al. 1999), the maternal nutritional status may have a more complex impact through microbial transmission on the metabolic programming of the child than previously anticipated. Additionally, the finding that the adiponectin concentration in the colostrum reflects maternal nutritional status and diet during pregnancy, and considering that the colostrum adiponectin may be involved in energy balance regulation in the infant also long-term, the results here would indicate that long-term health benefits for children may be conferred by balanced maternal nutrition during pregnancy and lactation and by appropriate development of the child`s gut microbiota.

6.8. The strengths and limitations of this study

One clear strength in this series was the double-blind, placebo-controlled design of both prospective follow-up studies. Especially the design of the mother-infant nutrition and probiotic study, in which the aim was to optimize maternal dietary intake and metabolism to promote maternal health and to reduce the risk of disease, allergic as well as metabolic, in the child is unique. Moreover, the high proportion of women having allergic diseases may be considered a strength of these studies, since according to current knowledge similar environmental influences underlie the increasing prevalence of obesity and allergic diseases, i.e. low-grade systemic inflammation (Weiss 2005 and Shore 2008). Interestingly, previous studies reporting data on non-insulin-dependent diabetes or GDM do not report the frequency of allergic diseases at all, although atopy and allergic disease affect a significant proportion of the Western population (Haahtela et al. 2008).

The prevalence of GDM was surprisingly high (**I**), mainly due to the diagnostic criteria for the disorder used in Finland during the study years 2002-2005. Universal acceptance of the diagnostic criteria for GDM, has however, not yet been achieved and national care guidelines dictate the type of screening and threshold selection in conjunction with the population-specific profile (Yogev et al. 2009). Nevertheless, elevated maternal glucose levels during pregnancy, even if within the normal nondiabetic range, have been shown to be related to larger offspring birth size and an increased risk of interventional deliveries (HAPO Study Cooperative Research Group, et al. 2008). Hence, the findings in our study invite acceptance of new universal guidelines for the type of threshold selection of GDM. Since this present study population comprised metabolically healthy women, the results probably underestimate the beneficial impact of the probiotics used in the prevention of GDM and call for further multidiscliplinary and clinical trials especially in

at-risk populations, the better to understand the mechanism by which probiotics and gut microbiota interact with the host and to identify the best perinatal dietary matrix.

In studies **II** and **IV** we measured all multimeric forms of adiponectin together, i.e. the total adiponectin concentration. This might be a limitation, since high-molecular-weight (HMW) adiponectin has been proposed to be the biologically active form of the hormone (Hara et al. 2006), although data also exist supporting the superiority of total adiponectin concentration in assessing metabolic variables (Blüher et al. 2007).

In study **IV** the main shortcoming was the limited number of study subjects, this further affecting the statistical analyses in lessening their power as well as also leading to incidental findings. These results certainly call for further multidiscliplinary trials designed to better understand the mechanism by which colostrum adiponectin could be involved in neonatal metabolic programming *via* the LPS-LBP ligand and the CD14 / TLR-4. Furthermore, longitudinal studies with a larger study population are needed to verify these very preliminary results.

7. SUMMARY AND CONCLUSIONS

The results of this present series suggest that a balanced maternal nutritional environment combined with gut microbiota modulation with probiotics can ameliorate perinatal energy homeostasis with long-term health implications conferred on both mother and child. This was demonstrated among metabolically healthy pregnant women for whom probiotic-supplemented perinatal dietary counselling was initiated during the first trimester of pregnancy. The probiotic intervention contributed to a lower incidence of GDM and the dietary intervention to more optimized fetal growth in GDM-affected pregnancies, i.e. pregnancies involving a risk of fetal overgrowth. Moreover, the dietary intervention was shown to elicit increased adiponectin concentrations in the colostrum, a result also demonstrated to be related to a balanced maternal nutritional status and a reduced risk of overweight development in the child during a 10 years´ follow-up. When the perinatal probiotic intervention was initiated during the third trimester of pregnancy, it tended to restrain excessive weight gain during the first years of life, the impact being most pronounced among children subsequently becoming overweight. In a substudy of this same population, there was also evidence that the amount of fecal Bifidobacteria had been lower at the age of 3 months among children who were overweight compared to children who were normal-weight at the age of 10 years. Additionally, the results of this sdudy provided clinical evidence for the safety of early perinatal probiotic intervention as well as of the postnatal probiotic intervention in a high-risk population, namely VLBW infants.

More specifically, the following results were obtained:

1. The basis for overweight development in a child is laid down during the perinatal period.
2. Perinatal probiotic intervention reduces the risk of GDM in pregnant women and restrains excessive weight gain in children during the first years of life.
3. Perinatal dietary counselling prevents excessive fetal weight gain in GDM-affected pregnancies.
4. Maternal diet and nutritional status during pregnancy determine the colostrum adiponectin concentration.
5. The initial microbial and nutritional environment in terms of early Bifidobacteria microbiota and colostrum adiponectin concentration as well as serum sCD14 levels, deviate in children subsequently becoming overweight and normal-weight.
6. The early perinatal probiotic intervention with LGG and *Bifidobacterium lactis* Bb12 in term infants as well as the postnatal probiotic intervention with LGG in VLBW infants can be considered safe.

To conclude, the results obtained here showed that a cost-effective nutraceutical approach such as perinatal intensive dietary counselling may carry long-term health benefits for both mother and child. These benefits were further strengthened when probiotic supplementation was combined with a healthy diet. The findings support the conception of the involvement of initial microbial and nutritional environment in the metabolic programming of the child. The colostrum adiponectin content was shown to be dependent on maternal diet and nutritional status during pregnancy, this being a putative explanation for the metabolic imprinting of overweight propensity from mother to offspring. Furthermore, the clinical data obtained here would indicate that changing the gut microbiota by means of probiotics may participate in the control of individual energy homeostasis. Specific strategies for modifying the gut microbiota to enhance Bifidobacteria may thus emerge as an action to reduce the incidence of overweight development and as a corollary restrain the Western life-style disease epidemic. The putative health-ameliorating effects of probiotics can be considered to be most pronounced during perinatal or early postnatal life, i.e. during the critical period when the composition of the gut microbiota and the immunological responsiveness is being consolidated.

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9. REFERENCES

Abt MC, Artis D. The intestinal microbiota in health and disease: the influence of microbial products on immune cell homeostasis. Curr Opin Gastroenterol 2009;25:496-502.

Ajuwon KM, Spurlock ME. Adiponectin inhibits LPS-induced NF-κB activation and IL-6 production and increases PPARγ2 expression in adipocytes. Am J Physiol Regul Integr Comp Physiol 2005;288:R1220-5.

Allen SJ, Jordan S, Storey M, Thornton CA, Gravenor M, Garaiova I, et al. Dietary supplementation wit Lactobacilli and Bifidobacteria is well tolerated and not associated with adverse events during late pregnancy and early infancy. J Nutr 2010;140:483-8.

Amar J, Burcelin R, Ruidavets JB, Cani PD, Fauvel J, Alessi MC, et al. Energy intake is associated with endotoxemia in apparently healthy men. Am J Clin Nutr 2008;87:1219-23.

Anderson RC, Cookson AL, McNabb WC, Kelly WJ, Roy NC. *Lactobacillus plantarum* DSM 2648 is a potential probiotic that enhances intestinal barrier function. FEMS Microbiol Lett 2010;309:184-92.

Arita Y, Kihara N, Ouchi M, Takahashi K, Maeda K, Miyagawa J, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 1999;257:79-83.

Armougom F, Henry M, Vialettes B, Raccah D, Raoult D. Monitoring bacterial community of human gut microbiota reveals an increase in *Lactobacillus* in obese patients and *Methanogens* in anorexic patients. PLoS ONE 2009;4:e7125

Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol 2008;8:411–20.

Bach JF. The effect of infection on the susceptibility to autoimmune and allergic diseases. N Eng J Med 2002;347:911-20.

Barker DJ. Maternal nutrition, fetal nutrition, and disease in later life. Nutrition 1997;13:807-13.

Barker DJ. The developmental origins of adult disease. J Am Coll Nutr 2004;6:588S-95S.

Barker DJ, Osmond C, Forsen TJ, Kajantie E, Eriksson JG. Trajectories of growth among children who later have coronary event. N Engl J Med 2005;353:1802-9.

Baumgart DC, Dignass AU. Intestinal barrier function. Cupp Opin Clin Nutr Metab Care 2002;5:685-94.

Becker W, Lyhne N, Pedersen AN, Aro A, Fogelholm M, Phorsdottir P, et al. Nordic nutrition recommendations 2004 – integrating nutrition and physical activity. Scand J Nutr 2004;48,178-87.

Bell MJ, Ternberg JL, Feigin RD, Keating JP, Marshall L, Barton L, et al. Neonatal necrotising enterocolitis: Therapeutic decisions based upon clinical staging. Ann Surg 1978;187:1-7.

Berg AH, Combs TP, Scherer PE. ACRP30/ adiponectin: an adipokine regulating glucose and lipid metabolism. Trends Endocrinol Metab 2002;13:84-9.

Blüher M, Brennan AM, Kelesidis T, Kratzsch J, Fasshauer M, Kralisch S, et al. Total and high-molecular weight adiponectin in relation to metabolic variables at baseline and in response to an exercise treatment program: comparative evaluation of three assays. Diabetes Care 2007;30:280-5.

Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M, et al. Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Infect Dis 2003;36:775-80.

Braddon FE, Rodgers B, Wadsworth ME, Davies JM. Onset of obesity in a 36 year birth cohort study. Br Med J (Clin Res Ed) 1986;293:299-303.

Brandtzaeg P, Halstensen TS, Kett K, Krajci P, Kvale D, Rognum TO, et al. Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes. Gastroenterology 1989;97:1562-84.

Brandtzaeg P. History of oral tolerance and mucosal immunity. Ann N Y Acad Sci 1996;778:1-27.

Brandtzaeg P. Development and basic mechanisms of human gut immunity. Nutr Rev 1998;56:S5-8.

Brandtzaeg P. Mucosal immunity: induction, dissemination, and effector functios. Scand J Immunol 2009;70:505-15.

Brandtzaeg P. The mucosal immune system and its integration of the mammary glands. J Pediatr 2010;156(2 Suppl):S8-15.

Bronsky J, Karpísek M, Bronská E, Pechová M, Jancíková B, Kotolová H, et al. Adiponectin, adipocyte fatty acid binding protein, and

epidermal fatty acid binding protein: proteins newly identified in human breast milk. Clin Chem 2006;52:1763-70.

Brun P, Castagliuolo I, Di Leo V, Buda A,Pinzani M, Palù G, et al. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 2007;292:G518-25.

Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 2004;101:15718-23.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science 2005;307:1915-20.

Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A 2007;104:979-84.

Calder PC. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot Essent Fatty Acids 2006;75:197-202.

Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007a;56:1761-72.

Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008;57:1470-81.

Cani PD, Delzenne NM. Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. Curr Opin Pharmacol 2009;9:1-7.

Cani PD, Knauf C, Iglesias MA, Drucker DJ, Delzenne NM, Burcelin R. Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor. Diabetes 2006;55:1484-90.

Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia 2007b;50:2374-83.

Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 2009;58:1091-103.

Caplan MS. Probiotic and prebiotic supplementation for the prevention of neonatal necrotizing enterocolitis. J Perinatol 2009;Suppl 2:S2-6.

Catalano PM, Farrell K, Thomas A, Huston-Presley L, Mencin P, de Mouzon SH, et al. Perinatal risk factors for childhood obesity and metabolic dysregulation. Am J Clin Nutr 2009;90:1303-13.

Cebra JJ. Influences of microbiota on intestinal immune system development. Am J Clin Nutr 1999;69:1046S-51S.

Chiba Y, Shida K, Nagata S, Wada M, Bian L, Wang C, et al. Well-controlled proinflammatory cytokine responses of peyer´s patch cells to probiotic *Lactobacillus casei*. Immunology 2010;130:352-62.

Christensen HR, Frøkiær H, Pestka JJ. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol 2002;168:171-8.

Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med 2010;16:228-31.

Claud EC, Savidge T, Walker WA. Modulation of human intestinal epithelial cell interleukin-8 secretion by human milk factors. Pediatr Res 2003;53:419-25.

Claud E, Walker WA. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. FASEB J 2001;15:1398-403.

Claus SP, Tsang TM, Wang Y, Cloarec O, Skordi E, Martin FP, et al. Systemic multicompartmental effects of the gut microbiome on mouse metabolic phenotypes. Mol Syst Biol 2008;4:291.

Cole TJ. Fitting smoothed centile curves to reference data (with discussion). J R Stat Soc Ser A Stat Soc 1988;151:385-418.

Cole TJ, Bellizzi MC, Flegal Km, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ 2000;320:1240-6.

Cole TJ, Green PJ. Smoothing reference centile curves: the LMS method and penalized likelihood. Stat Med 1992;11:1305-19.

Cole TJ, Freeman JV, Preece MA. British 1990 growth reference centiles for weight, height, body mass index and head circumference fitted by maximum penalized likelihood. Stat Med 1998;17:407-29.

Collado MC, Isolauri E, Laitinen K, Salminen S. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. Am J Clin Nutr 2008a;88:894-9.

Collado MC, Isolauri E, Salminen S. Specific probiotic strains and their combinations counteract adhesion of *Enterobacter sakazakii* to intestinal mucus. FEMS Microbiol Lett 2008b;285:58-64.

Combs TP, Berg AH, Obici S, Scherer PE, Rossetti L. Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. J Clin Invest 2001;108:1875-81.

Commane DM, Shortt CT, Silvi S, Cresci A, Hughes RM, Rowland IR. Effects of fermentation products of pro- and prebiotics on trans-epithelial electrical resistance in an in vitro model of the colon. Nutr Cancer 2005;51:102-9.

Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG. Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118. Proc Natl Acad Sci U S A 2007;104:7617-21.

Corthésy B. Roundtrip ticket for secretory IgA: role in mucosal homeostasis? J Immunol 2007;178:27-32.

Corvalán C, Uauy R, Kain J, Martorell R. Obesity indicators and cardiometabolic status in 4-y-old children. Am J Clin Nutr 2010;91:166-74.

Creely SJ, McTernan PG, Kusminski CM, Fisher M, Da Silva NF, Khanolkar M, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. Am J Physiol Endocrinol Metab 2007;292:E740-7.

Cruz JR, Cano F, Caceres P. Association of human milk sIgA antibodies with maternal intestinal exposure to microbial antigens. Adv Exp Med Biol 1999;310:193-7.

Cummings JH, Antoine JM, Azpiroz F, Bourdet-Sicard R, Brandtzaeg P, Calder PC, et al. PASSCLAIM--gut health and immunity. Eur J Nutr 2004;43 Suppl 2:II118-II73.

Dietz WH. Critical periods in childhood for the development of obesity. Am J Clin Nutr 1994;59:955-9.

Donato KA, Gareau MG, Wang YJ, Sherman PM. *Lactobacillus rhamnosus* GG attenuates interferon-{gamma} and tumor necrosis factor-{alpha}-induced barrier dysfunction and proinflammatory signalling. Microbiology 2010 Jul 3 [Epub ahead of print]

Dong H, Rowland I, Tuohy KM, Thomas LV, Yaqoob P. Selective effects of *Lactobacillus casei* Shirota on T cell activation, natural killer cell activity and cytokine production. Clin Exp Immunol 2010;161:378-88.

Duncan SH, Lobley GE, Holtrop G, Ince J, Johnstone AM, Louis P, et al. Human colonic microbiota associated with diet, obesity and weight loss. Int J Obes (Lond) 2008;32:1720-4.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal flora. Science 2005;308:1635-8.

Ekelund U, Ong K, Linné Y, Neovius M, Brage S, Dunger DB, et al. Upward weight percentile crossing in infancy and early childhood independently predicts fat mass in young adults: the Stockholm Weight Development Study (SWEDES). Am J Clin Nutr 2006;83:324-30.

Eriksson J, Forsen T, Osmond C, Barker D. Obesity from cradle to grave. Int J Obes 2003;27:722-7.

Ewaschuk JB, Diaz H, Meddings L, Diederichs B, Dmytrash A, Backer J, et al. Secreted bioactive factors from *Bifidobacterium infantis* enhance epithelial cell barrier function. Am J Physiol Gastrointest Liver Physiol 2008;295:G1025-G34.

Fantuzzi G. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol 2005;115:911-9.

Favier CF, Vaughan EE, De Vos WM, Akkermans AD. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 2002;68:219-26.

Field CJ. The immunological components of human milk and their effect on immune development in infants. J Nutr 2005;135:1-4.

Fleissner CK, Huebel N, Abd El-Bary MM, Loh G, Klaus S, Blaut M. Absence of intestinal microbiota does not protect mice from diet-induced obesity. Br J Nutr 2010 May 5:1-11. [Epub ahead of print]

Floch MH, Walker WA, Guandalini S, Hibberd P, Gorbach S, Surawicz C, et al. Recommendations for probiotic use--2008. J Clin Gastroenterol 2008;42:S104-8.

Food and Agriculture Organization of the United Nations, World Health Organization. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria (October 2001, Retrieved November 2009). [www.who.int/entity/foodsafety/publications/fs_management/en/probiotics.pdf "Health and Nutritional Properties

of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria"].

Galdeano CM, Perdigon G. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. Clin Vaccine Immunol 2006;13:219-26.

García-Lafuente A, Antolín M, Guarner F, Crespo E, Malagelada JR. Modulation of colonic barrier function by the composition of the commensal flora in the rat. Gut 2001;48:503-7.

Gibson GR, Probert HM, Van Loo J, Rastall RA, Roberfroid M. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev 2004;17:259-75.

Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 1995;125:1401-12.

Gillman MW, Rifas-Shiman SL, Camargi CA Jr, Berkey CS, Frazier AL, Rockett HR, et al. Risk of overweight among adolescens who were breastfed as infants. JAMA 2001;285:2461-7.

Griffiths EA, Duffy LC, Schanbacher FL, Qiao H, Dryja D, Leavens A, et al. In vivo effects of bifidobacteria and lactoferrin on gut endotoxin concentration and mucosal immunity in Balb/c mice. Dig Dis Sci 2004;49:579-89.

Groschwitz KR, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. J Allergy Clin Immunol 2009;124:3-20.

Grönlund MM, Arvilommi H, Kero P, Lehtonen OP, Isolauri E. Importance of intestinal colonisation in the maturation of humoral immunity in early infancy: a prospective follow up study of healthy infants aged 0-6 months. Arch Dis Child Fetal Neonatal Ed 2000;83:F186-92.

Guarner F, Schaafsma GJ. Probiotics. Int J Food Microbial 1998;39:237-8.

Gueimonde M, Kalliomäki M, Isolauri E, Salminen S. Probiotic intervention in neonates – will permanent colonization ensue? J Pediatr Gastroenterol Nutr 2006a;42:604-6.

Gueimonde M, Sakata S, Kalliomäki M, Isolauri E, Benno Y, Salminen S. Effect of maternal consumption of *Lactobacillus* GG on transfer and establishment of fecal bifidobacterial microbiota in neonates. J Pediatr Gastroenterol Nutr 2006b;42:166-70.

Gueimonde M, Laitinen K, Salminen S, Isolauri E. Breast milk: a source of bifidobacteria for infant gut development and maturation? Neonatology 2006c;92:64-6.

Haahtela T, von Hertzen L, Mäkelä M, Hannuksela M; Allergy Programme Working Group. Finnish allergy programme 2008-2018 – time to act and change the course. Allergy 2008;63:634-45.

Haller D, Antoine JM, Bengmark S, Enck P, Rijkers GT, Lenoir-Wijnkoop I. Guidance for substantiating the evidence for beneficial effects of probiotics: probiotics in chronic inflammatory bowel disease and the functional disorder irritable bowel syndrome. J Nutr 2010;140:690S-7S.

HAPO Study Cooperative Research Group, Metzger BE, Lowe LP, Dyer AR, Trimble ER, Chaovarindr U, et al. Hyperglycemia and adverse pregnancy outcomes. N Engl J Med 2008;358:1991-2002.

Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, Ebinuma H, et al. Measurement of the high-molecular weight form of adiponectin in plasma is useful for the prediction of insulin resistance and metabolic syndrome. Diabetes Care 2006;29:1357–62.

Harte AL, da Silva NF, Creely SJ, McGee KC, Billyard T, Youssef-Elabd EM, et al. Elevated endotoxin levels in non-alcoholic fatty liver disease. J Inflamm (Lond) 2010;7:15.

Haversen L, Ohlsson BG, Hahn-Soric M. Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cell via NF-κB. Cell Immunol 2002;220:83-95.

He F, Morita H, Ouwehand AC, Hosoda M, Hiramatsu M, Kurisaki J, et al. Stimulation of the secretion of pro-inflammatory cytokines by *Bifidobacterium* strains. Microbial Immunol 2002;46:781-5.

Hirai C, Ichiba H, Saito M, Shintaku H, Yamano T, Kusuda S. Trophic effect of multiple growth factors in amniotic fluid or human milk on cultured human fetal small intestinal cells. J Pediatr Gastroenterol Nutr 2002;34:524-8.

Holt PG, Jones CA. The development of the immune system during pregnancy and early life. Allergy 2000;55:688-97.

Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. Science 2001;292:1115–8.

Horbar JD. The Vermont Oxford Network: evidence-based quality improvement for neonatology. Pediatrics 1999;103(Suppl):350-9.

Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006;444:860-7.

Hui LL, Schooling M, Leung SS, Mak KH, Ho LM, Lam TH, et al. Birth weight, infant growth, and childhood body mass index: Hong Kong´s children

of 1998 birth cohort. Arch Pediatr Adolesc Med 2008;162:212-8.

Hyslop NE, Kern KC, Walker WA. Lysozyme in human colostrum and breast milk. In: Osserman EF, Canfield RE, Boyshok S, editors. Lysozyme. New York. Academic Press; 1974. p. 449-62.

Isolauri E, Joensuu J, Suomalainen H, Luomala M, Vesikari T. Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by *Lactobacillus casei GG*. Vaccine 1995;13:310-2.

Isolauri E, Kaila M, Arvola T, Majamaa H, Rantala I, Virtanen E, et al. Diet during rotavirus enteritis affects jejunal permeability to macromolecules in suckling rats. Pediatr Res 1993a;33:548-53.

Isolauri E, Kalliomäki M, Rautava S, Salminen S, Laitinen K. Obesity – extending the hygiene hypothesis. Nestle Nutr Workshop Ser Pediatr Program. 2009;64:75-85; discussion 85-9, 251-7.

Isolauri E, Majamaa H, Arvola T, Rantala I, Virtanen E, Arvilommi H. *Lactobacillus casei* strain GG reverse increased intestinal permeability induced by cow milk in suckling rats. Gastroenterology 1993b;105:1643-50.

Izcue A, Powrie F. Special regulatory T-cell review: Regulatory T cells and the intestinal tract—patrolling the frontier. Immunology 2008;123:6-10.

Jia W, Li H, Zhao L, Nicholson JK. Gut microbiota: a potential new territory for drug targeting. Nat Rev Drug Discov 2008;7:123-9.

Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, et al. Is meconium from healthy newborns actually sterile? Res Microbiol 2008;159:187-93.

Johansen FE, Pekna M, Norderhaug IN, Haneberg B, Hietala MA, Krajci P, et al. Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. J Exp Med 1999;190:915-21.

Johnson-Henry KC, Donato KA, Shen-Tu G, Gordonpour M, Sherman PM. *Lactobacillus rhamnosus* strain GG prevents enterohemorrhagic *Escherichia coli* O157:H7-induced changes in epithelial barrier function. Infect Immun 2008;76:1340-8.

Johnson-Henry KC, Hagen KE, Gordonpour M, Tompkins TA, Sherman PM. Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. Cell Microbiol 2007;9:356-67.

Kadooka Y, Sato M, Imaizumi K, Ogawa A, Ikuyama K, Akai Y, et al. Regulation of abdominal adiposity by probiotics (*Lactobacillus gasseri* SBT2055) in adults with obese tendencies in a randomized controlled trial. Eur J Clin Nutr 2010;64:636-43.

Kahn BB, Flier JS. Obesity and insulin resistance. J Clin Invest 2000;106:473-81.

Kaila M, Isolauri E, Soppi E, Virtanen E, Laine S, Arvilommi H. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain. Pediatr Res 1992;32:141-4.

Kalliomäki M, Antoine JM, Herz U, Rijkers GT, Wells JM, Mercenier A. Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of allergic diseases by probiotics. J Nutr 2010;140:713S-21S.

Kalliomäki M, Collado MC, Salminen S, Isolauri E. Early differences in fecal microbiota composition in children may predict overweight. Am J Clin Nutr 2008;87:534-8.

Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomized placebo-controlled trial. Lancet 2001;357:1076-9.

Kankaanpää PE, Salminen SJ, Isolauri E, Lee YK. The influence of polyunsaturated fatty acids on probiotic growth and adhesion. FEMS Microbiol Lett 2001;194:149-53.

Karlsson MR, Johansen FE, Kahu H, Macpherson A, Brandtzaeg P. Hypersensitivity and oral tolerance in the absence of a secretory immune system. Allergy 2009;65:561-70.

Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev 2003;8:223-46.

Kitchens RL, Thompson PA. Modulatory effects of sCD14 and LBP on LPS-host cell interactions. J Endotoxin Res 2005;11:225-9.

Kraehenbuhl JP, Corbett M. Keeping the gut microbiota at bay. Science 2004;303:1624-5.

Kubota N, Yano W, Kubota T, Yamauchi T, Itoh S, Kumagai H, et al. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab 2007;6:55-68.

Labeta MO, Vidal K, Mors JE. Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. J Exp Med 2002;191:1807-12.

Laitinen K, Hoppu U, Hämäläinen M, Liderborg K, Moilanen E, Isolauri E. Breast milk fatty acids may link innate and adaptive immune regulation: analysis of soluble CD14, prostaglandin E_2, and fatty acids. Pediatr Res 2006;59:723-7.

Laitinen K, Poussa T, Isolauri E; Nutrition, Allergy, Mucosal Immunology and Intestinal Microbiota Group. Probiotics and dietary counselling contribute to glucose regulation during and after pregnancy: a randomised controlled trial. Br J Nutr 2009;101:1679-87.

Lawror DA, Fraser A, Lindsay RS, Ness A, Dabelea D, Catalano P, et al. Association of existing diabetes, gestational diabetes and glycosuria in pregnancy with macrosomia and offspring body mass index, waist and fat mass in later childhood: findings from a prospective pregnancy cohort. Diabetologia 2010;53:89-97.

Lebeer S, Vanderleyden J, De Keersmaecker SC. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev microbiol 2010;8:171-84.

Lemieux I, Pascot A, Prud´homme D, Alméras N, Bogaty P, Nadeau A, et al. Elevated C-reactive protein: another component of the atherothrombotic profile of abdominal obesity. Arterioscler Thromb Vasc Biol 2001;21:961-7.

Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 2005;102:11070-5.

Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. Science 2008;320:1647-51.

Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444:1022-3.

Li S, Shin JJ, Ding EL, van Dam RM. Adiponectin levels and risk of type 2 diabetes: a systematic review and meta-analysis. JAMA 2009;302:179-88.

Liepke C, Adermann K, Raida M, Mägert HJ, Forssmann WG, Zucht HD. Human milk provides peptides highly stimulating the growth of bifidobacteria. Eur J Biochem 2002;269:712-8.

Lievin V, Peiffer I, Hudault S, Rochat F, Brassart D, Neeser JR, et al. Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut 2000;47:646-52.

Lilly DM, Stillwell RH. Probiotics: growth-promoting factors produced by micro-organisms. Science 1965;47:747-8.

Lin PW, Stoll BJ. Necrotizing enterocolitis. Lancet 2006;368:1271-83.

Litonjua AA, Gold DR. Asthma and obesity: common early-life influences in the inception of disease. J Allergy Clin Immunol 2008;121:1075-84.

Lotz M, Gütle D, Walther S, Ménard S, Bogdan C, Hornef MW. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. J Exp Med 2006;203:973-84.

Lucas A. Programming by early nutrition: an experimental approach. J Nutr 1998;128 (Suppl 2):401S-6S.

Luoto R, Matomäki J, Isolauri E, Lehtonen L. Incidence of necrotizing enterocolitis in very-low-birth-weight infants related to the use of *Lactobacillus* GG. Acta Paediatr 2010;99:1135-8.

Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA. Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells in vitro. Gut 2003;52:827-33.

Mackie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr 1999;69:1035S-45S.

Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science 2004;303:1662-5.

Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 2001;121:580–91.

Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, et al. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. Nat Med 2002;8:731–7.

Martin LJ, Woo JG, Geraghty SR, Altaye M, Davidson BS, Banach W, et al. Adiponectin is present in human milk and is associated with maternal factors. Am J Clin Nutr 2006;83:1106-11.

McCarthy A, Hughes R, Tilling K, Davies D, Smith GD, Ben-Shlomo Y. Birth weight; postnatal, infant, and childhood growth; and obesity in young adulthood: evidence from the Barry Caerphilly Growth Study. Am J Clin Nutr 2007;86:907-13.

McFarlin BK, Johnston CA, Tyler C, Hutchison AT, Kueht ML, Reeves R, et al. Inflammatory markers are elevated in overweight Mexican-American children. Int J Pediatr Obes 2007;2:235-41.

McGuckin MA, Eri R, Simms LA, Florin TH, Radford-Smith G. Intestinal barrier dysfunction in inflammatory bowel diseases. Inflamm Bowel Dis 2009;15:100-13.

Medellin-Peña MJ, Wang H, Johnson R, Anand S, Griffiths MW. Probiotics affect virulence-related gene expression in *Escherichia coli* O157:H7. Appl Environ Microbiol 2007;73:4259-67.

Membrez M, Blancher F, Jaquet M, Bibiloni R, Cani PD, Burcelin RG, et al. Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. FASEB J 2008;22:2416-26.

Meng D, Newburg DS, Young C, Baker A, Tonokonogy SL, Sartor BB, et al. Bacterial symbionts induce a FUT2-dependent fucosylated miche on colonic epithelium via ERK and JNK signaling. Am J Physiol Gastrointest Liver Physiol 2007;293:G780-7.

Metchnikoff E. The prolongation of life: optimistic studies. London: Heinemann. 1907.

Metzger BE, Coustan DR. Summary and recommendations of the fourth international workshop-conference on diabetes mellitus. The organizing committee. Diabetes Care 1998;21 (Suppl 2):B161-7.

Moreau MC, Ducluzeau R, Guy-Grand D, Muller MC. Increase in the population of duodenal immunoglobulin A plasmocytes in axenic mice associated with different living or dead bacterial strains of intestinal origin. Infect Immun 1978;21:532-9.

Mshvildadze M, Neu J, Shuster J, Theriaque D, Li N, Mai V. Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. J Pediatr 2010;156:20-5.

Muccioli GG, Naslain D, Bäckhed F, Reigstad CS, Lambert DM, Delzenne et al. The endocannabinoid system links gut microbiota to adipogenesis. Mol Syst Biol 2010;6:392.

Nadal I, Santacruz A, Marcos A, Warnberg J, Garagorri M, Moreno LA, et al. Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents. Int J Obes 2009;33:758-67.

Nanthakumar N, Fusunyan RD, Sanderson IR, Walker WA. Inflammation in the developing human intestine: a possible pathophysiologic basis for necrotizing enterocolitis. Proc Natl Acad Sci U S A 2000;97:6043-8.

Neish AS. Microbes in gastrointestinal health and disease. Gastroenterology 2009;136:65-80.

Nenci A, Becker C, Wullaert A, Gareus R, van Loo G, Danese S, et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature 2007;446:557-61.

Neutra MR, Mantis NJ, Kraehenbuhl JP. Collaboration of epithelial cells with organized mucosal lymphoid tissues. Nat Immunol 2001;2:1004-9.

Newburg DS. Human milk glycoconjugates that inhibit pathogens. Curr Med Chem 1999;6:117-27.

Newburg DS, Walker WA. Protection of the neonate by the innate immune system of developing gut and of human milk. Pediatr Res 2007;61:2-8.

Newburg DS, Woo JG, Morrow AL. Characteristics and potential functions of human milk adiponectin. J Pediatr 2010;156(2 Suppl):S41-6.

Ng SC, Hart AL, Kamm MA, Stagg AJ, Knight SC. Mechanisms of action of probiotics: recent advances. Inflamm Bowel Dis 2009;15:300-10.

Noble A. Do we have a memory of danger as well as antigen? Trends Immunol. 2009;30:150-6.

Nordic Working Group on Diet and Nutrition. Nordic nutrition recommendations. Scand J Nutr 1996;40:155-61.

Ohishi A, Takahashi S, Ito Y, Ohishi Y, Tsukamoto K, Nanba Y, et al. *Bifidobacterium* septicemia associated with postoperative probiotic therapy in a neonate with omphalocele. J Pediatr 2010;156:679-81.

Ohland CL, MacNaughton WK. Probiotic bacteria and intestinal epithelial function. Am J Physiol Gastrointest Liver Physiol 2010;298:G807-19.

Okamoto Y, Kihara S, Funahashi T, Matsuzava Y, Libby P. Adiponectin: a key adipocytokine in metabolic syndrome. Clin Sci 2006;110:267-78.

Otte JM, Podolsky DK. Functional modulation of enterocytes by Gram-positive and Gram-negative microorganisms. Am J Physiol Gastrointest Liver Physiol 2004;286:G613–G26.

Owen CG, Martin RM, Whincup PH, Smith GD, Cook DG. Effect of infant feeding on the risk of obesity across the life course: a quantitative review of published evidence. Pediatrics 2005;115:1367-77.

Owen CG, Martin RM, Whincup PH, Smith GD, Cook DG. Does breastfeeding influence risk of type 2 diabetes in later life? A quantitative analysis of published evidence. Am J Clin Nutr 2006;84:1043-54.

Owen CG, Whincup PH, Kaye SJ, Martin RM, Davey Smith G, Cook DG, et al. Does initial breastfeeding lead to lower blood cholesterol in adult life? A quantitative review of the evidence. Am J Clin Nutr 2008;88:305-14.

Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol 2007;5:e177.

Parassol N, Freitas M, Thoreux K, Dalmasso G, Bourdet-Sicard R, Rampal P. *Lactobacillus casei* DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic *Escherichia coli*-infected T84 cells. Res Microbiol 2005;156:256-62.

Parker LC, Prince LR, Sabroe I. Translational mini-review series on Toll-like receptors: networks regulated by Toll-like receptors mediate innate and adaptive immunity. Clin Exp Immunol 2007;147:199-207.

Parsons TJ, Power C, Manor O. Foetal and early life growth and body mass index from birth to early adulthood in 1958 British cohort: longitudinal study. BMJ 2001;323:1331-5.

Perez PF, Doré J, Leclerc M, Levenez F, Benyacoub J, Serrant P, et al. Bacterial imprinting of the neonatal immune system: lessons from maternal cells? Pediatrics 2007;119:e724-32.

Pesonen M, Kallio MJ, Siimes MA, Elg P, Björksten F, Ranki A. Cord serum immunoglobulin E as a risk factor for allergic symptoms and sensitization in children and young adults. Pediatr Allergy Immunol 2009;20:12-18.

Pettitt DJ, Forman MR, Hanson RL, Knowler WC, Bennett BH. Breastfeeding and incidence of non-insulin-dependent diabetes mellitus in Pima Indians. Lancet 1997;350:166-8.

Piccini MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S. Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. Nat Med 1998;4:1020-4.

Piirainen T, Isolauri E, Lagström H, Laitinen K. Impact of dietary counselling on nutrient intake during pregnancy: a prospective cohort study. Br J Nutr 2006;96:1096-104.

Pineiro M, Asp NG, Reid G, Macfarlane S, Morelli L, Brunser O, et al. FAO technical meeting on prebiotics. J Clin Gastroenterol 2008;42:S156-9.

Planchon SM, Martins CA, Guerrant RL, Roche JK. Regulation of intestinal epithelial barrier function by TGF-β1. Evidence for its role in abrogating the effect of a T cell cytokine. J Immunol 1994;153:5730-9.

Preidis GA, Versalovic J. Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. Gastroenterology 2009;136:2015-31.

Qamar A, Aboudola S, Warny M, Michetti P, Pothoulakis C, LaMont JT, et al. *Saccharomyces boulardii* stimulates intestinal immunoglobulin A immune response to *Clostridium difficile* toxin A in mice. Infect Immun 2001;69:2762-5.

Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microbicidal proteins involved in innate immunity. Cell 2004;118:229-41.

Rautava S, Walker WA. Commensal bacteria and epithelial cross talk in the developing intestine. Curr Gastroenterol Rep 2007;9:385-92.

Rautava S, Walker WA. Academy of breastfeeding medicine founder´s lecture 2008: Breastfeeding--an extrauterine link between mother and child. Breastfeed Med 2009;4:3-10.

Reigstad CS, Lunden GÖ, Felin J, Bäckhed F. Regulation of serum amyloid A3 (SAA3) in mouse colonic epithelium and adipose tissue by the intestinal microbiota. PloS ONE 2009;4:e5842.

Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. Science 1996;271:1723-6.

Rijkers GT, Bengmark S, Enck P, Haller D, Herz U, Kalliomaki M, et al. Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. J Nutr 2010;140:671S-6S.

Rinne M, Kalliomäki M, Salminen S, Isolauri E. Probiotic intervention in the first months of life: short-term effects on gastrointestinal symptoms and long-term effects on gut microbiota. J Pediatr Gastroenterol Nutr 2006;43:200-5.

Rolland-Cachera MF, Deheeger M, Bellisle F, Sempe M, Guilloud-Bataille M, Patois E. Adiposity rebound in children: a simple indicator for predicting obesity. Am J Clin Nutr 1984;39:129-35.

Rosenbloom AL. Physiology of growth. Ann Nestlé 2007;65:97-108.

Round JL. Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev 2009;9:313-23.

Ruan X, Shi H, Xia G, Xiao Y, Dong J, Ming F, et al. Encapsulated Bifidobacteria reduced bacterial translocation in rats following hemorrhagic shock and resuscitation. Nutrition 2007;23:754-61.

Ruemmele FM, Bier D, Marteau P, Rechkemmer G, Bourdet-Sicard R, Walker WA, et al. Clinical evidence for immunomodulatory effects of probiotic bacteria. J Pediatr Gastroenterol Nutr 2009;48:126-41.

Sakata T. Stimulatory effect of short-chain fatty acids on epithelial cell proliferation in the rat intestine: a possible explanation for trophic effects of fermentable fibre, gut microbes and luminal trophic factors. Br J Nutr 1987;58:95-103.

Sakata H, Yoshioka H, Fujita K. Development of the intestinal flora in very low birth weight infants compared to normal full-term newborns. Eur J Pediatr 1985;144:186-90.

Salminen MK, Rautelin H, Tynkkynen S, Poussa T, Saxelin M, Valtonen V, et al. *Lactobacillus* bacteremia, clinical significance, and patient outcome, with special focus on probiotic *L. rhamnosus* GG. Clin Infect Dis 2004a;38:62-9.

Salminen MK, Tynkkynen S, Rautelin H, Poussa T, Saxelin M, Ristola M, et al. The efficacy and safety of probiotic *Lactobacillus rhamnosus* GG on prolonged, noninfectious diarrhea in HIV patients on antiretroviral therapy: a randomized, placebo-controlled, crossover study. HIV Clin Trials 2004b;5:183-91.

Salminen MK, Tynkkynen S, Rautelin H, Saxelin M, Vaara M, Ruutu P, et al. *Lactobacillus* bacteremia during a rapid increase in probiotic use of *Lactobacillus rhamnosus* GG in Finland. Clin Infect Dis 2002;35:1155-60.

Salminen S, Nybom S, Meriluoto J, Collado MC, Vesterlund S, El-Nezami H. Interaction of probiotics and pathogens – benefits to human health? Curr Opin Biotechnol 2010;21:157-67.

Saltiel AR, Kahn CR. Insulin signaling and the regulation of glucose and lipid metabolism. Nature 2001;414:799-806.

Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, et al. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A 2008;105:16767-72.

Sanderson IR, Walker WA. Uptake and transport of macromolecules by the intestine: possible role in clinical disorders (an update). Gastroenterology 1993;104:622-39.

Santacruz A, Collado MC, García-Valdés L, Segura MT, Martín-Lagos JA, Anjos T, et al. Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. Br J Nutr 2010;104:83-92.

Santacruz A, Marcos A, Wärnberg J, Marti A, Martin-Matillas M, Campoy C, et al. Interplay between weight loss and gut microbiota composition in overweight adolescents. Obesity 2009;17:1906-15.

Satokari R, Grönroos T, Laitinen K, Salminen S, Isolauri E. *Bifidobacterium* and *Lactobacillus* DNA in the human placenta. Lett Appl Microbiol 2009;48:8-12.

Savino F, Petrucci E, Nanni G. Adiponectin: an intriguing hormone for paediatricians. Acta Paediatr 2008;97:701-5.

Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem 1995;270:26746-9.

Schlee M, Harder J, Köten B, Stange EF, Wehkamp J, Fellermann K. Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. Clin Exp Immunol 2008;151:528-35.

Schwiertz A, Taras D, Schäfer S, Beijer S, Bos NA, Donus C, et al. Microbiota and SCFA in lean and overweight healthy subjects. Epidemiology 2009;18:190-5.

Semple RK, Soos MA, Luan J, Mitchell CS, Wilson JC, Gurnell M, et al. Elevated plasma adiponectin in humans with genetically defective insulin receptors. J Clin endocrinol Metab 2006;91:3219-23.

Seth A, Yan F, Polk DB, Rao RK. Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 2008;294:G1060-9.

Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid induced insulin resistance. J Clin Invest 2006;116:3015-25.

Shore SA. Obesity and asthma: possible mechanism. J Allergy Clin Immunol 2008;121:1087-93.

Siegrist CA, Aspinall R. B-cell responses to vaccination at the extremes of age. Nat Rev Immunol 2009;9:185-94.

Sierro F, Dubois B, Coste A, Kaiserlian D, Kraehenbuhl JP, Sirard JC. Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated

migration of dendritic cells. Proc Natl Acad Sci U S A 2001;98:13722-7.

Sjögren YM, Tomicic S, Lundberg A, Böttcher MF, Björkstén B, Sverremark-Ekström E, et al. Influence of early gut microbiota on the maturation of childhood mucosal and systemic immune responses. Clin Exp Allergy 2009;39:1842-51.

Skinner AC, Steiner MJ, Henderson FW, Perrin EM. Multiple markers of inflammation and weight status: cross-sectional analyses throughout childhood. Pediatrics 2010;125:e801-9.

Stettler N, Zemel BS, Kumanyika S, Stalling VA. Infant weight gain and childhood overweight status in a multicenter, cohort study. Pediatrics 2002;109:194-9.

Stoll LL, Denning GM, Weintraub NL. Endotoxin, TLR4 signaling and vascular inflammation: potential therapeutic targets in cardiovascular disease. Curr Pharm Des 2006;12:4229-45.

Sudo N, Sawamura S, Tanaka Y, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J Immunol 1997;159:1739-45.

Summerbell CD, Waters E, Edmunds LD, Kelly S, Brown T, Campbell KJ. Interventions for preventing obesity in children. Cochrane Database Syst Rev 2005;(3):CD001871.

Szajewska H. Probiotics and prebiotics in preterm infants: Where are we? Where are we going? Early Hum Dev. 2010;86:81-6.

Takahashi K, Sugi Y, Hosono A, Kaminogawa S. Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. J Immunol 2009;183:6522-9.

Takamura N, Okubo T, Sonoyama K. *Lactobacillus plantarum* strain No. 14 reduces adipocyte size in mice fed high-fat diet. Exp Biol Med (Maywood) 2010;235:849-56.

Tam CS, Clément K, Baur LA, Tordjman J. Obesity and low-grade inflammation: a paediatric perspective. Obes Rev 2010;11:118-26.

Tedelind S, Westberg F, Kjerrulf M, Vidal A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. World J Gastroenterol 2007;13:2826-32.

Tiihonen K, Ouwehand AC, Rautonen N. Effect of overweight on gastrointestinal microbiology and immunology: correlation with blood biomarkers. Br J Nutr 2009;103:1070-8.

Tissier H. Traitment de infections intestinales par la methode de la flore bacterienne de I´intestin. C R Soc Biol 1906;60:359-61.

Tsatsanis C, Zacharioudaki V, Androulidaki A, Dermitzaki E, Charalampopoulos I, Minas V, et al. Adiponectin induces TNF-α and IL-6 in macrophages and promotes tolerance to itself and other pro-inflammatory stimuli. Biochem Biophys Res Commun 2005;335:1254-63.

Tsatsanis C, Zacharioudaki V, Androulidaki A, Dermitzaki E, Charalampopoulos I, Minas V, et al. Peripheral factors in the metabolic syndrome: the pivotal role of adiponectin. Ann N Y Acad Sci 2006;1083:185-95.

Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes 2007;56:1986-98.

Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe 2008;3:213-23.

Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457:480-4.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027-31.

Vael C, Desager K. The importance of the development of the intestinal microbiota in infancy. Curr Opin Pediatr 2009;21:794-800.

van Elburg RM, Uil JJ, de Monchy JG, Heymans HS. Intestinal permeability in pediatric gastroenterology. Scand J Gastroenterol 1992;194 (Suppl):19-24.

Velagapudi VR, Hezaveh R, Reigstad CS, Gopalacharyulu P, Yetukuri L, Islam S, et al. The gut microbiota modulates host energy and lipid metabolism in mice. J Lipid Res 2010;51:1101-12.

Vuillermin PJ, Ponsonby AL, Saffery R, Tang ML, Ellis JA, Sly P, et al. Microbial exposure, interferon gamma gene demethylation in naïve T-cells, and the risk of allergic disease. Allergy 2009;64:348-53.

von der Weid T, Bulliard C, Schiffrin EJ. Induction by a lactic acid bacterium of a population of CD4(+) T cells with low proliferative capacity that produce transforming growth factor beta

and interleukin-10. Clin Diagn Lab Immunol 2001:8:695-701.

von Kries R, Koletzko B, Sauerwald T, von Mutius E, Bameft D, Grunert V, et al. Breast feeding and obesity: cross sectional study. BMJ 1999;319:147-50.

Waldram A, Holmes E, Wang Y, Rantalainen M, Wilson ID, Tuohy KM, et al. Top-down systems biology modeling of host metabotype-microbiome associations in obese rodents. J Proteome Res 2009;8:2361-75.

Walker WA. Transmucosal passage of antigens. In: Schmidt E, editor. Food Allergy. New York: Vevey/Raven Press 1988:15-32.

Walker WA. Mechanisms of action of probiotics. CID 2008;46 (Suppl 2):S87-S91.

Walker WA. Immunology. Curr Opin Gastroenterol 2009;25:491-5.

Wang Z, Xiao G, Yao Y, Guo S, Lu K, Sheng Z. The role of bifidobacteria in gut barrier function after thermal injury in rats. J Trauma 2006;61:650-7.

Weiss ST. Obesity: insight into the origins of asthma. Nat Immunol 2005;6:537-9.

Wellen KE, Hotamisgil GS. Obesity-induced inflammatory changes in adipose tissue. J Clin Invest 2003;112:1785-8.

Weyermann M, Beermann C, Brenner H, Rothenbacher D. Adiponectin and leptin in maternal serum, cord blood, and breast milk. Clin Chem 2006;52:2095-102.

Weyermann M, Brenner H, Rothenbacher D. Adipokines in human milk and risk of overweight in early childhood: a prospective cohort study. Epidemiology 2007;18:722–9.

Whitaker KL, Jarvis MJ, Beeken RJ, Boniface D, Wardle J. Comparing maternal and paternal intergenerational transmission of obesity risk in a large population-based sample. Am J Clin Nutr 2010;91:1560-7.

WHO: Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, 2001 http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf.

WHO: Guidelines for the evaluation of probiotics in foods, 2002 http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

WHO Multicentre Growth Reference Study Group. *WHO Child Growth Standards: length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: methods and development*. Geneva, Switzerland: World Health Organization;2005. www.who.int/childgrowth/standards/bmi_for_age/en/index.html, www.who.int/growthref/who2007_bmi_for_age/en/index.html

Willemsen LE, Koetsier MA, Balvers M, Beermann C, Stahl B, van Tol EA. Polyunsaturated fatty acids support epithelial barrier integrity and reduce IL-4 mediated permeability in vitro. Eur J Nutr 2008;47:183-91.

Willemsen LE, Koetsier MA, van Deventer SJ, van Tol EA. Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E(1) and E(2) production by intestinal myofibroblasts. Gut 2003;52:1442–7.

Wolvers D, Antoine JM, Myllyluoma E, Schrezenmeir J, Szajewska H, Rijkers GT. Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of infections by probiotics. J Nutr 2010;140:698S-712S.

Woo JG, Guerrero ML, Altaye M, Ruiz-Palacios GM, Martin LJ, Dubert-Ferrandon A, et al. Human milk adiponectin is associated with infant growth in two independent cohorts. Breastfeed Med 2009;4:101-9.

Wu K, Bi Y, Sun K, Wang C. IL-10-producing type 1 regulatory T cells and allergy. Cell Mol Immunol 2007;4:269-75.

Wulster-Radcliffe MC, Ajuwon KM, Wang J, Christian JA, Spurlock ME. Adiponectin differentially regulates cytokines in porcine macrophages. Biochem Biophys Res Commun 2004;316:924-9.

Yamaguchi N, Argueta JG, Masuhiro Y, Kagishita M, Nonaka K, Saito T. Adiponectin inhibits Toll-like receptor family-induced signaling. FEBS Lett 2005;579:6821-6.

Yogev Y, Metzger BE, Hod M. Establishing diagnosis of gestational diabetes mellitus: Impact of the hyperglycemia and adverse pregnancy outcome study. Semin Foetal Neonatal Med 2009;14:94-100.

Zareie M, Johnson-Henry K, Jury J, Yang PC, Ngan BY, McKay DM, et al. Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress. Gut 2006;55:1553-60.

Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci U S A 2009;106:2365-70.