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Präbiotikasupplementation Schwangerer und ihre Wirkung auf die mütterliche und kindliche Darmflora sowie auf ausgewählte fetale Immunparameter - eine randomisierte, doppelblinde, placebo-kontrollierte Pilotstudie

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
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Effects of prebiotic supplementation during pregnancy on maternal & neonatal gut microbiota, as well as on selected foetal immune parameters - a randomised, double-blind, placebo-controlled pilot study

Thesis
for obtaining the doctoral degree in human biology
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1. INTRODUCTION

1.1 The gastrointestinal tract

For a long time the primary function of the gastrointestinal tract (GIT) had simply been considered to digest and absorb nutrients and to excrete waste end products [1, 2]. The assertion of Josh Billings "a good reliable set of bowels is worth more to a man than any quantity of brains" (1818-1875) represents a milestone in the knowledge of the digestive tract. Today, the GIT is known to play a central role in general well being and health [3]. With up to 400 m2, it is not only the largest body area interacting with the environment, but also the largest organ of immune defence in the human body [4]. The GIT is a complex, heavily populated and diverse ecosystem [5]. The microbial consortium (microbiota) consists of up to 10 Exponential 14 (10E14) microorganisms (mainly bacteria, but also fungi and protozoa) [6]. More than 500 different bacterial species coexist in the human colon. The exact numbers remain to be defined since less than 30% of the microorganisms can be cultured with current microbiological methods [7, 8]. It is estimated that about 40 species make up 99% of all isolated bacteria [9, 10]. Remarkably, these species belong to only eight of the 55 known bacterial divisions, with the *Firmicutes*, *Bacteroides* and *Actinobacteria* being the most widely represented [11]. Other bacteria that have been identified in high numbers include bifidobacteria, lactobacilli, coliforms, methanogens and dissimilatory sulfat-reducing bacteria (**Figure 1**) [7].

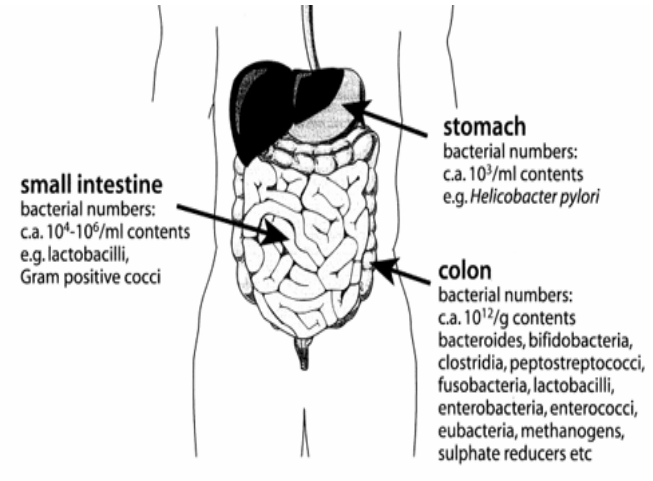


Figure 1. Diagrammed representation of the human GIT showing approximate bacterial numbers in each region [12].

Molecular tools based on 16S ribosomal desoxyribonucleic acid (rDNA) sequence similarities such as fluorescent in-situ hybridization (FISH), denaturing and temperature gradient gel electrophoresis (DGGE and TGGE), quantitative dot blot hybridization and restriction fragment length polymorphism (RFLP) have helped to overcome limitations of conventional microbiological plating methods [13-15]. The majority of the intestinal microorganisms have a strictly anaerobic metabolism [16]. Their metabolic end-products are short chain fatty acids (SCFA), mainly acetate, propionate, butyrate and a variety of other metabolites including products such as lactate, pyruvate, ethanol, hydrogen, succinate [5, 17]. SCFA serve as an energy source for the mucosal cells or are further metabolized by other bacterial species. The composition of the microbiota is affected by many different environmental factors such as diet, medication, stress, age and general living conditions [18]. The sequence in which bacteria settle in a niche is also determined by other factors, including host genetics, immune surveillance, and others [19]. During the birth process and rapidly thereafter, microbes from the mother and surrounding environment colonize the gut of the newborn [20]. Initially, facultative anaerobic strains such as *Escherichia coli* (*E. coli*) and *Enterococcus* predominate [21, 22] in the neonatal gut. They create a highly reduced environment that promotes the colonisation of strict anaerobes, including *Bifidobacterium*, *Bacteroides* and *Clostridium* species [21, 23]. Other anaerobes get successively established, resulting in a highly diverse and stable microbiota in adult age [24]. Studies have shown that vaginally delivered infants acquire *Bifidobacterium* and *Bacteroides* species faster than infants delivered by caesarean section [25, 26] and have a lower risk to develop atopic diseases [27-29]. Furthermore, breastfed infants have higher numbers of bifidobacteria than bottle fed children [30, 31] and seem to suffer less frequently from allergic diseases [32-35].

Evidence is increasing that the gut microbiota not only modulates the mucosal physiology and barrier function but also systemic and inflammatory responses [36, 37], as well as tolerance to innocuous antigens [38-40]. However, due to the dynamic complexity of the human microbiota, it has been difficult to establish the existence of clear associations between specific microbes and health. An understanding of the dynamics and physiologic functions of the microbiota is still in its infancy, but progress in biosciences supports the hypothesis that beyond providing nutrition, diet may also modulate various functions in the body that are relevant to health [41]. Growing knowledge of the gut microbiota and its interactions with the immune system has led to the development of special dietary strategies that serve to sustain or even improve normal GIT microbiology. Both prebiotics and probiotics are popular substances that have been developed to target the GIT microbiota.

1.2 Probiotics

The history of probiotics dates back as far as the first intake of fermented milk over 2000 years ago. In a Persian version of the Old Testament (Genesis 18:8) it states that "Abraham owed his longevity to the consumption of sour milk" [42, 43]. However, it was not until early in the 20th century that scientists like E. Mechtnikoff and H. Tissier developed the concept of what we now know as probiotics [44, 45]. The term "probiotic", meaning "for life" is derived from the Greek language. It was first used by Lilly and Stillwell [46] in 1965 to describe "substances secreted by one mircoorganism, which stimulated the growth of another" and thus was opposed to the term "antibiotic" [43]. Today probiotics are defined as "live microbial feed supplements, which beneficially affect the host by improving its intestinal microbial balance" [47].

A microbial strain is defined as probiotic [48], if it is
- of human origin
- non-pathogenic (documented clinical safety)
- resistant to technical processing (shelf-life, stability)
- resistant to gastric acid, bile salts and pancreatic enzymes
- able to adhere to intestinal epithelial tissue
- able to survive and colonize the intestinal tract
- able to produce antimicrobial substances
- able to a modulate immune responses (clinically validated and documented health effects).

Probiotics are mainly lactic acid producing bacteria (lactobacilli, lactococci, streptococci, enterococci, bifidobacteria) [5] but also some *Bacillus* species and non-pathogenic yeasts such as *Saccharomyces* (*S. boulardii, S. cerevisae*) and *Aspergillus* species. [49]. The most common probiotics belong to the genera *Lactobacillus* (*L. casei, L. acidophilus, L. rhamnosus, L. plantarum, L. johnsonii, L. reuteri*) and *Bifidobacterium* (*B. bifidum, B. lactis, B. longum, B. brevis*) and new strains are being isolated regularly [50].
The health effects attributed to probiotics are numerous and there is some evidence that probiotics are beneficial for the treatment and prevention of certain diseases [51, 52]. For example, two meta-analyses concluded that probiotics can be used to prevent antibiotic-associated diarrhoea in adults and children [53, 54]. Furthermore, there are indications that probiotics play an important role in the treatment of *Clostridium difficile*-associated diarrhoea [55, 56], acute rotavirus [57] and other infectious diarrhoea [58].

Several studies have investigated the efficacy of probiotics in the prevention of traveller's diarrhoea in adults, but methodical drawbacks such as compliance with treatment and problems in follow-up limit the validity of these conclusions. Clinical trials have also shown that probiotics can prevent allergic symptoms, especially atopic dermatitis [59-62]. Besides, probiotics can be used as therapy to reduce severity and frequency of allergic rhinitis [63, 64], and to decrease severity and duration of the common cold [65], as well as winter infections [66]. Furthermore, clinical studies have shown that probiotics can lead to an improvement in inflammatory bowel diseases such as Crohns' disease [67], ulcerative colitis [68], pouchitis [69], *Helicobacter pylori* infections [70, 71] and reduce cholesterol, as well as triacylglycerol plasma concentrations [72]. These numerous effects can hardly be explained by a unifying hypothesis based on a single mechanism. It is suggested that probiotic bacteria may exert different effects based on specific capabilities and enzymatic activities of different microbes, even within one species [73]. For example, benefits may arise from improved resistance to pathogens induced through the production of inhibitory substances, blocking of adhesion sites, competition for substrates and direct or indirect modulation of specific and non-specific immunity [74]. In uncontrolled studies *B. bifidum* Bb12 (1E10 colony forming units (CFU) per day) and *L. acidophilus* (7E10 CFU/day), each supplement given to 14 volunteers for three weeks doubled the numbers of peripheral white blood cells with phagocytic activity [75]. Similarly, *Lactobacillus rhamnosus* subspecies GG (LGG) has been shown to have immune modulatory effects. For examples LGG lowered pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) in allergic children [76]. In another study, administration of LGG for five weeks to healthy volunteers significantly decreased TNF- α, interleukin-6 (IL-6) and in part interferon-gamma (IFN-γ) cytokine secretion by peripheral blood cells following stimulation, whereas the IL-10 and in part IL-4 cytokine secretion was increased [77]. However, long-term intake is necessary to induce beneficial effects, especially since probiotic bacteria are rapidly cleared from faeces once daily intake ceased [78]. After four days intake they remained in 16% of the individuals and were undetectable eight days after cessation of supplementation. This demonstrates one of the key problems of probiotic bacteria: the inability of the fed probiotic strain to colonise the colon permanently and thereby become an integral part of the microbial community. To overcome these limitations, alternative approaches were developed which aimed at a selective stimulation of certain beneficial bacteria resident in the gut (like bifidobacteria and lactobacilli) by providing them with a specific growth substrate, called a "prebiotic".

1.3 Prebiotics

The term prebiotic was introduced by Gibson and Roberfroid [12] in 1995 who exchanged "pro" for "pre", which means "before" or "for". They defined prebiotics as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon". Preferred targets are species belonging to the lactobacilli and bifidobacteria genera.

To classify a substrate as "prebiotic" the following criteria must be fulfilled [79]: it must
- be neither hydrolysed nor absorbed in the upper part of the GIT
- be selective for one or more potentially beneficial commensal bacteria in the large intestine and encourage their growth and metabolism
- alter the colonic microenvironment towards a healthier composition
- induce beneficial luminal or systemic effects that are advantageous to the host.

Non-digestible carbohydrates (NDC), some peptides and proteins, as well as certain lipids possess prebiotic properties [5]. *In vitro* and *in vivo* data have been published for NDC like lactulose, inulin type-fructans, galactooligosaccharides (GOS), fructooligosaccharides (FOS), as well as xylo-, isomalto-, gluco- and soya-oligosaccharides [41]. According to the Nomenclature Committee of the IUB (NC-IUB) and the IUB-IUPAC Joint Commission on Biochemical Nomenclature (JCBN) [80] inulin is a beta (2-1) fructan (chain length 2 to 60 units; average degree of polymerisation (DPav) = 12); its partial enzymatic hydrolysis product is called oligofructose (OF, chain length 2 to 8; DPav = 4, **Figure 2**).

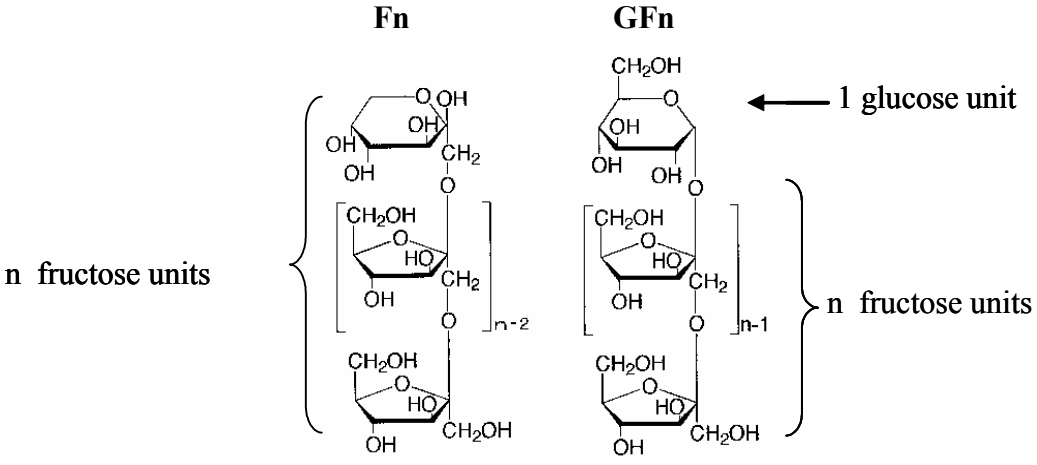


Figure 2. Structure of inulin. Inulin is a polysaccharide consisting of beta (2-1) fructosyl fructose units (Fn) with normally, but not necessarily, one glucopyranose unit at the reducing end (GFn). G, glucose; F, fructose; n, number of fructose moieties [81].

Inulin and OF are storage carbohydrates in many plants including wheat, onion, banana, chicory [79]. Commercial forms of inulin can be extracted from natural sources like chicory root or synthesized enzymatically from sucrose [82]. *In vitro* studies have indicated that inulin and OF are preferably fermented by bifidobacteria [83-85] and human *in vivo* studies have confirmed the bifidogenic effect of OF [86-88]. Bifidobacteria possess the cell-bound enzyme ß-fructofuranosidase that allows the preferred utilization of fructooligosaccharides [89] and offers this genus a competitive advantage over other bacteria in the human gut. The liberated fructose moiety is further metabolized in the "bifidus" pathway. Physiologic effects of NDC include increase of SCFA production, mineral absorption and vitamin B synthesis [90].

1.4 The immune system - an overview

Immune responses fall broadly into two categories, innate and adaptive immune responses, which work together synergistically [91]. The innate (also termed natural or naive) immune system provides the first line of defence against invading organisms without the need of prior exposure to their antigens. The cells that mediate innate immunity mainly include phagocytic cells (macrophages and neutrophils), inflammatory cells (basophils and mast cells), dendritic cells and natural killer cells. The adaptive (also specific or acquired) immune system develops during an individual's life time, is antigen-specific and more efficient upon secondary restimulation. It is mediated by lymphocytes (T and B cells) and their products (such as cytokines and antibodies) and depends on the presentation of foreign antigens by professional antigen presenting cells (APCs) in association with molecules of the major histocompatability complex (MHC). Both humoral- and cell-mediated mechanisms are involved in the adaptive immune system. Humoral immunity is mediated by antibody secreting B cells, while cellular immunity depends on T lymphocytes.

$CD4^+$ expressing T cells are called T helper cells (Th) and recognize antigens presented in the context of MHC class II molecules. $CD8^+$ expressing T cells are called cytotoxic T cells (Tc) and recognize antigens presented by MHC class I molecules [92].

Dendritic cells (DC) play a pivotal role in the initiation of immune responses by priming adaptive immunity. Immature DCs migrate through the blood stream and home to various peripheral tissues in search of pathogens. Pattern recognition receptors (PRR) such as Toll-like receptors (TLRs), lectin or intra-cellular nucleotide-oligomerization domain (NOD) receptors recognize various pathogen-derived molecules, known as pathogen-associated molecular patterns (PAMPs) and lead to an activation of DCs (**Figure 3**).

Upon activation and antigen uptake, DCs down-regulate their endocytotic activity and up-regulate expression of MHC, as well as adhesion and co-stimulatory molecules, such as $CD80^+$ and $CD86^+$, on their surface [93]. Mature DCs then migrate to areas of the draining lymph node (into the T cell areas of the secondary lymphoid organs), where they present antigen-derived peptides in the context of MHC to the T cell receptor (TCR) of naive ($CD45RA^+$) T cells (signal 1, **Figure 3**). Depending on the nature of the antigen, on the types of co-stimulatory molecules, such as $CD40^+$, $CD80^+$, $CD86^+$ (signal 2), and on the composition of the T cell polarizing factors, e.g. cytokines, chemokine ligands (signal 3), present during the initiation of the T cell response, different T effector subsets develop [94].

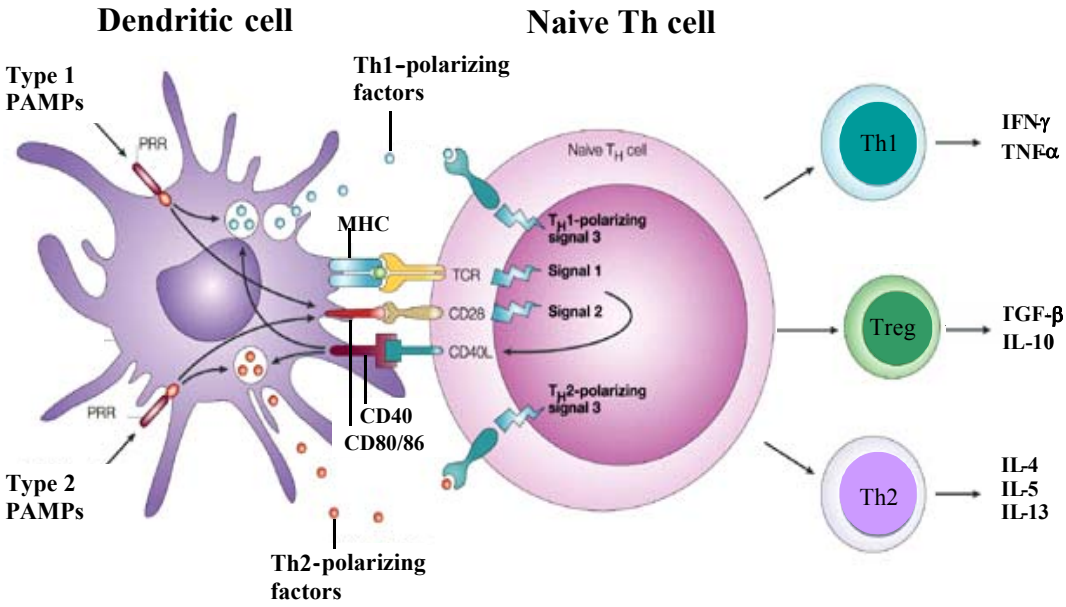


Figure 3. T helper cell stimulation and Th1/Th2 cell polarization requires three dendritic cell derived signals. Signal 1 is the "antigen-specific signal". It is mediated through the binding of pathogen-associated molecular patterns (PAMPS) to specialized pattern recognition receptors (PRRs) and the triggering of T cell receptor (TCR) by MHC associated peptides. Signal 2 is the "co-stimulatory signal", mainly mediated by the interaction of CD80 and CD86 on DCs with CD28 on T cells. Signal 3 is the "polarizing signal" that is mediated by various soluble or membrane bound factors, such as cytokines and chemokine ligands, that promote the development of Th1 or Th2 cells, respectively (adapted from [94]).

Based on their cytokine profiles different functional T helper cell subsets are distinguished [94, 95]: $CD4^+$ T helper 1 (Th1) cells produce IFN-γ, and to a lesser extent, IL-2 and IL-12 that activate macrophages and cytotoxic T cells. They are responsible for cell-mediated inflammatory responses against intra-cellular pathogens and viruses and mediate delayed type hypersensitivity [96]. $CD4^+$ T helper 2 (Th2) cells synthesize IL-4, IL-5, IL-13 and mediate humoral responses by activating B cells, mast cells and eosinophils against extra-cellular pathogens (e.g. parasites). Both Th1 and Th2 cells down-regulate each other's function and over-activation of either pattern can cause disease [97].

Similarly, $CD8^+$ Tc cells can be divided into Tc1 and Tc2 cells [98]. In addition to Th1/Tc1 and Th2/Tc2 cells a third type of regulatory T cells exists. These include several subsets such as natural T reg cells and inducible TR1 and Th3 cells [99, 100]. Regulatrory T cells suppress the function of effector cells by releasing the inhibitory cytokine, transforming growth factor-beta (TGF-ß), and the anti-inflammatory cytokine, IL-10. They play an important role in the maintenance of immune tolerance and suppression of immune responses by auto-reactive lymphocytes. After priming, clonal expansion and differentiation, the different effector T cell subsets acquire new migratory capacities that enable them to re-enter circulation and home to the sites of infection.

1.5 Chemokines & chemokine receptors

Homing and migration of leukocytes is a very dynamic process which is orchestrated by the regulated expression of different chemokines and chemokine receptors [101].

Chemokines (chemotactic cytokines) are small structurally related peptides of 8-10 kilo Dalton (~ 100 aminoacids) [102] that function through seven trans-membrane coupled G protein receptors, so called chemokine receptors (CKRs) [103]. They have two major roles in leukocyte migration: induction of chemotaxis and activation of integrins [104].

The human chemokine system currently includes more than 50 chemokines and 20 CKRs [105]. Chemokines can be divided according to their amino-terminal (NH2-terminal) cysteine motifs into four structural subfamilies: C (α), CC (β), CXC (γ) and CX3C (δ) subfamilies [106-108] (**Table 1**).

Chemokine receptors	Chemokine ligands	Functional classification of chemokine ligands
CXC chemokine receptors		
CXCR3	IP-10, MIG & I-TAC	inflammatory
CXCR4	SDF-1/PBSF	homeostatic
CXCR5	BLC/BCA-1	homeostatic
CC chemokine receptors		
CCR1	MCP-3, MIP-1α & RANTES	inflammatory
CCR2	MCP-1, MCP-2, MCP-3 & MCP-4	inflammatory
CCR3	MCP-3, MCP-4, Eotaxin 1 & 2, RANTES	inflammatory
CCR4	TARC & MDC	both
CCR5	MIP-1α, MIP-1β & RANTES	inflammatory
CCR6	LARC	both
CCR7	MIP-3β, ELC & SLC	homeostatic
CCR8	I-309	unknown
CCR9	TECK	homeostatic
CX_3 chemokine receptor		
CX_3CR1	Fractalkine	inflammatory
C chemokine receptor		
XCR1	Lymphotactin	unknown

Table 1. Summary of some chemokine receptors and their known ligands. Functional classification of chemokine ligands into inflammatory and homeostatic chemokines. Some chemokines belong to both subfamilies (adapted from [105, 109]). Systematic nomenclature for human chemokines can be accessed at http://cytokine.medic.kumamoto-u.ac.jp/.
IP-10, interferon-γ inducible protein 10 (CXCL10); MIG, monokine-induced by interferon γ (CXCL9); I-TAC, interferon-inducible T cell - α chemoattractant (CXCL11); SDF, stroma cell derived factor1 (CXCL12); PBSF, pre B cell growth stimulating factor; BLC, B lymphocyte chemoattractant (CXCL13); BCA-1, B cell attracting chemokine 1 (CXCL13); MCP-1, monocyte chemoattractant protein 1 (CCL2); MCP-2 (CCL8); MCP-3 (CCL7); MCP-4 (CCL13); MIP-1〈 macrophage inflammatory protein 1〈 (CCL3); RANTES, regulated on activation normal T cell expressed and secreted (CCL5); Eotaxin-1 (CCL11); Eotaxin-2 (CCL24); TARC, thymus- and activation-regulated chemokine (CCL17); MDC, macrophage-derived chemokine (CCL22); LARC, liver- and activation-regulated chemokine (CCL20); ELC, Epstein Barr virus–induced receptor ligand chemokine (CCL19); SLC, secondary lymphoid tissue chemokine (CCL21); I-309 (CCL1); TECK, thymus-expressedchemokine (CCL25); Fractalkine (CX3CL1).

Another classification uses physiologic features (conditions of production and cellular distribution of receptors) and divides chemokines into functional subfamilies to distinguish between inflammatory (inducible) and homeostatic (constitutive, housekeeping) lymphoid chemokines [110, 111]. For example, CCR7 and CCXR4 are known to promote homing of naïve T cells and mature DCs to secondary lymphoid organs (lymph nodes, Peyer's patches). In contrast, effector T cells down-regulate CCR7 expression and, depending on the polarizing signals, up-regulate typical Th1 CKRs, like CXCR3 and CCR5, or Th2 CKRs, like CCR3, CCR4, CCR8 and CRTH2. It must be noted that no single CKR is expressed exclusively within one subset [104], and that the combinatorial expression of CKRs fine tunes the specificity of leukocyte migration [105]. The appropriate expression of chemokines and their receptors facilitates encounters between DC, T and B cells and thereby promotes the development of effective adaptive immune responses.

1.6 Immune modulatory effects of probiotics & prebiotics

Several recent reviews summarized the available experimental evidence for the immune modulatory effects of probiotics [74, 75, 112, 113]. Thus, experimental and human studies showed that probiotics affect host resistance to intestinal infections, as well as a number of immune cell functions. This includes enhancement of phagocytic activity of peripheral blood leukocytes [75] and natural killer cell activity [113], stimulation of both non-specific secretory IgA [112, 114], as well as increase of cytokine production *in vivo* (IFN-γ, IFN-α, IL-2) and by peripheral blood mononuclear cells *ex vivo* (IL-1β, TNF-α, IL-6, IL-10, IFN-α, IFN-γ) [77, 115, 116]. However, the exact underlying immune mechanisms are generally not well defined [117]. Similarly, many studies have focused on the effects of prebiotics. Three randomized clinical trials (RCT) in infants approved by the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) committee found that oligosaccharides softened stool consistency, increased stool frequency [118-120] and even lowered faecal pH values, in a dose dependent manner [119]. These studies could also show short increases in the total number of bifidobacteria in stools. However, knowledge on the long-term health consequences of increased bifidobacterial counts is limited at present and data on the efficacy and safety of prebiotics are scare [121]. None of the RCTs assessed clinically important functional outcomes (e.g. immune or inflammatory modulation) and long-term benefits (e.g. GIT infections or allergic diseases). Furthermore, it is unclear whether prebiotics directly or indirectly modulate immune responses, for example by altering the composition of the intestinal flora or by producing SCFA [122]. Therefore, the ESPGHAN committee concluded that no general recommendations on the use of oligosaccharide supplementation in infancy as a prophylactic or therapeutic measure can be made [121]. Consequently, multidisciplinary approaches are necessary to analyse the interaction of probiotics and prebiotics with the immune system.

2. AIMS

2.1 Objectives

The objective of the thesis was to investigate the influence of dietary long-term supplementation with prebiotics in the last trimenon of pregnancy on the composition of the maternal gut and vaginal microbiota and on the neonatal gut colonization in a clinical study. In particular, the following hypotheses were tested in this clinical pilot study:

Long-term supplementation of pregnant women with short-chain galacto-oligosaccharaides (GOS) and long-chain fructo-oligosaccharides (FOS)

1) affects the growth of bifidobacteria and lactobacilli in the maternal gut
2) stabilizes the vaginal colonization with lactobacilli
3) influences the gut colonization of the neonate with bifidobacteria and lactobacilli
4) affects the immune system of the neonate.

2.2 Outcome parameters

Maternal primary outcome variables were the relative numbers of bifidobacteria and lactobacilli within the gut microbiota before and after prebiotic supplementation (throughout the 3rd trimester of pregnancy). Maternal secondary outcomes were stool frequency, stool consistency and vaginal pH values as a proxy for vaginal lactobacilli colonization. In addition, acceptance, tolerance and potential side effects that may be associated with the long-term use of prebiotics during pregnancy were assessed.

Primary neonatal outcomes were the analyses of the successive development of neonatal microbiota (day 5, day 20 and day 182). Secondary outcomes included a comprehensive phenotypical and functional analyses of cord blood (CB) lymphocytes to assess potential effects on the immune system.

3. STUDY DESIGN, MATERIAL & METHODS

3.1 Study design

To address these hypotheses a randomised double-blind placebo-controlled pilot study was designed as depicted in **Figure 4**.

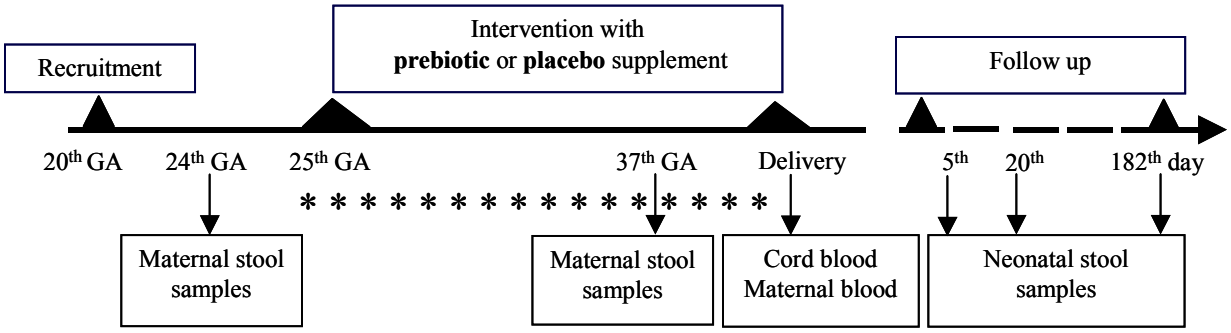


Figure 4. Study design: * Weekly check of stool frequency and consistency, regurgitation, and vaginal pH values. Gestational age (GA).

3.1.1 Study participants

Apparently healthy pregnant women with uncomplicated pregnancies booked for vaginal delivery at the Obstetrics' Hospital or the Department of Obstetrics and Gynaecology Großhadern of the Ludwig-Maximilians-University, Munich, Germany, were approached and invited to participate in the study. Women aged between 18 - 45 years were only included if they planned to deliver in one of the study hospitals and did not intend to donate CB stem cells. Allergies were not considered as an exclusion criterion.

Exclusion criteria were gestational age (GA) above the 24th week at enrolment, acute or chronic illnesses, chronic inflammatory bowel diseases (e.g. Crohn's disease, ulcerative colitis), gestational diabetes, intake of anti-inflammatory drugs (glucocorticoides, acetylsalicylic acid, non-steroidal anti-inflammatory drugs) or antibiotics and regular consumption of pre- or probiotic supplements. Participants were further excluded once they discontinued supplementation for longer than two days and for more than two times. Neonates with obvious malformation, perinatal asphyxia (5 min APGAR score < 7.0; CB pH < 7.2), or with clinical or laboratory signs of a neonatal or maternal infection at delivery were excluded.

The study protocol was approved by the local Ethics Committee of the Bavarian College of Physicians (Bayerische Landesärztekammer; # 03041). The CONSORT guidelines [123] (recommendations for improving the quality of reports of parallel-group randomized trials) were followed. After a careful explanation of the study details, written informed consent was obtained from the participants.

3.1.2 Dietary intervention

Pregnant women were randomized to receive daily either a prebiotic supplement containing oligosaccharides (GOS and FOS) (prebiotic group) or a placebo supplement containing maltodextrin (placebo-group). Participants received the study supplement (provided by NUMICO Research, Friedrichsdorf, Germany), free of charge, delivered to their homes. The complete amount of supplement necessary for at least 15 weeks of supplementation was provided in seven packages each containing 50 sachets. Each sachet contained 6 gram (g) of supplement and was blinded with a special code: "N" or "O". Detailed consumption instructions were given on the label of each sachet.

Six g of the water-soluble powder product contained either 3 g of GOS/FOS (Raftiline HP, provided by NUMICO Research, Friedrichsdorf, Germany) in a ratio of 9:1 (prebiotic supplement) or 6 g of maltodextrin (placebo supplement) (**Table 2**).

	Prebiotic (%)	**Placebo (%)**
Galactose	0.76	-
Glucose	14.39	-
Lactose	15.15	-
FOS (Fructooligosaccharides)	**4.91**	**-**
GOS (Galactooligosaccharides)	**45.45**	**-**
Maltodextrin	19.34	100

Table 2. Composition of the prebiotic and the placebo supplement (per 100 mL).

Maltodextrin was chosen as the placebo control because, in contrast to oligosaccharides, it is completely digested in the upper intestinal tract and does not interfere with the metabolic activity of the colonic flora. The sensory and other characteristics of the prebiotic and placebo preparation were virtually indistinguishable.

Women were instructed to consume three sachets per day by mixing the supplement with 200 mL of water or milk starting from the 25th week of gestation until delivery. They were further asked to maintain their habitual diet throughout the intervention period. To provide some dietary variation, the participants were allowed to use non-probiotic yogurt to mix the supplement.

3.1.3 Randomization & allocation

The sample size was based on the described concentration of bifidobacteria in faecal samples. With a sample size of at least 15 participants per group, it is possible to detect a mean difference of 30% in bifidobacteria, with a probability of 80% and a (2-sided) significance level of 0.05 [30]. A computerized randomization list using a 4 block design (total sample size: 30; number of treatments: 2) was used to implement the random allocation sequence.
The randomization was generated by NUMICO Research, Germany. The volunteers were allocated by sequential numbers at study entry to one of the two supplementation groups, and the allocated number was subsequently kept throughout the study. Participants who failed to complete the study (e.g. in case of revocation of written consent), or dropped out (e.g. because of medical problems or discontinuation of supplementation before study end) were replaced according to the randomization list. We assumed that the study withdrawal rate would be at least 25%. Based on this, we estimated that at least 19 participants must be enrolled into each group. In the case of > 30% missing data the concerned subject was excluded from the study and subsequent statistical data evaluation. All study personnel and participants were blinded to treatment assignments for the duration of the study. The code was revealed to the researchers once data collection and laboratory analyses were complete.

3.1.4 Questionnaires

At study entry, the atopic status of the family (mother, father and siblings) was assessed in a standardized personal face-to-face interview that included information on allergic diseases (allergic asthma (diagnosed by a doctor), hay fever, seasonal allergies, atopic eczema, urticaria, food allergies) and smoking habits (appendix: **9.6.1 Questionnaire 1**). Self-assessed questionnaires were used to collect weekly data on gastrointestinal bowel behaviour (stool frequency, stool consistency, incidence of regurgitation) and vaginal pH-values (CarePlan VpH) throughout the study (appendix: **9.6.2 Questionnaire 2** and **9.6.3 Questionnaire 3**). Participants were contacted at three week intervals via telephone to promote adherence to the protocol and to assess occurrence of side effects and complaints. The phone numbers of the study coordinators were available to the participants at all times to ask questions and/or to report perceived problems or concerns. At delivery, birth details were collected from the hospitals' maternal birth records, i.e. information about gravity, parity, gravity risks, maternal weight and height, pregnancy complications, mode of delivery and delivery complications (proteinuria, blood pressure, occurrence of eclampsia, and estimated blood loss). Furthermore,

foetal anthropometric measures such as birth weight and length, head circumference and APGAR scores were assessed. At study end, compliance, overall tolerance and acceptance, as well as occurrence, intensity and duration of complaints and side effects, such as abdominal pain, distension and flatulence were assessed (appendix: **9.6.4 Questionnaire 4**). Protocol adherence was checked by counting the number of returned sachets at the end of the study. When the infants were six months of age (day 182) a standardized follow-up telephone interview was conducted with the participants (appendix: **9.6.5 Questionnaire 5**). The follow-up questionnaire was adapted from the GINI (German Infant Nutritional Intervention Programm) study [32, 124] and included a series of questions regarding feeding habits, medication, infections and atopic dermatitis of the neonate.

3.1.5 Study samples

Stool sampling:
Two stool samples were taken from the mother before supplementation begin (before the 25th week of gestation) and an additional two samples shortly before expected delivery (**Figure 4**). Furthermore, two stool samples from the neonate were taken after 5 and 20 days post partum and at the age of six months (~ day 182). Women were asked to freeze the samples at - 20 °C within 20 min after collection and to inform the study coordinators. Frozen samples were transported with cool packs by taxi and stored at - 20 °C until analyses.

Blood sampling:
Placental venous CB was collected to assess the neonatal immune function (see methods below). The CB samples were collected into 7.5 mL ethylene diamine tetra acetate (EDTA) and 7.5 mL lithium heparin (LH) blood collection tubes from the placental vein immediately after delivery. Maternal peripheral blood was collected into 7.5 mL EDTA-tubes after delivery by venous puncture (**Figure 4**).

3.2 Materials

3.2.1 Instruments & software

Instrument [Software]	Company
Personal Computer (PC) with [Windows XP office package] [SPSS V12] [Reference Manager]	IBM, Heidelberg, Germany
BD FACSCanto™ with [BD FACSDiva™ Software]	BD Biosciences, Heidelberg, Germany
Bio-Plex Suspension Array Reader with [Bio-Plex-Manager Software™ V 3.0]	BioRad, Laboratories Hercules, Carlif. USA
Blood counter: XT-1800i™ Haematology Analyser	Sysmex America, Inc. Mundelein, USA
Rotanta centrifuge 460 R	Hettich, Tuttlingen, Germany
Eppendorf table centrifuge 5415C	Eppendorf, Hamburg, Germany
Research pipettes (0-2 μL, 2-20 μL, 10-100 μL, 100-100 μL)	Eppendorf, Hamburg, Germany
Eppendorf Easypet 4421 (pipetboy)	Eppendorf, Hamburg, Germany
Flow Titertek®Multichannel Pipette (50-300 μL)	Flow Titertek Labsystems, Finnland
Vortex-Genie 2	Scientific Industries, Bohemia, NY, USA
96-well Vacuum pump	BioRad, Laboratries GmbH, Munich, Germany
Rotamax 120	Heidolph Instruments GmbH & Co KG, Schwabach, Germany
Carbon dioxide (CO_2) Incubator BB6060	Hereaus Instruments, Thermo Electron Corporation, Langenselbold, Germany
LaminAir ® HBB 2472	Hereaus Instruments, Thermo Electron Corporation, Langenselbold, Germany
+ 4 °C Fridge	Gerlingen-Schillerhöhe, Germany
- 20 °C Freezer	Siemens, Munich, Germany
- 80 °C Freezer	Kendro, Thermo Electron Corporation, Langenselbold, Germany

Table 3. Instruments and software.

3.2.2 Consumables

Consumables	Company
S-Monovette® 7.5 mL K3E (EDTA-tubes)	Sarstedt, Nümbrecht, Germany
S-Monovette® 7.5 mL LH (Lithium-Heparin tubes)	Sarstedt, Nümbrecht, Germany
Sarstedt Serological pipettes (5 mL, 10 mL, 25 mL)	Sarstedt, Nümbrecht, Germany
epT.I.P.S. Standard (2-200 μL, 500-1250 μL)	Eppendorf, Hamburg, Germany

Eppendorf cups (1.5 mL, 2 mL)	Eppendorf, Hamburg, Germany
Cellstar® PP Test Tubes (15 mL, 50 mL)	Greiner bio one, Frickenhausen, Germany
BD Falcon™, 5 mL Polystyrene Round Bottom Tube	BD Biosciences Discovery, Heidelberg, Germany
Tissue Culture Plate, 24-well, flat bottom	BD Labware, Heidelberg, Germany
Spatula containers (polyethylene stool sample tubes)	Engelbrecht, Edermünde, Germany
Cool pads	Mack Ges.m.b.H, Altenmarkt Austria
CarePlan® VpH test gloves	Inverness medical Unipath Diagnostics, Cologne, Germany

Table 4. Consumables.

3.2.3 Reagents

Reagents	**Company**
Aqua bidestileta	Gibco, Invitrogen Life Technologies; Karlsruhe, Germany
RPMI 1640	Gibco, Invitrogen Life Technologies; Karlsruhe, Germany
Trizol® LS Reagent	Invitrogen Life Technologies, Karlsruhe, Germany
Phosphat-Buffered-Saline (PBS) pH 7.2 - $CaCl_2$ - $MgCl_2$	Gibco, Invitrogen Life Technologies; Karlsruhe, Germany
BD FACSFlow	BD Biosciences, Heidelberg, Germany
BD FACSClean	BD Biosciences, Heidelberg, Germany
BD FACS™ Lysing Solution (10x)	BD Biosciences, Heidelberg, Germany
Staphylococcal enterotoxin B (SEB)	Sigma Chemical Co., Deisenhofen, Germany
Beta-lactoglobulin (BLG)	Sigma Chemical Co., Poole, UK
Concavalin A (Con A)	Sigma Chemical Co., Poole, UK
Ovalbumin (OVA)	Sigma Chemical Co., Poole, UK
Lipopolysaccharid (LPS)	Sigma Chemical Co., Poole, UK
Dermatophagoides pteronysinus 1 (Der p1) affinity purified	Indoor Biotechnologies, Cardiff, UK
Bio-Plex Suspension Array System 8plex: TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-10, GM-CSF, G-CSF 4plex: IL-6, IL-8, IL-10, MCP-1, MIP-1ß IL-7, IL-5, IL-12, IL-13, IL-15	BioRad, Laboratories, Hercules, Carlifornia, USA

Table 5. Reagents.

3.2.4 Antibodies

For flow cytometric staining anti-human monoclonal antibodies labelled with fluorescein isothiocyante (FITC), phycoerythrin (PE), phycoerythrin-cyanin 5 (PC5), or allophycocyanin (APC) were used in the following dilutions (**Table 6**).

Antibodies	Fluorochrome	Dilution	Isotype	Company
CD45RA	PC5	1 : 4	MouseIgG2b,k	BD Biosciences
CD45RO	APC	1 : 4	IgG2ak	BD Biosciences
CD4	APC	1 : 2	Mouse IgG1,k	BD Biosciences
CD8	APC	1 : 2	MouseIgG1,k	BD Biosciences
CD14	FITC	1 : 2	Mouse IgG2a,k	BD Biosciences
CD69	FITC	pure	Mouse IgG1,k	BD Biosciences
CD8	FITC	1 : 2	RPA-T8	BD Biosciences
CD25	PE	1 : 2	MouseIgG1,k	BD Biosciences
TLR2	PE	1 : 2	Mouse IgG2a,k	eBiosciences
TLR4	PE	1 : 2	MouseIgG2a,k	eBiosciences
CRTH2	PE	1 : 2	ratIgG2a	Miltenyi Biotec
CCR1	PE	1 : 2	Mouse IgG2B	R&D Systems GmbH
CCR2	PE	1 : 2	Mouse IgG2B	R&D Systems GmbH
CCR4	PE	1 : 2	IgG1,k	BD Biosciences
CCR5	PE	1 : 2	Mouse IgG2a,k	BD Biosciences
CCR6	PE	1 : 2	Mouse IgG2B	R&D Systems GmbH
CCR7	PE	1 : 4	Mouse IgG2A	R&D Systems GmbH
CCR8	PE	pure	Rat IgG2b	R&D Systems GmbH
CCR9	PE	pure	Mouse IgG2A	R&D Systems GmbH
CXCR3	PE	1 : 4	MouseIgG1,k	R&D Systems GmbH
CXCR4	PE	1 : 2	IgG2A	R&D Systems GmbH
CXCR5	PE	1 : 2	Mouse IgG2B	R&D Systems GmbH
IgG1 / IgG1	FITC/PE	pure	-	BeckmannCoulter
IgG1 / IgG2a	FITC/PE	pure	-	BeckmannCoulter

Table 6. Antibodies used for flow cytometric staining (BD Biosciences, Heidelberg, Germany; eBioscience, San Diego, USA; Miltenyi Biotec, Bergisch-Gladbach, Germany; R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany; Beckman Coulter, Krefeld, Germany).

3.3 Methods

3.3.1 Microbial stool sample analyses

Collected samples were sent on dry ice to the Department of Biomedical Research, (NUMICO Research, Wageningen, Netherlands) where DNA isolations, DAPI, FISH and qPCR analyses were preformed (by Monique Haarman and Dr. Jan Knol). Stool samples were thawed and the pH was measured directly with a Handylab pH meter (Schott Glas, Mainz, Germany) equipped with an Inlab 423 pH electrode (Mettler-Toledo, Columbo, U.S.A.). Samples were diluted 10-fold in milliQ and homogenized with a stomacher (IUL Instrument, Barcelona, Spain). The suspensions used for DNA extractions were subsequently frozen at
- 20 °C until further analyses

For the fluorescent *in-situ* hybridization (FISH) analysis 1 mL of the homogenized faecal suspension was fixed in 3 mL freshly prepared 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) and incubated overnight at + 4 ^{o}C. The total number of bacteria was determined by 4´,6-diamino-2-phenylindole (DAPI) staining and the percentage of bifidobacteria and lactobacilli were determined by fluorescence in situ hybridisation (FISH), as described [30, 125] with some slight modifications.

Prior to the DNA isolation, the homogenized faecal samples were thawed at room temperature. The DNA isolations were carried out according to manufacturer's instructions (NucliSense Isolation Extraction Kit; BioMerieux, Boxtel, The Netherlands).

For the relative quantification of the genera *Bifidobacterium* and *Lactobacillus* and the different bifidobacterial and lactobacilli species, duplex 5' nuclease (qPCR) assays were used [126, 127]. Briefly, with different primer and probe combinations (appendix: **9.4 Primers and Probes used for the duplex 5' nuclease assays**), a temperature profile consisting of two minutes (min) at + 50 °C, 10 min at + 95 °C, followed by 45 cycles of 15 seconds (sec) at + 95 °C and 60 °C for one min was run on an ABI Prism 7900HT (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Thereafter, the percentages were determined according to Liu et al. [128]. The minimum detection threshold of FISH was 10E6 cells per g wet weight of faeces and the detection limit of the qPCR analyses was 0.00001%.

3.3.2 Flow cytometry

CB anti-coagulated with EDTA was processed and analysed within 4 h after blood collection. Optimised amounts of fluorochrome-conjugated antibodies were added to 50 μL whole CB (**Table 7**). For phenotypical analyses of T lymphocytes 4-colour cytometry with PE-conjugated monoclonal antibodies against different chemokine receptors (CKR), was used in combination with monoclonal antibodies against the naive lymphocyte marker CD45RA- (labelled in PC-5), the T helper cell marker $CD4^+$ (labelled in APC) and the T cytotoxic cell marker $CD8^+$ (labelled in FITC) (tubes 3 - 14). In addition, activation makers (CD25 and CD69) were assessed on $CD4^+$ and $CD8^+$ cells (tubes 15 and 16) and Toll-like receptors (TLR2 and TLR4) were analysed on $CD14^+$ expressing monocytes (tubes 1 and 2). Appropriate isotype controls of immune globulin 1 (IgG1) and 2a (IgG2a) were always included (tubes 17 and 18). After addition of the respective antibodies to each tube, samples were mixed for 30 sec and incubated for 20 min at + 4 °C in the dark.

Tube number	**PC5** Phycoerythrin-cyanin 5	**APC** Allophycocyanin	**FITC** Fluorescein isothiocyante	**PE** Phycoerythrin
1	CD45RA	CD4	CD14	TLR2
2	CD45RA	CD4	CD14	TLR4
3	CD45RA	CD4	CD8	CCR1
4	CD45RA	CD4	CD8	CCR2
5	CD45RA	CD4	CD8	CCR4
6	CD45RA	CD4	CD8	CCR5
7	CD45RA	CD4	CD8	CCR6
8	CD45RA	CD4	CD8	CCR7
9	CD45RA	CD4	CD8	CCR8
10	CD45RA	CD4	CD8	CCR9
11	CD45RA	CD4	CD8	CXCR3
12	CD45RA	CD4	CD8	CXCR4
13	CD45RA	CD4	CD8	CXCR5
14	CD45RA	CD4	CD8	CRTH2
15	CD45RA	CD4	CD69	CD25
16	CD45RA	CD8	CD69	CD25
17	CD45RA	CD45RO	IgG1	IgG1
18	CD45RA	CD45RO	IgG1	IgG2a

Table 7. Antibody combinations used for CB staining.

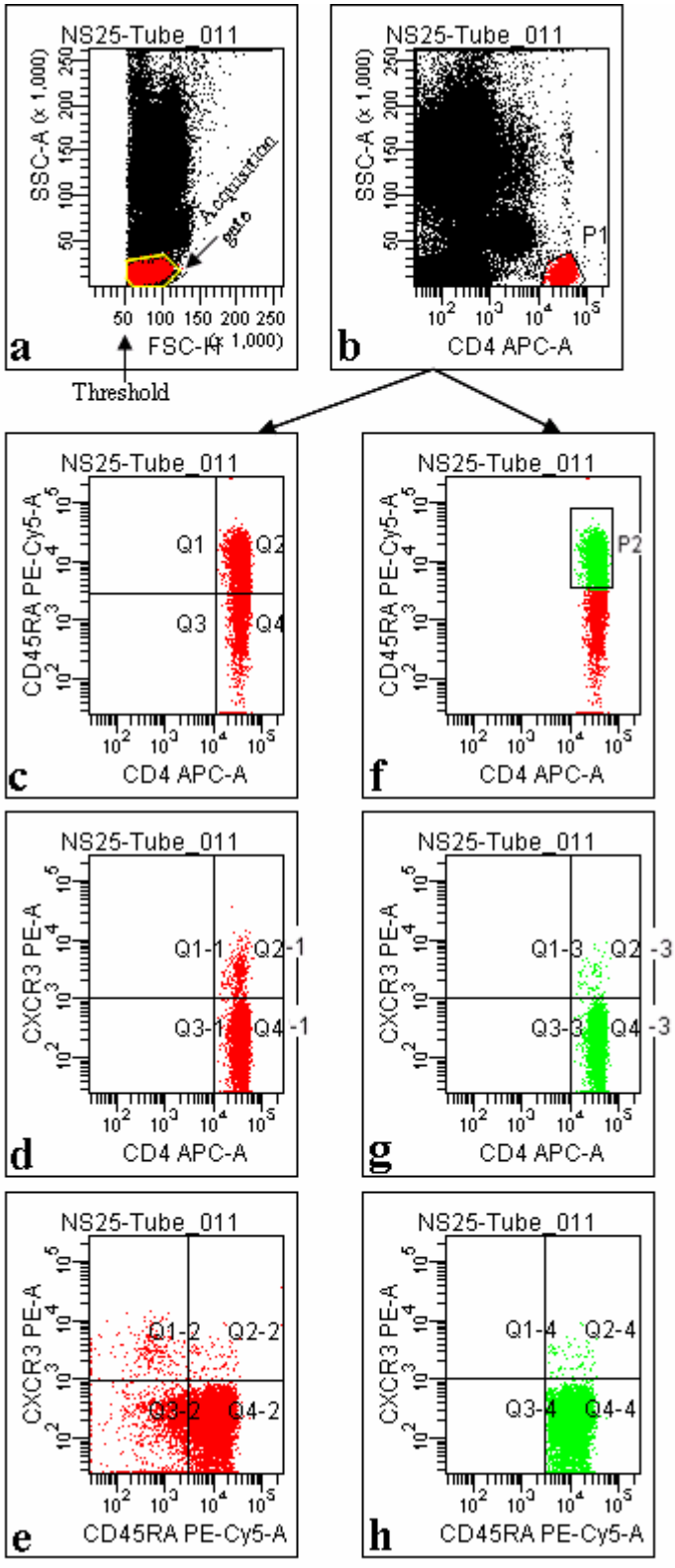
Stained cells were washed twice by centrifugation (3 min, 3000 rpm) with 1 mL PBS to remove unbound antibodies. Contaminating erythrocytes were lysed using 0.3 mL BD FACS™ 1x lysing solution. Samples were analysed immediately afterwards. Cells were acquired on a BD FACS-Canto flow cytometer (equipped with a 488-nm blue laser and a 635-nm red diode laser for multicolour measurements) and analysed using BD FACS-Diva Software. Initial gating with forward scatter (FSC) on the x-axis and side scatter (SSC) on the y-axis served to exclude debris and dead cells (threshold = 50).

An acquisition gate was set according to forward and side light scattering cell properties to collect only the lymphoid population (**Figure 5a**). A minimum of 10,000 lymphocytes was acquired (tubes 3 - 14). Same antibody batches were used to reduce intra-assay variability. In addition, laser and photomultiplier parameters were kept constant for all experiments.

CB contains nucleated red blood cells (NRBCs), which do not get lysed by lysis solution and which overlap with FSC-SSC characteristic of the lymphocytes. Therefore, a gate was set on $CD4^+$ expressing cells in the $CD4^+$-SSC plot (P1 in red) to exclude NRBCs from analyses in all samples (**Figure 5b**). After defining a cut-off value according to the isotype controls (tubes 17 and 18), the frequency of $CD45RA^+$ (quadrant 2, Q2), CKR^+ (Q2-1) and $CKR^+CD45RA^+$ (Q2-2) expressing cells were analysed within the $CD4^+$ population (**Figure 5c, 5d, 5e**).

A second gate (P2 in green) was set on $CD4^+CD4RA^+$ cells (**Figure 5f**) to determine the frequency of CKR^+ expressing cells within this subset (Q2-3 = Q2-4, **Figure 5g, 5h**). The same analyses procedure was performed for determining the frequency of CKR^+ expressing $CD8^+$ and $CD8^+CD45RA^+$ cells.

Since CXCR4 is a heterogeneously expressed receptor on lymphocytes (tube 12), a gate was set on $CXCR4^+$ expressing cells in the $CXCR4^+$-SSC plot and the mean fluorescence intensity (MFI) was determined on the different $CD4^+$ and $CD8^+$ T cell subsets. The gating strategy for $CXCR4^+$ is comparable to the gating strategy for TLR^+ expression on $CD14^+$ monocytes.



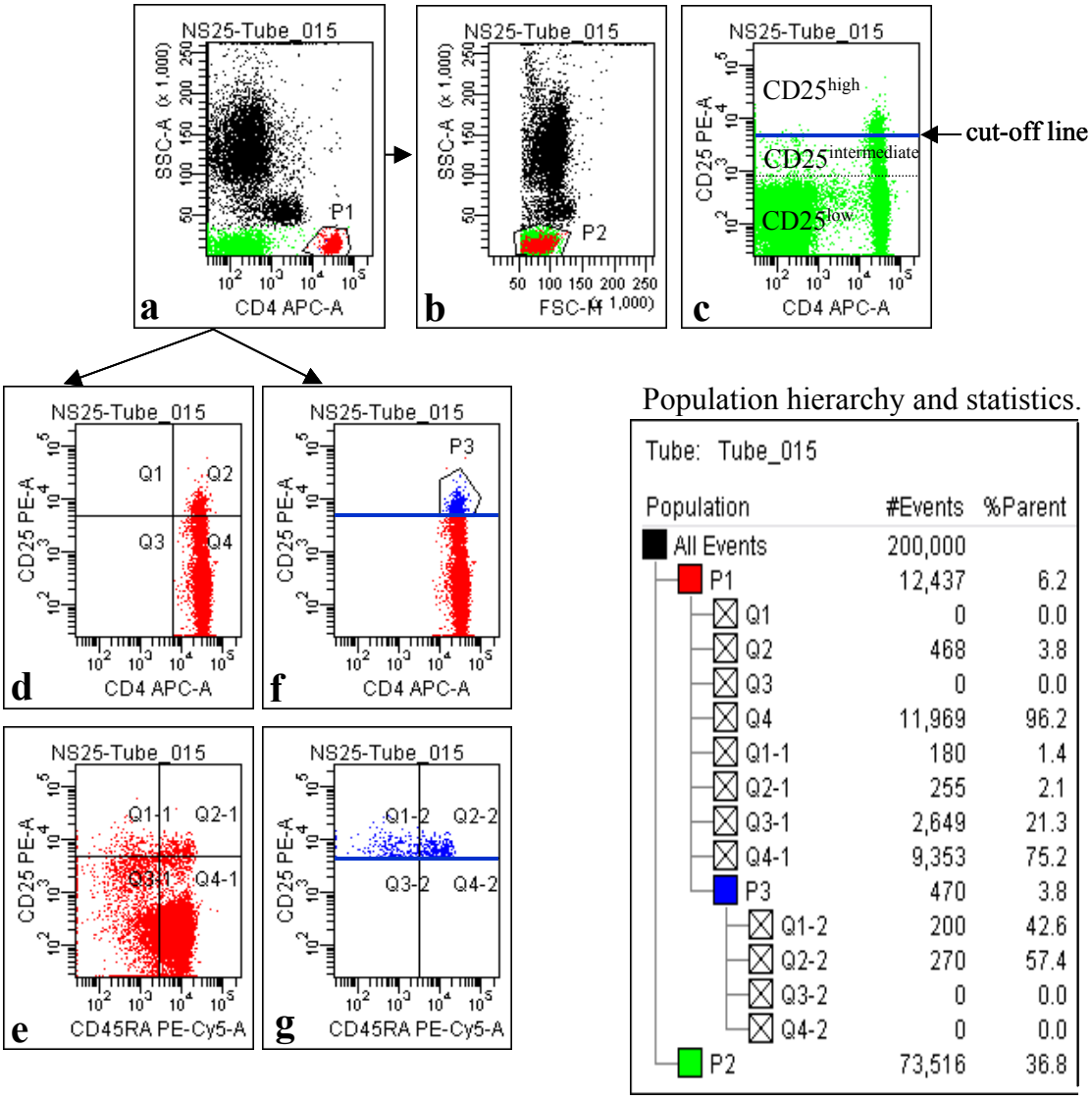
Population hierarchy and statistics.

Tube: Tube_011

Population	#Events	%Parent
All Events	200,000	
P1	10,195	5.1
Q1	0	0.0
Q2	8,576	84.1
Q3	0	0.0
Q4	1,619	15.9
Q1-1	0	0.0
Q2-1	296	2.9
Q3-1	0	0.0
Q4-1	9,899	97.1
Q1-2	221	2.2
Q2-2	83	0.8
Q3-2	1,425	14.0
Q4-2	8,466	83.0
P2	8,390	82.3
Q1-3	0	0.0
Q2-3	74	0.9
Q3-3	0	0.0
Q4-3	8,316	99.1
Q1-4	0	0.0
Q2-4	75	0.9
Q3-4	0	0.0
Q4-4	8,315	99.1

Figure 5. Gating strategy for determining the frequency of CKR^+ expressing $CD4^+$ cells. An acquisition gate was set on the lymphocytes according to the FSC and SSC (**a**). A gate was set on $CD4^+$ cells (P1 in red) in a $CD4^+$-SSC plot (**b**) and the frequencies of $CD45RA^+$ (**c**) and CKR^+ (**d**) and $CKR^+CD45RA^+$ (**e**) expressing $CD4^+$ cells was analysed within this gate. A second gate (P2 in green) was set on the $CD4^+CD45RA^+$ cells (**f**) to analyse the frequency of CKR^+ expressing cells (**g, h**) within the $CD4^+CD45RA^+$ population. Population hierarchy and statistics (number (#) of events and their percentages (%) in each gate (P) and quadrant (Q)) are shown in the table besides.

To determine the frequency of $CD4^+CD25^{high}$ expressing T cells a gate (P1 in red) was set on $CD4^+$ expressing cells in the $CD4^+$-SSC plot (**Figure 6a**). To discriminate between $CD25^{high}$ T cells (which presumably represent T regulatory cells) from $CD25^{intermediate}$ activated effector T cells, CD25 expression on $CD8^+$ cells was used as internal control. Therefore, a backward gating strategy was applied to set a second gate (P2 in green) on lymphocytes (**Figure 6b**) and CD25 expression on $CD4^+$ and $CD8^+$ cells was compared (**Figure 6c**). $CD8^+$ ($CD4^-$) cells express almost exclusively intermediate levels of CD25, whereas $CD4^+$ T cells express CD25 with high ($CD25^{high}$) or intermediate ($CD25^{intermediate}$) intensities [129]. Only $CD4^+$ cells expressing CD25 with higher intensities than the $CD8^+$ cells (above blue cut off line) were included in the analysis. The frequency of $CD25^{high}$ (Q2, **Figure 6d**) and of $CD45RA^+CD25^{high}$ expressing cells (Q2-1, **Figure 6e)** were determined within the $CD4^+$ population. A second gate (P3 in blue) was set on the $CD4^+CD25^{high}$ subset and the frequency of $CD45RA^+$ cells (Q2-2, **Figure 6f**) within this subset was determined (tube 15). Gating strategy was confirmed by analyses of $CD8^+CD25^+CD45RA^+$ labelled cells (tube 16).



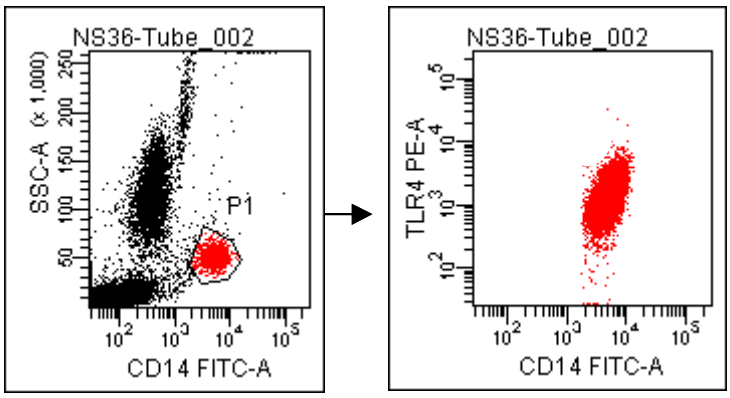
Population hierarchy and statistics.

Tube: Tube_015

Population	#Events	%Parent
All Events	200,000	
P1	12,437	6.2
Q1	0	0.0
Q2	468	3.8
Q3	0	0.0
Q4	11,969	96.2
Q1-1	180	1.4
Q2-1	255	2.1
Q3-1	2,649	21.3
Q4-1	9,353	75.2
P3	470	3.8
Q1-2	200	42.6
Q2-2	270	57.4
Q3-2	0	0.0
Q4-2	0	0.0
P2	73,516	36.8

Figure 6. Gating strategy for determining the frequency of $CD4^+CD25^{high}$ T regulatory cells. A gate (P1 in red) was set on $CD4^+$ cells in the $CD4^+$-SSC plot (**a**). For discrimination between $CD25^{high}$, $CD25^{intermediate}$ and $CD25^{low}$ cells a cut-off line (blue) was set between the $CD25^{high}$, $CD25^{intermediate}$ cells by comparing the CD25 expression levels on $CD4^+$ and $CD8^+$ cells (**c**). For this a gate (P2 in green) was set on the lymphocyte population (**b**). In a first analyses the frequency of $CD25^{high}$ (**d**) and $CD45RA^+CD25^{high}$ (**e**) expressing cells within the $CD4^+$ population were determined. A second gate (P3 in blue) was set on $CD4^+CD25^{high}$ population (**f**) and the frequency of $CD45RA^+$ expressing cells within this subset was determined (**g**). Population hierarchy and statistics (number (#) of events and their percentages (%) in each gate (P) and quadrant (Q)) are shown in the table besides.

To determine the expression of TLRs on monocytes, an acquisition gate (P1 in red) was set on the $CD14^+$ expressing cells in the $CD14^+$-SSC plot (**Figure 7**). The MFI of TLR2 and TLR4 was determined within this gate (tubes 1 and 2).



Population hierarchy and statistics.

Tube Name: Tube_002

Population	Parent ...	#Events	%Parent	%Total	CD14 F... Mean	TLR4 P... Mean
All Events	####	200,000	####	100.0	423	488
CD14+TLR+ Monocytes	All Eve...	8,864	4.4	4.4	5,068	1,705

Figure 7. Gating strategy for TLR^+ expression on $CD14^+$ monocytes. A gate was set on the $CD14^+$ cells in the $CD14^+$-SSC plot and MFI of TLR was determined within this gate. Population hierarchy and statistics (number (#) of events and their percentages (%) in each gate (P) and quadrant (Q)) are shown in the table below.

3.3.3 Simulation assay

Lithium heparinized CB samples were diluted 1 in 5 with RPMI-1640. Adapting a technique by E. Miles et al. [130] aliquots of 1 mL diluted CB were stimulated with the mitogen concavalin A (Con A 50 μg/mL), the food allergens beta-lactoglobulin (100 μg/mL BLG), ovalbumin (100 μg/mL OVA), the bacterial stimuli lipopolyssaccharid (0.1 μg/mL LPS), staphylococcal enterotoxin B (0.1 μg/mL SEB), the inhalative house dust mite allergen *Dermatophagoides pteronyssinus* (10 μg/mL Der p1) or medium alone in a 24-well culture plate. Stimuli were pre-aliquoted for each experiment to reduce intra-assay variability. Cells were incubated at + 37 °C in a 5% CO2-atmosphere for 24 h and 48 h. Supernatants were collected after centrifugation (3000 rpm for 5 min) and aliquots of 200 μL were stored for cytokine analyses at - 80 °C.

3.3.4 Multiplex cytokine array

The cytokine content of the stimulated CB supernatants was measured with a human multiplex, particle-based, flow cytometric assay. In short, premixed dyed 5.5 μm polystyrene beads (50 μL per well) coated with target capture antibodies specific for different cytokines (5000 beads per cytokine) were incubated with premixed cytokine standards or sample (50 µL) supernatants in 96-well filter plates. Plates were shaken for 30 sec at high speed (1000 rpm) and then incubated at room temperature for 30 min at low speed (300 rpm). Following incubation and washing, the bound cytokines were detected with premixed biotinylated detection antibodies (50 μL per well). After incubation and washing, streptavidin-phycoertythrin (25 μL streptavidin-PE per well) was added to the wells and the plates were shaken again for 10 min at room temperature. Finally, beads were washed, resuspended in 125 μL of Bio-Plex cytokine assay buffer and read by the Bio-Plex Suspension Array Reader (flow cytometric microtiter plate reader system). Data were analysed using Bio-Plex-Manager Software^{TM} (V 3.0) with 5 PL (5 parameter logistic regression algorithms) curve fits. The unknown concentrations were deduced from the cytokine standard curves and values above detection limits were excluded from further analyses.

Detection limits for the cytokines were 2 - 32000 pg/mL. TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, IL-13, IL-15, GM-CSF, G-CSF concentrations were directly measured without further dilution of the supernatants. Supernatants were diluted 1:10 in RPMI to determine the concentrations of IL-6, IL-8, MCP-1 and MIP-1ß. The differences in cytokine expression, as compared to the negative control, were determined and concentrations

were normalized to the number of lymphocytes (for IL-2) and leukocytes (for all other cytokines) obtained from the whole blood counts (pg/mL/10E3 cells).

3.3.5 Statistical analyses

Continuous data were tested by Kolmogorov-Smirnov test to determine if they were normally distributed. Normally distributed data were analyzed by the two-tailed t-test. For non-parametric data a two-tailed Mann-Whitney *U*-test (MW*U*) test was used. Descriptive results are expressed as median with ranges. Intra-group comparisons (compared to baseline) were performed using paired Wilcoxon test. For ordinal and nominal (categorical) data, the percent frequencies were calculated and the differences were determined using Pearson Chi square (χ^2) test. Differences within one group (intra-group comparisons) were calculated by the McNemar test. For repeated measurements, Friedmans test was used to assess differences within one group over time. Bacterial data were analysed as (a) continuous data and (b) dichotomous data (i.e. detected or not detected). Correlations (between mother and infant faecal bifidobacteria and lactobacilli colonization) were assessed using Spearman's rank correlation test. The bacterial diversity index (DI) was calculated using the following equation DI = (A/B) x 100, with "A" representing the number of species detected in a sample and "B" representing the total number of analyzed species (for bifidobacteria n=9 and for lactobacilli n=8). The bacterial similarity index (SI) between mother and infant pairs was determined using the following equation: SI = (C/D) x 100, with "C" representing the number of mother-infant species that matched, i.e. that were detectable in both mother and infant (species that were detected only in the mother or only in the infant were considered as not matching) and "D" representing the number of all species which were either positive in the mother and/or the infant. A probability level of below 0.05% was considered to be statistically significant.

To correct for multiple testing, the significance value was set to 0.003 (17 parameters tested by FACS; 17 bacterial species analysed by FISH and qPCR) or 0.004 (12 cytokines measured by Bioplex). Due to the exploratory nature of this study, we did not wish to exclude any important relationships by using stringent correction factors for multiple analyses. However, we recognize the potential for type 1 error, and data have been interpreted conservatively in this respect. Accordingly, our discussion has focussed on differences that are most likely to be of biological significance (namely differences of $p<0.001$).

4. RESULTS

4.1 Recruitment of study participants and sample collection

A total of 200 pregnant women between the 20th and 24th week of gestation were assessed for eligibility (**Figure 8**). Of these, 75 women were not interested in participating in the study. From the remaining 125 women, 77 women were excluded because they did not fulfil the inclusion criteria: 37 planned to deliver at other hospitals, 22 planned to donate stem cells and 18 needed medications.

Thus, 48 women consented to participate and were randomly assigned to one of the two supplementation groups. From these, seven women dropped out because of personal or medical reasons (movement to another city, delivery in another hospital, complications during pregnancy, gestational diabetes, anti-inflammatory medication). Five women were excluded because of non-compliance with the study procedures (interruption of supplementation for longer than two days and for more than two times), which was unrelated to the supplement assignment. Additionally, three women did not complete the study because they did not tolerate the supplementation: one woman from the prebiotic group suffered from constipation and bloating and two women from the placebo group stopped supplementation because they suffered from diarrhoea or reflux. Thus, 33 women completed the study (17 participants in the prebiotic group and 16 participants in the placebo group (drop out rate 31%). All women started supplementation at the 25th week of gestation up to delivery. Recruitment started in March 2004 and continued until July 2005. Follow-up ended in January 2006.

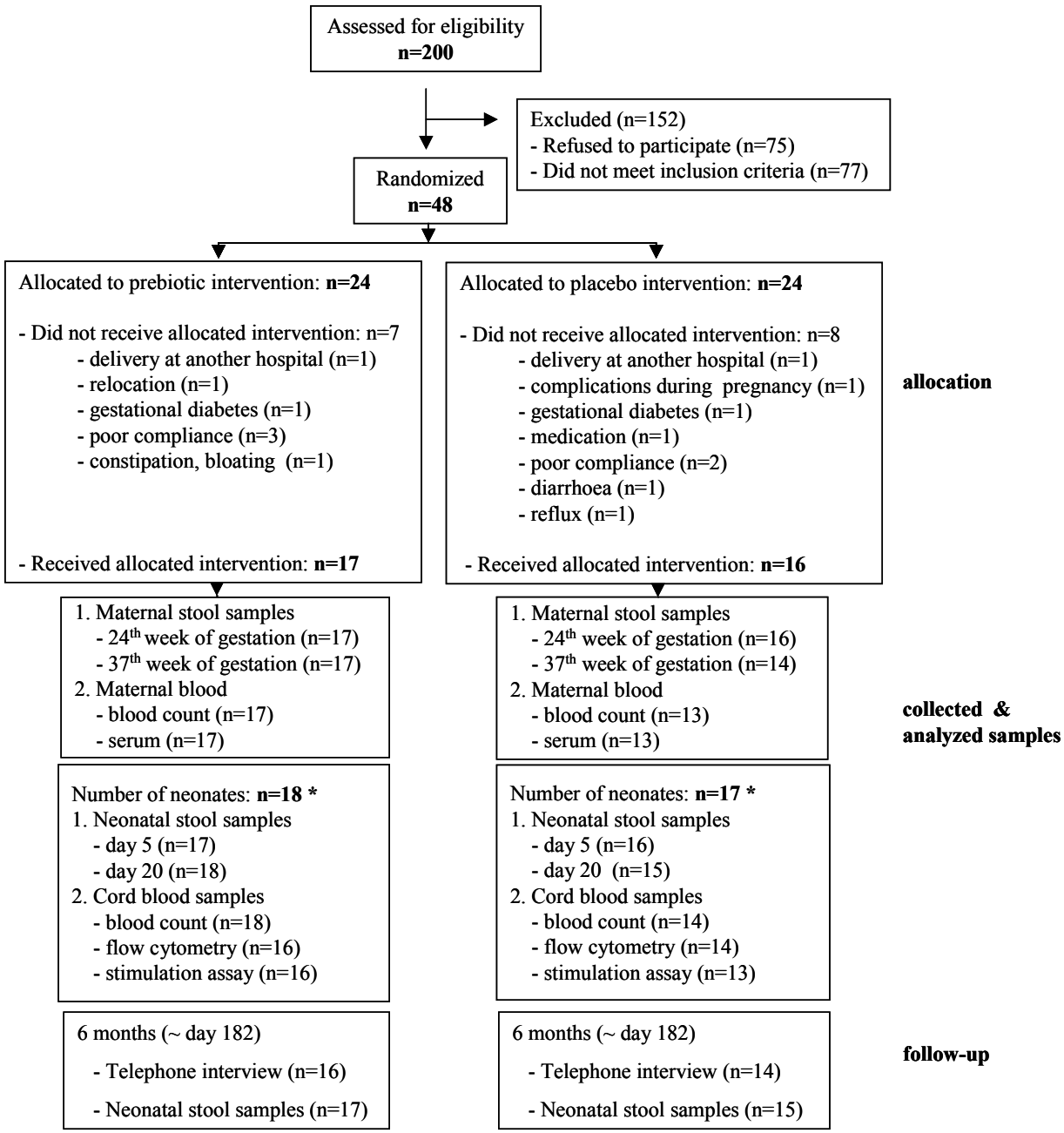


Figure 8. Flowchart of participant recruitment, sample collection and follow-up.
* one twin pregnancy in each group.

Explanation for the missing stool samples:

Maternal stool samples at the 37th GA: two mothers from the placebo group forgot to immediately freeze the stool samples after collection. Neonatal stool samples from day 5: one mother from the prebiotic and one from the placebo group were unable to collect a sample from the diapers of their babies. Neonatal stool samples from day 20: one mother from the placebo group forgot to immediately freeze the neonatal stool sample after collection and one sample from the placebo group did not contain enough material for analysis. Neonatal stool samples from day 182: one neonatal sample form the prebiotic group and two from the placebo group were not obtained due to loss of contact to participating women.

Explanation for the missing blood samples:

Maternal blood samples at delivery (blood count and serum): three mothers from the placebo group delivered at other hospitals. Cord blood samples at delivery (blood count, serum, flow cytometry, stimulation assay): three mothers from the placebo group delivered at other hospitals. Two CB tubes from the prebiotic group did not contain enough material for performing the flow cytometric analyses and the stimulation assay. One additional CB tube from the placebo group did not contain enough material for performing the stimulation assay.

Explanation for the missing follow-up interviews:

Contact was lost to one woman from the prebiotic and two women from the placebo group.

4.2 Analyses of questionnaires

4.2.1 Baseline characteristics

The baseline characteristics of the study population are summarized in **Table 8**. At study entry, participants did not differ in age and smoking habits. Paternal allergies and allergies in siblings showed no significant differences between both groups (data not shown).

Mother	**Prebiotic** (n=17)	**Placebo** (n=16)	P
Age at study entry[1]			0.096
median	33	35	
(range)	(23 - 48)	(22 - 43)	
Allergies			
allergic asthma	4 (23.5)	3 (18.7)	0.737
hay fever	7 (41.2)	9 (56.3)	0.387
seasonal allergies	6 (35.3)	8 (50.0)	0.393
atopic eczema	6 (35.3)	5 (31.3)	0.805
urticaria	4 (23.5)	2 (12.5)	0.412
food allergies	6 (35.3)	4 (25.0)	0.520
Smoking			0.965
yes	1 (5.9)	1 (6.3)	
no	16 (94.1)	15 (93.7)	

Table 8. Baseline characteristics of the study group. [1]Results are expressed as median (range) for continuous variables and numbers of subjects (%) for dichotomous variables. Differences between both groups were calculated with MW*U* test for continuous data or χ^2 test for dichotomous data.

4.2.2 Study population at delivery

Apart from two women with twin pregnancy (one gemini in the prebiotic-group and one in the placebo group) all women had a singleton pregnancy. All participants, except three women from the placebo group, delivered at one of the two study hospitals. Infants were born between the 36th - 41st week of gestation. Four participants from the prebiotic group (one with a twin pregnancy) and one participant from the placebo group delivered by caesarean section. No significant differences concerning body mass index (BMI), parity risks, maternal blood loss, mode of anaesthesia, and maternal blood count parameters (leukocytes, %-lymphocytes, haemoglobin) were observed between the groups (data not shown). The supplement was equally well accepted by all participants who completed the study. The median duration of supplementation was 3.4 - 3.8 months. All 33 participants reported full compliance with the intervention regime (none interrupted supplementation for longer than two days and for more than two times). Overall tolerance of both supplements was good. Complaints about reflux, bloating and abdominal pain were reported equally in both groups (**Table 9**).

No major side effects of the treatment were observed. All women stated that dissolving the powder supplement in fluids three times daily was too time consuming and claimed that they would have preferred taking capsules instead. Birth data were obtained from the all except three neonates whose mothers did not deliver at one of the two study hospitals. After supplementation, no significant differences were observed between both groups concerning APGAR, umbilical pH, as well as birth weight and length. Three infants from the prebiotic group and two from the placebo group were treated with antibiotics in the first three weeks of life for four to seven days. Antibiotic treatment was necessary because of amnion infection (n=4).

Mother	**Prebiotic** (n=17)	**Placebo** (n=16)	P
Pregnancy[1]			0.965
singleton	16 (94.1)	15 (93.8)	
gemini	1 (5.9)	1 (6.3)	
Gestational age			0.083
median	39.57	40.14	
(range)	(36.7 - 41.4)	(36.4 - 41.5)	
Delivery mode			0.292
spontaneous	11 (64.7)	14 (56.0)	
caesarean section	4 (23.5)	1 (6.3)	
vacuum extraction	2 (11.8)	1 (6.3)	
Interruption of supplementation			0.512
never	11 (64.7)	13 (81.3)	
once	3 (17.6)	2 (12.5)	
twice	3 (17.6)	1 (6.3)	
more than twice & longer than 2 days	0 (0.0)	0 (0.0)	
Tolerance			0.337
very good	8 (47.1)	11 (68.8)	
good	7 (41.2)	3 (18.8)	
not so good	1 (5.9)	2 (12.5)	
bad	1 (5.9)	0 (0.0)	
Complaints			0.554
yes	7 (41.2)	5 (31.3)	
no	10 (58.8)	11 (68.7)	
Intensity of complaints			0.717
good to bear	4 (23.5)	2 (12.5)	
bearable	2 (11.8)	1 (6.3)	
almost unbearable	1 (5.9)	2 (12.5)	
unbearable	0 (0.0)	0 (0.0)	
Duration of complaints			0.500
short	2 (11.7)	2 (12.5)	
up to 1 hour	1 (5.8)	2 (12.5)	
more than one hour	3 (17.6)	0 (0)	
the whole day	1 (5.8)	1 (6.3)	
Stool irregularities			0.188
yes	8 (47.1)	13 (81.3)	
no	9 (52.9)	3 (18.7)	

Newborn	Prebiotic (n=18)	Placebo (n=17)	P
Gender[1]			0.615
girl	8 (44.4)	8 (47.1)	
boy	10 (55.6)	9 (52.9)	
APGAR (5 min)			1.00
median	10	10	
(range)	(10 - 10)	(10 - 10)	
APGAR (10 min)			1.00
median	10	10	
(range)	(10 - 10)	(10 - 10)	
Umbilical pH			0.511
n[2]	18	16	
median	7.34	7.34	
(range)	(7.1 - 7.4)	(7.2 - 7.4)	
Birth weight (g)			0.947
median	3368	3370	
(range)	(2345 - 4080)	(2590 - 3855)	
Birth length (cm)			0.127
median	51.0	52.0	
(range)	(47.0 - 54.0)	(49.0 - 64.0)	

Table 9. Study population at delivery. [1]Results are expressed as median (range) for continuous variables and numbers of subjects (%) for dichotomous variables. [2]Numbers of subjects (n) are only indicated when they differ from the total number of subjects in each group. Differences between both groups were calculated with MW*U* test for continuous data or χ^2 test for dichotomous data.

Vaginal pH values remained stable at an acidic pH of around four during the supplementation period and no significant difference was observable between the prebiotic and placebo supplemented group (**Figure 9**).

Although stool consistency seemed to become softer towards study end in the prebiotic group, no significant differences were observed between both groups (data not shown). The stool frequency (**Figure 10**) and the regurgitation frequency (data not shown) did not differ significantly between both groups.

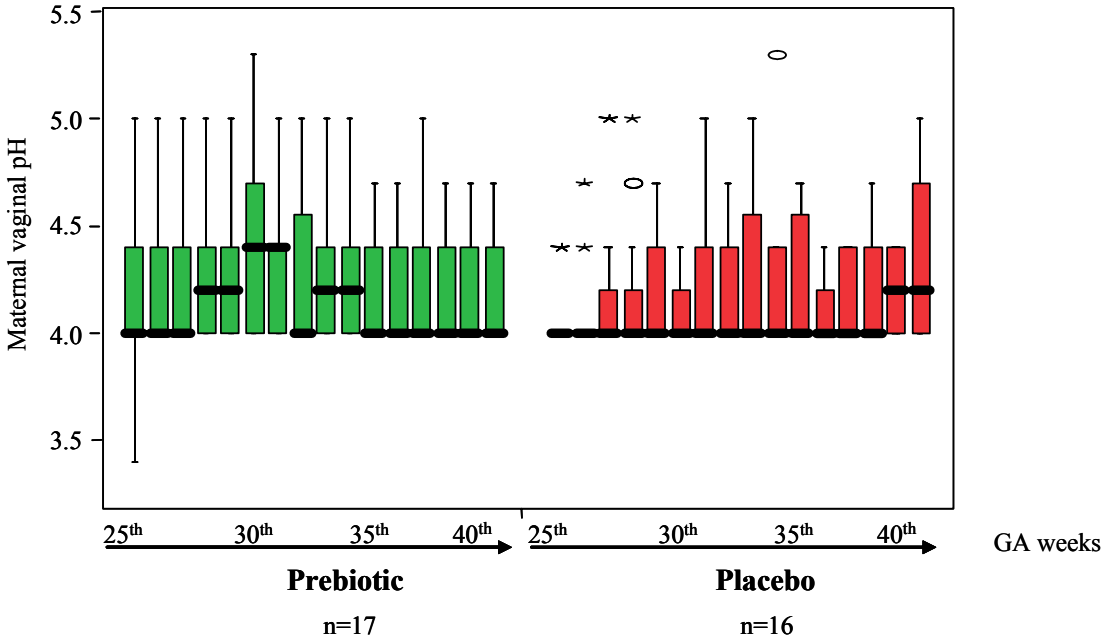


Figure 9. Average vaginal pH values in the prebiotic and placebo group during the supplementation period. Data are shown as box plots (black bar: median, boxes: inter-quartile range (IQR), T-bars: range,* and ° : outliers). Differences between both groups at each time point were assessed using MW*U* test. Differences within one group over time were assessed using Friedmans test.

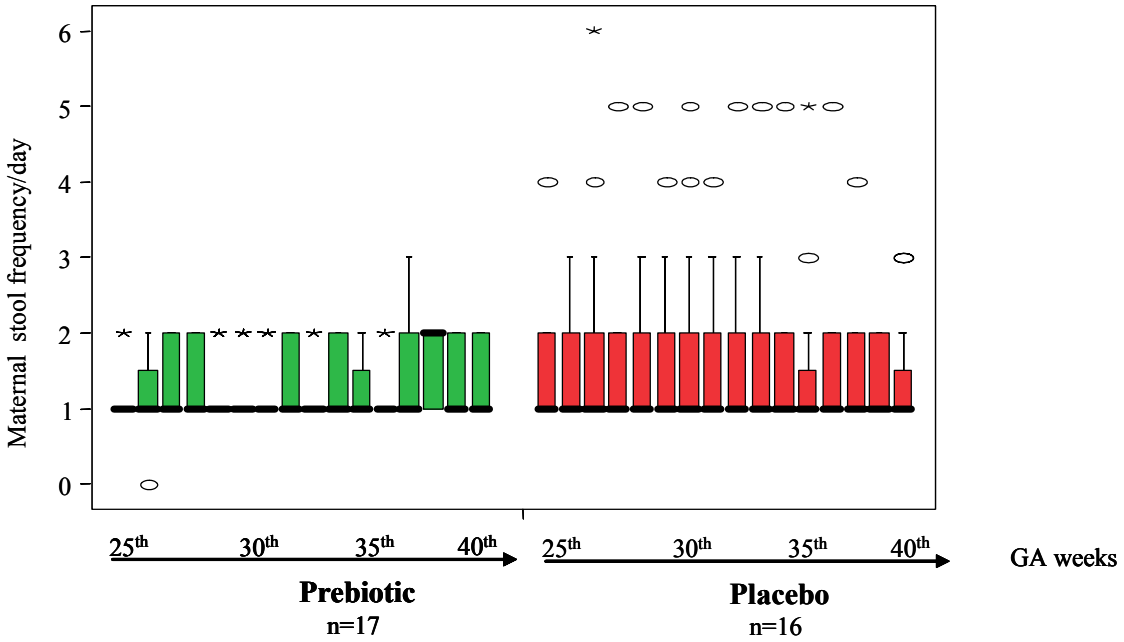


Figure 10. Average stool frequency per day in the prebiotic and placebo group during the supplementation period. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and ° : outliers). Differences between both groups at each time point were assessed using MW*U* test. Differences within one group over time were assessed using Friedmans test.

4.2.3 Follow-up interview

A follow-up interview was carried out with 30 participants (16 from the prebiotic group and 14 from the placebo group) and data were obtained from 17 and 15 infants, respectively. Contact to three women (one woman from the prebiotic and two women from the placebo group) and their children was lost. No statistically significant differences concerning feeding habits were observed between both supplementation groups (**Table 10**). In the first three days of life all neonates were breast fed and only three neonates (two from the prebiotic and one from the placebo group) received additional formula milk.

Infant	**Prebiotic** (n=17)	**Placebo** (n=15)	P
Breast milk in the first 3 days[1]			1.000
yes	17 (100)	15 (100)	
no	0 (0.0)	0 (0.0)	
Formula milk in the first 3 days			0.621
yes	2 (11.8)	1 (6.7)	
no	15 (88.2)	14 (93.3)	
Breast milk in the first 6 months			0.279
yes	17 (100)	14 (93.3)	
no	0 (0.0)	1 (6.7)	
Formula milk in the first months			0.337
yes	6 (35.3)	3 (20.0)	
no	11 (47.8)	12 (80.0)	
Exclusive breast feeding at the moment			0.968
yes, exclusively	2 (15.4)	2 (16.7)	
yes, but not exclusively	11 (84.6)	10 (88.3)	
no	4 (23.5)	3 (20.0)	
Solid foods			0.088
n[2]	15	13	
yes	12 (80.0)	13 (100)	
no	3 (20.0)	0 (0.0)	
Atopic eczema in the first 6 months			0.170
yes	2 (12.8)	0 (0.0)	
no	15 (88.2)	15 (100)	
Antibiotic treatment in the first 3 weeks			0.349
yes	3 (17.6)	1 (6.7)	
no	14 (82.4)	14 (93.3)	
Antibiotic treatment in the last 5 months			0.145
yes	2 (11.8)	0 (0.0)	
no	15 (88.2)	17 (100)	

Table 10. Feeding habits, atopic eczema and antibiotics in the first 6 months of life. Data were assessed retrospectively in an interview. [1]Results are expressed as numbers of subjects (%). Differences between both groups were calculated with χ^2 test. [2] Numbers of subjects (n) are only indicated when they differ from the total number of subjects in each group.

Similarly all infants from the prebiotic group and 93.3% of the infants from the placebo group predominantly received breast milk in the first six months of their life. 35.3% of the infants in the prebiotic group and 20% of the infants in the placebo group received additional formula milk. After half a year, two infants from each group were still exclusively breast fed. Most infants had already started taking supplementary food. Two participants from the prebiotic reported an atopic eczema (confirmed by a doctor's diagnosis) in their infants at the age of three and five months, respectively. Two mothers from the prebiotic group reported that their infants received antibiotics during the last five months due to an infection. Cephachlor was given to one neonate for 10 days at the age of four weeks and Clarithromycin was given to another neonate for seven days at the of age six weeks.

4.3 Analyses of stool samples

A total of 33 maternal stool samples (17 from the prebiotic and 16 from the control group) were collected before supplementation start (before the 25th week of gestation).
After supplementation (shortly before expected delivery) 31 stool samples (17 from the prebiotic and 14 from the placebo group) were obtained: two samples from the placebo group were not immediately frozen after collection and had to be excluded from analyses.
From the 35 neonates (one gemini in each supplementation group) 33 stool samples (17 from the prebiotic and 15 from the placebo group) were collected at day 5: one participant from each group was unable to collect any faecal sample from the baby because the sample was completely sucked up into the diaper. Thirty four neonatal stool samples (18 from the prebiotic and 16 from the placebo group) were collected at day 20: one participant from the placebo group forgot to immediately freeze the neonatal sample after collection. Additionally, one neonatal sample from day 20 form the placebo group did not contain enough material. After half a year (~ day 182) 32 neonatal stool samples (17 from the prebiotic and 15 from the placebo group) were collected: three neonatal stool samples (one from the prebiotic group and two from the placebo group) were not available due to loss of contact to participants. Collected samples were analysed by FISH and qPCR. The percentage of bifidobacteria and lactobacilli as determined by FISH and qPCR analyses showed no statistical differences. Data from both quantification methods (qPCR versus FISH) correlated significantly with each other [correlation for bifidobacteria > 0.904 and for lactobacilli > 0.942; $p<0.001$; Spearman Rho].

4.3.1 Maternal stool samples

Maternal baseline stool pH values (i.e. at the 25th week of gestation) did not differ significantly [p=0.564; MW*U*] between both intervention groups (**Figure 11**). Prebiotic supplementation was not associated with significantly increased stool pH values as compared to baseline [p=0.619, paired Wilcoxon] and as compared to placebo [p=0.905; MW*U*]. No significant difference between both groups was observed in the change of stool pH (Δ stool pH) from before to after supplementation [p=0.592; MW*U*].

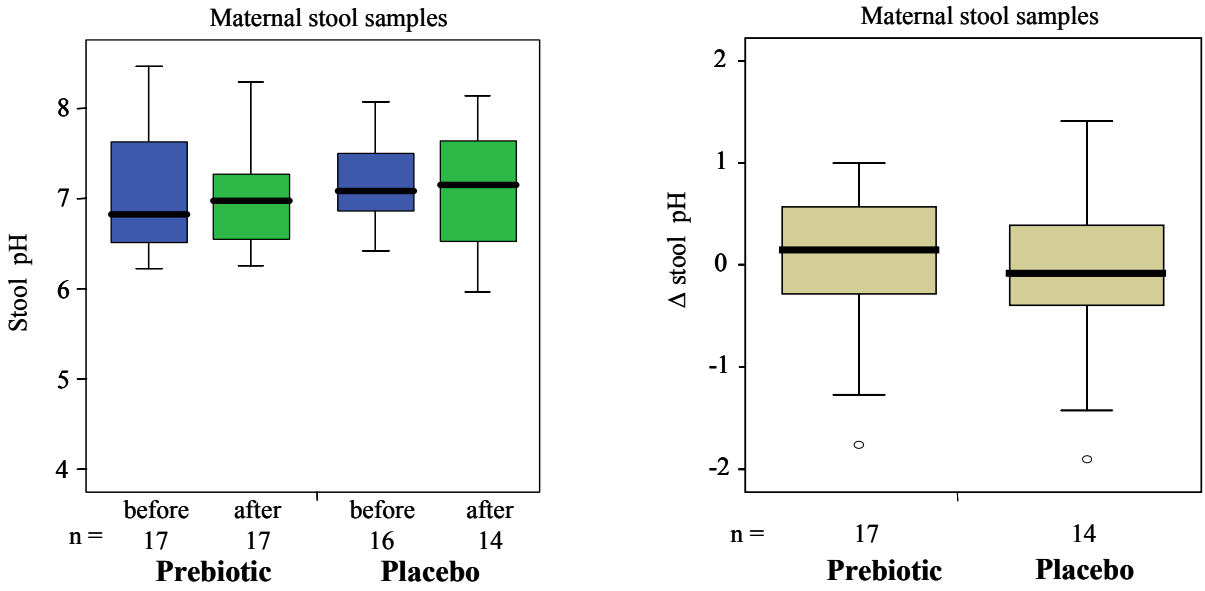


Figure 11. PH values in maternal stool samples before study start (before) and after supplementation (after) in the prebiotic and placebo supplemented group. The difference in stool pH before and after supplementation is shown as Δ stool pH. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, °: outliers). Differences between both groups were assessed using MW*U* test. Differences within one group before and after supplementation (intra-group comparisons) were assessed with paired Wilcoxon test.

At study entry, the total number of bacteria (as determined by DAPI) in the maternal samples was significantly lower [p=0.017; MWU] in the prebiotic group as compared to placebo (**Figure 12**). Prebiotic supplementation was not associated with significantly increased bacterial numbers as compared to study entry [p=0.227; paired Wilcoxon] and to the placebo group [p=0.427; MWU]. A significant increase [p=0.029; MWU] in the change of total bacterial counts (Δ total bacterial count) was observed in the prebiotic group as compared to the placebo group.

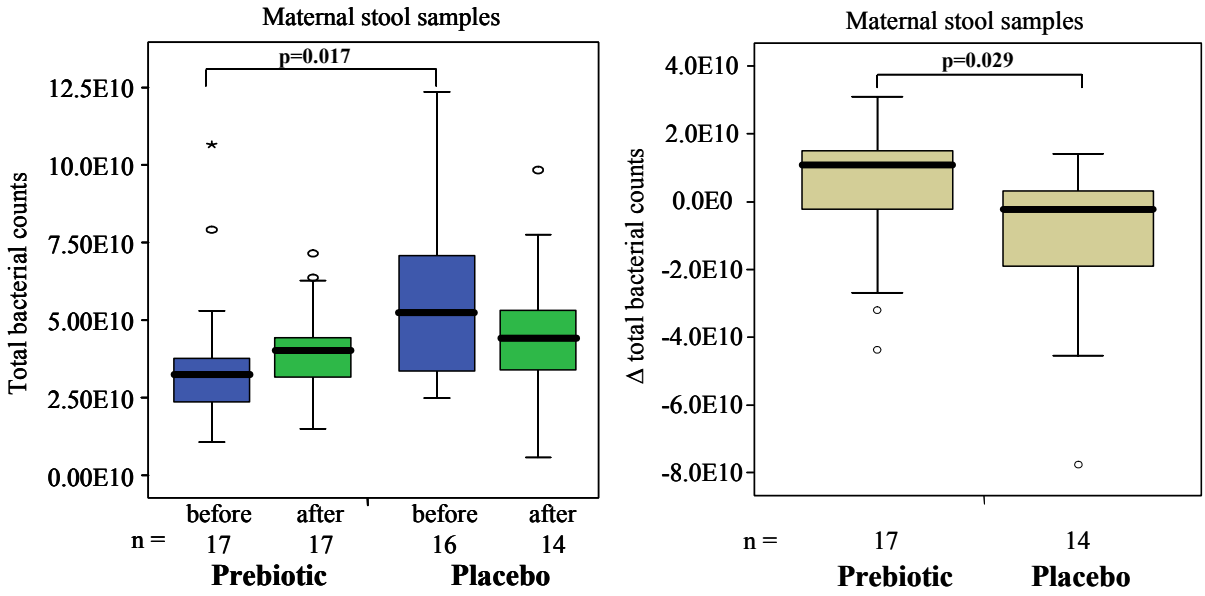


Figure 12. Total bacterial counts in maternal stool samples before and after supplementation in the prebiotic and placebo group (determined by DAPI). The difference in total bacterial counts before and after supplementation is shown as Δ total count. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and ^{o}: outliers). Differences between both groups were assessed using MW*U* test. Differences within one group before and after supplementation (intra-group comparisons) were assessed with paired Wilcoxon test.

The absolute numbers of bifidobacteria or lactobacilli (bacteria/mL) were determined from the percentage of bifidobacteria or lactobacilli (from qPCR analyses) and the total counts of bacteria (if available). Baseline bifidobacteria numbers per g faeces (bacteria/mL) were significantly lower [p=0.014; MW*U*] in the prebiotic group as compared to placebo (**Figure 13**). Prebiotic supplementation significantly increased the numbers of bifidobacteria/mL as compared to baseline [p=0.001: paired Wilcoxon] but not as compared to placebo [p=0.122; MW*U*]. A significant increase [p=0.024; MW*U*] in the change of absolute bifidobacteria numbers (Δ bifidobacteria/mL) was observed in the prebiotic group as compared to the placebo group.

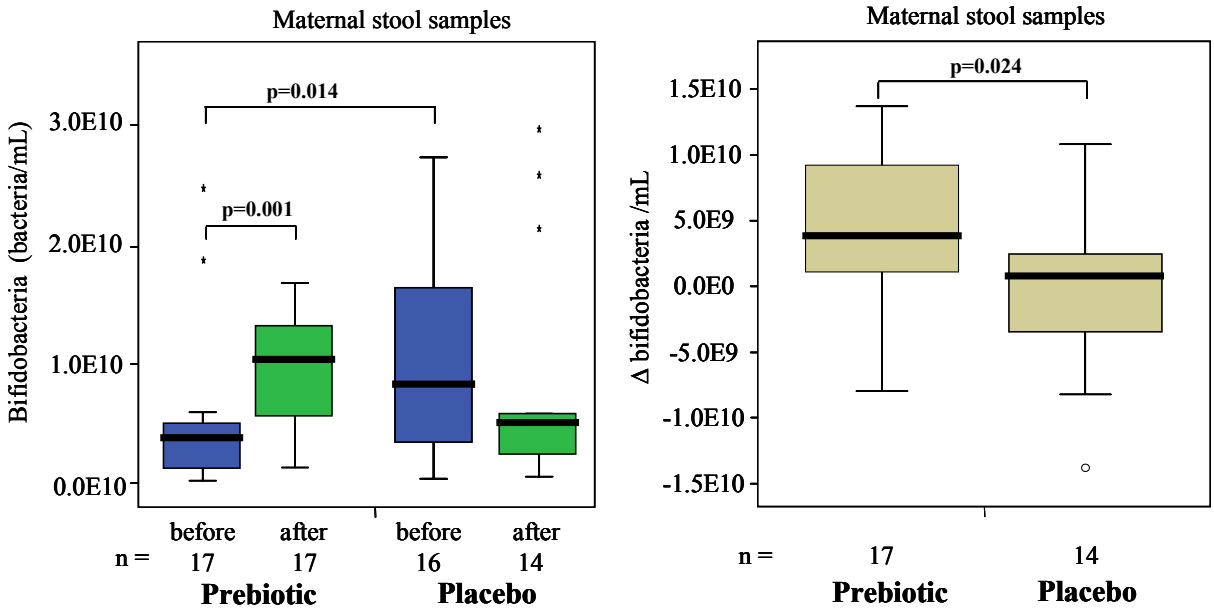


Figure 13. Absolute numbers of bifidobacteria (bacteria/mL) in maternal stool samples before and after supplementation in the prebiotic and placebo supplemented group (determined by qPCR). The difference in absolute numbers of bifidobacteria (bacteria/mL) before and after supplementation is shown as Δ bifidobacteria/mL. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and ° : outliers). Differences between both groups were assessed using MW*U* test. Differences within one group before and after supplementation (intra-group comparisons) were assessed with paired Wilcoxon test.

No statistical significant difference [p=0.476; MW*U*] between both supplementation groups was observed when analysing the numbers of lactobacilli per g faeces (bacteria/mL) at study start in maternal stool samples (**Figure 14**). Prebiotic supplementation had no effect on the numbers of lactobacilli (bacteria/mL) as compared to study start [p=0.75; paired Wilcoxon] and to placebo [p=0.224; MW*U*]. No difference in the change of absolute lactobacilli numbers (Δ lactobacilli/mL) was observed in the prebiotic group as compared to the placebo group [p=0.512; MW*U*].

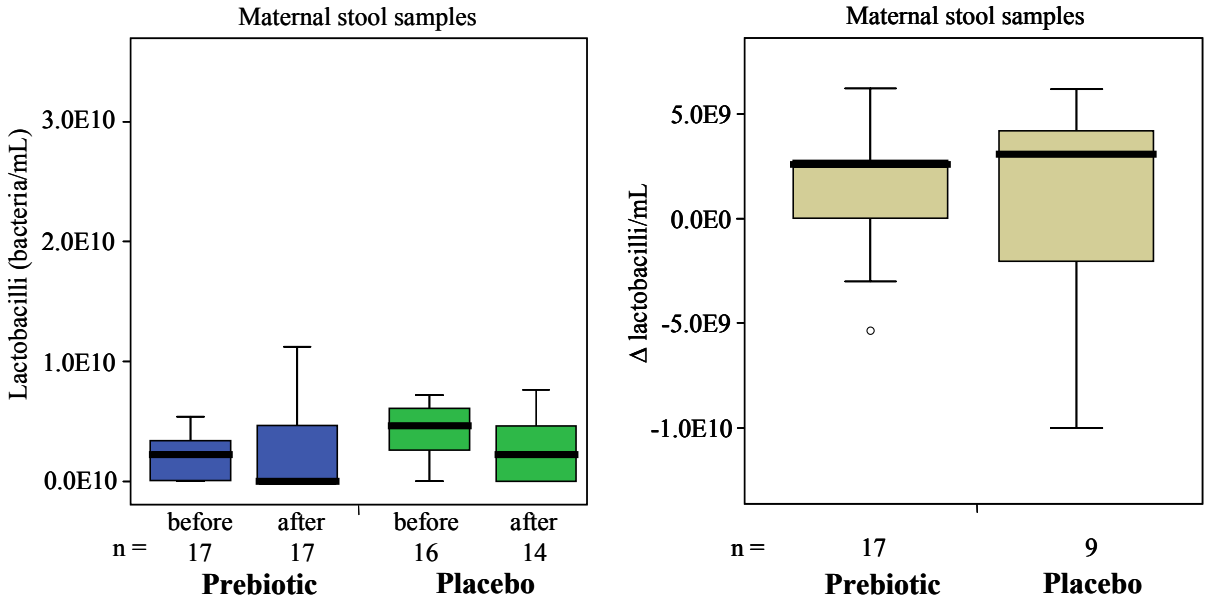


Figure 14. Absolute numbers of lactobacilli (bacteria/mL) in maternal stool samples before and after supplementation in the prebiotic and placebo supplemented group (determined by qPCR). The difference in absolute numbers of lactobacilli (bacteria/mL) before and after supplementation is shown as Δ lactobacilli/mL. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, ^{o} : outliers). Differences between both groups were assessed using MW*U* test. Differences within one group before and after supplementation (intra-group comparisons) were assessed with paired Wilcoxon test.

Baseline percentages of bifidobacteria within total bacteria did not differ significantly [p=0.105; MW*U*] between both supplementation groups in the maternal samples (**Figure 15**). At study end, the percentage of bifidobacteria were significantly higher in the prebiotic group as compared to study start [p≤0.001; paired Wilcoxon] and as compared to the placebo group [p=0.026; MW*U*]. Both results were confirmed by FISH analyses [p=0.009 and p=0.008, respectively]. Similarly, a significant increase [p=0.011; paired Wilcoxon] in the difference of percentages of bifidobacteria (Δ % bifidobacteria) was observed in the prebiotic group as compared to the placebo group. This result was confirmed by FISH analyses [p≤0.001].

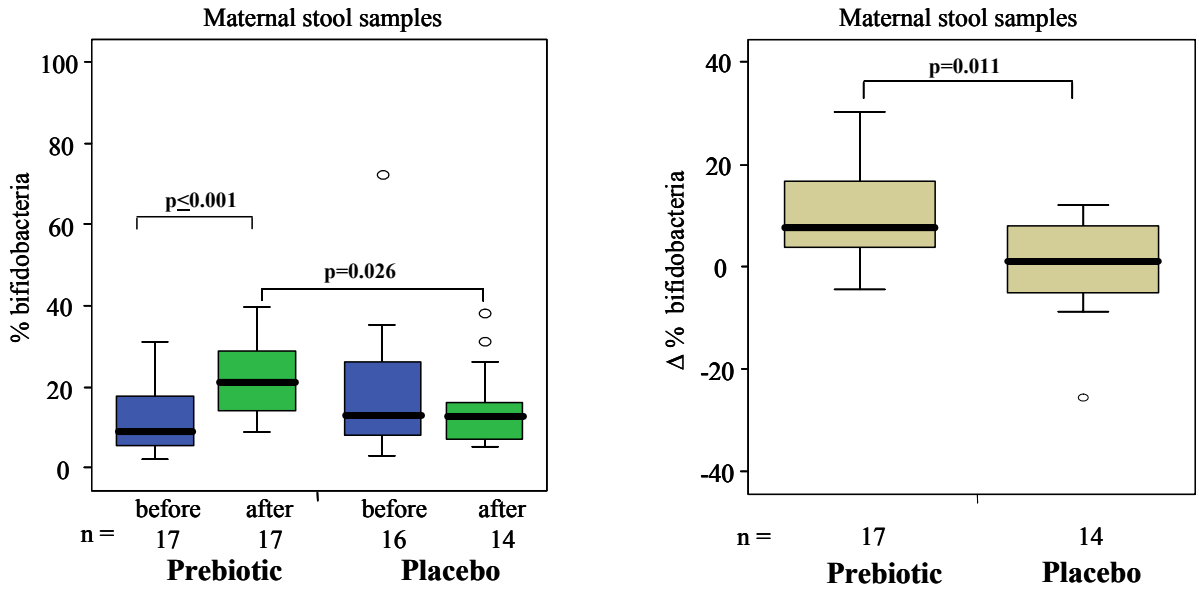


Figure 15. Percentages of bifidobacteria within total bacteria in maternal stool samples before and after supplementation in the prebiotic and the placebo group. The difference in percentages of bifidobacteria (%) before and after supplementation is shown as Δ % bifidobacteria. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, ° : outliers). Differences between both groups were assessed using MW*U* test. Differences within one group before and after supplementation (intra-group comparisons) were assessed with paired Wilcoxon test.

Baseline percentages of lactobacilli within total bacteria did not differ significantly [p=0.139; MW*U*] between both supplementation groups in the maternal samples (**Figure 16**).

Prebiotic supplementation had no effect on the percentages of lactobacilli as compared to study start [p=0.210; paired Wilcoxon] and to placebo [p=0.370; MW*U*]. These results were confirmed by FISH analyses. No difference in the change of percentages of lactobacilli (Δ % lactobacilli) was observed in the prebiotic group as compared to the placebo group [p=0.953; MW*U*]. This result was confirmed by FISH analyses.

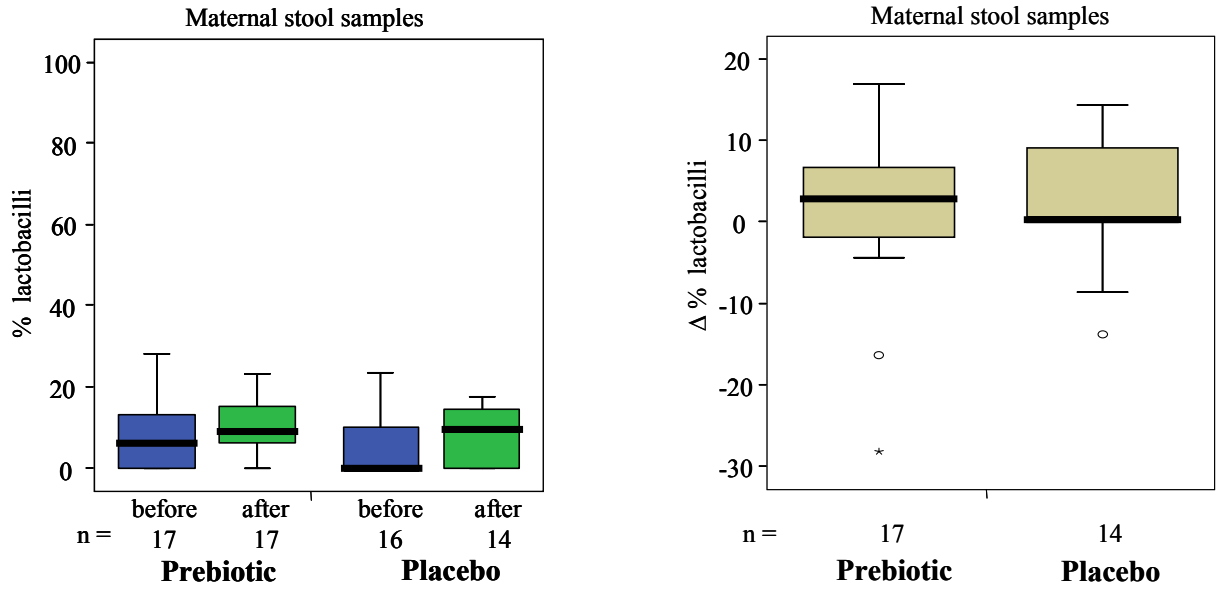


Figure 16. Percentages of lactobacilli within total bacteria in maternal stool samples before and after supplementation in the prebiotic and the placebo group. The difference in percent of lactobacilli (%) before and after supplementation is shown as Δ % lactobacilli. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and ° : outliers). Differences between both groups were assessed using MW*U* test. Differences within one group before and after supplementation (intra-group comparisons) were assessed with paired Wilcoxon test.

The percentage of subjects positive for the different bifidobacteria and lactobacilli species showed considerable inter- and intra-individual differences (**Table 11**).

B. catenulatum, *B. infantis* and *B. adolescentis* were the species most commonly characterized in maternal stool samples, both before after supplementation, in the two groups. No significant increase in the percentage of subjects positive for these bifidobacterial species was observed in the prebiotic group as compared to before supplementation begin and as compared to placebo. Before supplementation begin higher percentages of *B. breve* were observed in the prebiotic group as compared to the placebo group [p=0.04; χ^2]. This was confirmed by higher percentages of *B. breve* [p=0.032; MW*U*] as determined by qPCR analyses.

L. acidophilus, L. paracasei and *L. fermentum* were the species most commonly characterized in maternal stool samples, before and after supplementation, in two groups. Before supplementation begin higher percentages of *L. casei* [p=0.038; χ^2] and *L. paracasei* [p=0.019; χ^2] were observed in the prebiotic group as compared to the placebo group. This was confirmed by higher percentages of *L. casei* [p=0.042; MW*U*] and *L. paracasei* [p=0.015; MW*U*] as determined by qPCR analyses in the prebiotic group as compared to the placebo at study start. However, these differences were not significant upon taking multiple testing into consideration. Taken together, prebiotic supplementation had no detectable effect on maternal bifidobacterial and lactobacilli microbiota as compared to baseline (intra-group comparison) and as compared to placebo (inter-group comparison).

Mother	**Prebiotic**		**Placebo**	
% of subjects positive for	before (n=17)	after (n=17)	before (n=16)	after (n=14)
B. adolescentis	29.4	47.1	37.5	50.0
B. angulatum	11.8	17.6	18.8	26.6
B. animalis	11.8	23.5	0.0	0.0
B. bifidum	17.6	23.5	25.0	42.9
B. breve	35.3 [a]	35.3	6.3 [a]	42.9
B. catenulatum	70.6	70.6	50.0	64.3
B. dentium	5.9	0.0	6.3	0.0
B. infantis	58.8	70.6	56.3	71.6
B. longum	23.5	11.8	6.3	7.1
L. acidophilus	58.8	76.5	31.3	42.9
L. casei	23.5 [b]	5.9	0.0 [b]	0.0
L. delbrueckii	11.8	35.3	12.5	21.4
L. fermentum	35.3	11.8	18.8	35.7
L. paracasei	41.2 [c]	41.2	6.3 [c]	14.3
L. plantarum	0.0	0.0	18.8	21.4
L. reuteri	11.8	17.6	0.0	7.1
L. rhamnosus	11.8	17.6	18.8	14.3

Table 11. Percentage of mothers positive for specific bifidobacterial and lactobacilli species. Bacteria were quantified in maternal stool samples taken before and after supplementation. Differences between the prebiotic and placebo supplemented group were calculated by χ^2 test ([a] p=0.04,[b] p=0.038,[c] p=0.019; not significant after correction for multiple testing). Differences between both sampling time points within one supplementation group were calculated by McNemar test.

4.3.2 Neonatal stool samples

Baseline neonatal stool sample pH values at day 5 did not differ significantly [p=0.179; MW*U*] between both groups (**Figure 17**). Prebiotic intervention was not associated with increased stool pH values at day 20 or after half a year (~ day 182) as compared to baseline [p=0.407 or p=0.256; paired Wilcoxon] and as compared to placebo [p=0.574 or p=0.737; MW*U*]. A significant increase was observed when comparing pH values from day 20 with day 182 in the prebiotic group [p=0.024; paired Wilcoxon]. Caesarean delivery is known to influence the composition of the neonatal gut microbiota. Therefore, an analysis where infants born by caesarean delivery were excluded from analyses (five from the prebiotic and one from the placebo group) was performed. After exclusion of neonates born by caesarean sections the difference between day 20 and day 182 in the prebiotic was no longer present. No difference in the change of stool pH (Δ stool pH) was observed in the prebiotic group as compared to the placebo group at the respective time points [p=0.249; p=0.874 and p=0.299 respectively; MW*U*] (data not shown).

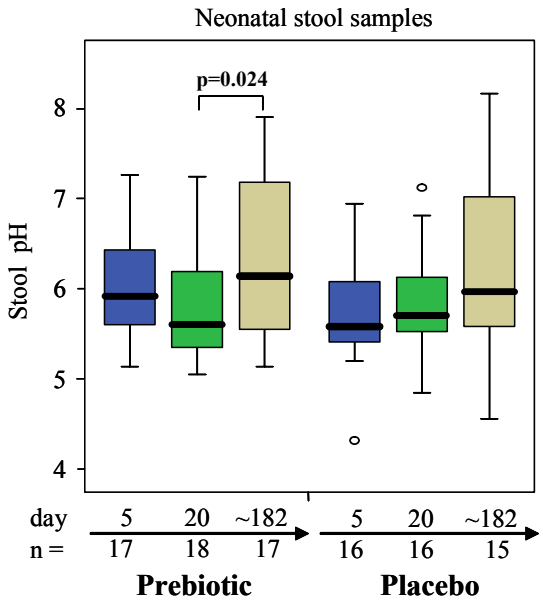


Figure 17. PH values in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and placebo supplemented group. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, ^{o}: outliers). Differences between both groups were assessed using MW*U* test. Differences within one group before and after supplementation (intra-group comparisons) were assessed with paired Wilcoxon test.

The **total numbers of bacteria** (DAPI) in neonatal stool samples did not differ significantly between both supplementation groups at day 5 [p=0.805; MW*U*], day 20 [p=0.154; MW*U*] and day 182 [p=0.728; MW*U*], (**Figure 18**). Differences in total counts and intra-group comparisons over time were not determined due to too small numbers of matching pairs for statistical analyses.

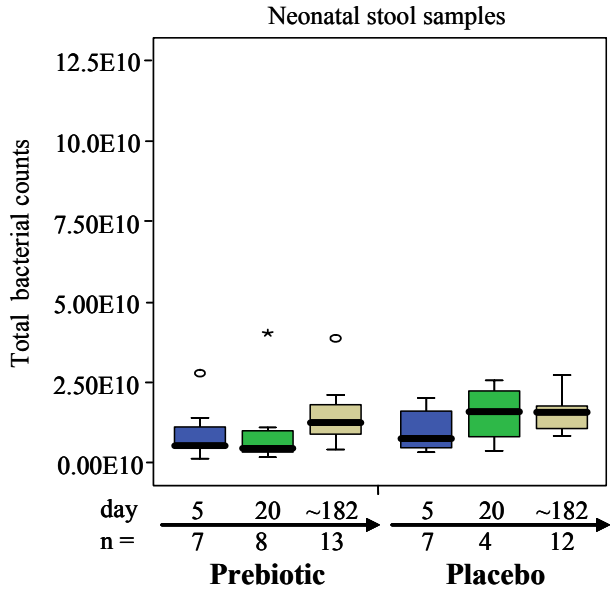


Figure18. Total bacterial counts in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and placebo supplemented group (determined by DAPI). Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and ^{o}: outliers). Differences between both groups were assessed using MW*U* test.

The absolute numbers of bifidobacteria or lactobacilli (bacteria/mL) were determined from the percentages of bifidobacteria or lactobacilli (from qPCR analyses) and the total counts of bacteria (if available). Baseline bifidobacteria numbers per g faeces (bacteria/mL) at day 5 did not differ significantly [p=0.110; MW*U*] between both groups (**Figure 19**). Significantly lower numbers of bifidobacteria (bacteria/mL) were observed at day 20 in the prebiotic group as compared to the placebo group [p=0.027; MW*U*]. After excluding neonates born by caesarean sections the difference seen between the prebiotic and the placebo group was no longer present. No statistical significant difference between both supplementation groups was observed for the numbers of bifidobacteria (bacteria/mL) at day 182 [p=0.870; MW*U*]. Differences in absolute numbers of bifidobacteria (Δ bifidobacteria/mL) before and after supplementation and intra-group comparisons over time were not determined due to too small numbers of matching pairs for statistical analyses.

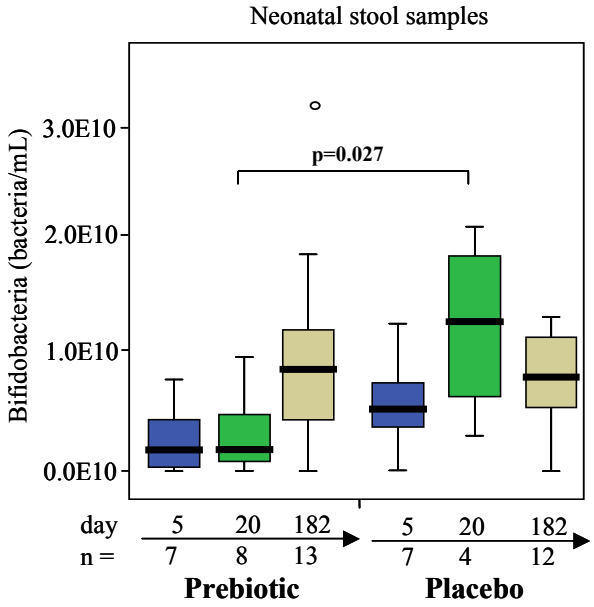


Figure 19. Absolute numbers of bifidobacteria (bacteria/mL) in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and placebo supplemented group (determined by qPCR). Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, ^{o} : outliers). Differences between both groups were assessed using MW*U* test.

At day 5 the prebiotic supplemented group showed significantly higher numbers of lactobacilli (bacteria/mL) [p=0.043; MW*U*] as compared to the placebo supplemented group (**Figure 20**). After exclusion of neonates born by caesarean sections this difference was no longer present. No significant differences between both groups were observed in samples taken at day 20 [p=0.058; MW*U*] and day 182 [p=0.213; MW*U*].

Differences in absolute numbers of lactobacilli (Δ lactobacilli/mL) before and after supplementation and intra-group comparisons over time were not determined due to too small numbers of matching pairs for statistical analyses.

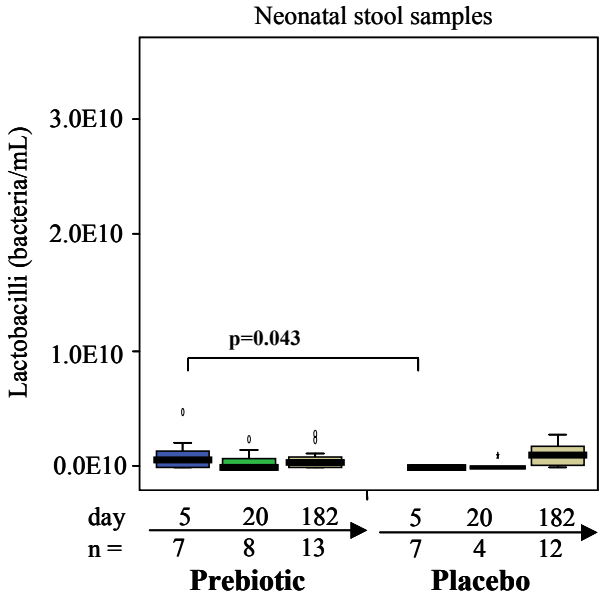


Figure 20. Absolute numbers of lactobacilli (bacteria/mL) in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and placebo supplemented group (determined by qPCR). Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and ^{o} : outliers). Differences between both groups were assessed using MW*U* test.

The analyses of the percentage of bifidobacteria within total bacteria at the three different time points revealed no significant difference [p=0.072; p=0.376, p=0.925 MW*U*; respectively] between both supplementation groups (**Figure 21**). Within the prebiotic group a significant increase [p=0.010; paired Wilcoxon] in the percentage of bifidobacteria was observed when comparing day 5 with day 182. This effect was only seen in the percentages of bifidobacteria determined by qPCR but not by FISH analyses. In addition, this difference was no longer present after exclusion of neonates born by caesarean sections.

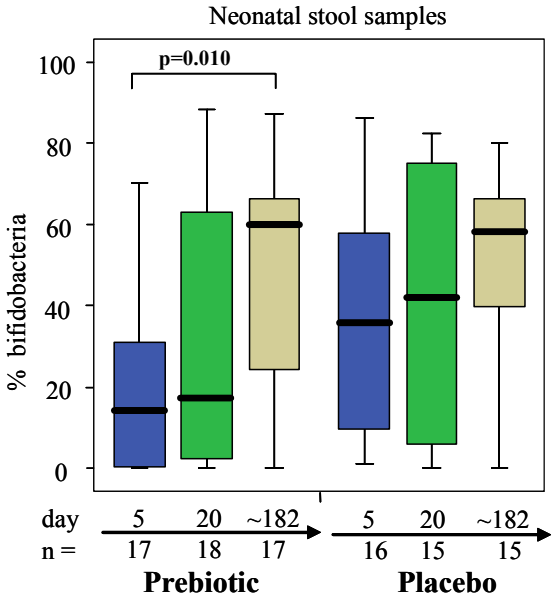


Figure 21. Percentages of bifidobacteria within total bacteria in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and the placebo supplemented group. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range). Differences between both groups were assessed using MW*U* test. Differences within one group before and after supplementation (intra-group comparisons) were assessed with paired Wilcoxon test.

Similarly, no significant difference was observed between both supplementation groups (**Figure 22)** when comparing the percentages of lactobacilli at day 5 [p=0.145; MWU] and day 182 [p=0.454; MW*U*]. Samples from day 20 tended to be a bit higher in the prebiotic group as compared to the placebo group [p=0.049; MW*U*]. This effect was only seen in the percentages of lactobacilli determined by qPCR but not by FISH analyses. In addition, this difference was no longer present after exclusion of neonates born by caesarean sections. Intra-group comparisons showed significantly higher percentages of lactobacilli at day 5 as compared to day 20 [p=0.012; paired Wilcoxon] in the prebiotic group determined by qPCR. This effect was not confirmed by FISH analyses and was no longer present after exclusion of neonates born by caesarean section. In addition, a significant increase in the numbers of

lactobacilli was observed in the placebo group [p=0.037; paired Wilcoxon] when comparing day 20 with day 182. This effect was confirmed by FISH analyses [p=0.020; paired Wilcoxon] and remained after excluding the children born by caesarean section [p=0.038; paired Wilcoxon].

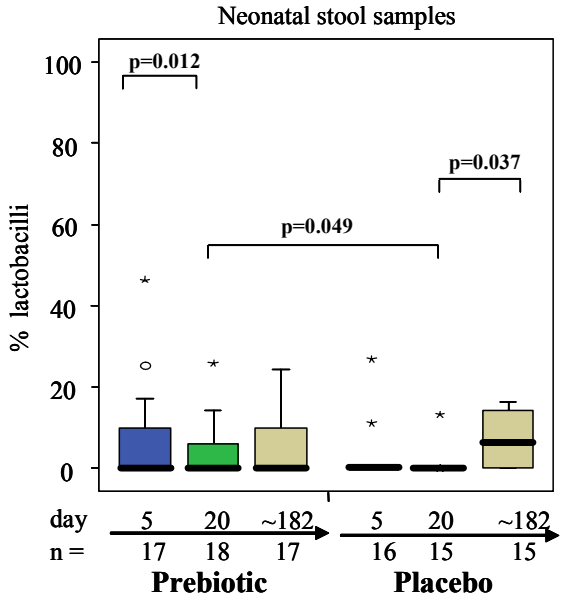


Figure 22. Percentages of lactobacilli within total bacteria in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and the placebo supplemented group. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and ^{o} : outliers). Differences between both groups were assessed using MW*U* test. Differences within one group before and after supplementation (intra-group comparisons) were assessed with paired Wilcoxon test.

The percentages of subjects positive for the different *Bifidobacterium* and *Lactobacillus* species showed considerable inter- and intra-individual differences in neonatal stool samples from both groups (**Table 12**). *B. infantis* followed by *B. breve, B. catemunaltum and B. bifidum* were among the most prevalent bifidobacterial species characterized in neonatal stool samples from day 5, day 20 and day 182, in both groups. The analyses of the different bifidobacterial species at the three different time points revealed no significant differences between both supplementation groups. Similarly intra-group comparisons showed no significant changes in the bifidobacterial species composition. At day 5, day 20 and day 182 *L. acidophilus, L. paracasei* and *L. fermentum* were among the most dominant lactobacilli species characterized in neonatal stool samples. At day 5 higher percentages of *L. acidophilus* were observed in the prebiotic group as compared to the placebo group [p=0.04; χ^2]. The difference was not observed when analysing the % qPCR data [p=0.100; MW*U*] or excluding children born by caesarean sections. In the placebo group intra-group comparisons revealed an increase in the percentage of subjects positive for *L. acidophilus* when comparing day 20 and day 182 respectively [0.016; McNemar]. This difference remained after exclusion of

children born by caesarean sections [p=0.016; McNemar] but could not be confirmed by qPCR analyses and [p=0.110; paired Wilcoxon]. It however, must be kept in mind that these differences were not significant upon taking multiple testing into consideration.

Neonate	**Prebiotic**			**Placebo**		
% of subjects positive for	Day 5 (n=17)	Day 20 (n=18)	Day 182 (n=17)	Day 5 (n=16)	Day 20 (n=15)	Day 182 (n=15)
B. adolescentis	11.8	16.7	11.8	31.3	20.0	13.3
B. angulatum	5.9	0.0	0.0	12.5	13.0	0.0
B. animalis	5.9	0.0	0.0	0.0	0.0	0.0
B. bifidum	29.4	50.0	35.3	56.3	50.0	46.7
B. breve	76.5	77.8	82.4	75.0	66.7	50.0
B. catenulatum	47.1	38.9	23.5	37.5	20.0	6.7
B. dentium	0.0	0.0	0.0	0.0	0.0	0.0
B. infantis	100	77.8	76.5	100	86.7	93.3
B. longum	25.0	27.8	29.4	25.0	13.3	20.0
L. acidophilus	52.9 [a]	27.8	41.2	18.8[a]	13.3 [b]	66.7 [b]
L. casei	17.6	16.7	29.4	6.3	6.7	20.0
L. delbrueckii	0.0	0.0	0.0	6.3	0.0	0.0
L. fermentum	35.3	22.2	11.8	12.5	13.3	26.7
L. paracasei	52.9	27.8	29.4	81.3	6.7	33.3
L. plantarum	17.6	11.1	5.9	6.3	0.0	0.0
L. reuteri	5.9	0.0	5.9	0.0	0.0	0.0
L. rhamnosus	0.0	0.0	0.0	0.0	0.0	0.0

Table 12. Percentage of neonates positive for specific bifidobacterial and lactobacilli species. Bacteria were quantified in neonatal stool samples taken at day 5, day 20 and day 182. Differences between the prebiotic and placebo supplemented group were calculated by χ^2 test ([a] p=0.04; not significant after correction for multiple testing). Differences between the three sampling time points day ([b]p=0.016) within one supplementation group were calculated by McNemar test.

4.3.3 Microbiota in maternal & neonatal samples

4.3.3.1 Diversity index

Maternal stool samples after, but not before, supplementation showed a significantly higher bifidobacterial diversity as compared to lactobacilli diversity in the prebiotic group [p=0.039; paired Wilcoxon]. In contrast, the diversity of bifidobacteria was higher in the placebo group both before and after supplementation [p=0.020; paired Wilcoxon]. Similarly, the neonates of both groups showed a significantly higher diversity of bifidobacteria species than lactobacilli species at all analysed time points [$p \leq 0.016$; paired Wilcoxon]. Maternal stool samples showed no differences in bifidobacterial and lactobacilli diversity in relation to the intervention group, either before or after supplementation begin (**Table 13**). Similarly, the samples taken from infants at day 5, day 20 and day 182 exhibited no significant differences in bifidobacterial and lactobacilli diversity between both groups (**Table 13**). When assessing

the samples from the mothers before supplementation begin with the samples from infants at day 5, significant differences in the bifidobacterial diversity were found in the placebo group [p=0.006; paired Wilcoxon] but not in the prebiotic supplemented group [p=0.163; paired Wilcoxon]. For both groups, no differences were found in the bifidobacterial diversity when comparing maternal samples before supplementation begin with neonatal samples at day 20, and when comparing maternal samples after supplementation with neonatal samples at days 5, 20 and 182, respectively (**Table 13**). In both groups, no significant differences in the lactobacilli diversity were found when comparing maternal samples before supplementation begin with neonatal samples from day 5. In contrast, significant differences were observed in both groups when comparing the diversity indices from maternal samples before supplementation with neonatal samples from day 20 [p=0.043 and p=0.035 respectively; paired Wilcoxon] and day 182 in the prebiotic group [p=0.033; paired Wilcoxon].

	Bifidobacteria			**Lactobacilli**		
Diversity index (%)	Prebiotic	Placebo	P	Prebiotic	Placebo	P
Mother before			0.272			0.055
n	17	16		17	16	
median[1]	22	22 [a]		25 [b, g]	0 [c]	
(range)	(11 - 77)	(11 - 44)		(0 - 50)	(0 - 50)	
Mother after			0.670			0.289
n	17	14		17	14	
median	22	33		22 [e]	22 [d,f]	
(range)	(11 - 77)	(11 - 55)		(0 - 44)	(0 - 44)	
Infant day 5			0.520			0.123
n	17	16 [a]		17	16	
median	33	33		37	0 [d]	
(range)	(11 - 66)	(22-55)		(0 - 50)	(0 - 63)	
Infant day 20			0.897			0.125
n	18	15		18	15	
median	27	33		0 [b,e]	0 [c,f]	
(range)	(0 - 55)	(0 - 55)		(0 - 44)	(0 - 33)	
Infant ~ day 182			0.983			0.658
n	17	15		17	15	
median	33	33		0 [g]	22	
(range)	(0 - 55)	(11 - 55)		(0 - 44)	(0 - 33)	

Table 13. Bifidobacterial and lactobacilli diversity indices in mothers receiving prebiotic or placebo supplementation and their infants. Maternal stool samples were taken before and after supplementation and neonatal stool samples were taken at day 5, day 20 and day 182. The bacterial diversity index was calculated using the following formula DI = (A/B) x 100, with A representing the number of species detected in a sample and B representing the total number of analysed species (n=9 bifidobacteria and n=8 lactobacilli). [1]Results are expressed as median (range). Differences between both supplementation groups were calculated with MW*U* test. Differences between maternal-infant samples within one supplementation group were calculated with paired Wilcoxon test. Identical superscripts indicate significant differences ([a] p=0.006, [b] p= 0.043, [c] p= 0.035, [d] p= 0.046, [e] p= 0.030, [f] p= 0.008, [g] p=0.033).

The comparison of the samples from the mothers after supplementation with the samples from the infant at day 5 revealed significant differences in lactobacilli diversity in the placebo group [p=0.046; paired Wilcoxon]. In both the prebiotic and the placebo group, the comparison of the samples from the mothers after supplementation with the samples from the infant at day 20 revealed significantly higher values in maternal samples [p=0.030, p=0.008; paired Wilcoxon].

4.3.3.2 Correlations

The analysis of the species correlating in maternal samples after supplementation with neonatal samples from day 5, day 20 and day 182 revealed no correlation for B. *breve, B. dentium, B. infantis, B. longum, L. paracasei and L. rhamnosus.* In contrast, a significant correlation between maternal samples after supplementation and neonatal samples from day 5 was observed in both groups for *B. adolescentis, B. angulatum*, *B. bifidum, B. catenulatum* and *L. fermentum* (**Table 14**). *B. animalis, B. catenulatum* and *L. reuteri* correlated only in the prebiotic group, while *B. infantis* and *B. plantarum* correlated only in the placebo group at day 5. The correlation of *L. fermentum* remained in both groups up to day 20 and in the prebiotic group up to day 182. The correlation between maternal samples after supplementation and neonatal samples at day 5 observed for *B. adolescentis* remained up to day 20 and day 182 in the prebiotic group. At day 182, a correlation was found in the prebiotic group for *B. bifidum* and *L. reuteri*. In the placebo group, only *B. infantis* correlated in neonatal samples from day 182 with maternal samples after supplementation. When comparing maternal samples after supplementation with neonatal samples from day 5, the total number of correlating species was identical in the prebiotic (n=7) as compared to the placebo supplemented group (n=7, **Table 14**). The total number was reduced in the prebiotic group (n=2) and in the placebo group (n=6) after exclusion of neonates born by caesarean sections (data not shown). The total number of species correlating in maternal samples after supplementation and neonatal samples from day 20 decreased in the prebiotic (n=2) and the placebo group (n=1) as compared to day 5 (**Table 14**), but remained the same after excluding the children born by caesarean sections (data not shown).

Similarly, the total number of species correlating in maternal samples after supplementation and neonatal samples from day 182 was decreased in the prebiotic (n=4) and the placebo group (n=1) as compared to day 5 (**Table 14**). After exclusion of caesarean section born infants, the number was further reduced in the prebiotic group (n=1) and in the placebo group (n=0, data not shown).

Correlation of species as determined by qPCR	**Mother after - Infant day 5**		**Mother after - Infant day 20**		**Mother after - Infant ~ day 182**	
	Prebiotic	Placebo	Prebiotic	Placebo	Prebiotic	Placebo
B. adolescentis			0.500			
Correlation[1]	0.509	0.735	0.034		0.516	
P	0.036	0.001			0.003	
B. angulatum						
Correlation	0.613	0.643				
P	0.008	0.009				
B. animalis						
Correlation	0.537					
P	0.026					
B. bifidum						
Correlation	0.692	0.586			0.512	
P	0.002	0.021			0.035	
B. catenulatum						
Correlation	0.559	0.599				
P	0.019	0.018				
B. infantis						
Correlation		0.651				0.515
P		0.008				0.049
L. delbrueckii						
Correlation						
P						
L. fermentum					0.637	
Correlation	0.491	0.546	0.661	0.575	0.005	
P	0.045	0.035	0.002	0.031		
L. plantarum						
Correlation		0.619				
P		0.013				
L. reuteri						
Correlation	0.613				0.613	
P	0.008				0.008	
Total number of correlating species	**7**	**7**	**2**	**1**	**4**	**1**

Table 14. Infant-mother correlations for specific bifidobacterial and lactobacilli species (as determined by qPCR) in mothers receiving prebiotic or placebo supplementation. Correlations were determined between samples after supplementation in mothers and at day 5 in children, after supplementation in mothers and at day 20 in children, as well as after supplementation in mothers and at day 182 in infants. [1]Correlations were calculated by Spearman rank test.

4.3.3.3 Similarity index

The similarity indices for bifidobacteria, lactobacilli, as well as bifidobacteria plus lactobacilli obtained from maternal samples before supplementation with neonatal samples from days 5, day 20 and day 182 did not differ significantly between both groups (data not shown). No differences between both supplementation groups were observed when comparing the similarity indices of bifidobacteria, lactobacilli or bifidobacteria and lactobacilli from maternal samples after supplementation with samples from the infant at day 5, day 20 or day 182, respectively (**Table 15**). Overall the bifidobacterial similarity indices were higher in both the prebiotic and placebo supplemented group as compared to the lactobacilli similarity indices.

	Mother after - infant day 5			**Mother after – infant day 20**			**Mother after - infant ~ day 182**		
Similarity index (%)	Prebiotic	Placebo	P	Prebiotic	Placebo	P	Prebiotic	Placebo	P
Bifidobacteria			0.176			0.349			0.223
n	17	15		18	14		17	15	
median[1]	50	60		33	55		33	40	
(range)	(0-100)	(25-100)		(0-100)	(0-100)		(0-75)	(0-100)	
Lactobacilli			0.622			0.764			0.777
n	17	10		18	9		16	14	
median	0	0		0	0		0	0	
(range)	(0-75)	(0-100)		(0-75)	(0-100)		(0-75)	(0-67)	
Bifidobacteria & lactobacilli			0.134			0.312			0.383
n	17	15		18	14		17	15	
median	40	43		23	38		25	29	
(range)	(0-82)	(20-83)		(0-73)	(0-75)		(0-75)	(0-67)	

Table 15. Infant-mother bifidobacterial, lactobacilli and bifidobacterial plus lactobacilli similarity indices (%) obtained for samples taken from the mothers after delivery and from the infants at day 5, day 20 and day 182 in the prebiotic and placebo supplemented group.
SI = (C/D) x 100, with C representing the number of mother-infant species that matched, i.e. that were detectable in both mother and infant and D representing the number of all species which were either positive in the mother, the infant or both. [1]Results are expressed as median (range). Differences between both supplementation groups were calculated with MW*U* test.

4.4 Analyses of CB samples

4.4.1 Flow cytometry data

From the 35 neonates 30 EDTA CB samples (16 from the prebiotic group and 14 from the placebo group) were collected immediately after delivery: no EDTA CB samples were available from the three placebo supplemented women, who delivered at other hospitals. In addition, two CB tubes from the prebiotic group did not contain enough material for performing the flow cytometric analyses. All obtained samples were analysed by flow cytometry. Samples from antibiotic treated babies (n=5, 3 from the prebiotic and 2 from the placebo group) were retrospectively excluded from statistical analyses, so that 25 samples (13 from the prebiotic group and 12 from the placebo group) were included in the statistical analyses.

4.4.1.1 Chemokine receptor expression on $CD4^+$ & $CD8^+$ T cell subsets

CB cells were labelled with anti-CD4, anti-CD8 (to distinguish naive T helper from cytotoxic T cells), anti-CD45-RA (to distinguish naive from memory T cells) and antibodies directed against different chemokine receptors (CKRs). The $CD4^+/CD8^+$ ratio did not differ significantly [p=0.647; MW*U*] between the prebiotic group (median: 3.6; range: 1.0 - 7.8) and the placebo group (median: 3.5; range: 1.3 - 12.8).

$CD4^+$ and $CD8^+$ cells were predominantly found to be naïve expressing $CD45RA^+$ cells, while only a very small subset expressed the $CD45RO^+$ memory marker. The frequency of $CD45RA^+$ or $CD45RO^+$ expressing $CD4^+$ or $CD8^+$ T cells did not differ between both supplementation groups (**Table 16**).

	$CD4^+$ T cells			**$CD8^+$ T cells**		
	Prebiotic (n=13)	Placebo (n=12)	P	Prebiotic (n=13)	Placebo (n=12)	P
% CD45RA[1]			0.242			0.399
median	83.8	85.3		91.2	92.95	
(range)	(62.7 - 95.8)	(79.4 - 97.3)		(83.3 - 98.5)	(78.5 - 99.5)	
% CD45RO			0.149			0.221
median	13.2	12.8		8.8	7.05	
(range)	(4.0 - 37.3)	(0.0 - 20.6)		(1.5 - 16.7)	(0.5 - 15.2)	

Table 16. Frequency of $CD45RA^+$ and $CD45RO^+$ expressing cells within the $CD4^+$ and $CD8^+$ CB T cell population in the prebiotic and placebo group. [1]Results are expressed as median (range). Differences between both groups were calculated with MW*U* test.

To address the potential of chemokine responsiveness of CB T cells, the cell surface expression of different CKRs was examined within $CD4^+$ and $CD8^+$ T cell subsets. The frequency of CKR^+ expressing $CD4^+$ CB T cells revealed no significant differences between both supplementation groups (**Figure 23**). Overall the frequency of CCR1, CCR2, CCR5, CCR6, CCR8, CCR9, CRTH2, CXCR5 expressing $CD4^+$ T cells was low (< 1%). In contrast, the frequency of CCR4 and CXCR3 expressing $CD4^+$ T cells was slightly higher (< 6%). Consistent with their known tropism for secondary lymphoid tissues through high endothelial venules, most $CD4^+$ naive T cells expressed CCR7 (median of both groups 94.9%) and CXCR4 (median of both groups 100%). Since CXCR4 expression was heterogeneously spread through the lymphocyte gate, the MFI of CXCR4 on $CD4^+$ was analysed. No significant difference [p=0.055; MW*U*] was observed between the prebiotic (median: 1340; range: 1048 - 3162) and the placebo group (median: 2139; range: 609 - 18165) for the MFI of $CXCR4^+$ on $CD4^+$ cells.

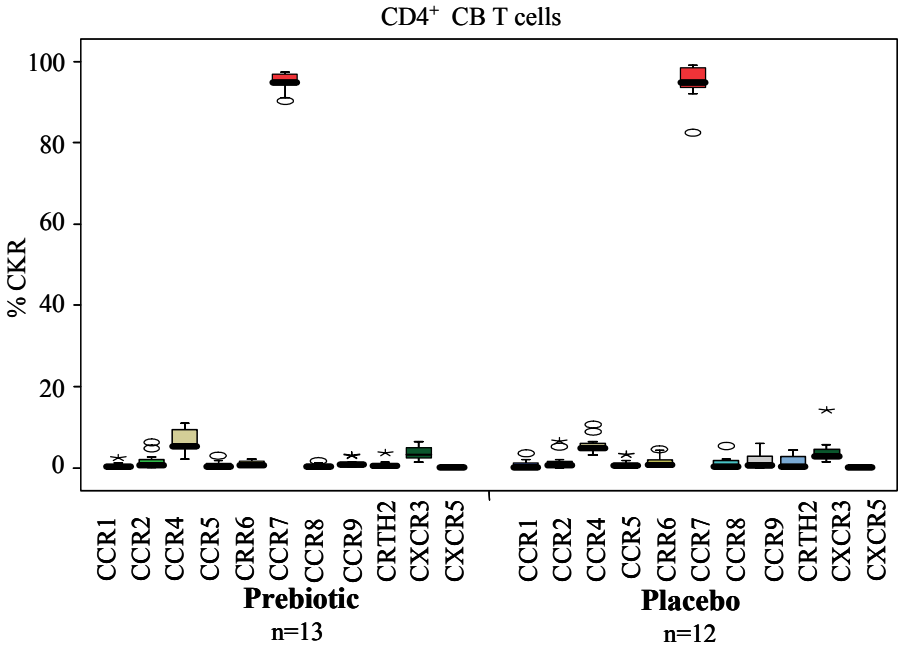


Figure 23. Frequency of CKR^+ expressing cells within the $CD4^+$ CB T cell population in the prebiotic and placebo group. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and °: outliers). Differences between both groups were calculated with MW*U* test.

The analyses of the frequency of CKR^+ expressing $CD8^+$ CB T cells showed no significant differences between both groups (**Figure 24**). The frequency of CCR1, CCR2, CCR4, CCR5, CCR6, CCR8, CCR9, CRTH2, CXCR5 expressing $CD8^+$ T cells was low (< 5%) as compared to CCR7 (median of both groups 88.3%) and CXCR4 (median of both groups 100%).

The MFI of $CXCR4^+$ on $CD8^+$ T cells showed no significant difference between the prebiotic (median: 3187; range: 1731 - 6369) and the placebo supplemented group (median: 4656; range: 916 - 31750). The MFI of CXCR4 was significantly higher on $CD8^+$ T cells than on $CD4^+$ T cells [$p \leq 0.001$; paired Wilcoxon].

Apart from CCR4 and CCR7, which were expressed at higher frequencies on $CD4^+$ T cells, all other CKRs were expressed at significantly higher frequencies on $CD8^+$ T cells [$p \leq 0.003$; paired Wilcoxon]. CXCR3 was expressed at a higher frequency on $CD8^+$ T cells (median of both groups 82.6%) than on $CD4^+$ T cells [$p \leq 0.001$; paired Wilcoxon]. A subset analyses revealed, that CXCR3 was mainly found on naïve $CD8^+$ T cells (71.6% of the $CD8^+CD45RA^+$ cells expressed CXCR3).

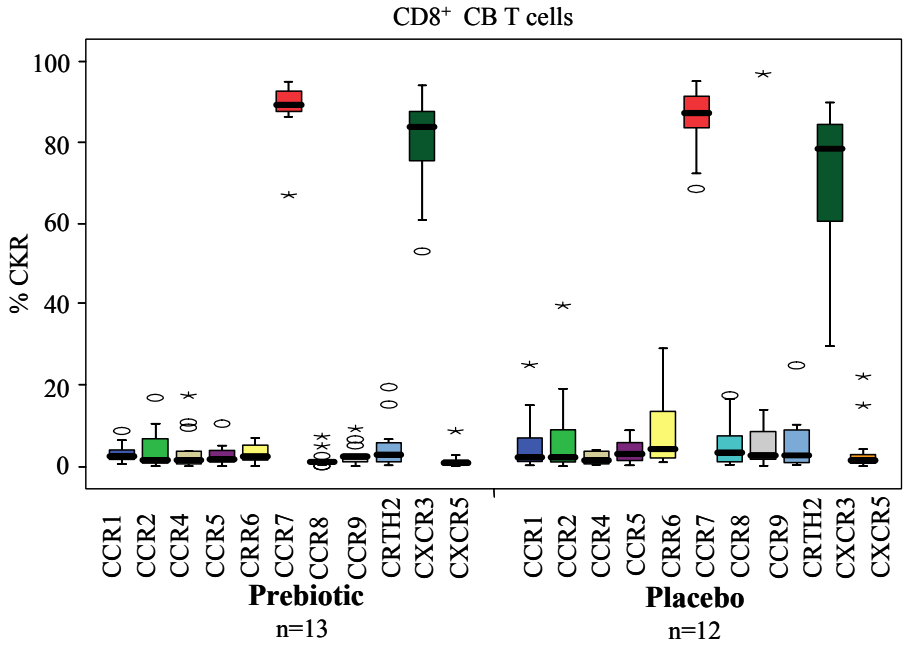


Figure 24. Frequency of CKR^+ expressing cells within the $CD8^+$ CB T cell population in the prebiotic and placebo group. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and °: outliers). Differences between both groups were calculated with MW*U* test.

A further subset analyses of the frequency of CKR^+ expressing $CD4^+CD45RA^+$ and $CD8^+$ $CD45RA^+$ T cells revealed no significant differences between both groups (data not shown).In order to determine whether a Th2 bias is present in CB the ratios of Th1:Th2 associated CKRs were calculated within the $CD4^+$ and $CD8^+$ subsets. The ratios of CXCR3:CCR4, CXCR3:CCR8, CCR5:CCR4 and CCR5:CCR8 revealed no significant differences between both groups (**Table 17**).

Th1:Th2 ratio	CD4+ T cells			CD8+ T cells		
	Prebiotic (n=13)	Placebo (n=12)	P	Prebiotic (n=13)	Placebo (n=12)	P
CXCR3:CCR4			0.786			0.389
median (range)	0.56 (0.21 - 1.55)	0.58 (0.35 - 3.30)		47.07 (4.79 - 219.25)	43.32 (16.03 - 233.0)	
CXCR3:CCR8			0.807			0.176
median (range)	6.00 (2.08 - 25.00)	8.77 (0.90 - 43.0)		68.64 (8.34 - 219.67)	17.15 (2.19 - 285.67)	
CCR5:CCR4			0.142			0.295
median (range)	0.09 (0.00 - 1.36)	0.14 (0.04 - 0.75)		1.13 (0.05 - 7.50)	2.54 (0.2 - 8.71)	
CCR5:CCR8			0.463			0.156
median (range)	1.00 (0.00 - 8.00)	1.83 (0.11 – 18.0)		1.02 (0.5 - 35.00)	0.51 (0.09 - 9.89)	

Table17. Th1:Th2 ratio on $CD4^+$ and $CD8^+$ CB T cells in the prebiotic and placebo group. Differences between both groups were calculated with MW*U* test.

4.4.1.2 $CD25^{high}$ expression on $CD4^+$ T cell subsets

The analyses of the frequency of $CD25^{high}$ expressing $CD4^+$, $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T cell subsets revealed no significant differences between both groups (Figure 25).

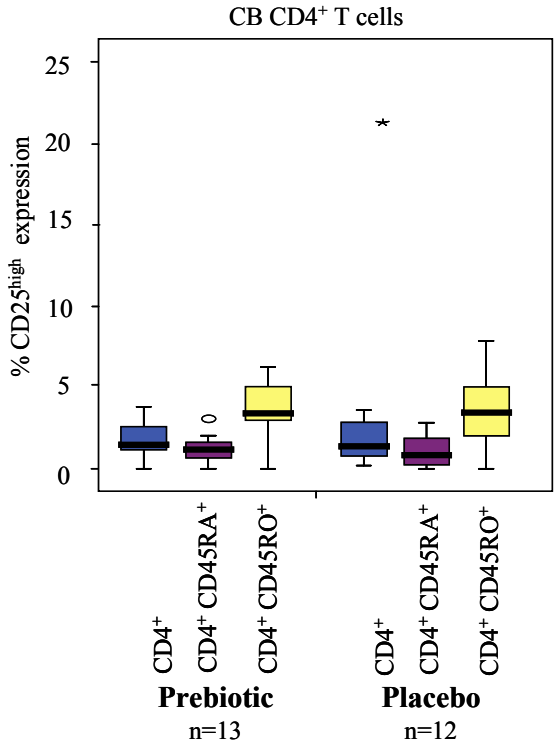


Figure 25. Frequency of $CD25^{high}$ expressing cells within different $CD4^+$ subsets of CB T cells in the prebiotic and placebo group. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and °: outliers). Differences between both groups were calculated with MW*U* test.

The frequency of the early expression marker $CD69^+$ on $CD4^+$ T cells was low in both the prebiotic group (median: 0.2; range: 0.0 - 0.4) and the placebo group (median: 0.3; range 0.0 - 1.0) [p=0.488; MWU].

4.4.1.3 $TLR2^+$ & $TLR4^+$ expression on CD14+ monocytes

The MFI of $CD14^+$ monocytes showed no significant difference [p=0.050; MW*U*] between the prebiotic group (median: 3906, range: 2911 - 7408) and the placebo group (median: 5501; range: 2090 - 9959). The MFI of $TLR2^+$ was higher [p=0.019; MW*U*] in the placebo group, while $TLR4^+$ was equally expressed in both groups (**Figure 26**). However, upon taking into multiple testing into consideration, this difference was not significant.

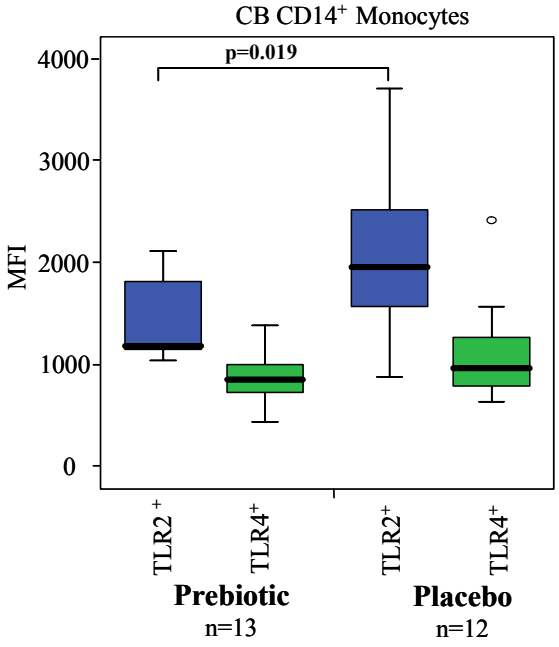


Figure 26. MFI of $TLR2^+$ and $TLR4^+$ on $CD14^+$ CB monocytes in the prebiotic and placebo group. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, ^{o} : outliers). Differences between both groups were calculated with MW*U* test.

4.4.2 Multiplex array data

From 35 neonates, 29 Li-Heparin CB samples (16 from the prebiotic group and 13 from the placebo group) were immediately collected after delivery: no CB samples tubes were available for the stimulation assay from the three placebo supplemented mothers, who delivered at other hospitals. In addition, two CB tubes from the prebiotic group and one from the placebo group did not contain enough material for performing the stimulation assay. All samples were analysed. Samples from antibiotic treated babies (n=5, three from the prebiotic and two from the placebo group) were retrospectively excluded from the analyses. Two further CB samples from the placebo group had to be excluded from statistical analyses because of high background cytokine concentrations in the negative control. Background could have been caused by cross-contamination of the stimuli on the plate adjacent to the well containing the negative control. In all other cases, CB cells which were not stimulated over the culture period (negative control) showed no or very low cytokine expression (background responses). Twenty two CB culture supernatants (13 from the prebiotic and 9 from the placebo group) were included in the statistical analyses.

For all stimuli, IL-2 concentrations were based on the number of lymphocytes (pg/mL/10E3 leukocytes), while all other analyzed cytokines were based on the number of leukocytes (pg/mL/10E3 lymphocytes). The concentrations of IL-5, IL-7, IL-12, IL-13 and IL-15 were low or below detection limit regardless of the stimuli used (data not shown). TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-10, GM-CSF, G-CSF concentrations in the supernatants were directly measured without further dilution, while IL-6, IL-8, IL-10, MCP-1, MIP-1ß supernatants were diluted 1:10.

4.4.2.1 Cytokine expression in Der p1, BLG, LPS & OVA stimulated samples

The analyses of the cytokines IL-1ß, IL-4, IL-10, IFN-γ, TNF-α, GM-CSF, G-CSF revealed that Der p1, as well as BLG, OVA and LPS induced similar cytokine expression patterns after 24 h. Der p1, BLG, OVA, and LPS showed approximately equal amounts of IL-1β and G-CSF, comparably lower amounts of IL-10, IFN-γ, TNF-α, GM-CSF and very low concentrations of IL-2 and IL-4 (**Figure 27**). Except for G-CSF, which was expressed at higher levels in the Der p1 [p= 0.012; MW*U*], BLG [p=0.03; MW*U*] and OVA [p=0.008; MW*U*] stimulated samples from prebiotic group, all other analysed cytokines revealed no significant differences between both supplementation groups. However, upon taking multiple testing into consideration, these differences were not significant.

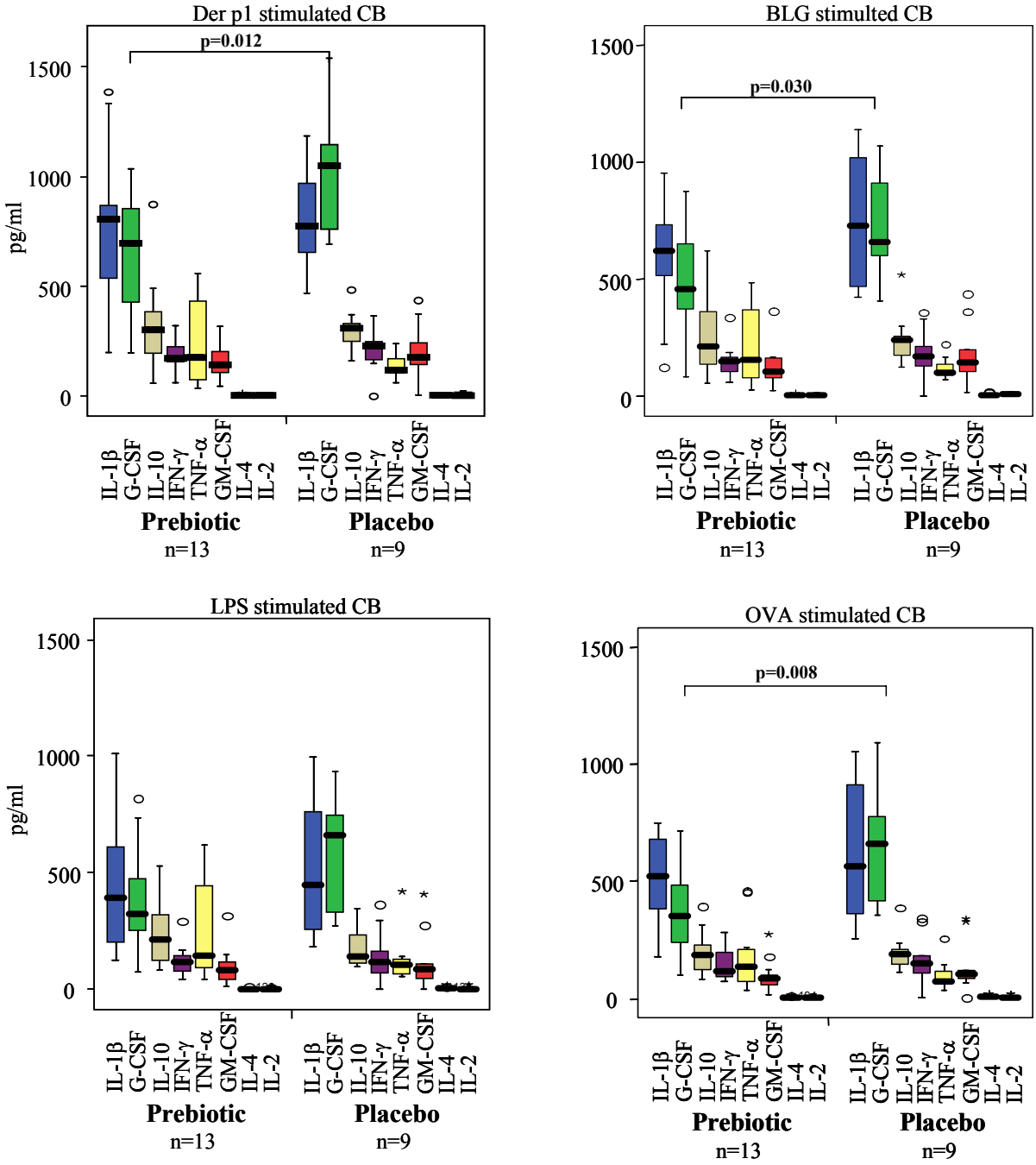


Figure 27. Cytokine expression levels after stimulation of whole CB samples from the prebiotic and the placebo group with Der p1, BLG, LPS, OVA for 24 h. For IL-1ß, G-CSF, IL-10, IFN-γ, TNF-α, GM-CSF and IL-4 concentrations are shown in pg/mL/10E3 leukocytes and for IL-2 in pg/mL/10E3 lymphocytes. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and ^{o}: outliers). Differences between both groups were calculated with MW*U* test.

IL-6 was induced at very high concentrations in Der p1, BLG, LPS and OVA stimulated samples (**Figure 28**), whereas IL-8, MCP-1 and MIP-1ß were induced at much lower levels after 24 h. Samples from both supplementation groups revealed no significant differences after 24 h.

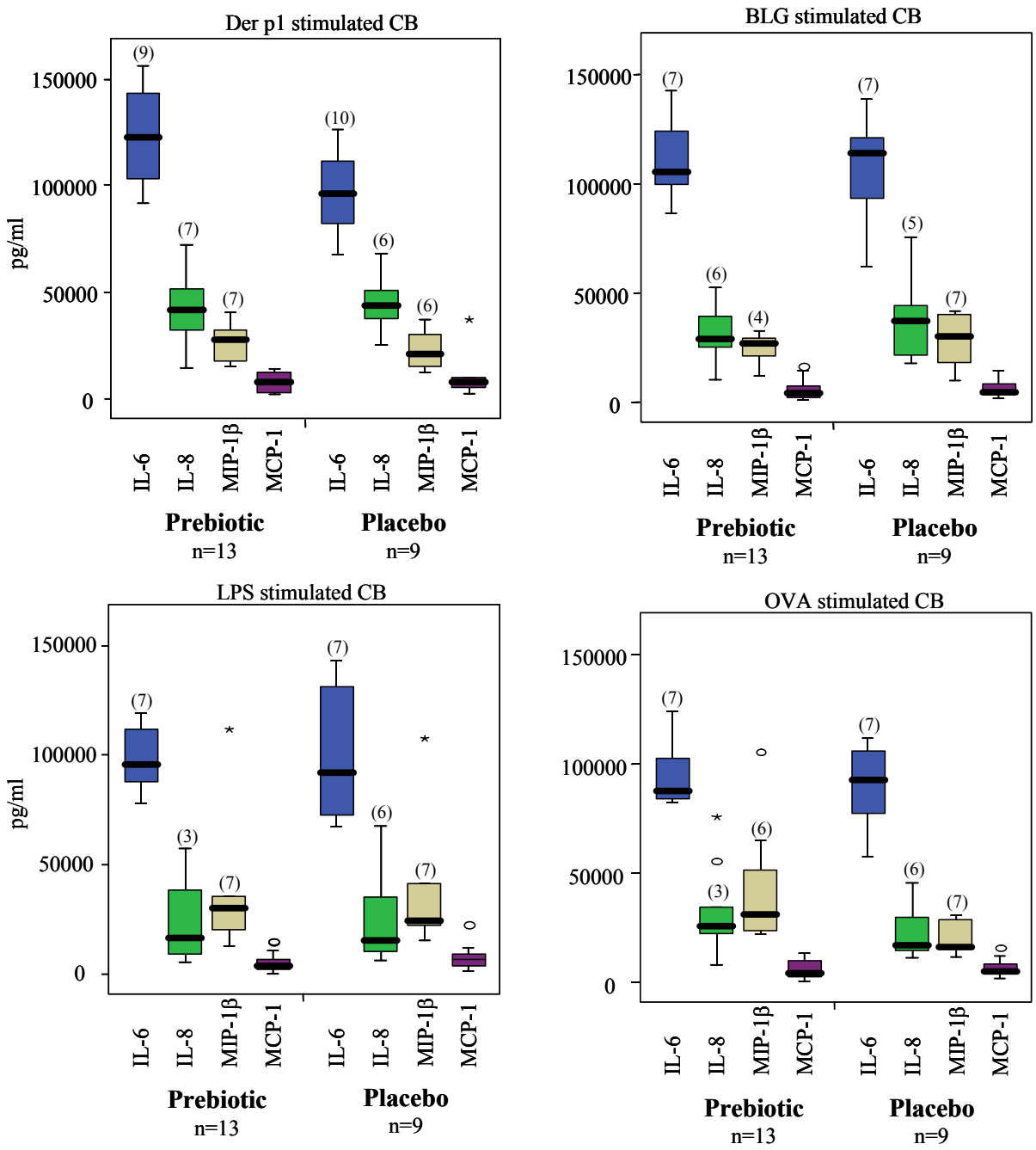


Figure 28. Cytokine expression levels (pg/mL) after stimulation of whole CB samples from the prebiotic and the placebo group with Der p1, BLG, LPS, OVA for 24 h. For IL-6, IL-8, MIP-1ß and MCP-1 concentrations are shown in pg/mL/10E3 leukocytes. The numbers in parentheses reflect the number of samples with cytokine concentrations above detection limit (not used as data for statistical calculation). Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and °: outliers). Differences between both groups were calculated with MW*U* test.

4.4.2.2 Cytokine expression in Con A & SEB stimulated samples

Con A and SEB were potent IL-2 inducers in contrast to Der p1, BLG, LPS and OVA, which did not induce IL-2 after 24 h (**Figure 29**). The highest IL-2 concentrations were detected in Con A stimulated samples, while IFN-γ, GM-CSF, G-CSF, IL-1β, IL-10 and TNF-α were induced at lower levels. SEB induced only low amounts of IL-2 and none of the other cytokines. No significant differences were observed between both supplementation groups after 24 h.

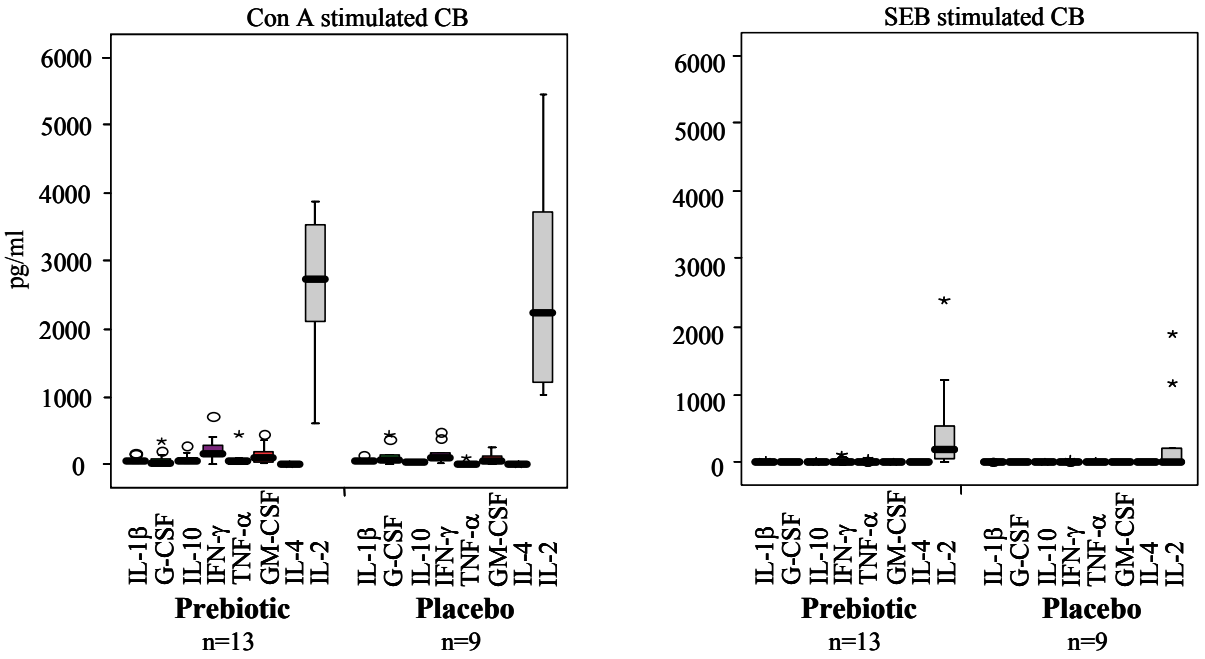


Figure 29. Cytokine expression levels (pg/mL) after stimulation of whole CB samples from the prebiotic and the placebo group with Con A and SEB for 24 h. For IL-1ß, G-CSF, IL-10, IFN-γ, TNF-α, GM-CSF and IL-4 concentrations are shown in pg/mL/10E3 leukocytes and for IL-2 in pg/mL/10E3 lymphocytes. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and °: outliers). Differences between both groups were calculated with MW*U* test.

IL-6, MIP-1ß and IL-8 were induced at lower or similar levels in Con A stimulated samples as compared to samples stimulated with Der p1, BLG, LPS and OVA after 24 h (**Figure 30**).

In comparison to SEB and all other stimuli, Con A induced the highest amounts of MCP-1. From all stimuli, SEB induced the lowest IL-6, IL-8, MCP-1 and MIP-1ß concentrations after 24 h. No differences were observed between both supplementation groups after 24 h.

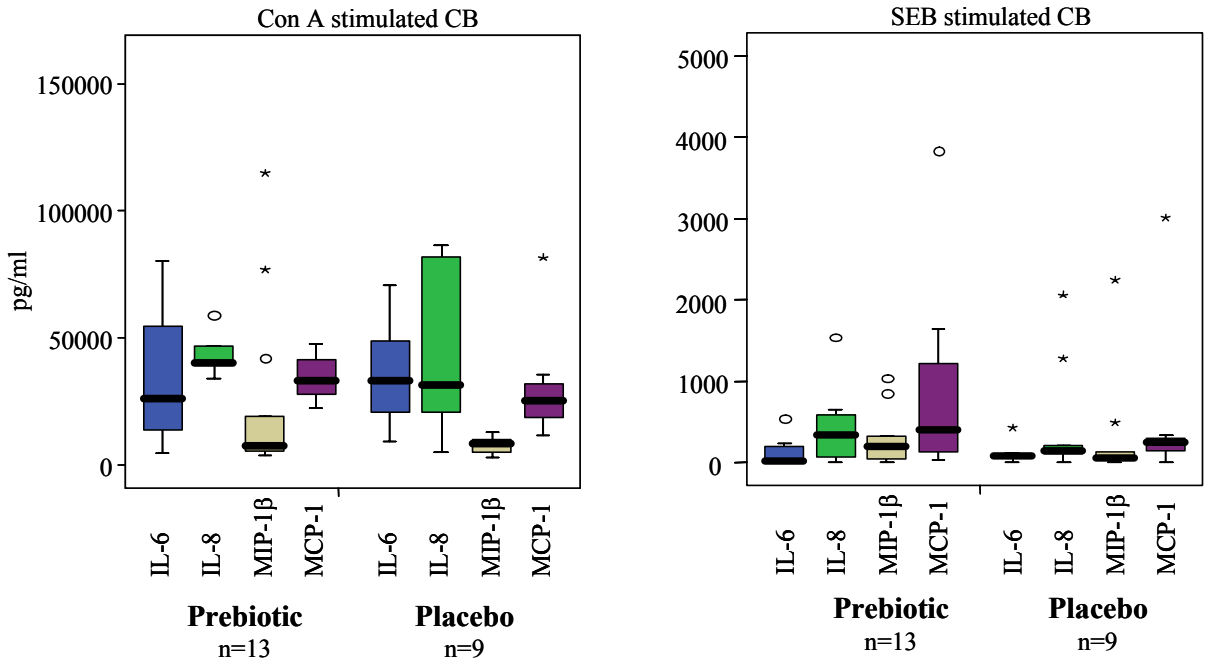


Figure 30. Cytokine expression levels (pg/mL) after stimulation of whole CB samples from the prebiotic and the placebo group with Con A and SEB for 24 h. For IL-6, IL-8, MIP-1ß and MCP-1 concentrations are shown in pg/mL/10E3 leukocytes. Data are shown as box plots (black bar: median, boxes: IQR, T-bars: range, * and ^{o}: outliers). Differences between both groups were calculated with MW*U* test.

For the calculation of the Th1:Th2 ratio, IL-4 concentrations below detection limit were set to 1.0 pg/mL. The ratio of INF-γ to IL-4 (Th1:Th2) calculated for each cytokine showed no significant differences between both groups (data not shown). Supernatants from the 48 h stimulation assay were not further analysed.

5. DISCUSSION

This double-blind randomised placebo-controlled pilot intervention study (with 33 participants) investigated the effects of prebiotic supplementation during the last trimenon of pregnancy on the maternal gut and vaginal microbiota. In addition, the influence on the neonatal gut colonization and selected foetal immune parameters was assessed.
Self-assessment based questionnaires were used to determine the overall long-term acceptance and tolerance of prebiotic supplementation during pregnancy. Supplementation of pregnant women with 3x3 g of prebiotics (GOS/FOS : 9/1) per day was well tolerated. No apparent adverse effects were observed. As expected, abdominal discomfort (flatulence, constipation, diarrhoea, reflux) was reported rarely and only by a few participants.
Only a few double-blind placebo-controlled human trials with prebiotics have been conducted so far and a direct comparison with other intervention studies was not possible due to differences in prebiotic preparations (FOS, GOS, neosugar, inulin), doses and duration of supplementation, study participants and study design. Nevertheless, our results are in accordance with studies in human adults, which have shown that intake doses of prebiotics in the range of 4-15 g per day for 2 to 5 weeks [86, 87] were generally well tolerated by healthy adult participants, while intake doses as high as 30 g/day (0.5 g/kg) have been associated with gastrointestinal side effects, in particular flatulence [131].

5.1 Microbiota analyses

5.1.1 Maternal vaginal pH

Previous studies have found that the vagina is normally protected by lactobacilli. Lactobacilli maintain an acidic environment and produce substances (e.g. lactic acid, hydrogen peroxide and bacteriocins) that inhibit the activity of other microorganisms. Bacterial vaginosis is one of the most common infections that occurs in pregnant women [132]. It is associated with an elevated vaginal pH (more than 4.5) and an altered vaginal microbiota. Bacterial vaginosis is characterized by a decrease of the normally predominant lactobacilli species and an increase in potentially pathogenic anaerobic, facultative and gram negative microorganisms (e.g. *Candida*, *Gardnerella*, *Mycoplasma*, *Ureoplasma*, *Streptococcus* species and *E. coli*). Studies have shown that bacterial vaginosis can lead to a two-fold increased risk of preterm labour and spontaneous abortion [132, 133]. The balance can be restored with antibiotics, by direct application of probiotic lactobacilli (*L. rhamnosus* GR-1, *L. fermentum* B-45 and RC-14) into

the vagina or by oral consumption of lactobacilli containing fermented dairy products [134, 135]. However, according to a PubMed search (containing the terms: clinical study, prebiotics, inulin, oligofructose and bacterial vaginosis, vaginal pH; from 1996-2006), no studies on the effects of oral prebiotic supplementation on vaginal pH (restoration or stabilization of pH values) have been conducted so far. The concept of vaginal pH self-measurement using gloves to screen for disturbances in the vaginal milieu during pregnancy has previously been shown to efficiently prevent prematurity [136].

Throughout the intervention study, we observed no significant differences in self-assessed vaginal pH values between the prebiotic and the placebo supplemented group. Since no vaginal fluid samples were collected and analysed, no conclusions on the numbers of lactobacilli and consequently on a potential stabilization of the vaginal microbiota can be drawn.

5.1.2 Maternal stool samples

During the study period, no effects of prebiotic supplementation on bowel habits including stool frequency and consistency, as well as stool pH were observed.

Some intervention studies with prebiotics reported stimulation of colonic motility and decrease of transit time [137], normalization of stool consistency (relief of constipation or loose stool) [84], increase of stool weight [86], and decrease of pH in faecal slurry cultures [85], while others found no changes in stool pH, as well as stool frequency [88, 138].

These conflicting data might be attributed to differences in daily prebiotic intake, study population and duration of supplementation period. However, in general OF and inulin have been shown to have few effects on bowel habits of adults, other than increasing output [139]. Since faecal output and SCFA were not measured in our study, we can draw no conclusions on the effects of prebiotics on stool weight and SCFA profiles. In addition, no food protocols were collected. Therefore, it is not clear, whether the lack of prebiotic effect on stool frequency and consistency was due to changes in life style, including diet and fluid intake, (e.g. magnesium, folate or iron pills and laxative consumption) or due to general changes of intestinal motility and physical inactivity connected to pregnancy.

It is becoming increasingly clear that there is considerable variation between individuals with respect to both the diversity of the colonic microbiota [140] and the response of the microbiota to prebiotic consumption [141]. The total counts and the basal numbers of bifidobacteria per g faeces (bacteria/mL) differed significantly between both supplementation

groups before study begin, while the numbers of lactobacilli were very similar. The results of total counts determined by DAPI staining are in good agreement with values obtained by other investigators [142]. On the other hand, considerable variations have been observed when comparing our results with culturable total counts. Culturable total counts are often mentioned in intervention studies [86] and have been reported to be 5-10 times lower than the total cell counts [125, 142] as determined by DAPI. These variations may be explained by differences in media and cultivation methods (aerobe or anaerobe conditions), as well as differences in detection sensitivity (conventional plating techniques versus the currently available modern culture independent molecular based detection methods). Differences (Δ) in total bacterial counts (DAPI) and numbers of bifidobacteria (bacteria/mL as well as % of total bacteria) determined by qPCR were significantly higher in the prebiotic supplemented group than in the placebo group. This was confirmed by an increase of the bifidobacterial numbers in total bacterial load as determined by FISH. In contrast, we observed no changes in the numbers (bacteria/mL) and the percentages of lactobacilli (determined by qPCR and FISH). Our results agree with a number of *in vitro* [85] and *in vivo* studies using OF [12, 84]. Intake doses of FOS, which have elicited a bifidogenic effect in adults, ranged from 4 to 15 g/day (i.e. 0.05 - 0.11 g/kg/day) and were already observed after 2 to 5 weeks, independent of chain length or GOS or FOS type [84, 86-88]. Gibson et al. [86] showed that supplementation of 15 g OF/day to strictly controlled diets of healthy adults for 15 days caused a significant increase in the mean viable counts of bifidobacteria, but not of lactobacilli. Interestingly, the numbers of bifidobacteria declined significantly, when OF was withdrawn, indicating that the increase was transient and directly attributable to the addition of OF to the diet. In addition, OF had little effect on the total viable counts of aerobes and anaerobes. Roberfroid et al. [41] reported that the degree of prebiotic stimulus is in part dependent on the initial amount of the endogenous probiotic flora and that there is no clear dose-effect relationship in term of log increases of bifidobacterial counts in the human gut after FOS supplementation. He showed that the lower the initial number of bifidobacteria the greater the increase (whatever the daily dose, within a range of 4-20 g or more) [41] and stated that only one log-fold increase in bifidobacteria can be considered as an indication for a modification of the intestinal flora. However, since faecal counts of bifidobacteria vary considerably within the general population (from 10E7 to 10E9), such dose-effect relationships are difficult to observe [41].

We found considerable inter- and intra-individual differences in microbial bifidobacteria and lactobacilli composition in both supplementation groups. This is in agreement with observations made by other investigators, who analysed 16S RNA fingerprints and found that

the predominant bacterial community of each individual was host specific and stable [10, 140]. Similarly, high fluctuations in the species and strain composition, as well as the numbers over time, have been shown by others, reporting that the relative proportions can vary considerably in time depending on the individual [142-144]. Despite the inter- and intra-individual variations, no indications for a change in bifidobacterial and lactobacilli composition at a single species level (inter-group comparisons and intra-group comparisons) were observed. This indicates a stronger impact of the indigenous microbiota and shows that the microbiota in adults remains rather constant, despite changes in dietary intake.

Before supplementation *B. catenulatum,* followed by *B. infantis* and *B. adolescentis* were the most frequent species in maternal samples in both groups of our study. The percentages were determined according to Liu et al. [128]. Due to different quantification techniques, direct comparisons of our results with data from other studies were not possible. However, the overall proportions of enumerated bifidobacteria were consistent with the current knowledge obtained by both culture and molecular-based methods, showing that in most adults the bifidobacterial community is a combination of one to four species: *B. adolescentis, B. longum*, *B. catenulatum and B. bifidum* [145, 146]. For example, He F. et al. [147] analysed bifidobacterial species composition in healthy adults and identified 51 bifidobacterial strains, most belonging to the species *B. adolescentis*, *B. breve*, *B. infantis* and *B. longum*. In contrast, *B. longum*, *B. catenulatum* and *B. adolescentis* were the most commonly characterized species in a study with healthy Japanese adults [142]. The same species were also observed in a study with Finnish pregnant women [148]. The observed heterogeneity may be attributed to different quantification methods or nutritional and lifestyle habits, as well as genetic and environmental differences. Therefore, caution should be taken when interpreting the results, especially, when it comes to strain and species numbers.

Identification of lactobacilli by traditional plating culture methods is known to be more difficult, as compared to bifidobacteria, and enumeration has been shown to depend on plating techniques e.g. selective culture media and incubation conditions [149]. Lactobacilli constitute less than 1% of the total bacterial community and considerable variability in the numbers and species of lactobacilli has been shown between individuals [15]. *L. acidophilus* was the species most commonly characterized in both groups, before and after supplementation, followed by *L. paracasei, L. fermentum* and *L. casei*. Consistent with our results, *L. acidophilus*, *L. casei* and *L. fermentum,* but also *L. salivarius*, *L. plantarum*, and *L. reuteri* have been shown to be typical representatives which can be isolated from human intestine [15, 146].

Overall, we found a significant increase in bifidobacterial numbers in the prebiotic supplemented group. The faecal samples from mothers receiving prebiotic supplementation showed a large variety of bifidobacterial and lactobacilli species, but the profiles were very similar to that of the control group. The levels of the different species showed that long-term prebiotic supplementation (with 9 g GOS/FOS per day in a ratio of 9:1) did not selectively stimulate the growth of one particular species in the intestinal microbiota. However, effects of prebiotics on other predominant genera in the large bowel, including *Clostridium-Coccoides-Eubacterium* group, *Bacteroides* group or *Atopobium* cluster, were not analysed. In addition, it should be noted that our picture has been biased in favour of the analysed *Bifidobacterium* and *Lactobacillus* species detectable by FISH and qPCR primers and that many other species are still unknown [7, 140].

5.1.3 Neonatal stool samples

Trials in which prebiotic supplementation has been evaluated in infants differed from ours in study design and study population. To our knowledge, most trials with prebiotics directly supplemented term or preterm infants for a certain time period and compared the microbiota with that of infants fed standard formula [118-120, 150]. Only a few studies included breastfed children as a control group [126, 127, 151, 152]. Since all neonates in our study were breastfed, we searched for studies, which included breastfed infants as a control group in their investigations. However, due to different sampling time points and quantification techniques, direct comparisons of our results with data from other studies were not possible.

Neonatal stool pH ranged between 5.6 - 6.1 and showed no significant differences between both groups, when analysing samples taken at day 5, day 20 and after half a year. In accordance, stool pH from three month old breastfed infants was shown to range around of 5.8 [152]. Changes in neonatal stool frequency and consistency were not assessed; therefore no conclusions on bowel behaviour can be drawn from our study.

For a long time it was assumed that the maternal vagina is the source of the bacteria [23, 25] that initially colonize the neonatal GIT. However, plasmid profiling showed that lactobacilli inhabiting the maternal vagina did not colonize the infant digestive tract [20]. Evidence for the transmission of faecal isolates of the bifido- and enterobacteria from the mother to the infant exist. There are indications that the faeces at 10 days after birth would contain bacteria that had truly colonized the digestive tract of the infant [20].

In our study, total bacterial counts, numbers of bifidobacteria and lactobacilli (bacteria/mL), as well as the percentages of bifidobacteria and lactobacilli, revealed no significant differences between both groups, at day 5, day 20 and day 182 after exclusion of children born by caesarean sections. Due to different stool sampling time points in other studies a direct comparison of our data was not possible. For example, one supplementation study found 67.6±4.1% of bifidobacteria in the breastfed infant group at the age of three months using FISH analyses [152]. In another study, the percentages of bifidobacteria in exclusively breastfed infants aged 28-90 days were 63.3±7.7% and increased to 90.3±3.6% after a six week follow-up using both FISH and qPCR analyses [126]. Similarly, the percentages of lactobacilli increased from 0.8±0.3 to 4.1±1.5% [127] after six weeks. In our study, the analyses of the percentages of subjects positive for a certain bifidobacterial or lactobacilli species showed that *B. infantis* followed by *B. breve*, *B. catenulatum*, *B. bifidum* and *B. longum* were not only the most prevalent bifidobacteria species characterized at day 5 and day 20 in both groups, but also the bifidobacterial species that occurred at the highest percentages (% qPCR). Similarly, *L. paracasei*, *L. acidophilus* and *L. fermentum* were among the most prevalent strains detected at day 5 and day 20. No significant differences were found between both groups, except for the percentages of subjects positive for *L. acidophilus* at day 5. However, this result was not confirmed by qPCR analyses. After half a year, the most frequent isolated stains were *B. infantis*, *B. breve*, *L. acidophilus* and *L. paracasei*, while *B. angulatum*, *B. animalis*, *B. dentium*, *L. delbrukeii* and *L. rhamnosus* were never found.

These results are in agreement with Haarman et al. [126, 127], who reported that *B. infantis*, *B. breve* and *B. longum*, as well as *L. acidophilus* and *L. paracasei* were the most dominant species in one to three month old breastfed infants. In addition, *B. bifidum,* as well as *L. reuteri*, *L. gasseri*, *L. rhamnosus* and *L. fermentum* are also commonly present in infants [153]. However, our data are in contrast to a recent Finnish study by Gueimonde et al. analysing the bifidobacterial species composition in infants and their mothers [148]. In this study, pregnant women received LGG or placebo for four weeks before and three weeks after delivery. Stool samples were taken from the mother before supplementation and at study end. In addition, stool samples from the infants were collected at day 5 and day 21. *B. adolescentis* (~ 30.8%), *B. infantis*, *B. longum* and *B. catenulatum* (all 15.4%) were reported to be the most frequently detected species at day 5, while *B. longum* (~ 25%) and *B. breve*, *B. bifidum* and *B. adolescentis* (~ 12.5%) were found to be the most frequent detected strains after 21 days in the breastfed placebo group. Lactobacilli were not assessed in this study.

Our results are also in contrast to data published by Ahrne S. et al. [149], who found *L. rhamnosus* to be the most common isolated lactobacilli strain (~ 21%) in six-month old breastfed Swedish children. In both studies methodological differences (detection sensitivity of the applied analyses method), country dependent differences and genetic variations may have contributed to the observed differences in neonatal microbiota. Favier et al. [154] studied the molecular succession of bacterial communities in neonates using denaturing and temperature gel gradient electrophoresis (DGGE, TGGE) and showed a colonization by bifidobacterial species on the 3rd to 4th day of life. The profiles during the first few days of life were simple but became more complex as the bacterial diversity increased with time. *Streptococcus* and *Enterococcus s*pecies were mainly found during the breast feeding period, while the colonization of *Ruminococcus* and *Clostridium* or *Enterobacter* species was strongly dependent on the time point of formula introduction, milk withdrawal and solid food introduction. Similarly, Sakata et al. [155] reported that the intestine was first colonized by enterobacteria and streptococci in term infants in the first few days, while bifidobacteria became dominant at day four. Since other bacterial strains were not analysed in our study, we cannot draw any conclusions about differences in the overall bacterial community. Gronlund et al. [26] found that the faecal bifidobacterial colonization of infants born by caesarean delivery was delayed. *Bifidobacterium*-like bacteria and *Lactobacillus*-like bacteria colonization rates reached the rates of vaginally delivered infants at one month and 10 days, respectively. Infants born by caesarean delivery were also significantly less often colonized with bacteria of the *Bacteroides fragilis* group than vaginally delivered infants [25, 26, 156]. Due to the small sample size of the infants born by caesarean sections (five infants from the prebiotic group versus one from the placebo group), we did not further analyse and compare the bacterial colonization patterns of this subgroup with vaginally delivered infants.

5.1.4 Comparison of maternal & neonatal microbiota

The diversity index of bifidobacteria and lactobacilli species in infant samples from day 5, day 20 and day 182 did not differ significantly between both groups. To our knowledge no comparable study analysing both bifidobacteria and lactobacilli diversity indices has been published thus far. In our study, the numbers of bifidobacteria at all analysed time points were significantly higher than the numbers of lactobacilli in both groups. This is in accordance with studies showing that bifidobacteria dominate over lactobacilli in this age group [157, 158].

We observed that in both supplementation groups the lactobacilli diversity index, but not bifidobacterial diversity index, was significantly higher in maternal samples taken shortly before delivery, as compared to infant samples collected at day 20. This is in contrast to Gueimondes results [148], who only observed in the placebo group a significantly higher bifidobacterial diversity when comparing maternal stool samples taken after delivery and neonatal stool samples taken at day 21. However, it must be stated that a direct comparison with our data with Gueimondes data [148] was not possible due to differences in supplementation (prebiotic GOS:FOS supplementation in our study versus probiotic LGG supplementation in Gueimondes study).

The analyses of the total number of species correlating in maternal stool samples after supplementation with neonatal samples at day 5 revealed no significant differences between the prebiotic and the placebo group. At day 20 the total number of correlating species was lower as compared to day 5 and tended to be slightly higher in the prebiotic group than in the placebo group. We found no indications that the prebiotic-induced bifidobacterial colonization of the maternal intestine affected the colonization of the infant. This is in contrast to results published by Schultz et al. [159], who showed that pregnant women consuming LGG capsules during the last weeks of their pregnancy specifically conferred the strain to their vaginally delivered children. Interestingly, LGG was not only detectable at the age of one month but was also found to colonize the infant at the age of six months. Similarly, Gueimode et al. [148] found significant correlations in the breastfed placebo group for *B. adolescentis*, *B. longum* and *B. catenulatum* when comparing maternal samples taken three weeks after delivery with neonatal stool samples taken at day 21 from breastfed infants. A direct comparison of our data with the data from this study was not possible due to differences in the analyses procedure used for determining correlations. While we used the % qPCR data, Gueimonde et al. [148] used the % of subjects positive for a species to calculate the correlations between maternal and infant samples.

We found no significant differences between both supplementation groups when comparing the similarity index of bifidobacteria and lactobacilli, respectively at any of the time points analysed. This is in contrast to Gueimonde et al. [148], who reported significantly higher similarity in the placebo, as compared to the LGG group, when analysing stool samples from mothers after delivery with neonatal stool samples at three weeks. This similarity was not seen when samples taken from the mother before delivery were compared with samples taken from the infant at day 5. Again, it must be stated that a direct comparison with our data was

not possible due to differences in supplementation (prebiotics versus probiotics) and analyses procedures used for determining similarity indices.

Overall, the analyses of neonatal stool composition showed no significant differences between both supplementation groups, indicating that neonatal microbiota does not reflect the differences induced through prebiotic supplementation in maternal microbiota. This further indicates that factors other than the maternal bacterial colonization patterns (e.g. breast feeding or physiological and immunological characteristics of the infant) may dominate and be more important than the maternal inoculation effect. However, it should be noted that a large group of bifidobacteria and lactobacilli species in faecal samples is still unknown and that the distribution of these unknown species in neonatal stool might still differ between both groups [160]. In addition, no conclusions can be made concerning other bacterial strains including *Bacteroides*, *Enterococcus* and *Clostridium* species since these were not analysed in neonatal samples. If and to what extent increased maternal bifidobacterial counts could affect the colonization of the neonatal gut remains to be determined in further studies.

5.2 CB analyses

Only a few data are available concerning the role of prebiotics in modulating the immune system. The few studies that have examined the effects of prebiotic fibres on the immune system were conducted in animals (rats, mice, dogs). However, indications for modifications of the gut microbiota and thus of the gut-associated lymphoid system are becoming more and more evident in animals [82]. As reviewed by Schley et al. [161], prebiotic supplementation in animals was associated with increased lymphocyte proliferation and lymphocyte numbers in GALT and peripheral blood. Additionally, increases in IgA secretion or IgA cells in GALT, decreases in $CD4^+/CD8^+$ ratio in spleen and increases in the phagocytic function of intra-peritoneal macrophages have been reported. So far, only a few human studies have investigated the effects of inulin and oligofructose alone, or in combination with other dietary supplements, on immune competence. Prebiotic supplementation resulted in minor changes of systemic immune functions, such as decreases in phagocytic activity, but no data are available on the effects of prebiotics on the GALT in humans [162].
One reason why only little attention has been given to the effects of nutritional interventions on immune functions is the current lack of a single immune marker to predict the outcome of a dietary intervention in humans [163]. The difficulty in choosing the optimal combination of

suitable *ex vivo* and *in vivo* immune markers in order to assess the effects of nutrition intervention studies on the activities of immune key cells, has been discussed in a review by Albers et al. [163]. Biological relevance, sensitivity and practical feasibility must be taken into consideration when choosing immune function assays. In search for markers detectable at birth, numerous investigators analysed umbilical CB to assess the immunologic status of the newborn. The methodologies applied in such analyses include the assessment of cellular functions by cell culture, measurement of various immunologic mediators in the circulation by ELISA and phenotype by flow cytometry. Immune responses have been shown to be Th2 polarized in the foetus and successively become Th1 dominated in the first year of life [164]. Most investigators use CB mononuclear cells (CBMCs) isolated by density gradient centrifugation from whole blood for phenotypical and functional characterisation of lymphocytes. However, ficoll density centrifugation and extensive washing have been shown to remove the cells from their endogenous cytokine and cellular milieu [165], thereby affecting T cell function, cytokine production and surface molecule expression [166]. Purification techniques may also introduce *ex vivo* manipulation and give opportunity for artefacts. Therefore, it is recommended to reduce experimental manipulation to get accurate results reflecting the true *in vivo* situation. Since numerous investigators have reported success using whole blood assays to examine the activation state and the cytokine production capacity of various immune cell populations, we undertook an exploratory examination of whole CB immune parameters. For the phenotypical characterisation of lymphocytes, we used chemokine receptor (CKR) markers as surrogate markers, to screen for different Th1/Tc1 and Th2/Tc2 populations in un-stimulated whole CB samples. In addition, we screened for changes in the population of cells producing either Th1 or Th2 cytokines using a whole blood stimulation assay.

5.2.1 Phenotypical characterisation of CB

Ex vivo phenotypic analyses using multi-parameter flow cytometry can provide information on the activation and differentiation status of lymphocytes and their functional properties. Several T cell analyses comparing whole blood and purified peripheral blood mononuclear cells (PBMC) have shown methodologically different results including variations in the $CD4^+/CD8^+$ ratios [167] and chemokine receptor expression [168]. Although cryopreserved PBMCs [169, 170] are often used in clinical studies for practical reasons, direct analyses of whole blood samples is the method of choice, as cells remain largely un-manipulated [171-

174]. However, two problems of whole blood cultures have been reported in the literature. First of all, prevention of correct staining when using whole blood is a potential downside associated with this procedure. For instance, soluble cell surface proteins in whole blood may interact with marking antibodies. Furthermore, soluble ligands for cell surface proteins may prevent antibody binding to the same protein and plasma proteins may interact with the peptides used for identification of antigen-specific cells [175]. Secondly, there have been conflicting reports about the content and functional properties of CB lymphocytes. Discrepancies are influenced by technical factors, including the fact that some investigators did not sufficiently exclude NRBCs (potentially as high as 30%) from the lymphocyte gate. This may lead to inaccurate estimates of lymphocyte subsets in various reports [174]. Additionally, gating strategy and analyses procedure can be a significant source of variability. Due to small sample amounts, we decided to study surface molecule expression and cytokine production by leukocytes in freshly drawn CB samples and tried to reduce variability by standardizing sample processing and analyses procedures. Samples were analysed within 4 h after collection. Staining conditions (reagent quality and batches), as well as flow cytometric parameter settings were kept identical for all samples. In addition, the analyses procedures were kept constant. For some markers (e.g. CXCR4 and TLR2) gating and setting of limits varied slightly from sample to sample, so that an individual fine sample analyses was required. We set the lymphocyte gate using both the SSC combined with the expression of either $CD4^+$ or $CD8^+$. This avoids the problem created by NRBCs since erythrocytes express neither $CD4^+$ nor $CD8^+$. A direct comparison of our data with published data was not possible due to differences in sample processing (storage conditions) and gating strategy (e.g. surface marker expression is often showed as percentage within a lymphocyte population or percentage within the $CD3^+$ T cell population instead of percentage within the $CD4^+$ or $CD8^+$ populations). We observed a $CD4^+/CD8^+$ ratio of 3.55 in whole CB lymphocytes and found no significant differences between both groups. This finding is consistent with data published by Canto et al. [170], who found a ratio of 3.19 in CBMCs. For a further characterisation of the CB T lymphocytes, we analysed the expression of the leukocyte membrane glycoprotein CD45 and found no significant differences between both groups. CD45 exists in two different isoforms, as determined by mRNA splicing. Primary responses are mediated by unprimed T cells expressing $CD45RA^+$, while recall responses are mediated by $CD45RO^+$ expressing cells [176]. We observed that most CB lymphocytes cells expressed the $CD45RA^+$ isoform which is typical for naïve, unprimed T cells. This is in accordance with data published by Szabolcs et al. [174] and by others [170, 173, 177], showing that the majority of the CB T lymphocytes

are phenotypically and functionally immature. Correspondingly, only a small portion expressed the $CD45RO^+$ memory phenotype. The memory function of the low numbers of $CD45RO^+$ T cells found in the foetal circulation remains undetermined [178].

Previous studies have shown that increasing antigenic exposure from the maternal environment by dia-placental [179] and trans-placental passage [164, 180, 181] can lead to activation and differentiation of Th0 cell to Th1/Tc1 or Th2/Tc2 cells. Therefore, we analysed a broad spectrum of Th1/Tc1- and Th2/Tc2- related CKRs on $CD4^+$ and $CD8^+$ CB T cells. All analysed CKRs on $CD4^+$ and $CD8^+$ CB T cells subsets showed no significant differences between both groups. Consistent with the observation that Th1/Th2 chemokine receptors are mainly expressed on memory T cells [169], we observed little or no CCR1, CCR2, CCR5, CCR6, CCR8, CCR9, CRTH2 and CXCR5 expression on $CD4^+$ and $CD8^+$ T cells. In contrast, CCR4 was expressed at higher frequencies on $CD4^+$ than on $CD8^+$ T cells. Interestingly, CXCR3 was expressed at much lower frequencies on $CD4^+$ than on $CD8^+$ T cells. This is in accordance with Campbell et al. [182], who demonstrated that CXCR3 expression in PBMCs is heavily restricted to $CD4^+CD45RO^+$ cells, while experienced and naïve $CD8^+$ cells expressed CXCR3 at similar frequencies. We observed that CCR7 and CXCR4 were expressed on the majority of naïve CB T cells. This corresponds with data from the HLDA8 Workshop panel report [172], showing no expression of CCR1, CCR5 CCR9 on CB $CD4^+$ cells, very low CXCR3 frequencies on $CD4^+$ T cells and stated that CXCR4 was always co-expressed on $CD4^+$ CB T cells. The activation-induced molecule CD69, is known to be rapidly up-regulated within hours after antigenic stimulation. Both $CD4^+$ and $CD8^+$ CB T cells expressed CD69 at very low levels. No indications for significant differences between the two supplementation groups were observed. In accordance with our results, the expression of the early activation marker CD69 on $CD3^+CD4^+$ and $CD3^+CD8^+$ cells from term neonates has been shown to be barely detectable [183].

A prerequisite to study T reg cells is the selection of $CD4^+$ T cells expressing high levels of the late activation marker CD25 [184, 185]. In line with others, we found three subsets of CD25 expressing $CD4^+$ CB T cells: $CD4^+CD25^{low}$, $CD4^+CD25^{intermediate}$ and $CD4^+CD25^{high}$ T cells [129]. In opposite to $CD4^+CD25^{low}$ and $CD4^+CD25^{intermediate}$ T cells fractions, which do not exhibit regulatory properties [185, 186], the regulatory function is confined to the $CD4^+CD25^{high}$ T lymphocytes. The $CD4^+CD25^{intermediate}$ T cell subset contains recently activated and proliferating T cells, which transiently up-regulate CD25 [187]. We found that 1.5% of the $CD4^+$ T cells expressed CD25 at high levels. No significant differences were observed between both supplementation groups. A further subset analyses showed that 1.2%

of the $CD4^+CD45RA^+$ and 3.4% of the $CD4^+CD45RO^+$ cells expressed CD25 at high levels. In human adult peripheral blood 2-3% of the $CD4^+$ T cells are $CD4^+CD25^{high}$ T reg cells. Although a natural naive T regulatory subset (0.2 - 3.3% Nn T reg) exists [184], most of the T reg cells are confined to the CD45RO memory T cell compartment [188] in adults.

In contrast, the majority of preterm and term CB T reg cells have a naive phenotype [178, 186] and are able to suppress proliferation of polyclonally activated $CD25^-$ (responder) T cells in *in vitro* assays [189]. Differences in gating strategy might explain the different observations between our data and that of other groups. For example, Godfrey et al. [190] found that about 5% of the CB T cells distinctly expressed $CD25^{high}$, while others found lower levels [184]. Similar results were found by Valmori et al. [184], who reported that up to 7% of the total $CD4^+$ T cells and that more than 90% of the $CD4^+CD45RA^+$ T cells in CB expressed CD25. This implies that $CD45RA^+$ naive foetal T reg cells undergo antigen-mediated activation during foetal or postnatal development [191]. Isolation of T regs by beads or FACS sorting to analyse functional properties (suppressive function) of CB T regs in *in vitro* assays was not possible in our study, due to small samples volumes.

Since TLRs have been implicated in the recognition of bacterial cell wall products and possibly bridge innate and acquired immunity [192], we investigated the expression of $TLR2^+$ and $TLR4^+$ on $CD14^+$ monocytes. TLR4 recognizes LPS (lipid A) of Gram-negative bacteria with $CD14^+$ being required as an accessory molecule for efficient LPS signalling. In contrast, TLR2 signals in response to a wide range of lipopeptides and is believed to be involved in the recognition of a wide variety of infectious pathogens and their products including yeast cell walls, mycobacteria, whole Gram-positive bacteria, lipoproteins, glycolipids and peptidoglucyan [193]. Signaling by TLRs initiates acute inflammatory responses by induction of antimicrobial genes and inflammatory cytokines and chemokines [193].

We found MFI values of $CD14^+$ on monocytes ranging between 3906 in the prebiotic group and 5501 in the placebo group. The MFI of $TLR2^+$ tended to be higher on $CD14^+$ monocytes than the MFI of $TLR4^+$. Our findings are in contrast to other studies, although the overall tendency is similar (MFI $CD14^+$> $TLR2^+$>$TLR4^+$). For example, Drohan et al. [194] found that the MFI of $CD14^+$, $TLR2^+$ and $TLR4^+$ were much lower on un-stimulated whole CB monocytes. Similarly, Amoudruz et al. [195] showed that un-stimulated CBMC samples from children with mothers having no allergy had much lower $CD14^+$, $TLR2^+$ and $TLR4^+$ MFIs. Variations might be explained by differences in antibody sensitivities, sample storage or processing.

5.2.2 Functional characterisation of CB

The ability of CB cells to proliferate and produce cytokines in response to a variety of environmental antigens (e.g. food antigens, LPS and inhalative allergens) has been described by many investigators [196-198]. The lack of previous antigenic experience might be, in part, responsible for the reduced capacity to respond to antigens [199], as compared to memory or effector T cells. In addition, CB responses to allergens indicated that allergen-responsive T cells in neonates lacked the fine tuned specificity of adult memory cells [200].

Although several studies have demonstrated that cytokine concentrations in whole blood cultures are comparable to those in PBMC cultures, whole blood assays showed less inter-assay variability [166]. In addition, preparation techniques have been shown to cause an *ex vivo* alternation of quantitative cytokine mRNA levels (5-9 fold up-regulation of mRNA levels from cytokines like IL-2, IL-4, TNF-α) thereby leading to different expression profiles in blood samples [201]. Whole blood culture systems can be used as an effective method to obtain reliable and consistent information on the status of cells, especially since the true *in vivo* milieu of cytokines, as well as cellular and non-cellular constituents are retained.

Therefore, we adapted an experimental set up by Miles et al. [130], which can be easily applied to field studies for simple and standardized analyses of cytokine responses in small amounts of CB samples. The analysis of cytokine concentrations in supernatants in response to a specific stimulus is often used to characterize the nature of the T cell immunity. A large number of research studies have focused on general activation of cell cultures with mitogens like phytohaemagglutinin (PHA), Con A or superantigens to measure cytokine production. However, these results only describe an individual's general immune response. To better characterize the immune response to a specific pathogen or allergen, stimulation with an antigen-specific for a particular pathogen or allergen can be used. Therefore, we stimulated CB cells with a panel of stimuli (mitogen: Con A as a control antigen) and antigens (food and inhalative allergens) to determine the responses of CB cells to specific and non-specific stimuli. CB was diluted 1/5 in culture medium, since 1/5 and 1/10 dilutions have been shown to be optimal and necessary to prevent coagulation and allow cultivation for longer periods of time [165, 202]. Pre-aliqouted reagents were used for stimulation to standardize the assay and reduce variability. In addition, intra-assay precision was further increased by fast processing within 4 h after blood collection. As reviewed by Vignali et al. [203] cytokine production has been extensively investigated in the past using diverse assays which detect cytokines on different levels. Polymerase chain reaction (PCR) measures cytokines of the messenger

ribonucleic acid (mRNA) levels, intra-cellular cytokine staining (ICS) measures intra-cellular cytokines levels, enzyme-linked immunospot (EIPSOT) measures cell-associated cytokines, enzyme-linked immune assay (ELISA) and multiplex techniques measure extracellular cytokine concentrations [203]. Intra-cellular staining (to assess cytokine production on the single cell level by flow cytometry) and [3H] thymidine uptake (to measure proliferative responses) were not applicable in this study. Therefore, we assessed cytokine concentrations in culture supernatants using a multiplex method, which enables the simultaneous detection of many cytokines in one measurement and yields similar results to ELISA assays (concerning sensitivity and accuracy). However, this method brings along the disadvantage that the cellular source of cytokines produced by whole CB cells is not ascertainable. A direct comparison of our data with results obtained from other studies was not possible due to different experimental conditions: freshly isolated versus cryopreserved CBMCs [199, 204], undiluted versus diluted whole blood [130] and modes of activation (different stimuli, stimuli concentrations, incubation periods and cytokine detection methods).

A potential drawback of the whole CB assay is the variable number of leukocytes in whole blood culture, which can influence the amounts of detected cytokines. Therefore, we normalized the cytokine concentrations to the number of cells (leukocytes or lymphocytes) used in the assay. We observed that allergen- and mitogen-induced cytokine production 24 h post-stimulation showed typical cytokine expression patterns. The characteristic stimuli-induced cytokine profile might reflect differences in the cell signalling processes [130]. Overall, no significant differences concerning the stimuli-induced cytokine patterns of both groups were observed, after determining the concentration of each cytokine as pg/mL/leukocyte. As reported by Lagrelius et al. [205], different kinetic patterns can be distinguished when detecting cytokine concentrations at different sampling time points (one up to seven days) after stimulation with different stimuli: early peaking and then declining; early peaking to plateau levels; continuously increasing; and persisting levels. Therefore, it is recommended perform measurements when cytokine levels peak. However, finding a single time point that could be used to satisfy these criteria for all cytokines and stimuli studied is difficult [205]. In accordance with Miles et al. [130], we therefore measured the cytokine concentrations after 24 h. Extra-cellular cytokines in the whole blood culture system showed very low background levels in un-stimulated cultures. This has also been suggested by other groups using whole blood cultures [205]. Our data agree with results obtained by Miles et al. [130], who observed that Con A was a very potent IL-2 inducer. However, in contrast to our observations, Miles et al. [130] found Con A to be a more potent inducer of IFN-γ and IL-10

than IL-2. In accordance to Miles et al. [130], we found that Der p1 induced the highest IFN-γ production among the allergens (BLG and OVA) and that Der p1 was a better IL-10 inducer than Con A. In contrast, we observed that Der p1 was the most potent IFN-γ inducer of all stimuli, while Miles et al. [130] found that Con A induced the highest IFN-γ concentrations. As a conclusion, our data agree only partly with the data published by Miles et al. [130]. The observed variations could be explained by differences in detection sensitivity (ELISA versus Bioplex), data analyses (we normalized our data to the number of leukocytes or lymphocytes while Miles et al. [130] reported crude data) or sample processing. While all our samples were stimulated within 4 h after blood collection, Miles et al. [130], do not mention the time between sample collection and experiment begin. They acknowledge that apoptosis could have contributed to the pattern of cytokine appearance in two ways: “first: removal of cytokine producing cells by apoptosis” and “second: release of cytosolic components including cytokines into the medium”.

On the basis of the analysed immune parameters (CKR expression and cytokine responses upon stimulation of CB), we found no indications for any significant differences between both groups. This suggests that maternal prebiotic supplementation and subsequent bifidogenic alteration of maternal microbiota has no direct measurable effect on foetal immune parameters (as determined by phenotype and the reactivity of whole CB to different stimuli). However, it should be noted that due to sample size limitations other immune parameters, e.g. phagocytic or NK cell activity, were not assessed in this study.

5.3 Potential confounders & outlook

Pregnant women were randomised to one of the two study groups so that known and unknown confounders should be balanced evenly between the groups. Although, we do not expect to have to take confounding factors into account in our analyses, we analysed the distributions of several factors to test whether any imbalance occurred. The factors included those related to the family (age of parents, socio-economic status, ethnicity, number and age of siblings, parental smoking habits, mode of birth, birth weight and duration of breast feeding). We found that none of these parameters could have affected our results.

The fact that no reliable method exists to ensure regular intake of supplementation by participants is one weak point of this study. Another important drawback is the size of the study population (n=17 per group). However, our pilot study is the first study analysing a broad spectrum of immune markers in combination with different bifidobacterial and lactobacilli species. Although we found no significant effects on neonatal microbiota and foetal immune parameters, it should be noted that the lack of an observed effect on the analysed parameters does not allow the conclusion, that there is no effect.

So far, only one recent placebo-controlled RCT assessed the effect of prebiotic baby formulae on the incidence of atopic dermatitis in atopy-predisposed infants [206]. This study found a significant reduction of atopic dermatitis in the prebiotic group, as compared to the placebo group. However, how prebiotics modulate postnatal immune development by altering gut microbiota composition and what possible long-term implications (what potential) this might have on primary allergy prevention during pregnancy is unclear at present and needs to be assessed further in larger trails.

6. SUMMARY

To our knowledge, this is the first long-term, randomised, double-blind, placebo-controlled pilot study reporting the effects of prebiotic supplementation during pregnancy on mothers and their offspring. Prebiotic supplementation (at the given concentration) in the last trimenon of pregnancy was well tolerated and had a bifidogenic effect on the gut microbiota of pregnant women. Prebiotic supplementation had no significant effect on the numbers (bacteria/mL) or the percentages of lactobacilli within total bacteria and did not affect maternal stool frequency and consistency or vaginal pH values. In addition, there were no obvious differences between the prebiotic and the placebo supplemented groups concerning the neonatal bifidobacterial and lactobacilli microbiota or the screened CB immune parameters. Based on our present data, we conclude that direct administration of prebiotics to pregnant women cannot be considered as the method of choice to induce a bifidogenic effect in the microbiota of breastfed neonates. We assume that an increase of bifidobacteria in the neonatal gut can be achieved more effectively by directly supplementing infant formulae with prebiotics.

Therefore we, agree with the statement of the ESPGHAN committee and conclude that as long as there are no data on major clinical or long-term benefits (e.g. gastrointestinal infection, allergic disease) [121], “no general recommendations can be made on the use of prebiotic formulae in infancy”. If and to what extent formulae fed neonates could profit from a bifidogenic enriched maternal microbiota remains to be determined. Large multi-centre RCT addressing functional important outcomes (e.g. immune or inflammatory modulation) with validated clinical outcome measures are necessary in future to validate the importance of prebiotics as a prophylactic or therapeutic measure.

7. ZUSAMMENFASSUNG

Diese Studie ist die erste, randomisierte, doppel-blinde, placebo-kontrollierte Langzeitpilotstudie, welche die Wirkung der Präbiotika-Supplementation während der Schwangerschaft auf die Zusammensetzung der mütterlichen und kindlichen Darmmikroflora untersucht. Die Präbiotika-Supplementation im letzten Trimenon der Schwangerschaft zeigte eine gute Verträglichkeit und einen bifidogenen Effekt auf die Darmflora Schwangerer. Im Gegensatz dazu, hatte die Präbiotika-Supplementation keinen signifikanten Einfluss auf die Anzahl der Lactobazillen (Bakterien/mL) oder deren prozentualen Anteil an der Gesamtdarmmirkoflora. Ein Einfluss auf die mütterliche Stuhlfrequenz bzw. Konsistenz und auf die vaginalen pH-Werte war nicht nachweisbar. Die Präbiotika- und die Placebo-Supplementierten Gruppen unterschieden sich nicht bezüglich der neonatalen Entwicklung der Bifidobakterien- und Lactobazillen-Microflora sowie der untersuchten Nabelschnurparameter. Aus den vorliegenden Daten schließen wir, dass die mütterliche Präbiotika-Supplementation nicht zur Induktion eines bifidogenen Effekts in der Darmflora gestillter Kinder empfohlen werden kann. Es ist anzunehmen, dass eine Erhöhung der Bifidobakterien im Neugeborenendarm effektiver durch die direkte Supplementation der Babynahrung mit Präbioitika erreicht werden kann.

Aus diesem Grund, schließen wir uns dem Konsens des ESPGHAN Komitees an und kommen angesichts des Fehlens von Daten bezüglich wichtiger klinischer Langzeit Vorteile durch Präbiotika (z.B. gastrointestinale Infektionen, allergische Erkrankungen) [121], zu dem Schluss dass „keine generelle Empfehlung der Supplementation von Babynahrung mit Präbiotika“ gegeben werden kann. Ob und in welchem Ausmaß zugefütterte Neugeborene von einer mit Bifidobakterien angereicherten mütterlichen Flora profitieren, muss noch untersucht werden. Zukünftige randomisierte multizentrische klinische Studien an einem repräsentativen Kollektiv werden, bei Berücksichtigung funktioneller sowie validierter klinischer Parameter, den Stellenwert der Präbiotika-Supplementation als prophylaktische und gegebenenfalls therapeutische Maßnahme beleuchten.

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9. Appendix

9.1 List of abbreviations

Abbreviations	
%	Percent
Δ	Delta or difference(s)
#	Number(s)
~	Approximately
<	Less than
>	More than
°C	Degrees Celsius
µg	Microgram(s)
µL	Microliter(s)
10E3	10 exponential 3 = 1000
APC(s)	Antigen presenting cell(s)
APC-labelled	Allophycocyanin labelled
APGAR	American Pediatric Gross Assessment Record
B. bifidum	*Bifidobacterium bifidum*
BLG	Beta lactoglobulin
BMI	Body mass index
CB	Cord blood
CBMC	Cord blood mononuclear cell(s)
CD	Cluster of differentiation
CFU	Colony forming unit(s)
CKR	Chemokine receptor(s)
cm	Centimetre(s)
Con A	Concavalin A
DAPI	4',6-diamidino-2-phenylindol
DC	Dendritic cell(s)
Der p1	Dermatophagoides pteronysinus 1
DGGE	Denaturing gel gradient electrophoresis
DI	Diversity index
DPav	Average degree of polymerization
E. coli	*Escherichia coli*
e.g.	For example
EDTA	Ethylen diamine tetra acetate
ELISA	Enzyme-linked immune assay
ELISPOT	Enzyme-linked immunospot
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
et al.	And others
F	Fructose
FACS	Fluorescence activated cell sorting
FISH	Fluorescence in-situ hybridisation
FITC-labelled	Fluorescein isothiocyante labelled
FOS	Fructooligosaccharide(s)
FSC	Forward scatter
g	Gram(s)
G	Glucose
GA	Gestational age

GALT	Gut associated lymphoid tissue
G-CSF	Granulocyte colony-stimulating factor
GI	Gastrointestinal
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-monocyte colony-stimulating factor
GOS	Galactooligosaccharide(s)
h	Hour(s)
ICS	Intracellular cytokine staining
IFN-γ	Interferon-gamma
IgG1	Immune globulin G1
IL-6	Interleukin-6
IQR	Interquartile range(s)
kg	Klilogram(s)
L. acidophilus	*Lactobacillus acidophilus*
LGG	*Lactobacillus rhmnosus subspecies GG*
LH	Lithium heparin
LPS	Lipopolysaccharid
m2	Square meter(s)
MCP-1	Monocyte chemoattractant protein-1
MFI	Mean fluorescence intensity
mg	Milligram(s)
MHC	Major histocompatibility complex
min	Minute(s)
MIP-1ß	Macrophage inflammatory protein-1 beta
mL	Millilitre(s)
mRNA	Messenger ribonucleic acid
MW*U*	Mann-Whitney U-test
n	Number(s)
NDC	Non-digestible carbohydrate(s)
NH2-terminal	Amino-terminal
NK cell	Natural killer cell
nm	Nanometer(s)
NOD	Nucleotide-oligomerization domain
Nn T reg cells	Natural naïve T regulatory cells
NRBCs	Nucleated red blood cells
OF	Oligofructose
OS	Oligosaccahride(s)
OVA	Ovalbumin
P	Significance value
P1	Gate 1 (in FACS analysis)
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffered saline
PC5-labelled	Phycoerythrin-cyanin 5-labelled
PCR	Polymerase chain reaction
PE-labelled	Phycoerythrin-labelled
pg	Picogramm(s)
PHA	Phytohaemagglutinin
PRR	Pattern recognition receptor(s)
Q1	Quadrant 1 (in FACS analysis)
qPCR	Quantitative polymerase chain reaction

RCT	Randomized clinical trail(s)
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment ploymorphism
RNA	Ribonucleic acid
rpm	Rounds per minute
S	Svedberg
S. cerevisae	*Saccharomyces cerevisae*
SCFA	Short chain fatty acids
SEB	Staphylococcal enterotoxin B
sec	Second(s)
SI	Similarity index
SSC	Side scatter
T reg cell	T regulatory cell
Tc cell	T cytotoxic cell
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TGGE	Temperature gel gradient electrophoresis
Th cell	T helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
χ^2	Pearson chi square test

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Figure 12. Total bacterial counts in maternal stool samples before and after supplementation in the prebiotic and placebo group (determined by DAPI).

Figure 13. Absolute numbers of bifidobacteria (bacteria/mL) in maternal stool samples before and after supplementation in the prebiotic and placebo supplemented group (determined by qPCR).

Figure 14. Absolute numbers of lactobacilli (bacteria/mL) in maternal stool samples before and after supplementation in the prebiotic and placebo supplemented group (determined by qPCR).

Figure 15. Percentages of bifidobacteria within total bacteria in maternal stool samples before and after supplementation in the prebiotic and the placebo group.

Figure 16. Percentages of lactobacilli within total bacteria in maternal stool samples before and after supplementation in the prebiotic and the placebo group.

Figure 17. PH values in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and placebo supplemented group.

Figure 18. Total bacterial counts in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and placebo supplemented group (determined by DAPI).

Figure 19. Absolute numbers of bifidobacteria (bacteria/mL) in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and placebo supplemented group (determined by qPCR).

Figure 20. Absolute numbers of lactobacilli (bacteria/mL) in neonatal stool samples from day 5, day 20 and day 182 in the prebiotic and placebo supplemented group (determined by qPCR).

Figure 21. Percentages of bifidobacteria within total bacteria in neonatal stool samples from say 5, 20, and 182 in the prebiotic and the placebo supplemented group.

Figure 22. Percentages of lactobacilli within number total bacteria in neonatal stool samples from day 5, day 20, and day 182 in the prebiotic and the placebo supplemented group.

Figure 23. Frequency of CKR^+ expressing cells within the $CD4^+$ CB T cell population in the prebiotic and placebo group.

Figure 24. Frequency of CKR^+ expressing cells within the $CD8^+$ CB T cell population in the prebiotic and placebo group.

Figure 25. Frequency of $CD25^{high}$ expressing cells within different $CD4^+$ subsets of CB T cells in the prebiotic and placebo group.

Figure 26. MFI of $TLR2^+$ and $TLR4^+$ on $CD14^+$ CB monocytes in the prebiotic and placebo group.

Figure 27. Cytokine expression levels after stimulation of whole CB samples from the prebiotic and the placebo group with Der p1, BLG, LPS, OVA for 24 h. For IL-1ß, G-CSF,

IL-10, IFN-γ, TNF-α, GM-CSF and IL-4 concentrations are shown in pg/mL/10E3 leukocytes and for IL-2 in pg/mL/10E3 lymphocytes.

Figure 28. Cytokine expression levels (pg/mL) after stimulation of whole CB samples from the prebiotic and the placebo group with Der p1, BLG, LPS, OVA for 24 h. For IL-6, IL-8, MIP-1ß and MCP-1 concentrations are shown in pg/mL/10E3 leukocytes.

Figure 29. Cytokine expression levels (pg/mL) after stimulation of whole CB samples from the prebiotic and the placebo group with Con A and SEB for 24 h. For IL-1ß, G-CSF, IL-10, IFN-γ, TNF-α, GM-CSF and IL-4 concentrations are shown in pg/mL/10E3 leukocytes and for IL-2 in pg/mL/10E3 lymphocytes.

Figure 30. Cytokine expression levels (pg/mL) after stimulation of whole CB samples from the prebiotic and the placebo group with Con A and SEB for 24 h. For IL-6, IL-8, MIP-1ß and MCP-1 concentrations are shown in pg/mL/10E3 leukocytes.

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Table 16. Frequency of $CD45RA^+$ and $CD45RO^+$ expressing cells within the $CD4^+$ and $CD8^+$ CB T cell population in the prebiotic and placebo group.

Table 17. Th1:Th2 ratio on $CD4^+$ and $CD8^+$ CB T cells in the prebiotic and placebo group.

9.4 Primers and probes used for the duplex 5′ nuclease assays

Target	Primer or probe	Sequence (5′ → 3′)	T_m °C	% GC	BLAST identification no. or reference	Amplicon length (bp)
B. adolescentis	F_adol_IS	ATA GTG GAC GCG AGC AAG AGA	59	52	1015335678-6465-18906	71
	R_adol_IS	TTG AAG AGT TTG GCG AAA TCG	59	43	1015335740-7519-1624	
	P_adol_IS	CTG AAA GAA CGT TTC TTT TT[a]	69	30	1015335863-95222-17207	
B. angulatum	F_angul_IS	TGG TGG TTT GAG AAC TGG ATA GTG	59	46	1015336044-12581-14600	117
	R_angul_IS	TCG ACG AAC AAC AAT AAA CAA AAC A	59	32	1015336147-14351-29932	
	P_angul_IS	AAG GCC AAA GCC TC	70	57	1015488648-5575-2104	
B. bifidum	F_bif_IS	GTT GAT TTC GCC GGA CTC TTC	60	52	1015336612-215666-12828	105
	R_bif_IS	GCA AGC CTA TCG CGC AAA	60	56	1015336668-22451-30731	
	P_bif_IS	AAC TCC GCT GGC AAC A	70	56	1015336773-24053-3416	
B. breve	F_breve_IS	GTG GTG GCT TGA GAA CTG GAT AG	59	52	1015243936-11550-20833	118
	R_breve_IS	CAA AAC GAT CGA AAC AAA CAC TAA A	58	32	1015244110-13595-29514	
	P_breve_IS	TGA TTC CTC GTT CTT GCT GT	69	45	1015244238-15062-16853	
B. catenulatum	F_cate_IS	GTG GAC GCG AGC AAT GC	58	65	1015335268-99-20718	67
	R_cate_IS	AAT AGA GCC TGG CGA AAT CG	58	50	1015335364-1571-12175	
	P_cate_IS	AAG CAA ACG ATG ACA TCA	68	39	1015335455-2899-17859	
B. dentium	F_dent_IS	CCG CCA CCC ACA GTC T	59	71	1015399643-15856-19947	150
	R_dent_IS	AGC AAA GGG AAA CAC CAT GTT T	59	41	1015399751-16991-11210	
	P_dent_IS	ACG CGT CCA ACG GA	70	64	1015399833-18158-5198	
B. infantis	F_inf_IS	CGC GAG CAA AAC AAT GGT T[a]	58	47	1037961234-06371-14364	76
	R_inf_IS	AAC GAT CGA AAC GAA CAA TAG AGT T	58	36	1037961263-06691-25461	
	P_inf_IS	TTC GAA ATC AAC AGC AAA A[a]	69	32	1037961294-06967-17477	
B. longum	F_long_IS	TGG AAG ACG TCG TTG GCT TT	59	50	1015323391-27595-22257	109
	R_long_IS	ATC GCG CCA GGC	58	56	1015323469-28673-	

		AAA A[a]			23147	
	P_long_IS	CGC ACC CAC CGC A	68	77	1015488566-4529-13934	
All bifidobacteria	F_allbif_IS	GGG ATG CTG GTG TGG AAG AGA	60	57	1015399960-19603-31240	231[a]
	R_allbif_IS	TGC TCG CGT CCA CTA TCC AGT	60	57	1015400076-20827-17418	
	P_allbif_IS	TCA AAC CAC CAC GCG CCA	70	61	1015400166-21749-18424	
L. acidophilus	F_acid_IS	GAA AGA GCC CAA ACC AAG TGA TT	59	43	1089017502-26171-202965955840	85
	R_acid_IS	CTT CCC AGA TAA TTC AAC TAT CGC TTA	59	37	1089017571-27139-52545094772	
	P_acid_IS	TAC CAC TTT GCA GTC CTA CA	70	45	1089017717-29310-154296055415	
L. casei	F_case_IS	CTA TAA GTA AGC TTT GAT CCG GAG ATT T	59	36	1037022798-023495-2136	132
	R_case_IS	CTT CCT GCG GGT ACT GAG ATG T	59	55	1037022917-024843-29627	
	P_case_IS	ACA AGC TAT GAA TTC ACT TGC	70	38	1037022752-023005-20772	
L. delbrueckii	F_delb_IS	CAC TTG TAC GTT GAA AAC TGA ATA TCT TAA[a]	58	30	1089018504-4206-64529811906	94
	R_delb_IS	CGA ACT CTC TCG GTC GCT TT	58	55	1089018475-6841-166657768151	
	P_delb_IS	CCG AGA ATC ATT GAG ATC	68	44	1089018437-6309-163988227498	
L. fermentum	F_ferm_IS	AAC CGA GAA CAC CGC GTT AT	58	50	1036676682-09669-23287	88
	R_ferm_IS	ACT TAA CCT TAC TGA TCG TAG ATC AGT CA	58	38	1036676709-010209-2351	
	P_ferm_IS	TAA TCG CAT ACT CAA CTA A	68	32	1036676736-010547-20717	
L. paracasei	F_paca_IS	ACA TCA GTG TAT TGC TTG TCA GTG AAT AC	60	38	1038306417-016220-23561	80
	R_paca_IS	CCT GCG GGT ACT GAG ATG TTT C	60	55	1038306445-016796-3050	
	P_paca_IS	TGC CGC CGG CCA G	70	85	1038306524-018375-2626	
L. plantarum	F_plan_IS	TGG ATC ACC TCC TTT CTA AGG AAT	58	42	1038305707-03107-18756	144
	R_plan_IS	TGT TCT CGG TTT CAT TAT GAA AAA ATA[a]	58	26	1038305742-04177-12861	
	P_plan_IS	ACA TTC TTC GAA ACT TTG T	68	32	1038305778-04682-12880	
L. reuteri	F_reut_IS	ACC GAG AAC ACC GCG TTA TTT	59	48	1089025339-29395-129280047216	93
	R_reut_IS	CAT AAC TTA ACC TAA ACA ATC AAA GAT TGT CT	59	28	1089025385-30347-37558232754	
	P_reut_IS	ATC GCT AAC TCA ATT AAT	69	28	1089025413-30287-26112845854	
L. rhamnosus	F_rham_IS	CGG CTG GAT CAC CTC CTT T	59	58	1023708254-09591-2284	97

	R_rham_IS	GCT TGA GGG TAA TCC CCT CAA	59	52	1023708352-010389-16127	
	P_rham_IS	CCT GCA CAC ACG AAA	69	55	1023708453-011313-6655	
Lactobacillus spp.	F_alllact_IS	TGG ATG CCT TGG CAC TAG GA	58	55	1024485925-024664-30598	92
	R_alllact_IS	AAA TCT CCG GAT CAA AGC TTA CTT AT	58	35	1024478788-024701-16287	
	P_alllact_IS	TAT TAG TTC CGT CCT TCA TC	68	40	1024478009-017753-28422	
All bacteria	F_eub	TCC TAC GGG AGG CAG CAG T	59		Reference [160]	466[a]
	R_eub	GGA CTA CCA GGG TAT CTA ATC CTG TT	58			
	P_eub	CGT ATT ACC GCG GCT GCT GGC AC	70			

[a]In these cases, concessions to the probe and primer design had to be made (more than three consecutive nucleotides are the same or amplicon length is greater then 150 bp). According to Haarman et al. [126, 127].

9.5 Study sheets

9.5.1 Parent information sheet

PREBIOTICS AND THEIR INFLUENCE ON THE IMMUNE SYSTEM OF PREGNANT WOMEN AND THEIR NEWBORNS

Dear Parents,

During the last few years allergic diseases have become more and more prevalent among children. Although many investigations indicate that environmental influences during pregnancy are relevant for the development of allergies in later life, the exact reasons for this increase are still unclear. Among other factors, the predominance of certain gut bacteria like lactobacilli and bifidobacteria are supposed to have protective effects. With this letter we want to inform you about a study, which examines the influence of prebiotics on the gut microbiota and the immune system of pregnant women and their newborns.

This study is carried out at the Children's Hospital of the Ludwig-Maximilians-University of Munich, Department of Metabolic Disease and Nutrition, "Dr. von Haunerschen Kinderspital" under the supervision of Prof. Dr. Berthold V.Koletzko and Dr. Susanne Krauss-Etschmann.

Prebiotics are naturally occurring food carbohydrates, which we cannot digest but which specifically stimulate the growth of bifidobacteria and lactobacilli. These bacteria predominate in the gut of healthy breast fed infants and are supposed to protect against various enteric infections. In addition, lactobacilli protect the vagina from infections with fungi and other harmful bacteria. One of these harmful bacteria are streptococci, which cause almost no symptoms in pregnant women, but can lead to life threatening infections in newborns. According to newer results, it appears that the consumption of certain lactobacilli during infancy can reduce the occurrence of allergies in the first two years by almost 50%. Therefore, our study wants to examine the influence of dietary long-term supplementation with prebiotics during pregnancy on the composition of the maternal and neonatal gut microbiota. We also want to assess whether prebiotic supplementation stabilizes the vaginal colonization with lactobacilli and thereby reduces the incidence of bacterial vaginosis.

Furthermore, we want to analyse whether prebiotics can influence the immune system of newborns and lead to a reduction of allergy incidence.

If you are interested in participating in this study, we would like to ask you for your cooperation:

We will provide you with a prebiotic or maltodextrin supplementation in form of sachets. You will be consuming three sachets per day from the 25th week of gestation until delivery. Before study begin we will collect two stool samples form you. Additional two samples will be collected towards study end (shortly before delivery). We also ask you to weekly check your vaginal pH with the help of CarePlan VpH test gloves (which you will be receiving from us). Additionally, we ask you for a singular blood sample of 10 mL, which will be taken from you during the routine medical check-up at delivery.

Furthermore, placental venous cord blood will be collected at delivery to assess possible effects on the neonatal immune system. Later on, we will ask you for stool samples from your child at the 5th and 20th day of life. Serious risks for you and your child exist at no time point of the study. According to the regulations, however, a study participant insurance was signed (Aon Nederland V 001100031366). The data from the examinations will be saved electronically. Within the frame of the corresponding regulatory instructions, only the examining doctors, as well as authorized collaborators will have access to the confidential data, in which you and your child are mentioned by name. These persons are under professional discretion and are obliged to data protection. Anonymous data will only be passed on for statistical and scientific reasons.

Your participation in the study is voluntarily.

You can of course withdraw your participation agreement at any time point of the study without any reasons and disadvantages for you and your child. Questions about the content and subject of the study will be answered by Dr. Susanne Krauss-Etschmann and members of the study team at any time point (telephone: 089/51607706).

Your advantage:

During the study we will inform you about your and your child's stool sample results. If you decide to take part in the study, we kindly ask you to declare with your signature that you have been informed about the content and the course of the study and that you agree to participate.

Thank you very much!

9.5.2 Inclusion & exclusion criteria sheet

INCLUSION & EXCLUSION CRITERIA

Inclusion criteria:

- Healthy pregnant woman > 18 and < 40 years
- Signed informed consent
- No acute illness
- No treatment with antibiotics
- No regular consumption of probiotic or prebiotic supplements
- Planned delivery at the Obstetrics Hospital or the Department of Obstetrics & Gynaecology of the Ludwig-Maximilians-University of Munich
- Planned vaginal delivery

Exclusion criteria:

- Gestational age above the 24th week at enrolment
- Intention to donate cord blood stem cells
- Acute illness or chronic inflammatory bowel disease (Crohn's disease, ulcerative colitis or gestational diabetes)
- Allergies are not an exclusion criterion!!!!!
- Consumption of anti-inflammatory drugs (cortisone, acetylsalicylic acid, non-steroidal anti-inflammatory drugs)
- Planned caesarean section
- Perinatal asphyxia (5 min APGAR < 7.0; cord blood pH < 7.2)
- Clinical or laboratory signs for a neonatal or maternal infection at delivery
- Obvious malformation of the newborn
- Interruption of the supplementation for longer than two days and more than two times

9.5.3 Informed consent sheet

INFORMED CONSENT

PREBIOTICS AND THEIR INFLUENCE ON THE IMMUNE SYSTEM OF PREGNANT WOMEN AND THEIR NEWBORNS – A CLINICAL INTERVENTION STUDY

Surname: _____

First name: _____

Date of birth: _____

Code: _____

I here with confirm that the study conditions were completely explained to me.

A copy of the enclosed form, containing the study conditions was given to me.

All questions were answered to my satisfaction. Possible risks and disadvantages were explained to me. I know that I can now and at any other future time point ask questions considering the study and the examination results.

I consent that my health data are recorded within the scope of the clinical examination, that they can be viewed by authorized representatives of the sponsors of the clinical study, the monitoring authority or the federal authority and that they can – as far as the reference to my person is not recognizable from these data – be passed on to the sponsors of the clinical study and the above mentioned authorities for inspection reasons. I was assured that my and my child's data remain confidential at any time point.

Information concerning my or my child's identity will not be passed on or printed.

I know that I and my child can withdraw from the study at any time point.

I further understand that - if the study is not completed or if I withdraw from the study at any time point - the quality of the medical treatment for me and my baby at the Obstetrics Hospital of the Ludwig-Maximilians-University of Munich (I. UFK) will not be affected.

I agree to take part: Munich, the _____

_____ _____

Signature of the participant Signature of the informing staff

9.5.4 Instruction sheet

INSTRUCTIONS

Stool samples:

In your envelope you will find two tubes containing spatula containers for **stool sampling**.

Please use the spatula to collect a **small** amount of stool (~ size of a pea) and close the containers properly. Please label the tube with your **name** and **birth day**. Your data will be coded later on. Please store the tube **immediately** after sample withdrawal in the **freezer**.

There are definitely no hygienic risks with the correct withdrawal technique.

Please inform us as soon as you have collected the sample by calling: **089 / 5160 – 7706 or 089 / 5160 – 7796.** You can also leave a message on the answering machine. We will then come and collect the samples within 24 hours.

Questionnaires:

In the envelope you will find a **questionnaire.** Please fill out the questionnaire (1) as **complete as possible and bring it with you to the next check-up appointment**. There is evidence from many studies that there is a connection between the bacteriological colonization of the gut and the hygienic conditions or the current lifestyle in the early childhood. Furthermore, diseases and medication can be of some relevance. The asked questions are important and are based on medical examinations, which have already been performed.

General information:

If you have any further questions, e.g. concerning the questionnaire or the sample withdrawal, please ask us at your next check up appointment or directly call us at any time point. We assure that your personal data will be deleted, so that later on an assignment to the sample will not be possible.

Thank you very much for your cooperation!

9.6 Study questionnaires

9.6.1 Questionnaire 1: Allergies

ALLERGY QUESTIONNAIRE (Q1)

Name:					
Code & ID:		**Mother**		**Father**	
Nr.	**Questions about the parents**	Yes	No	Yes	No
1.	Do you or did you ever have asthma?				
2.	Has this been confirmed by a doctor?				
3.	Did you have an asthma attack in the last 12 month?				
4.	Do you take any medicine (inhalation, spray, pills) against asthma at the moment? If yes, which? _____				
5.	Do you or did you have hay fever, rhinitis or allergic conjunctivitis?				
6.	Do you or did you suffer - without having a cold - from a congested or running nose and/or swollen, itchy eyes and this regularly in the spring and summer time or almost every time you deal with specific fur or feather carrying animals?				
7.	Do you or did you ever suffer from neurodermatitis (also called endogenous eczema or atopic dermatitis)?				
8.	Do or did you have hives also called urticaria, with wheals like after a nettle contact/and or swelling of the lips and eyes?				
9.	Do you regularly react allergic to certain foods (urticaria, worsening of eczema, nausea, vomiting, diarrhoea, asthma)?				
10.	Do you regularly take medicine? If yes, which? _____ _____				
11.	Do you smoke? If yes how many cigarettes per day? _____				
	The following questions are referred to siblings (not half brothers and sisters) of your baby?				
12.	Does your baby have siblings (no half brothers and sisters)? If you have answered question 12 with no, please omit the following questions. If you have answered question 12 with yes, please answer the following questions:				

		Sibling 1		**Sibling 2**	
	Questions about the siblings:	Yes	No	Yes	No
13.	Did your child ever have asthma?				
14.	Did your child ever have hay fever?				
15.	Did your child have sneezing attacks, a running, congested, or itchy nose although he/she was not suffering from a cold?				
16.	Did your child ever have an itchy skin rash, which was more or less severe over the last 6 months?				
17.	Did your child ever have neurodermatitis? (also called endogenous eczema or atopic dermatitis)?				
18.	Does your child regularly suffer from certain food allergies (urticaria, worsening of eczema, nausea, vomiting, diarrhoea, asthma)?				

Thank you very much for your cooperation!

9.6.2 Questionnaire 2: Stool frequency, consistency & regurgitation

STOOL FREQUENCY, CONSISTENCY & REGURGITATION QUESTIONNAIRE (Q2)

Name:			
Code & ID:			
Please weekly fill out the following table: enter how often you suffered from regurgitation as well as your daily average stool frequency and consistency according to the codes below.			
Gestational age	**Regurgitation**	**Stool frequency**	**Stool consistency**
25th week			
26th week			
27th week			
28th week			
29th week			
30th week			
31st week			
32nd week			
33rd week			
34th week			
35th week			
36th week			
37th week			
38th week			
39th week			
40th week			
	1 = no regurgitation 2 = regurgitation good to bear 3 = regurgitation just to bear 4 = regurgitation almost not to bear 5 = regurgitation unbearable	average per day	1 = no stool 2 = very hard and painful stool 3 = hard stool 4 = soft and formed stool 5 = soft and non-formed stool 6 = diarrhoea

Thank you very much for your cooperation!

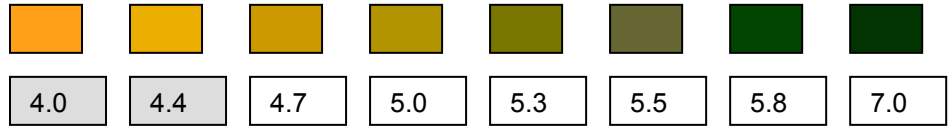
9.6.3 Questionnaire 3: Vaginal pH

VAGINAL PH QUESTIONNAIRE (Q3)

Name:	
Code & ID:	
Please measure your vaginal pH with the enclosed CarePlan VpH test gloves once a week and enter the vaginal pH value into the table with the help of the colour scale below.	
Gestational age	**Vaginal pH**
25th week	
26th week	
27th week	
28th week	
29th week	
30th week	
31st week	
32nd week	
33rd week	
34th week	
35th week	
36th week	
37th week	
38th week	
39th week	
40th week	

A disturbance of the vaginal microbiota is often accompanied by infections, which can increase the risk of early birth or abortion. In many cases measuring vaginal pH allows an early recognition of a disturbed flora. The weekly control of your vaginal pH during pregnancy can improve your chances for an early detection of an infection and a timely doctoral supervision. You should visit the doctor if the pH value is above 4.4.

Colour scale for reading the pH-values:



9.6.4 Questionnaire 4: Compliance & tolerance

COMPLIANCE & TOLERANCE QUESTIONNAIRE (Q4)

Dear study participant!

First of all we would like to thank you for taking part in this study.

The purpose of the following questionnaire is to find out how good pregnant women tolerate the supplementation. We therefore ask you to read the following questions carefully before answering them. Important: Most questions can be answered at the end of your pregnancy. Please note that the tables with the questions concerning regurgitation or reflux, stool frequency and consistency (questionnaire 2) as well as vaginal pH (questionnaire 3) must be answered weekly.

Name: _____

Code/ID: _____ **Date of birth:** _____

1. What is your martial status?

□ unmarried

□ married

2. What is your nationality? _____

3. If you are not from Germany, how long have you been living here? _____years

4. What is your highest school education?

□ No school education

□ CSE

□ Secondary school or similar certificate

□ Technical college

□ General qualification for university

5. What is your highest job qualification?

□ No job qualification

□ Finished apprenticeship

□ Master craftsman's certificate

□ Vocational school degree

□ Technical college degree

□ University degree

6. If you had stool irregularities, of what kind were they?

_____

_____

7. Did you stop taking the sachets for longer than 2 days?

□ Never

□ Once

□ Twice

□ More than twice

8. How good did you tolerate the sachets?

□ Very good

□ Good

□ Not so good

□ Not at all

9. If you did not tolerate the sachets so good or if you did not tolerate them at all:

Why was the tolerance not so good? What kind of problems or complaints did you have?

_____

_____

10. How intensive were your complaints?

□ I had no complaints

□ Good to bear

□ Bearable

□ Almost unbearable

□ Unbearable

11. If you had complaints, how long did these usually last?

□ Only short

□ Up to one hour

□ Several hours

□ The whole day

10. Did you stop taking the sachets before the end of your pregnancy?

□ Yes

□ No

13. If you stopped taking the sachets, what were the reasons?

_____

Thank you very much for your cooperation!

9.6.5 Questionnaire 5: Follow-up interview

FOLLOW-UP INTERVIEW (Q5 SHORT VERSION)

Name: _____

Code & ID: _____ **Date of birth:** _____

Interviewer ID: _____ **Date of interview:**_____

1. What is the name of your child? _____
2. When was your child born? _____
3. What kind of milk did your child get in the first 3 three days after birth?

□ Breast milk

□ Formulae milk

□ Others: _____

4. What kind of milk did your child get in the first 6 months?

□ Breast milk

□ Formulae milk

□ Others: _____

5. Are you still breast feeding at the moment?

□ Yes, exclusively

□ Yes, but not exclusively

□ No

6. Have you started giving solid food to your child?

□ Yes

□ No

7. If yes, what kind of solid food did you give your child? _____
8. How old was you child when your started giving solid food? _____
9. In the last 6 months, did your child ever suffer from

□ Cold

□ Fever

□ Cough

□ Atopic eczema

□ Food allergy

10. Did your child receive antibiotics in the first 3 weeks after birth?

□ Yes

□ No

11. If yes, which antibiotics did your child get? _____

Why? _____and for how long? _____and how often? _____

12. Did your child receive antibiotics in the last 5 months?

□ Yes

□ No

13. If yes, which antibiotics did your child get ?_____

Why? _____and for how long? _____and how often? _____

Thank you very much for your cooperation!

9.7 Acknowledgements

This work was performed at the Div. of Metabolic Disease and Nutrition, Children's Hospital LMUin the group of Prof. Dr. Berthold V. Koletzko under the supervision of Dr. Susanne Krauss-Etschmann, Clinical Cooperation Group "Paediatric Immune Regulation", of the GSF National Research Centre for Environment and Health, Institute of Molecular Immunology.
The study was supported by a grant from the "Child Health Foundation" and a grant by NUMICO Research.

First of all, I would like to thank Prof. B. V. Koletzko for giving me the opportunity to work on the study described in this thesis, for his constant interest in my work and for supporting me in getting the grant from "Child Health Foundation".

I am particularly grateful Prof. Dr. Dolores J. Schendel for employing me as a PhD student at the GSF Institute of Molecular Immunology, for giving me the opportunity to present my results at different GSF seminars and diverse conferences and for reviewing my thesis regarding the correct use of the English language.

Special thanks go to my supervisor Dr. S. Krauss-Etschmann, for her continuous support and encouragement during the last two and a half years, for her suggestions, ideas, discussions and her helpful advice in analysing and interpreting the data. I would like to thank you for giving me the opportunity to participate in scientific events and workshops and to contribute to other research projects of the CCG. Thanks also for showing me that successful research and having a family is not impossible.

I also want to thank Dr. Christopher Beermann and his team form NUMICO Research, Friedrichsdorf, Germany, for initiating the study and providing the study supplements, as well as Dr. Jan Knol and Monique Haarman from NUMICO Research, Wageningen, Netherlands, for performing the bacterial stool analysis and for helping me with the interpretation of the microbiological data.

Thanks also to Mrs. Hildegard Debertin for helping me with the recruitment of the study participants, as well as Prof. Dr. Franz Kainer, Dr. Dorothea Rjosk-Dendorfer and the nurses of the Obstetrics Hospital and the Department of Obstetrics & Gynaecology, LMU for

supporting me throughout the study. My sincere appreciation goes to the study participants for their willingness to participate in this pilot study and their cooperativeness throughout the whole clinical trail. I also want to thank Dr. Hans Demmelmair (Division of Metabolic Diseases and Nutritional Medicine, LMU) for the fruitful scientific discussions and for always helping me out whenever I faced problems. Many thanks go to PD Dr. Christine Falk (Institute of Molecular Immunology, GSF) for contributing her expertise with the Bioplex-assays.

I am also indebted to my colleagues for providing a stimulating and friendly environment in the lab. Thanks also to all my friends, who constantly supported and encouraged me and never grew tired of listening to my reports.

I dedicate this work to my parents and my brother. I whish to thank them for their ever-reliable encouragement and belief in me, for their patience and understanding and for always being there for me. Without them it would have been impossible for me to successfully finish my dissertation.

9.8 Curriculum vitae

Personal data

Name:	Rania SHADID
Address:	Marktplatz 4, 83714 Miesbach
Telephone:	0049-8025-1037 or 0049-0174-6583279
E-mail:	shadidrania@hotmail.com
Date of birth:	18.03.1977
Place of birth:	Tripolis
Martial status:	Single
Nationality:	German

Education

1997 - 2002	Master's degree in Biology (M.Sc.), Ludwig-Maximilians-University LMU, Munich, Germany Major subject: Microbiology Minor subjects: Biochemistry, Pharmacology & Toxicology, Ecology Experimental Master's Thesis at the, Department of Biochemistry, Gene Centre Munich, in the Laboratory of Professor Dr. R. Grosschedl Project: „Analysis of the specificity of LEF-1 SUMOylation by different E3-Ligases".
9/2003 - 6/2004	Intensive course Economies, Distance University Hagen (IWW)

Job Experience

12/2002 - 11/2003	Scientist at Tecan Munich GmbH, Proteomics Division
3/2004 - Present	Experimental/clincial PhD thesis at the Children's Hospital of the LMU in the Clinical Cooperation Group: CCG Paediatric Immune Regulation of the GSF Institute of Molecular Immunology, Munich, in the Laboratory of Dr. S. Krauss-Etschmann Project: „Effects of prebiotic supplementation during pregnancy on maternal and neonatal gut microbiota, as well as on selected foetal immune parameters - a randomized, double-blind, placebo-controlled pilot study".

Stipends

3/2004 - 9/2004	Grant from the charitable "Child Health Foundation"
6/2006	ESPGHAN Young Investigators Award

Languages Fluent in English and Arabic, French: school level

9.9 Publication and abstract list

POSTERS and PRESENTATIONS

Effect of maternal prebiotic supplementation on selected foetal immune parameters.
(presented at the 36th annual meeting of the DGfI German Society of Immunology, DGfI Congress 2005, Kiel, Germany)
R. Shadid, C. S. Falk, D. J. Schendel, C. Beermann, B. Stahl, S. Krauss-Etschmann, B. V. Koletzko; Immunobiology 201: p 420; 2005

Effect of maternal prebiotic supplementation on maternal and foetal microbiota as well as on selected neonatal immune parameters
(presented at the 39th annual meeting of the European Society for Paediatric Gastroenterology Hepatology and Nutrition, ESPGHAN Congress 2006, Dresden, Germany)
R. Shadid, M. Haarman, J. Knol, C. Beermann, D. Rjosk-Dendorfer, D. J. Schendel, B. V. Koletzko, S. Krauss-Etschmann; Journal of Pediatric Gastroenetrology and Nutrition 42(5); 2006

Comprehensive analysis of the T cell chemokine receptor expression profile in cord blood versus peripheral adult blood.
(presented at the 16th European Congress of Immunology, ECI Congress 2006, Paris, France)
R. Shadid, D. Rjosk-Dendorfer, D. Reinhardt, D. J. Schendel, S. Krauss-Etschmann; abstract book p: 472, PA-3138, 2006

Modulation of human dendritic cells by seven different probiotic strains.
(presented at the 16th European Congress of Immunology, ECI Congress 2006, Paris, France)
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SEMINARS

Effect of prebiotics on the immune system of pregnant women and their offspring
(presented at the GSF National Research Centre for Environment and Health, Institute of Molecular Immunology, GSF Seminar 2004, Großhadern)

Effect of prebiotics on the immune system of pregnant women and their babies
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Effect of prebiotics on the immune system of pregnant women and their babies
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NUHEAL-Study
(presented at the Childrens Hospital of the LMU, Division of Metabolic Diseases and Nutritional Medicine, LMU Seminar 2005, Munich)

Effects of prebiotic supplementation during pregnancy on the maternal and neonatal gut microbiota and selected fetal immune parameters
(presented at the Childrens Hospital of the LMU, Division of Metabolic Diseases and Nutritional Medicine, LMU Seminar 2006, Munich)

Immune effects of n-3 fatty acid supply in pregnancy
(presented at the Scientific Workshop on Early Nutrition and Immunity 2006, Obergurgl)
S. Krauss-Etschmann, R. Shadid, H. Demmelmair, B. V. Koletzko, NUHEAL study group

PUBLICATIONS (manuscripts submitted or in preparation)

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