Gut microbiota and allergies in children from farming families

The FARMFLORA cohort

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To Mathias, Ville & Gustav

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ABSTRACT

Allergies have increased in industrialized countries, including Sweden, especially in the 1970´s to 1990´s. An increasingly hygienic lifestyle with less exposure to bacteria and other microbes has been suggested as a contributing factor, based e.g. on epidemiological studies linking factors associated with increased microbial exposure, like farm living, older siblings or pets, to protection from later allergy. Microbial exposure in early life may stimulate the developing immune system in a way that promotes the maturation of tolerance mechanisms that prevent allergy. The gut microbiota is an important stimulus for the immune system, and its composition and development may have implications for later allergy. Here, we followed the gut colonization pattern by quantitative culture of fecal samples obtained over the first 18 months of life in the FARMFLORA birth cohort, consisting of children living on small dairy farms or in the same rural area but not on farms, in Southwest Sweden. Major facultative and anaerobic bacteria were identified, and we also studied the lactobacillus flora in more detail. The maturation of the gut microbiota was mirrored in an increasing ratio of anaerobic to facultative bacterial counts with increasing age. Colonization patterns were related to farm living, pet exposure, siblings and other exposures, and to allergy at 3 or 8 years of age. A farming environment was associated with an increased ratio of anaerobic to facultative bacteria in feces at 1 week of age and lower number of *E. coli*, indicating early acquisition of anaerobes able to suppress facultative bacteria. Farmers' infants also harbored *Clostridioides difficile* less frequently by 1 year of age, indicating a more mature gut microbiota able to suppress this

bacterium. Contact with household pets was associated with more frequent colonization by several anaerobic bacteria early in life. Being firstborn, delivered by cesarian section or exposed to antibiotics during delivery were associated with delayed colonization by typical fecal bacteria, higher numbers of facultative bacteria in the early gut microbiota, and less colonization by certain species of lactobacilli. Firstborn infants showed signs of delayed gut microbiota maturation, with increased colonization by *C. difficile* and a low ratio of anaerobic to facultative bacterial counts in the gut microbiota by one year of age. Allergy was negatively associated with a high ratio of anaerobic to facultative bacteria in feces and colonization by anaerobic bacteria early in life, and with several species of lactobacilli. Colonization by *C. difficile* was instead associated with an increased risk of allergy. Taken together, our findings suggest that farm living and household pets, but also older siblings have an impact on the early gut microbiota. The effects of these exposures on the gut microbiota may contribute to their protective effect against allergy development, but further studies are needed to confirm the observations made in this small birth cohort study.

Keywords: infant, microbiota, gut, farm, pets, allergy, siblings, bacterial culture

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Sammanfattning på svenska

Allergier har ökat i industrialiserade länder, inklusive Sverige, särskilt under senare delen av 1900-talet. En del i förklaringen tros ligga i en allt renare livsstil med ökad hygien, och därmed en minskad exponering för bakterier och andra mikrober. Barn som växer upp på bondgård har i flera epidemiologiska studier visat sig ha en lägre risk att utveckla allergi än andra, och detta har också setts hos barn med äldre syskon, och barn som växer upp med husdjur. Vad detta beror på är inte klarlagt, men en möjlig förklaring är att dessa barn i högre grad exponeras för mikrober än andra barn, och på så vis snabbare utvecklar en mer komplex, mogen tarmflora. Tarmfloran kan bidra till att stimulera immunsystemets utveckling så att det inte reagerar på ofarliga ämnen, och därmed bidra till att minska risken för allergiutveckling. Hos nyfödda sker en gradvis kolonisering av tarmen, där de första bakteriena som etablerar sig är de som klarar sig i såväl syrefattig som syrerik miljö, s.k. fakultativa bakterier, men med tiden domineras tarmfloran alltmer av anaeroba bakterier som trivs i den syrefattiga miljön i tarmen, och tarmflorans mognad speglas av en ökande andel anaeroba bakterier i jämförelse med fakultativa. I den här studien följde vi tarmflorans utveckling under de första 18 månaderna efter födseln hos barn från uppväxta på bondgård i sydvästra Sverige och andra barn från samma område på landet i FARMFLORA-kohorten. Tarmflorans utveckling och mognad relaterades till bland annat bondgård, äldre syskon och husdjur i familjen, samt till allergi vid 3 eller 8 års ålder. Vi kunde se att barn som växte upp på bondgård eller med husdjur, men även barn med äldre syskon hade en tarmflora med större andel anaeroba bakterier i förhållande till de fakultativa i avföringen redan vid 1 veckas ålder, och att barnen som växt upp på bondgård i mindre utsträckning var koloniserade med *C. difficile* vid ett års ålder, en bakterie som kan tolkas som ett tecken på en mindre mogen tarmflora. Vi kunde även se att flera anaeroba bakterier var vanligare hos barn med husdjur. Barn utan äldre syskon visade tecken på fördröjd mognad av tarmfloran, med en låg andel anaeroba bakterier i förhållande till fakultativa bakterier i tarmfloran vid ett års ålder. Vi kunde också se att de barn som inte var allergiska i större utsträckning hade en tarmflora med större andel anaeroba bakterier i förhållande till de fakultativa. Sammantaget tyder våra resultat på bondgårdsmiljö och husdjur, men även äldre syskon, påverkar den tidiga tarmfloran vilket kan bidra till dessa faktorers skydd mot allergiutveckling, men fler, större studier behövs för att bekräfta dessa resultat.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Ljung A*, Gio-Batta M*, Hesselmar B, Imberg H, Rabe H, Nowrouzian FL, Johansen S, Törnhage CJ, Lindhagen G, Ceder M, Lundell AC, Rudin A, Wold AE, Adlerberth I. Gut microbiota markers in early childhood are linked to farm living, pets in household and allergy. *PLoS One*. 2024;19(11):e0313078. doi:10.1371/journal.pone.0313078

II. Ljung A*, Gio-Batta M*, Hesselmar B, Imberg H, Rabe H, Nowrouzian FL, Johansen S, Wold AE, Adlerberth I. Major gut microbiota perturbations in firstborn infants compared to those with older siblings soon after delivery. BMC Pediatr. 2025 Oct 7;25(1):780. doi: 10.1186/s12887-025-06015-7.

III. Ljung A, Nowrouzian FL, Hesselmar B, Imberg H, Rabe H, Lindgren Å, Johansen S, Törnhage CJ, Lundell AC, Wold AE, Adlerberth I..Gut colonization in rural infants by certain species of lactobacilli is associated with reduced risk of allergy later in childhood. *Manuscript in preparation.*

*Shared first authorship

CONTENTS

ABBREVIATIONS

APC	Antigen presenting cell
CFU	Colony-forming unit
CoNS	Coagulase-negative staphylococci
DC	Dendritic cell
GEE	Generalized estimating equations
IgA, IgM, IgD	Immunoglobulin A, M, D
IL	Interleukin
LPS	Lipopolysaccharide
MLN	Mesenteric lymph node
PCR	Polymerase Chain Reaction
SCFA	Short chain fatty acid
SIgA	Secretory immunoglobulin A
Th cell	T helper cell
Treg	Regulatory T cell

INTRODUCTION

The human body is colonized by a large number of bacteria. These bacteria inhabit both the skin, the respiratory tract and the alimentary tract, the majority being so called commensal bacteria that normally does not cause disease. The gastrointestinal of an adult is inhabited by several hundred different species, forming a complex ecosystem, referred to as the gut microbiota. The development of the gut microbiota starts at birth, and the infant gut microbiota is far less complex than that of an adult. Changes in diversity and counts then take place over the first years of life and aerobic and facultative bacteria are successively outnumbered and replaced by anaerobic bacteria. The bacteria forming the gut microbiota are acquired from outside sources, and the composition can be influenced by, for instance, delivery mode and feeding pattern, and other environmental factors. The gut microbiota may also affect human health where, for instance, a low diversity of the microbiota in early childhood has been associated with later development of atopic diseases such as asthma and eczema. The mechanism behind this effect is not known, but one possible explanation is the potential of the microbiota to stimulate the developing immune system in early infancy and thereby promote the maturation of immunological tolerance mechanisms. Growing up on a dairy farm is associated with a lower risk of allergy development at school age, as is contact with household pets and older siblings. This effect might be due to an increased exposure to bacteria in the farming environment, from farm animals, pets or older children. In this thesis we investigated colonization by major groups of facultative and anaerobic bacteria and also in detail colonization by different species of lactobacilli in the gut microbiota over the first 18 months after birth in the FARMFLORA cohort in relation to a number of predefined environmental- and life style factors. We also aimed to identify potential associations between the colonization pattern by bacterial groups and species and a doctor's diagnosis of allergy at 3 and 8 years of age in this cohort.

BACTERIAL CLASSIFICATION AND STRUCTURE

It has been estimated that there are 10^7-10^9 individual species of bacteria and 4-6 x 10^{30} procaryote cells in the world (1, 2).The first observation of bacteria was made in 1674 by the Dutch naturalist Antonie van Leeuwenhoek in a self-made light microscope (3). He named them *animalcules*, "little animals", as their behaviour in the water drops examined in the microscope resembled the movements of animals.

Taxonomy, (from Greek: taxis ("arrangement"), and nomos ("law") is the systematic classification used for naming organisms based on shared characteristics. Each taxonomic group is further specified in taxonomic levels, where each level contains organisms with increasing similarities. This hierarchical system for organizing living organisms is based on the Linnaean classification system described in the work *Systema Naturæ*, written by Carl von Linné in the middle of the 18´th century, in which the taxonomic levels kingdom, class, order, genus and species were introduced. Early classifications of bacteria were based on morphology and biochemical traits. Today, the relationships between different bacterial groups are explored using molecular genetic techniques, giving new knowledge leading to the re-naming and re-classification of many bacteria.

In 1990, Carl Woese proposed the use of a three-domain system, with domain sorting above kingdom, dividing cellular life into Bacteria, Archeae and Eucaryota (Figure 1), based on his own phylogenetic studies performed in the 1960´s and 1970´s (4, 5). In addition to this, the taxonomic levels used today also include phylum, family and subspecies. The different taxonomic levels go from the broadest or "highest", i.e. domain, to kingdom, phylum, class, order, family, genus, species and sometimes subspecies. In this thesis, bacteria are mostly discussed at the genus or species level. The species name is composed of the genus name followed by the name specific for the species, i.e. *Escherichia* (genus), *Escherichia coli* (species).

Bacteria belong to the procaryotes, meaning they are unicellular organisms, that lack many compounds existing in eucaryotic cells, for instance nucleus, Golgi apparatus and endoplasmatic reticulum. Their genetic material consists of one single circular chromosome in the cytoplasm, which also contains ribosomes and is surrounded by the cytoplasmic membrane. Most bacteria also

have a cell wall, a structure that is not found in human cells and therefore have the potential to activate the immune system. The cell wall can be one of two types, and depending on the cell wall structure they stain differently in the traditional Gram-staining, which is the basis for the grouping of bacteria into two groups, Gram-positive and Gram-negative bacteria.

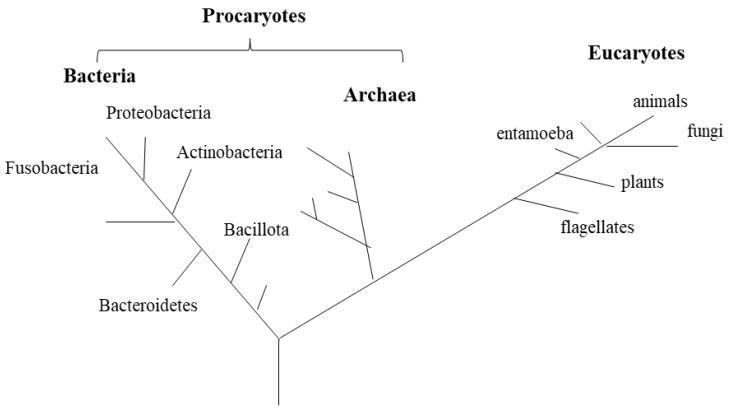

Figure 1: The tree of life. Illustration of the classification of cellular life into different groups. The eucaryotes on the right include organisms with a cell nucleus. The procaryotes on the left include organisms belonging to the domains Bacteria and Archaea, and are single-celled organisms with no cell nucleus

Gram-stain

The technique of Gram-staining was first published by Hans Christian Gram in 1884. This method is still widely in use, even though it has undergone some modifications since 1884 (6). Gram-staining is done in four steps, blue staining by crystal violet, fixation with iodine, alcohol treatment and counterstaining with saffranine.

In the first step, cultured bacteria are dyed using Crystal Violet followed by fixation with iodine. In the next step, the preparate is treated with alcohol, a decolorizing agent, and thereafter a red dye, Safranin is added. Cells that keep the blue color (from Crystal Violet) are defined as Gram-positive, while those

who lose the blue color when treated with alcohol and are colored red by Safranine and termed Gram-negative.

The reason why the cell is dyed blue or red is explained by the difference in structure of the cell wall of the Gram-positive and Gram-negative bacteria, schematically illustrated in Figure 2.

Gram-positive bacteria

Gram-positive bacteria have a thick cell wall, built up by up to 50 layers of peptidoglycan polymers, consisting of long chains of the alternating sugars N-acetylglucosamine and N-acetylmuramic acid, connected by peptide chains. Most Gram-positive bacteria also have teichoic acids and lipoteichoic acids anchored in their cell wall, that helps maintain the cell's structure and also have an antigenic function (7).

Gram-negative bacteria

Gram-negative bacteria, on the other hand, have a thin cell wall, consisting often of only one or a few layers of peptidoglycan covered by an outer membrane which is linked to the cell wall by lipoproteins. The outer membrane contains membrane proteins with various functions, and lipopolysaccharides, LPS. LPS, also called endotoxin, functions as a strong danger signal (8), i.e. a molecule that activates the innate immune system and evokes inflammation. LPS is made up by Lipid A, which is responsible for the strong immune stimulation by LPS (9, 10), a core region made up by sugars, divided in an inner core, attached to the Lipid A, and an outer core, and a variable, sometimes branched chain of repeating oligosaccharide units attached to the outer core, the O-chain, or O-antigen.

When bacteria are Gram-stained, the crystal violet and the iodine added in the staining procedure are believed to form complexes with proteins within the cells. These are trapped within the thick cell wall of the Gram-positive bacteria, giving the cell its blue color. In Gram-negative bacteria, which have little peptidoglycan, the lipid membranes are damaged by the alcohol during the staining, the membrane proteins are denaturated and the blue dye complexes leaks out, leading to decolorization. When counterstained with safranine, the cell is instead dyed red.

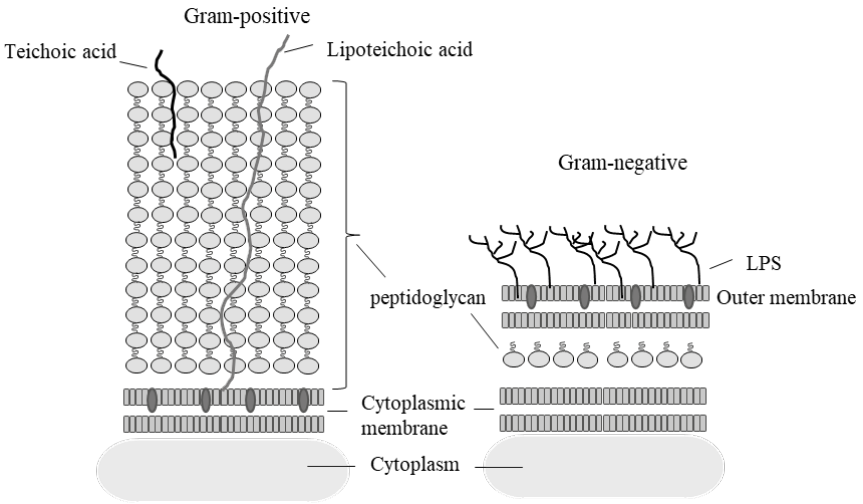

Figure 2: Schematic illustration of the Gram-positive and Gram-negative cell wall. Gram-positive bacteria (left) have a cell wall made up of up to 50 layers of peptidoglycan. The Gram-negative cell wall (right) is much thinner and surrounded by an outer membrane which contains lipopolysaccharide (LPS).

Aerobic, facultative anaerobic and obligate anaerobic bacteria

Bacteria are also described based on their growth characteristics and their use of oxygen in metabolism, and can be divided into obligate aerobic, facultative anaerobic or anaerobic bacteria.

Obligate aerobic bacteria like *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and *Micrococcus luteus* can only survive under aerobic conditions and rely on oxygen as the final electron acceptor in their energy production (aerobe respiration), where carbohydrates are turned into energy (ATP), carbon dioxide and water. Anaerobic bacteria cannot use oxygen in their metabolism and rely on other energy generating reactions. Their metabolism includes fermentation, where they convert organic substances like carbohydrates into lactic acid or short chain fatty acids (e.g. acetate, butyrate and propionate), alcohols or gases. These processes yield much less energy than aerobic respiration. Obligate anaerobic bacteria, including e.g. *Fusobacterium* and *Bacteroides*, cannot survive and many of them die rapidly in the presence of oxygen. This is because they have no or very low levels of the enzyme superoxide dismutase, which neutralizes reactive oxygen species in e.g.

aerobic bacteria. Some anaerobic bacteria can tolerate and even replicate in the presence of oxygen and are hence termed aerotolerant anaerobic bacteria. Lactobacilli, bifidobacteria and *Actinomyces* are examples of such bacteria. Facultative anaerobic bacteria grow and replicate under both aerobic and anaerobic conditions. When deprived of oxygen they have the ability to use fermentation for energy production but since aerobe respiration gives much more energy, they grow far better under aerobic conditions. Groups belonging to the facultative anaerobes are, among many, *Enterobacteriaceae,* staphylococci and enterococci, all common human colonizers in different body sites.

COLONIZATION OF THE HUMAN BODY

Microbiota is a word used to describe the microbial community (or collection of microbes) in a specific environment (11, 12). In humans, the term can be used for microbial communities at several different locations in and on the body and they may include, besides bacteria, yeasts and, in the gut, archae. The skin, nasopharynx and upper respiratory tract as well as the mouth, the gastrointestinal tract and the vagina all have a microbiota adapted to the environment characterizing each location, while the lower respiratory tract including the lungs, and the mucosa of the bladder and the uterus are normally sterile.

Skin

Bacteria colonizing the skin are mainly Gram-positive bacteria like coagulase negative staphylococci (CoNS, e.g. *S. epidermidis*, *S. hominis*) and *Staphylococcus aureus*, *Micrococcus*, corynebacteria, cutibacteria and propionibacteria, but also yeasts (13-15). The bacteria reside in superficial layers of epidermis, and in the upper parts of hair follicles and glandular ducts. The number and types of bacteria on the skin varies at different sites, where dry sites are less colonized, while moist and warm sites provide a more hospitable milieu for bacteria, and are more densely colonized (16-18).

Airways

The bacterial colonization in the nasopharynx and the upper respiratory tract is similar to that of the oral flora (see below) and includes commensal bacteria like *Streptococcus viridans*, CoNS and corynebacteria, but also potential pathogens, like *Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis,* all common in small children but to a lesser extent in adults. The sinuses and the middle ear, also part of the upper respiratory tract, are normally sterile.

Vagina

The vaginal microbiota of most of fertile women is dominated by lactobacilli, and the most commonly occurring species are *Lactobacillus crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* (19, 20). Lactobacilli are important for upholding the low pH in the vaginal mucosa due to their fermentation of sugars into lactic acid. The low pH also inhibits growth of other bacteria and colonization by lactobacilli may thereby help to protect against infections (21). Other bacteria that are common in the vaginal microbiota, but which are normally present in lower numbers, including anaerobes like for instance *Prevotella* spp (22). Streptococci may also be a part of the vaginal flora, and *Streptococcus agalactiae*, Group B- streptococci, can be transferred during vaginal delivery and cause infection in the new-born infant (23).

The alimentary tract

The alimentary tract is made up by the mouth, the oesophagus and the gastro-intestinal system, along with accessory organs such as salivary glands, pancreas and gallbladder. The bacterial colonization varies in richness and density from the mouth and through the gastrointestinal channel.

The mouth

The mouth contains several different surfaces for bacterial growth, where the tounge, cheeks, tonsils, teeth and dental pockets are all habitat to a large variety of bacteria. The most dominant species in the mucosa of the mouth are *Streptococcus mitis* and *S. oralis*. The genera *Neisseria*, *Veillonella*, *Eikenella* and *Prevotella* are found in high proportions in saliva and the back of the

tounge, gingiva and the cheeks, whereas *Actinomyces* are found on the gums, and lactobacilli can be found in the crypts of the tounge (24-27). Bacteria residing in the mouth are collected in the saliva and swallowed, transferring bacteria from the oral cavity to the stomach via the oesophagus.

Oesophagus

It is not known if the esophagus is actually colonized by any bacteria in healthy individuals, or if the bacteria isolated from this site only represent those swallowed with the saliva. The bacteria most commonly found belong to the genera *Streptococcus, Haemophilus, Neisseria*, *Prevotella* and *Veillonella* (28), i.e. bacteria common also in the oral cavity.

The stomach

The acidic content of the stomach is designed to be a defense against pathogens, and the low pH, varying between 1.5 to 3.5 (29), makes it a harsh environment for many bacteria. Few of the bacteria which are swallowed survive once they reach the stomach. One exception is the well known colonizer *Helicobacter pylori* which survives and colonizes the mucosa due to its ability to produce urease, which shields it from the stomach acid (30, 31). There are studies on the microbiota of the stomach using DNA-based analyses that suggest that other bacteria may colonize the stomach as well (32, 33). However, since DNA-based methods cannot discriminate between living and dead bacteria, this may lead to an overestimation of the variety of bacteria actually colonizing the stomach.

The small intestine

The small intestine is made up by the duodenum, jejunum and ileum. The counts of bacteria colonizing the small intestine is relatively low, especially in the upper bowel, and the colonizing bacterial groups are similar to those in the mouth, like streptococci, *Veillonella*, *Gemella* and *Rothia* (34). Near the stomach, peristalsis is rapid and the environment is rich in oxygen, and the bacterial groups found in the first parts of the small intestine are mainly facultatives and aerotolerant anaerobes, such as streptococci, lactobacilli and enterococci (35, 36). As peristalsis slows down, bacterial counts increase and, as these bacteria consume the oxygen, the atmosphere becomes more anaerobic

and anaerobic bacterial groups like *Bacteroides* and clostridia can colonize, especially near the ileocecal vault where the microbial composition more resembles that of the colon. The population levels increase gradually along the small intestine from 10^2 -10^4 bacteria/ml luminal content in the upper bowel, closest to the stomach, to approximately 10^5-10^8 bacteria/ml in the distal ileum (37, 38).

The large intestine

The microbiota of the colon is the most complex one in humans and microorganisms from all three domains are found. In the colon, the peristaltic movements are slower than in the small intestine, making it possible for bacteria to multiply and reach very high population counts. Differences in colonization within the colon also exists, where some bacterial groups are mainly found in the lumen, while other resides in the mucus layer (39-42).

Bacteria in the adult gut microbiota account for 60% of the fecal mass with 10^{11} bacteria/gram feces (43), and several hundred different species are normally present. The microbiota is dominated by anaerobic bacteria that outnumbers the facultatives by 100-1000:1 (44). Among the anaerobes, bacteria belonging to the phylum Bacillota (Firmicutes) are the most dominant in the adult microbiota, which is confirmed by both culture-based as well as DNA-based methods (45-47). Many species found within the Bacillota group belong to the Clostridial clusters, represented by, among others, *Clostridium*, *Ruminococcus*, *Peptostreptococcus* and *Faecalibacterium* (Table 1).

The Clostridial clusters hold both commensal bacteria and potential pathogens, like *C. perfringens* and *C. septicum*. Another example is *Clostridioides diffficile*, a sporeforming bacterium that is commonly found in infants, but rarely cause infections in this age group (48). Only a few percent of adults harbor *C. difficile* in low population counts in the gut, as it is outcompeted in their more species rich anaerobic microbiota (49). However, in adults, often following treatment with broad-spectrum antibiotics disrupting the gut microbiota, *C. difficile* can expand and reach high population counts, and toxin-producing strains can cause infections of varying severity in adult individuals (50).

Other gut colonizers within the phylum Bacillota are e.g. the genera *Veillonella* and *Lachnospira*, but also lactobacilli, which are described in more detail under a separate heading below. Several different species of lactobacilli can be found in the gut microbiota (51) where *L. plantarum*, *L. rhamnosus* and *L. paracasei* are among the most common (52).

The second largest group in the gut microbiota consists of bacteria belonging to the phylum Bacteroidota, including e.g. the genera *Bacteroides* and *Prevotella* (47). Different species of *Bifidobacterium*, belonging to the phylum Actinobacteria, are other important gut anaerobes.

Facultative anaerobic bacteria make up <1% of the gut microbiota, and members of the *Enterobacteriaceae* family (phylum Proteobacteria) and enterococci (phylum Bacillota) are the most common among the facultatives (38, 53, 54). In adults, *Escherichia coli* is the dominant species of the *Enterobacteriaceae*, but other Gram-negative enterobacteria, e.g. *Klebsiella* and *Enterobacter*, may also be present. Entercocci are Gram-positive bacteria and *Enterococcus faecalis* is the most common enterococcal species in the human gut. Streptococcal species like *S. bovis* and *S. agalctiae* may also be present. Approximately one third of adults may harbor staphylococci (phylum Bacillota) in low counts in the gut and one in ten may be colonized by *Staphylococcus aureus* (55), although these bacteria are mostly recognized as colonizers of the skin.

Table 1:Bacterial phylae and genera found in the colon

Phylum	Class	Genus	Gram
Anaerobic bacteria			
Bacillota	Clostridia	*Clostridium*	+
		Eubacterium	+
		Ruminococcus	+
		Faecalibacterium	+
		Roseburia	+
		Peptostreptococcus	+
		Clostridioides	+
	Bacilli	Lactobacilli*	+
	Negativicutes	*Veillonella*	-
		Megasphera	-
Bacteroidota	Bacteroidia	*Bacteroides*	-
		Parabacteroides	-
		Alistipes	-
Fusobacteriota	Fusobacteriia	*Fusobacterium*	-
Verrucomicrobiota	Verrucomicrobiae	*Akkersmania*	-
Actinomycetota	Actinomycetes	*Bifidobacterium*	+
Facultative bacteria			
Bacillota	Bacilli	*Enterococcus*	+
		Streptococcus	+
		Staphylococcus	+
Proteobacteria	γ-proteobacteria	*Escherichia*	-
		Klebsiella	-
		Enterobacter	-

**Species included in the group lactobacilli are further described in Table 2*

Lactobacilli

Lactobacilli, also belonging to the phylum Bacillota, are found in the gut microbiota as well as at other body sites. Many of the species previously included in the genus *Lactobacillus* have been assigned new genus names but are still referred to as lactobacilli (56), and genera and species commonly isolated from the human gut (51, 57) are shown in Table 2, according to the new nomenclature. Their main metabolism involves fermentation of carbohydrates to lactic acid, and most species are aerotolerant anaerobes. Lactobacilli are found not only in the human normal flora, but also in the gut microbiota of animals, on plants and in food, like dairy products, fermented foods and vegetables (56, 58-61).

Lactobacilli as probiotics

Probiotics, defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (62), is widely used today. Many probiotic preparations include one or several species of lactobacilli, since these bacteria are generally regarded as safe and health-promoting. There are clinical studies that have shown effects of probiotics containing lactobacilli e.g. for the prevention of antibiotic associated diarrhea (63), treatment of rotavirus diarrhea (64, 65), or for the prevention of eczema in childhood (66-68). The mechanisms behind the observed effects are not clear (69). In rare cases, probiotic lactobacilli may cause infection in the patient treated (70).

Table 2: Genera and species of lactobacilli found in the human gut

Genus	Species
Lactobacillus	*Lactobacillus delbruckeii* *Lactobacillus jensenii* *Lactobacillus acidophilus* *Lactobacillus crispatus* *Lactobacillus gasseri*
Lacticaseibacillus	*Lacticaseibacillus paracasei* *Lacticaseibacillus rhamnosus*
Ligilactobacillus	*Ligilactobacillus salivarius* *Ligilactobacillus ruminis*
Limosilactobacillus	*Limosilactobacillus reuteri* *Limosilactobacillus fermentum* *Limosilactobacillus mucosae*
Lactiplantibacillus	*Lactiplantibacillus plantarum*
Latilactobacillus	*Latilactobacillus sakei*

The table shows genera and species of Lactobacilli commonly isolated from the human gut (51, 57) according to their new nomenclature (56).

METHODS FOR CHARACTERIZATION OF THE GUT MICROBIOTA

Historically, bacteriology has been based on visibility, where bacteria have been observed either through a microscope or by the naked eye in broth or on agar plates. Since the first observations by van Leeuwenhoek in the 17´th century, techniques for growing and studying bacteria have developed into the culture media and methods used today. The first large studies of the gut microbiota from the 1970´s were culture based (55, 71-73), and many of the findings made still apply. However, not all bacteria, especially not all obligate

anaerobes, can be cultured. Based on studies comparing cell counts as determined microscopically and quantitative culture of the same bacterial community, it is estimated that 70-80% of bacteria in the gut are not readily cultured (36, 45). The development of DNA-based methods has given new insights to the composition of the human gut microbiota, especially when it comes to detection of fastidious and highly oxygen-sensitive anaerobes.

Culture-based methods

Bacterial culturing

Culture-based methods for identification of bacteria involves the use of different culture media and growth conditions suitable for the detection of different bacterial groups.

Facultative bacteria are cultured in air, while strict and aerotolerant anaerobes are cultured in an anaerobic atmosphere where nitrogen is used instead of oxygen. Some bacteria, like streptococci, grow best in a CO_2 -enriched atmosphere and bacteria like *Helicobacter* and *Campylobacter* are cultured in a microaerophilic gas environment with a maximum of 10 % oxygen. In addition to this, the choice of culture media affects the outcome. The use of non-selective media allows growth of a large variety of bacteria. Usually, a blood agar containing horse blood is used, like Columbia blood agar for facultative bacteria and Brucella blood agar for anaerobes.

The addition of selective media simplifies separation of different bacteria as the composition of the agar enhance or inhibit growth specific bacterial groups. Gram-negative bacteria, like members of the *Enterobacteriaceae* family, are often cultured on Drigalski-agar, which inhibits the growth of Gram positive bacteria, and there are other agars selective for e.g. streptococci, enterococci or staphylococci among others. The same principle applies for selective culture of different anaerobes.

The bacterial population counts in for example a fecal sample can be quantified in a procedure called quantitative culture. This means that a defined portion of the sample (e.g. 100 ml or mg) is resolved in a defined volume of sterile fluid (e.g. peptone water) and then serially diluted (e.g. 1:10) in tubes containing this fluid. A defined portion of each dilution is then spread on agar plates,

incubated and examined. The total number of colonies, or the number of each colony type can then be counted on a plate from a dilution yielding free-lying colonies, and the population counts of all bacteria combined or of each bacterial colony type in the sample can be calculated. Isolates of each colony type can then be subcultured and identified.

Methods for identification of bacterial isolates

Isolates from cultures can be identified to genus or species using several different methods. Colony morphology and growth on selective media under specific conditions and Gram staining can be used to make a rough identification and divide bacteria into groups. Further methods applied are the presence or absence of bacterial enzymes. For example, *S. aureus* has e.g. the enzyme coagulase that convert fibrinogen to fibrin, which is missing in most other staphylococcal species. All staphylococci have catalase, an enzyme that converts hydrogen peroxide to oxygen and water, while this enzyme is lacking in e.g. streptococci and enterococci.

Biochemical methods based on the detection of biochemical processes in the bacteria, like fermentation of sugars and other metabolic processes may be used. This is often called biotyping, and there are several commercial arrays available like API20E for fermenting gram-negative facultative bacteria, mainly species within the *Enterobacteriaceae* family, and Rapid id 32A for anaerobic bacteria.

Mass spectrometry is another method for identification, where Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) is frequently used. This method uses laser to create protein fragments from the selected bacteria, which are ionized and separated, and this creates unique protein profiles that are compared to a reference database for identification to the genus and/or species level.

DNA based methods for the identification of bacterial isolates

Polymerase chain reaction (PCR)

Bacterial genes or nucleotide sequencies which are specific for a genus or a species can also be used for the identification of bacterial isolates, using

polymerase chain reaction (PCR), a method introduced by Kary B. Mullis in 1983 (74, 75) PCR is a method for amplification of a specific nucleotid sequence, achieved by the use of forward and reverse primers with a specific nucleotide sequence, and a DNA-polymerase, the Taq-polymerase, a heat stable polymerase discovered in 1976 in the bacterium *Thermus aquaticus* (76). .

The procedure starts with denaturation of the double-stranded DNA in a heating step, into single stranded DNA. The temperature is then lowered, and the primers anneal to the corresponding nucleotide in the single stranded DNA followed by polymerization, forming a new copy of the double-stranded DNA. This step is then repeated, resulting in a doubling of DNA-fragments in every step.

Real-time PCR includes the use of a probe that is labeled with a fluorophore, and complementary to a nucleotide sequence located between the primers When the Taq-polymerase reaches the probe, the fluorophore is separated from the probe and emits a light signal. The procedure is repeated in cycles, and when the fluorescence from the probe reaches a certain level, it is detected by a sensor (77). The cycle number when the light signal is detected is referred to as the cycle threshold value (Ct value), which is inversed in relation to the number of targeted sequences in the sample, that is, a high number of the targeted sequences in the initial sample requires fewer cycles to reach the detection level.

The PCR product can also be identified using gel-electrophoresis, in which case, no probe is included. Agarose gel-electrophoresis is performed by placing the PCR-products in wells on an Agarose-gel and applying an electrical current at the top end of the agarose-gel. The DNA-fragments, which have a negative charge, are drawn to the positive charge at the bottom of the gel. The gel is dyed with ethidium bromide or a loading buffer, photographed under UV-light and inspected by the naked eye.

Sequencing of the bacterial 16S rRNA gene

The 16s rRNA is located on the small subunit of the bacterial ribosome and present in all bacteria. The gene encoding 16S rRNA is evolutionary stable but contains 9 variable regions, that give rise to genetic variations between

bacterial genera and often also between species within a genus. Sequencing of the 16S rRNA gene is commonly used for identification of bacterial isolates to the genus or species-level. Sequencing means that one determines the precise order of the nucleotides, Adenine (A), Thymine (T), Cytocine (C) and Guanine (G) in a DNA sequence, e.g. a part of a gene, a full gene or a whole genome. Before sequencing, the 16S rRNA gene must be amplified in order to obtain enough material. This requires extraction of DNA from the bacteria, which involves breaking the cell wall. DNA is then purified and the 16S gene, or parts of it, is amplified in PCR using specific primers. The amplicons, e.g.16S rRNA gene-sequences are then sequenced. Traditionally, this has been done using Sanger sequencing (78), where fluorescently labeled "stop"-nucleotides (A, T, C and G) are used to generate DNA fragments of all possible sizes with the gene to be sequenced as template. These fluorescent fragments are then separated based on their size, and as each stop nucleotide generates a specific fluorescence signal that can be detected, the sequence of the gene can be determined and compared with 16S rRNA-gene sequences for thousands of different bacterial species which have been published in public databases.

During the last decades, however, cheaper and much faster sequencing technologies than the Sanger method have been developed, termed next generation sequencing (NGS) methods. Today, these methods are often used for 16S rRNA gene sequencing of bacterial isolates, and they may also be used for sequencing of whole bacterial genomes (whole genome sequencing, WGS), as described below.

Whole genome sequencing of bacterial genomes

Within many bacterial genera, there is not enough sequence variation in the 16S rRNA-gene to allow identification below the genus level. In such cases, sequencing of the whole genome of the bacterial isolate may be required for species identification. Two commonly used sequencing methods are the Illumina and Ion Torrent technologies which allow the simultaneous sequencing of thousands of DNA-fragments.

Illumina sequencing (Illumina Inc, San Diego CA, USA), which was used for whole genome sequencing of some bacterial isolates in the present thesis, is a method first developed in the 1990s (79). To sequence the full genome of a bacterial isolate, DNA from a pure culture of the bacteria is first isolated and

purified. The DNA is cut into thousands of short fragments, and a short DNA-sequence (adapter) representing a primer binding site is coupled to the end of each fragment. This step is called library preparation. Thereafter, each fragment is amplified (by PCR) to yield a high number of copies which are attached to a so called flow cell, where the actual sequencing occurs. Fluorescens-labeled nucleotides, where each of the four different nucleotides has a unique label, are added to the flow cell in cycles. During each cycle, the labeled nucleotide which base-pairs to the next nucleotide on the DNA fragment to be sequenced will be incorporated into the growing DNA chains, and this will release its specific fluorescence signal, which is registered. All the thousands of different short DNA-fragments from the bacterial genome can be sequenced simultaneously in parallel sequencing reactions. The next step involves bioinformatic analysis, where all the short DNA sequences are aligned and assembled to finally yield (almost) the complete genome of the bacterial isolate.

Culture independent methods for characterization of the gut microbiota

Several methods have been developed to determine the composition of the gut microbiota without prior culturing. These are based on analyses of bacterial DNA present in e.g a fecal sample.

One approach that can be applied if the aim is to identify certain pre-defined bacteria in a fecal sample is to use primers recognizing genes (often the 16S rRNA gene) specific for selected genera or species in the gut (80). After extraction and purification of DNA from the fecal sample, the sample can be analyzed using Real-time PCR by adding the primers and probes designed for the specific bacteria one wishes to detect in the sample.

When the aim is to study the full set of bacteria in the gut microbiota, sequencing methods have largely taken over today. The method most commonly used for characterization of the gut microbiota is sequencing of the 16S rRNA gene (or commonly a part of this gene). The next generation sequencing technologies have made it possible to simultaneously sequence 16S rRNA genes from all the bacteria present in a fecal sample in relatively high populations. Examples of sequencing technologies frequently used are

Illumina, which was described briefly above and Ion Torrent (Thermo Fisher Scientific,inc, Waltham, Massachusets, US), in which the incorporation of new nucleotides into the nucleotide chain made with the DNA-fragment to be sequenced as template are detected by the release of hydrogen ions occurring in this reaction (81, 82).

After the amplification of all 16S rRNA-genes in a fecal sample and library preparation, thousands of 16S rRNA gene fragments can be sequenced in parallel using either technology. The information obtained from the sequencing process is then analyzed using bioinformatics, and compared to libraries containing known 16s rRNA sequences for different bacterial groups, forming a list of bacteria present in the original sample.

In recent years, whole genome sequencing has been used in some studies of the gut microbiota (83) which has also been made possibly thanks to the development within sequencing technologies. With this approach, all bacterial DNA is extracted from the fecal sample, and cut into short fragments, which are sequenced. The short sequences obtained are pieced together into longer sequences, or contigs. representing the genes from the different bacteria present in the sample. Certain genes identify the bacteria present in the sample, other genes give information on the metabolic reactions associated with different bacteria. Thus, both taxonomic and functional knowledge about the gut microbiota may be achieved. However, these analyses are very complex as extremely large amounts of data must be handled, and very expensive.

Culture - or -DNA-based methods?

Whether to use culture- or DNA-based methods for characterization of the microbiota is a matter of discussion. There are advantages and disadvantages of both methods, and some of the differences can be summarized as below.

A real advantage of sequencing-based methods, e.g 16S rRNA gene sequencing, is that all bacteria present in high abundances in the gut microbiota can be detected. There are, as mentioned above, a large number of fastidious and highly oxygen-sensitive anaerobic bacteria that are not readily cultured, and DNA-based methods provide very important information on the non-cultivable bacteria in the gut, and on gut microbiota diversity, even though new approaches, culturomics, have increased the possibilities to detect highly

fastidious bacteria also by culture (84). NGS-based methods are also much faster, and today also cheaper than culture-based methods, which are very laborious and time-consuming.

Culture only detects live bacteria, while all DNA-based methods like sequencing of the 16S rRNA gene or the use of species-specific primers to detect specific bacteria will include both living and dead bacteria. This may give a false impression of a colonizing microbiota when, in fact, a varying portion of the DNA-fragments from different bacteria may represent dead bacteria. Another problem is that the thickness of the gram-positive cell wall makes it more difficult to extract DNA from those cells, than from Gram-negative bacteria (85), which may create an image that Gram-negatives are more abundant than Gram-positives in the microbiota.

Another difference between the methodologies concerns the calculation of population counts. The use of quantitative culture permits a precise determination of the population levels of living bacteria in a sample, provided that culture media and conditions support their growth. When using 16S rRNA gene sequencing, the proportions of bacteria in relation to other groups in a sample are often given (relative abundance), but the population levels of different bacteria cannot be established, unless combined with other methods (86). Also, the 16S rRNA gene is present in a varying number of copies in different bacterial groups (87), and this is usually not adjusted for when calculating the relative abundance of different bacteria (86).

The use of selective media during culturing enables the detection and enumeration of some bacteria that may be present only in low population numbers in a fecal sample, e.g. staphylococci. Such bacteria may be missed in a 16S rRNAgene sequencing analysis of a fecal sample if the sequencing depth is not sufficient.

Regarding identification of bacteria, the use of culture based methods often provides better discrimination at the species level, since cultured isolates can be saved and analyzed using a range of different methods if necessary, including biotyping- or DNA-based methods with species-specific PCR or sequencing. When using the most commonly available methods for 16S rRNA microbiota analysis many bacteria can only be identified to the genus or family level which especially applies for e.g. *Enterobacteriaceae* and staphylococci

(85). However, the methods complement each other and combining the results from studies using either of these approaches give new insights into the composition of the gut microbiota.

ESTABLISHMENT AND DEVELOPMENT OF THE GUT MICROBIOTA IN INFANCY

Under normal circumstances, infants are sterile when leaving the womb, and the colonization starts at birth (88). The infant gut microbiota is much less complex than that of an adult and undergoes a gradual change over the first years (89-91) towards a more adult composition, with increasing anaerobic bacterial diversity and an increasing ratio of anaerobic to facultative bacterial population counts. During this period of gut microbiota development, the variability between individuals is also higher than in adults (90, 92).

The environment in the neonatal gut is rich in oxygen, and facultative bacteria able to survive and replicate in this aerobic milieu are the first to colonize. The facultative anaerobes first to establish are *E. coli* and other members of the *Enterobacteriaceae* family such as *Klebsiella* and *Enterobacter*, and Gram-positive facultative bacteria including e.g. enterococci and staphylococci (38, 89, 93, 94) including also *S. aureus* (95).

When these facultative bacteria grow and multiply, they consume the oxygen present in the gut, which in turn creates a more anaerobic environment. Once the oxygen levels are low enough, anaerobes can start to colonize and the first to establish are the more aerotolerant anaerobes such as bifidobacteria and lactobacilli followed by *Bacteroides* and *Clostridium* (89, 96) and during the first 6 months of age, bifidobacteria is ususally the dominant group (90), whereafter *Bacteroides* may become more abundant. In less industrialized countries, the genus *Prevotella* is often more common than *Bacteroides* (97).

As a result of the successive increase in population numbers of anaerobes, the counts of facultative bacteria start to diminish. Some groups, like *Klebsiella* and staphylococci are less adapted to the colon compared to *E. coli*. Therefore, *Klebsiella* mainly colonize infants (38) and population counts of staphylococci decrease more rapidly than those of *E. coli* (95). *Clostridioides difficile* is often found in the first year of life but is rare in older children and adults (49), reflecting that this species is outcompeted in an increasing complex gut

microbiota. From around 6 months of age, a variety of obligate anaerobic bacteria mostly belonging to the phylum Bacillota, like *Faecalibacterium* and *Ruminococcus* start to establish, and a complex and diverse microbiota with a more adult composition will successively take form. As the complexity of the gut bacterial composition increases, all facultative bacteria are successively outnumbered by anaerobes, and the ratio of anaerobic to facultative bacterial population counts increases, from approximately 1.5 :1 in infants to 100-10000:1 in adults (44, 73). The successive development of the infant microbiota with an increasing diversity of anaerobes and successively suppressed population counts of facultatives is often described as maturation and is reflected in the increasing ratio of anaerobic to facultative bacterial counts during early childhood.

SOURCES OF BACTERIA AND FACTORS OF INFLUENCE ON THE INFANT GUT MICROBIOTA

The bacteria colonizing the gut reach the intestines via the mouth. They may be acquired from various sources, and there are a number of factors that may influence the colonization pattern, and the composition and development of the microbiota.

Contact with bacteria during delivery

The infant's first contact with bacteria occurs at delivery. Infants born vaginally come in contact with the mother´s vaginal and often her fecal flora. Bacteria that are transferred from the maternal gut microbiota and shown to persist in the infant gut are *E. coli*, bifidobacteria and *Bacteroides* (98-100). The mother's vaginal flora may be a source of lactobacilli colonizing the infant (101-103), but lactobacilli may also be transferred from the maternal fecal microbiota (104, 105).

Infants born by cesarian section do not come in contact with these typical vaginal or gut bacteria at birth, which may delay colonization, especially by *E. coli*, *Bacteroides* and bifidobacteria (106, 107) and instead give way to other Enterobacteriaceae like *Klebsiella* and *Enterobacter* and enterococci in early infancy (93, 108). Cesarean-delivered infants are also more likely to carry skin bacteria like staphylococci than vaginally delivered ones soon after birth (102).

Among the anaerobes, clostridia are often acquired earlier by cesarean delivered infants than by infants delivered vaginally (93, 108). Cesarean-delivered infants are also more frequently colonized by C. difficile (109). Regarding bifidobacterial colonization, cesarean delivered infants have been shown to catch up in the first month, while *Bacteroides* may take up to a year to reach the same prevalence and population levels as in vaginally born infants (98, 110, 111). When observed, delayed acquisition of lactobacilli in caesarean delivered infants seems to be short-termed (107, 112).

Family members

Bacteria may also come from other members of the family, as the microflora in household members has been shown to resemble one another (113). For instance, skin bacteria like staphylococci are likely to reach the infant gut from the skin of the mother in connection with breastfeeding, or via parents' hands. *S. aureus* is a typical skin bacterium that is more common in the infant than the adult gut microbiota, and has been shown to be transferred from the parents and also to have the ability to persist in the infant gut, possibly due to little competition from traditional gut bacteria in the early gut microbiota (95, 114). There are studies indicating that colonization by typical gut bacteria like e.g. *E. coli* and *Bacteroides* species is delayed today as compared to the 1980:s in industrialized societies, possibly as a result of an increasingly hygienic lifestyle (98).

Bacteria from the parents' oral flora is also likely to pass from parents to infants, for instance when parents clean their childrens' pacifiers by putting them in their own mouth (115) or when infants try foods pre-chewed by the parents (116). Most likely oral bacteria are also transferred when kissing and cuddling the baby. Some oral bacteria may establish in the infant gut. For instance, similar species of lactobacilli may colonize the gut and the mouth (117) and the anaerobe *Veillonella* may also colonize at both sites (118-120).

Siblings may be another important source of bacteria, and children with older siblings have less clostridia but higher abundance of bifidobacteria and *Bacteroides* in the first month (93, 121). They also have a more developed and diverse gut microbiota (107, 121, 122), and have a higher ratio of anaerobe to facultative bacteria in the gut microbiota at one year of age (93). The age gap between siblings also seems to be of importance, even more than the number

of older siblings (121), with a shorter age gap to the older sibling being associated with larger effects on the gut microbiota. The observed effects may be due to a transfer of fecal bacteria from siblings, either from the siblings directly or via parents' hands.

Household pets

Household pets also have their own microbiota, which is readily transferred to the skin of other members of the household (113). This may also affect the gut microbiota of infants, where certain strains of bifidobacteria normally isolated from animals more frequently colonize infants with pets (123). In another study, *Ruminococcus* was enriched in infants with pets, and they also tended to have an increased species richness of Bacillota bacteria in their gut microbiota at 3 months of age (124). Pets may also carry bacteria from the environment with them into the house. The transfer of bacteria from pets to the infant most likely occurs upon direct contact with the fur and skin of pets, but bacteria may also reach the infant gut via transfer from the pets' mouth to the infants' hands.

Day care

Children who attend day care come in contact both with other children, and adults outside of the family. These new social contacts provide a source of bacteria that may establish and affect the composition of the microbiota. Children in day care develop a more diverse and species rich gut microbiota than others of the same age, that also resembles other children within the same group (125, 126).

Environment

Our physical environment is populated by microbes, like for instance lactobacilli and members of the *Enterobacteriaceae* family other than *E. coli*, such as *Klebsiella*. These bacteria can be found in various foods, like fruits and vegetables, and can establish in the infant gut. Clostridial spores are also frequent in various environments (127, 128). Further, fecal bacteria may contaminate the environment under poor hygienic conditions.

Geography and living standards are factors shown to have an impact on the developing microbiota, where infants in rich industrialized countries show delayed colonization by some typical gut bacteria (98) as compared to infants from low- or-middle-income countries, whereas e.g. *S. aureus,* foremost a skin bacterium, has become a more frequent gut colonizer (38, 98, 129). Differences in colonization by fecal bacteria in relation to living standards is also illustrated by early colonization by *E. coli* in cesarean delivered infants in Pakistan, who acquired these bacteria almost as early as vaginally delivered infants, and much earlier than Swedish infants (130, 131). Further, cesarean delivered preterm infants in Nigeria born in the early 1980´s acquired many fecal bacteria as early or almost as early as vaginally delivered preterm infants (132). There are also studies that have observed an earlier and more prevalent colonization with lactobacilli in infants of low- or middle-income countries, as compared to more industrialized, high-income countries (133-135).

Farm living

Children living on farms with animal husbandry are likely to come in contact with a larger variety of microbes than other children (136), which may affect gut microbiota establishment and development. However, few studies have described the gut microbiota of farmers´children, and there are no previous reports on their colonization pattern in the very first weeks of life.

A study of the gut microbiota of 5-13 years old children from farming or anthroposophic families and control children revealed that farmers´ children had higher abundances of some bacteria, putatively *Clostridium* or *Eubacterium* species, than other children, and fewer different dominant bacterial groups in their microbiota than children from anthroposophic families (137). A more recent study described that farmers' children have a more mature gut microbiota at one year of age than non-farmer´s children but reported no effect of farm exposure on the gut microbiota composition at 2 months of age (138). A third study analysed patterns of short chain fatty acids, products of anaerobic bacterial metabolism, in feces of the FARMFLORA children studied in the present thesis and showed that at three years of age the children living on farms had higher levels of iso-butyric, iso-valeric and valeric acid than the control children (139), indicating differences in gut microbiota composition.

Antibiotics

Exposure to antibiotics during delivery is another factor influencing the early gut microbiota. (140), and for example a low relative abundance of *Bacteroides* and high relative abundance of enterococci and *Clostridium* at 3 months with, differences persisting for up to 12 months, especially in sectio delivered infants have been observed(141). The influence of antibiotic treatment on the composition of the early gut microbiota is also observed in infants receceiving antibiotic treatment in the first days after delivery showed, where increased colonization rates by *Enterococcus* in the at 3 days of age, or reduced colonization by bifidobacteria and higher counts of *Enterobacteriaceae* at 1 month, (142), decreased numbers of *Bacteroides* and bifidobacteria (109) have been observed.

Breastmilk and formula

Breastfeeding or formula feeding may also influence the early gut colonization pattern and microbiota development. It is often said that breastfed infants have more lactobacilli and bifidobacteria than formula fed infants, but this is not a consistent finding (38). However, the relative proportion of bifidobacteria may be higher in breastfed infants, since they may have lower counts of other anaerobes, e.g. *Bacteroides* and clostridia (38). Regarding lactobacilli the results vary between studies. In the Swedish birth cohort ALLERGYFLORA colonization by lactobacilli during infancy was investigated in detail. Infants who still received some breastmilk at 6 months of age were more frequently colonized by lactobacilli and also tended to have higher population counts of these bacteria than completely weaned infants, and the only species observed to be more common in breastfed infants was *Lacticaseibacillus* rhamnosus (143).

Formula-fed infants have been observed *E. coli, Bacteroides*, *lactobacilli* and *C. difficile* to be more common than breastfed infants (109) while staphylococci may be more numerous in breastfed infants (38). Cessation of breastfeeding seems to affect the composition of the gut microbiota more than the introduction of other foods, decreasing the occurrence of bifidobacteria, staphylococci, and streptococci (144) and is also associated with an increased microbiota maturation with increasing the prevalence of anaerobic bacteria

belonging to phylum Bacillota (90). Cessation of breastfeeding also lead to increased levels of certain members in the *Lachnospiraceae* family (144).

GUT MICROBIOTA – FUNCTIONS AND EFFECTS ON THE HOST

Infections

The gut microbiota harbors a large population of commensal bacteria with low or no capacity to cause infections in healthy individuals, but also potential pathogens. The lining of the gut acts as a physical barrier, but a pathogen may cross, or translocate, over the epithelial barrier and cause bacteremia, or spread through the bloodstream to other sites of the body. This is more common for facultative bacteria such as *E. coli* and other members of the Enterobacteriaceae family, compared to the obligate anaerobes (145). Facultative gut bacteria may also cause infections if colonizing other body sites, like the urinary or respiratory tract. Even though obligate anaerobes residing in the gut rarely give rise to infections, they may, for example, cause appendicitis and diverticulitis. There are a large variety of bacteria in the gut microbiota that are normally considered harmless, like bifidobacteria, lactobacilli and coagulase negative staphylococci (CoNS). However, also these bacteria may cause opportunistic infections in immunocompromised hosts.

Colonization resistance

While the microbiota contains bacteria with the ability to cause infections, it also has an important function to serve as a first line of defense against pathogens, a mechanism called colonization resistance. Colonization resistance is mediated through competition for nutrients and physical space, and production of inhibitory compounds and which hinders colonization and/or expansion of potential pathogens (146). One example of how colonization resistance works is illustrated by infections caused by the spore-forming bacterium *C. difficile*. When the gut microbiota is disrupted, for instance after treatment with broad spectrum antibiotics, the metabolism is altered which permits *C. difficile* to expand and produce toxins. By administration of feces from healthy donors, fecal microbiota transplant (FMT), the balance in the microbiota is restored, leading to shrinking populations of *C. difficile*, and

regression of symptoms (147). The microbiota in infancy is a developing ecosystem, where new species establish continuously, and, consequently, the colonization resistance provided by this immature microbiota is poor. For example, the infectious dose of salmonella is lower in infants than in adults (148). Also, infants are much more frequently colonized by *C. difficile* than adults, although they do not develop *C. difficile* disease (49).

Metabolism

Gut bacteria are also an important part of the digestive system, and as they proliferate, they use both carbohydrates and proteins as nutrients, leading to production of different metabolites. The carbohydrates come from different sources. Food is one, where, in infants, breastmilk and formulas contain carbohydrates in the form of oligosaccharides which reach the colon. Dietary fibers from solid food is another source in older children and adults, as well as proteins and carbohydrates from shedded epithelial cells and mucus.

Fermentation of carbohydrates by anaerobic bacteria produce short chain fatty acids (SCFA), e.g. acetate, propionate and butyrate. The metabolism differs between bacterial groups meaning that the SCFAs that are produced depend on the composition of the gut microbiota as well as the diet. Also, metabolites produced by some bacteria function as nutrients to others, so called cross feeding (149, 150). For instance, acetate produced by certain bacterial species may be used in the metabolism of other species to produce butyrate.

Among the SCFAs produced by commensal gut bacteria, acetate is formed by bifidobacteria and *Bacteroides* spp (150, 151), while *Veillonellla*, among others, produce propionate (151), and butyrate can be produced by bacteria belonging to the Clostridial group (151). These fatty acids may have several effects on the host. Both butyrate and propionate are an important source of energy for colonic epithelial cells (152, 153), and butyrate has also been observed to increase mucus production (154), and both propionate and butyrate may also influence cell division and differentiation in epithelial cells (155).

Colonocytes may also synthesize glucose from propionic acid (152) which may be released to the blood stream and used by the host. The composition of the microbiota is also believed to be of importance in obesity, as demonstrated

in animal models, where colonization by certain bacterial groups in germ-free mice led to an increase in weight and body fat (156, 157).

Stimulation of the immune system

The gut microbiota is an important stimuli to the developing immune system after birth, as demonstrated in germ free mice where the lymphoid system is underdeveloped compared to colonized animals (158-160). One hypothesis is that the gut microbiota in infancy may stimulate the immune system in a way that promotes the maturation of immunological tolerance mechanisms which may counteract the development of allergy and other immune-mediated diseases (161, 162). This influence of the gut microbiota on the immune system and its possible role in protection from allergy development is discussed further in the sections below about the immune system and allergy, respectively.

THE IMMUNE SYSTEM

Our first defence against microbes are mechanical or chemical barriers, e.g. the superficial cell layers of the skin, mucous production at mucosal sites, ciliae on respiratory epithelial cells and acidic pH in the stomach. Skin and epithelia are also protected by the secretion of small peptides that may damage bacteria and other microbes – antimicrobial peptides (163, 164). Bacteria that manage to cross the epithelial barrier can be eliminated by the innate or adaptive immune system.

The innate immune system

The innate immune system is responsible for the immediate inflammatory response to bacteria and other microorganisms that cross epithelial barriers or reach normally sterile mucosal sites such as the bladder or the lungs. It involves e.g. macrophages and dendritic cells, that react to conserved structures specific to bacteria or other microbes, called pathogen-associated molecular patterns (PAMPs) (165), that bind to receptors on the cell surface, called pattern recognition receptors (PRRs) (166). The activation of macrophages in this way starts a chain of reactions that attracts neutrophils and cells producing cytotoxic compounds, natural killer-cells, to the site of infection. If a microbe is not

eliminated by the innate immune system, it is instead met by the adaptive, or acquired, immune system.

The adaptive immune system

The adaptive immune system is the next line of defence against pathogens and is mediated by lymphocytes, i.e. T- and B-cells. It takes longer to activate, but is more specific and also upholds memory against antigens. Each T- or B-cell has receptors that recognize one specific antigen, e.g. a structure on a specific bacterial species or strain. Upon encounter with their antigen and activation, such a cell may proliferate and give rise to a large number of cells with specificity for the same antigen. Both effector cells, which may directly fight e.g. a specific bacterial intruder, and memory cells, which are long-lived and rapidly activated next time the body encounters the same intruder, are developed.

T-cells are formed in the bone marrow from hematopoietic stem cells, and immature thymocytes are transported to the thymus for further development into CD8+ or CD4+ T-cells. After leaving the thymus, T-cells circulate in the blood and lymphoid vessels, and also accumulate in the spleen and lymphoid tissues. In the lymphoid tissue, naïve CD4+ T-cells are activated upon presentation to its specific antigen by antigen presenting cells (APC), i.e. dendritic cells and macrophages, and differentiate into effector T cells (T helper cells or regulatory T cells, depending on the microenvironment) and memory T-cells (167, 168).

B-cells also originate from hematopoetic stem cells and are produced in the bone marrow. After leaving this site, they mature in the spleen or lymph nodes upon contact with antigens presented by antigen presenting cells, APCs. Naïve B-cells express IgM and IgD (169) but after encounter with its antigen, with the simultaneous help from activated T helper cells, the B-cell starts to proliferate, and the B-cell receptor type is switched to IgG, IgA or IgE, a process called isotype switch (170, 171), and may thereafter differentiate into memory B cells or antibody-producing plasma cells.

T helper cells can differentiate into subtypes, e.g. Th1, Th2, and Th17 that have different functions, and the IgE immune response involved in allergic reactions (172) as well as in the defense against parasitic infections (173) is induced by

Th2 cells. Regulatory T cells may instead deactivate T helper cells and antigen-presenting cells.

The immune system in the gut

The gut associated lymphoid tissue, GALT, is made up by lymphoid tissue beneath the epithelial layer in the lamina propria (Fig 3), organized in the Peyer´s patches, mainly located in the distal ileum (174, 175), and in lymphoid patches in the colon (176). It also includes the mesenteric lymph nodes that drain lymph from the small and large bowel. Bacterial passage over the epithelial layer can be accomplished through various mechanisms. Translocation of bacteria may occur from the gastrointestinal tract over the epithelium (177) or via cells specialized in transporting antigens over the epithelia, like microfold cells, M-cells, which are located above the Peyer´s patches, (178). Antigen presenting cells like macrophages and dendritic cells, DCs, then have the ability to capture and transport antigens and present them to T and B cells in the Peyer´s patches or in the mesenteric lymph nodes (179).

The immune responses elicited in Peyer´s patches and colonic patches towards encountered bacteria are focused on the development of plasma cells that migrate to the lamina propria and produce IgA. The IgA antibodies are then bound to secretory component, transported over the gut epithelium and secreted to the lumen as secretory IgA, SIgA. In studies on Germ-free mice, it has been demonstrated that new colonizers of the gut can translocate and initiate immune responses in the Peyer's patches resulting in production of SIgA by plasma cells in the lamina propria. Once the IgA-response has developed, the translocation of the specific strain ceases, although these bacteria still persist in the gut (158), and a large portion of gut bacteria are coated with secretory IgA (180), indicating that they have elicited specific immune responses. The IgA-producing plasma cells in the human gut are not present in newborns infants but develop over the first weeks of life (181).

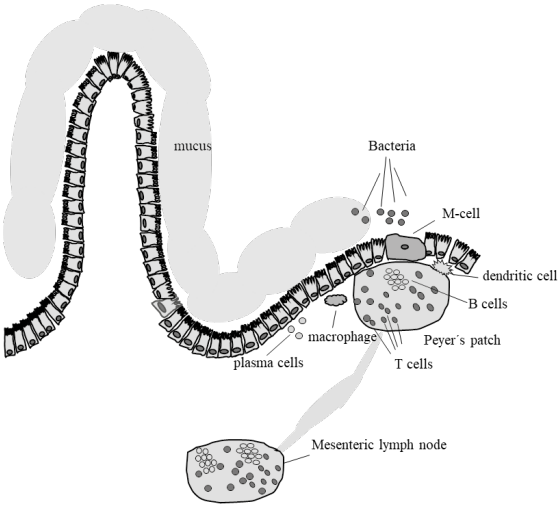

Figure 3: A schematic illustration of a Peyers´patch and a Mesenteric Lymph Node (MLN). The M-cell transports antigen over the epithelum. Antigen presenting cells (APC) like macrophages and dendritic cells, (DC), can then capture the antigens and present them to T and B cells in the Peyer´s patches or in the mesenteric lymph nodes.

IMMUNOLOGICAL TOLERANCE MECHANISMS

Central tolerance

Another important function of the adaptive immune system is to uphold tolerance against self-antigens and foreign but harmless antigens to avoid unwanted and potentially harmful reactions to such substances.

During the development of T-cells in the thymus, CD4+ T-cells are presented to a large variety of self-proteins, and T-cells that react with any of these antigens are eliminated, to make sure that the immune system does not react to our own cells, a mechanism called central tolerance (182). However, some CD4+ cells reacting very strongly with self-antigens are not eliminated, but instead develop into regulatory T-cells, Tregs, before leaving the thymus. Their function is to moderate the T-and B-cell responses to self-antigens (183) and Tregs depend on the transcription factor FOXP3 for their development and

function, (183, 184). Tregs may also produce anti-inflammatory cytokines like IL-10 (185) and TGF-b (186).

Oral tolerance

The normal immunological reaction to ingested or inhaled antigens from food or the surrounding environment is active ignorance, or tolerance. This may be achieved by feeding the antigen, which is also called oral (or mucosal) tolerance, a peripherally induced tolerance to harmless antigens. Oral tolerance was demonstrated in pigs already in 1911 (187), and later in mouse models (188-190). The first demonstration of oral tolerance in humans was published in 1994 where feeding of antigen led to reduced skin test reactivity towards this antigen (191). Regulatory T cells are important for upholding oral tolerance. In mouse models, low doses of antigen ingestion led to development of Tregs from naïve T-cells at mucosal sites (192), so called induced Tregs.

The gut microbiota and the immune system

The gut microbiota may stimulate the development of the adaptive immune system in early infancy. Although the mechanisms are not fully understood, it has been demonstrated in animal models that germfree mice have fewer and smaller Peyers´ patches and less developed mesenteric lymph nodes as compared to mice with a normal microbiota (158, 159). Further, they have fewer T-cells and IgA-producing plasma cells in the intestinal lamina propria than conventional mice (158, 193). In pigs, levels of IgA-antibodies in serum have been demonstrated to be higher in colonized as compared to germ-free animals at an early age (194).

The gut microbiota may also support the development of immunological tolerance mechanisms. Regulatory T cells in germ-free mice are less functional (195, 196) and germ-free mice have a more short-lived oral tolerance than colonized mice (161, 197). Studies have shown that colonization of germ-free animals with a defined commensal bacterial microbiota results in the expansion of Tregs in the colonic mucosa (198). Also, the administration of a mixture of strains belonging to clostridial clusters IV and XIV to mice resulted in the proliferation of Tregs in the colonic lamina propria (199). In a mouse study the administration of either a mixture of Clostridiales or Bacteroidales species stimulated the development of Tregs (200) and a very recent study

showed that a mixture of lactobacilli (*L. gasseri, L. reuteri* and *L. rhamnosus*) could increase Treg populations and promote tolerance in mice (201).
Also, some SCFAs produced during bacterial metabolism have the ability to stimulate the development of FOXP3+ regulatory T-cells and increase levels of IL-10, in different mouse models (202-204). Certain other bacterial products may also promote oral tolerance development. Oral feeding with *S. aureus* superantigens is linked to improved oral tolerance in mice (205) and is also shown to lead to an increased concentration of regulatory T cells in the gut mucosa (206).

SENSITIZATION AND ALLERGY

Allergies may have different underlying immunological mechanisms. An IgE-mediated allergy is an immune response involving production of IgE-antibodies towards harmless food or environmental antigens (allergens) which results in symptoms from one or more organ systems, whereas sensitization refers to an IgE-mediated immune response to an allergen which does not necessarily result in symptoms. Atopy refers to the predisposition in an individual to become sensitized and develop and IgE-mediated allergy.

Historically, allergy has been a rare illness, although there are descriptions of symptoms of asthma and rhinitis dating back to the 16`th century, then called “the rose cold” (207, 208). One of the first clinical descriptions of hay fever was made in 1819 in a letter to the Medico-Chirurgical society of London, titled ”Case of a periodical affection of the eyes and chest”, written by John Bostock, a medical doctor in London, based on self-experienced symptoms (209).

Since then, there has been a steady increase in allergies. In Sweden, allergic rhinitis, asthma, and eczema doubled in school children over a short period of time in the 1980´s (210) and in a more recent study from 2015 one third of 5 year-old children were reported to be affected by at least one of asthma, rhinoconjunctivitis or atopic dermatitis (211). Globally, according to the International Study of Asthma and Allergies in Childhood (ISAAC), 11.7%, 8.5% and 7.9% of 6-7-year olds had asthma, rhinoconjunctivitis or eczema, respectively, in 2012 (212). WHO estimated that 262 million people had asthma in 2019 world wide (213).

Sensitization

In some individuals, the ingestion or inhalation of harmless antigens from food or the environment leads to production of specific IgE-antibodies towards the allergen. This is called sensitization (214) and is the first step in the development of an IgE-mediated allergy. Sensitization may occur after inhalation or ingestion of the antigen, most often a protein, or by contact over an impaired skin barrier (215). The allergen is then detected by an antigen-presenting cell (APC), most often a dendritic cell (DC) and presented to naïve T cells. This activates the T-cell, which in turn activates B-cells to transform into IgE-producing plasma cells. The released IgE antibodies bind to mast cells in the tissue, which may then be activated the next time they come in contact with the allergen.

Allergy

In some, but not all, sensitized individuals a second contact with an allergen leads to development of an IgE-mediated allergy, with symptoms caused by the IgE-mediated immune response. Several different organ systems may be affected. Symptoms may include urticaria or rash on the skin, or oedema, (swelling and puffiness of the tissues) around the mouth and eyes. The gastrointestinal tract may also be affected, with vomiting and diarrhea, and the airways with itchy and runny eyes and nose, i.e. allergic rhinoconjunctivitis, and obstruction of the airways especially upon exhaling, i.e. asthma. In severe cases, an anaphylactic reaction can occur, which is a life-threatening condition if not treated. The allergic reaction starts when the allergen, inhaled or ingested, binds to two or more allergen-specific IgE-antibodies on the surface of the mast cell. Granulae containing inflammatory mediators, like histamine and proteases, are then released from the mast cell, leading to the symptoms connected with allergic disease as described above.

Although sensitization does not always lead to allergic disease, the two conditions are associated, with increased risk of asthma and rhinitis at 5 years of age in children who were sensitized at 18 months of age (216) and this elevated risk also relates to atopic dermatitis (217). Children with eczema combined with sensitization have an increased risk of developing asthma, and

the development of allergy, where one allergic disease may be replaced by another, is referred to as “the atopic march” (218).

FACTORS OF INFLUENCE ON ALLERGY DEVELOPMENT

The hygiene hypothesis for allergy development

The increase in allergies in the past decades cannot be explained by genetic changes in the population but is more likely to be the result of changes in the physical environment over the same time-period. In 1989, David Strachan presented a theory, commonly known as the hygiene hypothesis, in which he suggested that the spread of childhood infections somehow trained the immune system in a way that was protective against the development of allergy later in life. He based this on epidemiological observations that hay fever was less common in children with older siblings, and the level of protection increased with the number of older siblings (219, 220). The protective effect of siblings have since then been confirmed in a large number of studies (220). In the late 1990´s, serological studies on Italian recruits showed lower rates of asthma and allergic rhinitis in those with antibodies against Hepatitis A virus (221). Somewhat later, this was also shown for persons with antibodies against *Toxoplasma gondii* and *Helicobacter pylori* while there was no such connection for infections caused by respiratory viruses. This led to the theory that exposure to foodborne microbes that are spread via the fecal-oral route, interpreted as a sign of lower hygienic standard, are negatively associated with asthma and rhinitis, rather than respiratory viral infections (222),

Lack of microbial stimuli from gut microbes was also suggested to contribute to the increasing rates of allergy in industrialized societies (223), based on studies showing a change in gut colonization pattern of infants from industrialized countries over the past decades. Swedish infants were later colonized by commensal gut bacteria such as *E. coli* when compared to infants from Pakistan (131), and also had a slower turn-over of different strains of *E. coli* in their gut (130, 224). The delay in acquisition of *E. coli* and some other typical gut microbes as compared to studies of infants from industrialized societies in the 1970s along with an increase in staphylococci as early gut colonizers could to be the result of increased hygiene (98).

Farm living

Studies from Switzerland and Austria have demonstrated that children growing up on dairy farms have lower risk of allergy at school age than other children (225, 226). Children from three different rural areas in Switzerland showed lower risk for allergic rhinitis in farmers´ children than others from the same area (226), and around the same time, another study saw a reduced incidence of asthma and hay fever in Austrian children growing up on farms (225). In a large cross-sectional survey of school children from Austria, Switzerland and Germany, asthma and hay fever were inversely related to exposure to stables and the consumption of farm milk, and that this protective effect was stronger if the child was exposed to these factors at an early age (<1 year) (227). Close animal contact is another factor linked to the lower risk of allergy development in farmers' children (228). These findings are based on epidemiological studies, and the protective effect of farm living has been suggested to be mediated by an increased exposure to bacterial components, especially endotoxins in the farming environment (229, 230). Farmers´ children have also been shown to encounter a larger variety of microbes than children not living on farms(136) and to have a more mature gut microbiota at one year of age than non-farmer´s children (138).

Household pets

Growing up with pets also seems to have a protective effect against the development of allergy, and early contact with household pets is associated with a lower frequency of allergic rhinitis and asthma at school age (231, 232). This effect is seen especially in children growing up with more than one dog or cat, or with both a cat and a dog (233, 234). Having two or more dogs was also associated with reduced risk of a positive skin prick test at school age (235). It has been suggested that the dose-dependent effect, with less allergy the more cats and dogs the children are exposed to at an early age, may mimic a mini-farm environment, and hence be due to increased microbial exposure (234).

Other factors associated with allergy development

Another factor connected to allergy development is delivery mode, and the association has been shown in several studies, where children delivered by cesarian section are at higher risk of developing food allergy (236), asthma (237, 238) and allergic rhinitis (239). Antibiotic treatment of the child has also been associated with a diagnosis of allergic disease later in childhood. Treatment with more than one group of antibiotics is associated with increased risk, for asthma and allergic rhinitis (240, 241). Also, there are observations on associations between antibiotic treatment in infancy and later asthma development, where, for instance, an association between asthma in 12 -year old children and antibiotic treatment in the first week after delivery was observed in one study (242), while another saw an association between antibiotics administered during the first year of life and asthma later in childhood (243). Antibiotic treatment of the mother during pregnancy slightly increased the risk of atopic dermatitis in the offspring in another study (244).

Gut microbiota and allergy

The associations between the gut microbiota in early life and later allergy have been the subject of studies both regarding the presence of specific bacteria and the diversity of the infant gut microbiota. For instance, high population counts of *E coli* at one month of age have been associated with an increased risk atopic dermatitis while colonization by *C. difficile* has been linked to an increased risk of allergic sensitization, eczema and recurrent wheeze (80) or to a diagnosis of asthma, allergic rhinitis, atopic dermatitis and/or food allergy later in childhood (245). One study including infants in Sweden and Estonia observed associations between reduced early colonization by bifidobacteria or enterococci but increased carriage of *S. aureus* and allergy as diagnosed during the first years of life (246). Also, a group of lactobacilli including *L. rhamnosus, L. paracasei* and *L. casei* have been associated with protection in some studies (247, 248).

Low diversity in the early gut microbiota has in several studies been associated with an increased risk of developing allergic disease later in childhood. Atopic eczema during the first two years of life was associated with a low diversity of the infant's gut microbiota at one week (249, 250) and one month of age (251).

Low diversity at one months of age was also associated with an asthma diagnosis at school age (252). In later years, several studies analyzing the gut microbiota of infants by 16S rRNA gene sequencing have identified associations between delayed gut microbiota maturation at one year of age and later allergy (108, 138, 253, 254). Thus, an increasing number of studies lend support for the hypothesis that composition and development of the gut microbiota during infancy may have implications for later allergy.

AIM

Overall aims

- To describe the development of the gut microbiota over the first 18 months of life in rural children and how it relates to various lifestyle factors and exposures

- To investigate possible associations between the early gut colonization pattern and later allergy development in rural children

Specific aims

- To describe the gut colonization patterns of major groups of culturable gut bacteria over the first 18 months of life in children in the FARMFLORA birth cohort (Paper I)

- To explore if these colonization patterns differ in relation to farm-living and contact with household pets (Paper I), or in relation to birth order, delivery mode and exposure to antibiotics during delivery (Paper II)

- To investigate if the gut colonization patterns of major groups of culturable gut bacteria during infancy relate to allergy as diagnosed at 3 or 8 years of age in the FARMFLORA children (Paper I)

- To describe in detail the gut colonization patterns of individual species of lactobacilli over the first 18 months of life in the FARMFLORA cohort, and relate the colonization patterns to feeding mode and the exposures and lifestyle factors listed above (paper III)

- To explore whether gut colonization by any specific species of lactobacilli in infancy is associated with protection from allergy as diagnosed at 3 or 8 years of age in the FARMFLORA children (paper III)

PATIENTS AND METHODS

THE FARMFLORA COHORT

The FARMFLORA cohort included 65 children born at term in Skaraborg County in southwestern Sweden, 28 from families living on dairy farms with close animal contact, and 37 living in the same rural area but not on a farm (255). Pregnant women were recruited at maternity wards in the area and final inclusion in the study was done at birth. Data on farm living, household pets, older siblings and allergic heredity was collected at interviews, and data on delivery mode and antibiotic treatment of the mother during delivery was obtained from hospital records. Information on feeding patterns including breastfeeding was recorded by parents in journals and the information was collected at children's age 6, 12 and 18 months (256). At 3 and 8 years of age, the children were examined clinically by pediatricians and evaluated for allergic diseases (255, 257). Major characteristics of the cohort are shown in Table 3.

Diagnosis and prevalence of allergy in the FARMFLORA cohort

For the diagnose of allergy, the children were examined by a pediatrician at 3 and 8 years of age and evaluated for symptoms of asthma, atopic eczema, food allergy and allergic rhinoconjunctivitis (ARC). The criteria used for these diagnoses have previously been published (255, 257), and are listed below. Allergy was defined as having one or more of these diagnoses.

Asthma

Asthma at 3 years was defined as ≥3 episodes of wheezing, including at least one after 2 years of age, or ≥4 weeks of persistent wheeze, combined with a) wheezing between colds, eczema, ARC or food allergy; or b) improvement on inhalation treatment with glucocorticoids or leukotriene antagonists. At 8 years of age, asthma was defined as wheeze in the last year combined with: a) reversal of bronchial obstruction by β_2 agonist (>12%); b) bronchial hyper-

responsiveness upon methacholine challenge (PD 20 <0.6 mg); or c) ongoing asthma treatment with inhaled corticosteroids.

Allergic rhinoconjunctivitis (ARC)

A diagnosis of ARC at 3 or 8 years was based on typical symptoms from the eyes and/or the nose in the last year when exposed to pollen or animal dander, combined with a positive specific IgE-test or skin prick test to the suspected allergen(s).

Atopic dermatitis

A diagnosis of atopic dermatitis at 3 or 8 years of age was based on Williams´ criteria (258) or based on the presence of itching spots on typical locations that had come and gone for at least 6 months, including a period in the last year.

Food allergy

Food allergy was diagnosed based on the anamnesis (within the last 12 months) of an immediate or late-onset reaction occurring after intake of the suspected food and improvement upon its elimination from the diet, combined with any of the following: a) other signs of allergy; b) involvement of more than one organ system; or c) challenge tests, allergy tests or biopsies supporting the diagnosis.

Prevalence of allergy

In total, 11 out of 63 children who took part in the 3-year examination were allergic (255), and 10 out of the 48 children participating in the 8-year follow up (257). Five of the ten children who were diagnosed as allergic at 8 years of age, were allergic already at three years of age. Four of the 11 children who were allergic at age three were no longer allergic at age 8 and two were not examined at the later time point.

Table 3: Major characteristics of the full FARMFLORA cohort and of different subgroups

Child characteristics	Total cohort (N=65)	Farming family (N=28)	Pets in household (N=40)	Firstborn (N=29)	Cesarean delivery (N=10)	Antibiotics during delivery (N=10)	Girls (N=32)	Excl brf ≥ 4 mo (N=34)	Allergy 3 years (N=11)	Allergy 8 years (N=10)
Farming family	28 (43)		21 (52)	10 (35)	3 (30)	2 (20)	18 (56)	17 (50)	1 (9)	3 (30)
Pets in household[a]	40 (62)	21 (75)		19 (68)	7 (70)	6 (60)	19 (61)	19 (56)	5 (46)	5 (56)
Firstborn	29 (45)	10 (35)	19 (47)		7 (70)	9 (90)	14 (44)	15 (44)	5 (45)	7 (70)
Cesarean delivery	10 (15)	3 (11)	7 (18)	7 (24)		5 (50)	4 (13)	3 (9)	4 (36)	4 (40)
Antibiotics during delivery[b]	10 (15)	2 (7)	6 (15)	9 (31)	5 (50)		3 (9)	5 (15)	4 (36)	5 (50)
Girls	32 (48)	18 (64)	19 (47)	14 (48)	4 (40)	3 (30)		22 (65)	2 (18)	4 (40)
Excl brf ≥ 4 mo[c,f]	34 (52)	17 (61)	19 (48)	15 (52)	3 (30)	5 (50)	22 (69)		2 (18)	3 (30)
Allergy 3 years[d,f]	11 (17)	1 (4)	5 (13)	5 (17)	4 (40)	4 (40)	2 (7)	2 (6)		5 (50)
Allergy 8 years[e,f]	10 (21)	3 (17)	5 (19)	7 (29)	4 (44)	5 (50)	4 (16)	3 (12)	5 (56)	

[a] Data regarding household pets (cat and/or dog) is missing in one case. [b]Antibiotic treatment of the mother during delivery included cefuroxime (2 cases), cefuroxime and metronidazole (2 cases), benzylpenicillin (1 case) benzylpenicillin and cefuroxime (1 case), and erythromycin (2 cases). Data is missing in 2 cases. [c] Data on feeding pattern including breastfeeding was collected at 6, 12 and 18 mo of age (256) [d] 63 children were examined for allergy at three (255) and [e] 48 at eight years of age (257). [f] Data regarding allergy in relation to cohort characteristics have been published previously (255-257)

COLLECTION AND CULTURE OF FECAL SAMPLES (PAPER I)

Collection and transport of fecal samples

For analysis of the infants´gut microbiota, a rectal swab sample was collected by personel at the maternity ward at infant age 3 days, and fecal samples were collected by the parents at 1 and 2 weeks, and at 1, 2, 4, 6, 12 and 18 months of age (Fig. 4). The samples were placed in airtight bags in which an anaerobic atmosphere was created (AnaeroGen Compact, Oxoid Ltd, Basingstoke, UK) and these were transported to the laboratory where the samples were cultured within 24 hours of collection.

Quantitative culture of fecal samples (Paper I)

The rectal swab obtained at 3 days of age was cultured and incubated in air on one non-selective and three selective media for the detection of the facultative bacterial groups *Enterobacteriaceae*, staphylococci and enterococci, respectively (Fig. 4, Table 4). Fecal samples were cultured quantitatively for the detection and quantification of facultative and anaerobic bacteria, as previously described (98) (Fig. 4). The bacterial groups targeted included the facultatives mentioned above, and the anaerobic bacteria bifidobacteria, lactobacilli, *Bacteroides*, clostridia (anaerobic sporeformers) and *Clostridioides difficile*. Two non-selective media (for total facultative and total anaerobic bacterial counts, respectively) and media selective for each of the targeted bacterial groups, except for clostridia, were used (259-267). Clostridial spores were selected for by alcohol treatment of the fecal samples. The plates were incubated under aerobic or anaerobic conditions, as specified in Table 4.

Population counts of specific bacterial genera, groups or species were calculated from their growth on the selective agar plates intended for their isolation. Bacterial counts were calculated from dilutions yielding 10-100 free lying colonies on the agar plate. First, the number of colonies resembling each colony type identified on the agar plate was counted, and for each colony type, the number of colony forming units (CFU)/ml of the dilution was calculated. The population counts per gram of feces were obtained by dividing this number

by the dilution. The limit of detection was 330 ($10^{2.52}$) CFU/g feces. After subculture and identification of each colony type (see below), the population counts of all colony types identified as the targeted bacteria were summarized, yielding the population counts for the targeted bacterial group.
In a few cases, the targeted bacterial group failed to grow on its selective media but was instead isolated and identified on another type of media. In these cases the sample was registered as positive for this bacterial group, but its' population counts were not calculated.

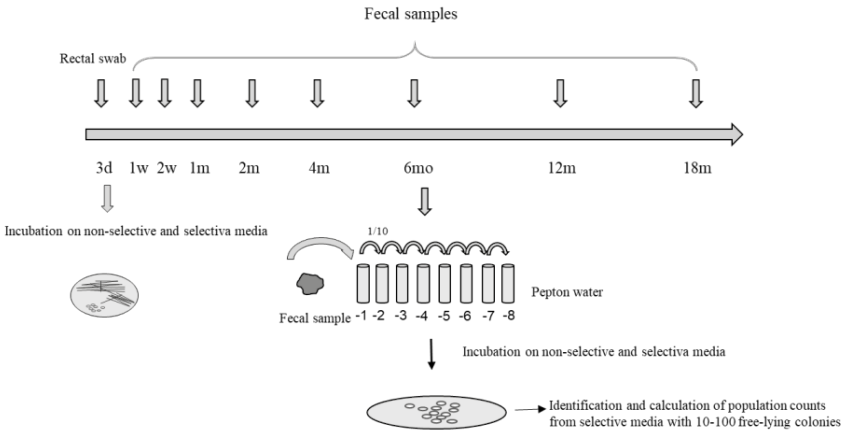

Figure 4: Rectal swabs were streaked directly on agar plates with non-selective and selective media for the detection of facultative bacteria. Fecal samples were cultured quantitatively for the detection and quantification of facultative and anaerobic bacteria. (98). From each fecal sample, 170 µl of feces was diluted in 1.5 ml sterile peptone water and serially diluted 1:10 eight times, whereafter 30 µL from appropriate dilutions were plated on two non-selective media, and agar plates selective for each of the targeted bacterial groups, except for clostridia. (259-267) Clostridial spores were selected for by alcohol treatment of the 1:10 dilution of the fecal samples, which were then further diluted and plated on non-selective media. The plates were incubated under aerobic or anaerobic conditions, as specified in Table 4

Total population counts of facultative and anaerobic bacteria were calculated from counting the total number of colonies on appropriate dilutions on non-selective media, Colombia and Brucella blood agar, respectively.

For the calculation of the total number of anaerobic bacteria, the colony types growing on anaerobically incubated Brucella blood agar were counted separately, isolated and subcultured in air, and the colony counts of isolates growing aerobically were subtracted from the total colony counts, before calculating the total anaerobic bacterial population counts in the sample. Exceptions were made for Gram-positive rods with faint growth under aerobic conditions, as both lactobacilli and bifidobacteria are aerotolerant and may show week growth under aerobic conditions.

IDENTIFICATION OF BACTERIA (PAPER I)

Each colony type growing on selective agar plates were separately enumerated, Gram-stained and subcultured for further identification to the genus or species level as specified in Table 4.

Drigalski agar (261) was used for the isolation of bacteria belonging to the *Enterobacteriaceae* family. Identification of these isolates to the genus or species level was performed using API 20E biotyping system (Bio-Merieux, Marcy l'Etoile, France) according to the manufacturer´s instructions. Staphylococci were isolated from Staphylococcus agar (262), and identification was based on their growth on this selective medium and their Gram-stained appearance, combined with a positive catalase test. The coagulase test was used to separate *Staphylococcus aureus* from coagulase-negative staphylococci (CoNS).
Enterococcosel agar plates (263) were used for the isolation of *Enterococcus* species, i.e. enterococci Enterococci were identified based on their typical Gram-stain appearance, and by their ability to hydrolyse esculin on the Enterococcosel agar plates.

Table 4: Culture media and culture conditions and methods used for the identification of bacterial genera and species

Culture medium	Bacteria identified	Culture condition	time (d)	Samples cultured F=fecal R=rectal	Methods for identification
Colombia blood	Total counts of facultative bacteria	Air	2	F, R	Aerobic growth
Drigalski	*Enterobacteriaceae*	Air	2	F, R	Gram-stain, API 20E[a]
Staphylococcus	*Staphylococcus*	Air	2	F, R	Gram-stain, catalase test, coagulase test
Enterococcosel	*Enterococcus*	Air	2	F, R	Gram-stain, Esculin test
Brucella blood	Total counts of anaerobic bacteria	Anaerobic	3	F	Anaerobic growth[b]
Bacteroides bile esculin	*Bacteroides*	Anaerobic	3	F	Gram-stain, Rapid ID 32A[a]
Beerens	*Bifidobacterium*	Anaerobic	3	F	Gram-stain Genus-specific Real-Time PCR (268)
Rogosa	Lactobacilli	Anaerobic	3	F	Gram-stain Multiplex group- and species specific PCR assays (51) WGS[d]
Brucella blood	*Clostridium*[c]	Anaerobic	3	F	Gram-stain, Rapid ID 32A[a]
CCFA agar	*Clostridioides difficile*	Anaerobic	3	F	Gram-stain, Rapid ID 32A[a]

The fecal swab obtained at 3 days of age was cultured in air for the identification of facultative bacteria. Dilutions of fecal samples were cultured in air and under anaerobic conditions for the identification of facultative and anaerobic bacteria. Two non-selective media (for total facultative and total anaerobic bacterial counts, respectively) and media selective for each of the targeted bacterial groups, except for clostridia, were used (259-267). [a] API biotyping system (Bio-Merieux, Marcy l'Etoile, France. [b] Facultative bacteria were subtracted from the total count. [c] For the isolation of anaerobic spore formers (clostridia), 200 µl of feces diluted 1/10 was mixed with 200 µl of 99% ethanol, shaken in room-temperature for 30 min, diluted 1:10 and cultured from -1, -3 and -5 dilutions. [d] Whole genome sequencing (WGS) (Illumina (Illumina Inc, San Diego CA, USA)) was used for the identification of some isolates of lactobacilli to the species level in paper III.

Regarding anaerobic bacteria, Bacteroides Bile Esculin agar (265) was used for the isolation of *Bacteroides* spp.*,* and spore forming clostridia were isolated from Brucella blood agar plates (264) seeded with ethanol treated dilutions of fecal samples. Colonies from CCFA agar plates, which are selective for *C. difficile* (260) were isolated and further analyzed for the identification of this species. These anaerobes, *Bacteroides* spp, clostridia*, and C. difficile* were identified to the species or genus level using Rapid ID 32 A biotyping system (Bio-Merieux, Marcy l'Etoile, France) according to the manufacturer´s instructions. Some putative clostridial isolates (spore-forming Gram-positive or Gram-variable rod-shaped anaerobic bacteria) failed to grow on subculturing and could not be subjected to Rapid ID 32 A biotyping, but were accepted as clostridia based on their Gram-stained appearance.
Putative bifidobacteria were isolated from Beerens agar (266) and putative lactobacilli from Rogosa agar (267). Different colony types were separately enumerated, subcultured and further identified using PCR based methods as described below. Some isolates of lactobacilli were identified to the species level using whole genome sequencing (see below).
In a few cases, putative bifidobacterial isolates died on subculture and could not be analysed further. These isolates were still defined as *Bifidobacterium* if showing typical bifid or club-shaped forms in the Gram-stained smear.

PCR for the identification of *Bifidobacterium* and lactobacilli (Paper I and III)

Primers and probes used in the analyses

The primers and probe used for identification of bifidobacteria and all primers used for the identification of lactobacilli to the group or species level have previously been published (51, 268). The primers used in the latter analyses are shown in Table 5.

Extraction of bacterial DNA

Extraction of DNA was performed by suspending bacterial colonies in Tris-EDTA buffer (Sigma-Aldrich Sweden AB, Sweden, Stockholm). The suspension was incubated for 10 min at 95ºC and centrifuged in room

temperature for 3 min, and the supernatant was used as DNA template. The same method for DNA extraction was used for both lactobacilli and bifidobacteria.

Real-time PCR for the identification of Bifidobacterium *(Paper I)*

Putative bifidobacterial isolates were identified to the genus level in a real-time PCR largely as described by Penders and coworkers (268). For the PCR, 1 µl of template DNA was added to 12.5 µl HotStarTaq Master Mix (Qiagen GmbH, Hilden, Germany), 0.15 µM probe, 0.3 µM of each primer and RNAse free H_2O in a final volume of 25 µL. The PCR was run on Applied Biosystems 7500 PCR systems (Applied Biosystems, Stockholm, Sweden), and the PCR program used was 95ºC for 10 minutes, followed by 95ºC for 15 seconds and 60ºC for 1 minute for 35 cycles. The results were analyzed using SDS 1.3 Software (Applied Biosystems). A Ct-value >30 was regarded as negative.

Multiplex PCRs for the identification of lactobacilli (Paper I and III)

Lactobacilli were analyzed in two steps for identification to the group or species level (51) (Table 5). The first multiplex PCR, termed PCR-G, identified 4 groups of lactobacilli common in the human gut microbiota, groups I-IV (51, 143). Isolates positive in group I could be directly identified as *L. delbrueckii*, since group I only included this species, but the PCR-G analysis was used only for the identification of lactobacilli as one large group, as reported in **Paper I**. In **Paper III**, isolates positive in any of the groups II-IV were identified to the species level using at least one of 5 species-specific multiplex-PCRs (PCR II:1, PCR II:2, PCR III, PCR IV:1, PCR IV:2). The groups and species identified by the different PCRs are shown in Table 5. The PCR assays were performed largely as previously described (51).

The same protocol was used for the PCR mixture in both the group-specific and the species-specific multiplex PCR assays. The PCR mixture contained 0.4 µl of template DNA, 12.5 µl HotStarTaq Master Mix (Qiagen GmbH, Hilden, Germany), 0.33 µM of each primer and RNAse free H_2O in a final volume of 25 µl.The PCR program was run at 95ºC for 15 min, followed by 35 cycles consisting of denaturation for 20 s at 95ºC and annealing for 2 min, a final cycle for extension for 5 min at 74ºC and cooling at 4º C. This program was used in all assays. The annealing temperature for the PCR-G was 55ºC while

the annealing temperature for the different species-specific PCRs differed depending in the primers used (Table 5).
The PCR products were dyed with Invitrogen Blue Gel Loading Buffer (Thermo Fisher Scientific inc, Waltham, Massachusets, US) and separated by gel electrophoresis, using Invitrogen E-Gel 2 % (Thermo Fisher Scientific,inc Waltham, Massachusets, US). The gel was photographed under UV-light and inspected by the naked eye.

Whole genome sequencing (WGS) for identification of lactobacilli (Paper III)

Isolates of lactobacilli that were positive in PCR-G but which could not be identified in the species-specific PCR-assays were analyzed at Research Institutes of Sweden (RISE), Borås, Sweden. WGS was performed using Illumina sequencing and the data obtained was analyzed using JSpeciesWS Online Service (RiboCon GmbH, Bremen, Germany) (269), as described in detail in **Paper III.**

Table 5: Primers used in multiplex PCRs for the identification of lactobacilli to the group or species level.

Lactobacilli				**Annealing temperature**
Group (PCR-G)	**Group-specific Primers (sequence 5´-3´) F=forward, R=reverse**	**Species within group** (PCR assay)	**Species-specific Primers (sequence 5´-3´) F=forward, R=reverse**	55 °C (PCR-G)
Group I[a]	F Ldel-7 (ACAGATGGATGGAGAGCAGA) R Lac-2 (CCTCTTCGCTCGCCGCTACT)	***L. delbrueckii***		55 °C
Group II	F Lu-1 (ATTGTAGAGCGACCGAGAAG) R Lac2 (CCTCTTCGCTCGCCGCTACT)	***L. Jensenii*** (PCR II:1)	F Ljen-3 (AAGAAGGCACTGAGTACGGA) R 23-10C (CCTTTCCCTCACGGTACTG)	55 °C
		L. acidophilus (PCR II:1)	F Laci-1 (TGCAAAGTGGTAGCGTAAGC) R 23-10C (CCTTTCCCTCACGGTACTG)	55 °C
		L. crispatus (PCR II:2)	F Lcri-1 (AGGATATGGAGAGCAGGAAT) R Lcri-2 (CAACTATCTCTTACACTGCC)	65°C
		L. gasseri (PCR II:2)	F Lgas-1 (AGCGACCGAGAAGAGAGAGA) R Lgas-2 (TGCTATCGCTTCAAGTGCTT)	65°C
Group III	F Lu-5 (CTAGCGGGTGCGACTTTGTT) R Lac2 (CCTCTTCGCTCGCCGCTACT)	***L. paracasei*** (PCR III)	F LU-5 (CTAGCGGGTGCGACTTTGTT) R Lpar-4 (GGCCAGCTATGTATTCACTGA)	62°C
		L. rhamnosus (PCR III)	F LU-5 (CTAGCGGGTGCGACTTTGTT) R RhaII (GCGATGCGAATTTCTATTATT)	62°C
Group IV	F Lu-3 (AAACCGAGAACACCGCGTT) R Lac2 (CCTCTTCGCTCGCCGCTACT)	***L. Salivarius*** (PCR IV:1)	F Lsal-1 (AATCGCTAAACTCATAACCT) R Lsal-2 (CACTCTCTTTGGCTAATCTT)	60°C
		L. reuteri (PCR IV:1)	F Lreu-1 (CAGACAATCTTTGATTGTTTAG) R Lreu-4 (GCTTGTTGGTTTGGGCTCTTC)	60°C
		L. Plantarum (PCR IV:1)	F Lpla-3 (ATTCATAGTCTAGTTGGAGGT) R Lpla-2 (CCTGAACTGAGAGAATTTGA)	60°C
		L. fermentum (PCR IV:2)	F Lfer-3 (ACTAACTTGACTGATCTACGA) R Lfer-4 (TTCACTGCTCAAGTAATCATC)	60°C

Lactobacilli were first identified as belonging to one of four different groups of lactobacilli, groups I, II, III and IV, using a mixture of four forward primers and one reverse primer (51) (PCR-G). [a]Group I only contains *L. delbrueckii* so this species was identified in the group-specific PCR. Isolates positive for Groups II, IIII or IV in this multiplex PCR were further analysed using species-specific primers for identification to the species level, using one or more species specific multiplex PCR assays (PCR II:1 – PCR IV:2) (51)

STATISTICS

For analyses of associations between bacterial variables and the exposures studied, three measures were used: **colonization**, defined as the presence or absence of bacterial groups in feces at each specific time; **bacterial population counts** in colonized infants (CFU/g feces); and the **ratio of anaerobic to facultative** bacterial population counts. Colonization, bacterial population counts, and the anaerobic/facultative ratio were determined at each sampling occasion between 1 week and 18 months of age. For the rectal swab obtained at 3 days of age, only colonization by facultative bacteria was determined.

Colonization by each bacterial group at each sampling occasion was presented as colonization rates. Bacterial population counts were presented as the median values in colonized children, and the anaerobic to facultative ratio as the median ratio of anaerobic/facultative population counts.

Bacterial variables were analyzed using Generalized Estimating Equations (GEE) to account for repeated measures within individuals. A Matérn covariance structure was specified to accommodate unequal time intervals between observations. Binary variables were analyzed using GEE with generalized least squares (working normal model) to estimate proportions and proportion differences between groups. Continuous outcomes were analyzed assuming a log-normal distribution to account for skewness. Robust standard errors (HC3 method) were applied to address potential violations of distributional assumptions. For comparisons at specific time points, time was treated as a categorical variable, and statistical significance was assessed using Wald Z-test for group difference based on the GEE model. GEE was also used for analysis of trends in population counts of each bacterial group from 1 week to 18 months (**Paper I**), but with time as a continuous variable.

The results for bacterial colonization were presented as the rate difference between the study groups (**Paper I**–**III**), and as fold changes for differences in bacterial population counts (**Papers I**–**III**) and in the anaerobic/facultative ratio (**Papers I** and **II**).

Study group characteristics were compared using Fisher's exact test for binary variables (**Papers I**–**III**), and the Mann-Whitney U test for numeric variables (**Paper II**). The Mann-Whitney U test was also applied to changes in bacterial

population counts between sampling points in **Paper III.** Statistical analyses were performed by using SAS/STAT Software, Version 9.4 (SAS Institute Inc., Cary, NC), and IBM SPSS Statistics, Version 29.0 (IBM Corp., Armonk, NY, USA)

RESULTS

Gut colonization by cultivable bacteria in the FARMFLORA cohort (Paper I)

In **Paper I**, the development of the gut microbiota in the FARMFLORA infants was described by determining the colonization rate by major groups of cultivable facultative and anaerobic bacteria over time, and their fecal population counts, during the first 18 months of life. Gut microbiota development was also studied by determining the ratio of anaerobic to facultative bacterial counts in fecal samples at different sampling times.

Colonization by facultative and anaerobic bacteria

At 3 days of age, the most commonly isolated bacteria were coagulase-negative staphylococci, which could be found in over 90 % of the samples obtained using the rectal swab (Table 6). Among the other facultatives, enterococci were isolated from approximately 70%, and *E. coli* from around half of the infants at that sampling time. This was followed by an increase in colonization by all facultative bacteria over the following weeks. However, the colonization rate of CoNS decreased somewhat after 2 weeks of age, and at 2 months enterococci were instead the most common facultative bacteria, isolated in 95% of samples. Colonization by *E. coli* increased quite slowly and was isolated from 90% of the infants at 4 months and continued to increase until 12 months of age (Table 6). All other facultatives had reached their highest colonization rates at 6 months, and remained at the same level, or decreased slowly thereafter. *Staphylococcus aureus* and members of the *Enterobacteriaceae* family other than *E. coli* (termed Non-*E.coli*), were less frequently isolated at all sampling occasions and were also the ones with clearly decreasing colonization rates after 6 months of age (Table 6).

As for the anaerobic bacteria, *Bifidobacterium* spp. were isolated from approximately 80% of the infants already at 1 week and were the most frequently isolated anaerobes still at 4 months of age. At 6 months, clostridia, which increased steadily towards this time point, was slightly more prevalent than bifidobacteria, although both groups were found in most infants. *Bacteroides* spp increased more slowly but was isolated from almost all infants by 12 months of age, whereas lactobacilli, which were found in approximately

60 % of the infants at 1 month, did not reach higher colonization rates. Bifidobacteria, clostridia and *Bacteroides* spp. were all isolated from virtually all children at 12 and 18 months of age. *Clostridioides difficile*, the only anaerobe identified to the species level, was isolated from no more than ~30% of infants at any time, with the highest colonization rates found at 6–12 months of age.

Table 6: Colonization frequencies and median fecal population counts in colonized infants.

Infant age	3d	1w	2w	1m	2m	4m	6m	12m	18m
% colonized									
Facultative bacteria									
E. coli	56	61	63	69	77	89	90	98	93
Non-*E. coli*	4	14	30	34	41	50	65	48	45
Enterococcus	70	75	80	86	95	92	100	98	95
CoNS	94	98	100	89	84	84	85	85	77
S. aureus	22	28	38	45	48	47	49	41	24
Anaerobic bacteria									
Bacteroides		44	43	47	55	57	69	92	98
Bifidobacterium		75	83	85	86	86	83	95	94
Lactobacilli		33	42	65	67	63	62	55	67
Clostridia		48	43	60	63	77	89	95	97
C. difficile		7	8	12	13	23	32	33	18
Median population counts									
Facultative bacteria									
E. coli		9.1	9.2	8.6	8.7	8.7	8.6	8	7.6
Non-*E. coli*		9.2	7.8	7.9	8.2	7.5	7.5	6.8	6.5
Enterococcus		7.1	7.5	7.1	6.9	8.2	7.7	6.5	6.2
CoNS		7.7	7.5	6	4.5	4.5	4.1	4.1	4.1
S. aureus		6.2	6	5.4	4.8	4.5	4.4	4.1	4
Anaerobic bacteria									
Bacteroides		8.4	8.8	9.2	9.1	9.8	9.5	9.4	9.5
Bifidobacterium		9.7	9.9	10	10	9.8	9.7	9.4	9
Lactobacilli		7.6	7.7	8.6	8.2	8.5	8	5.4	5.7
Clostridia		5.4	5.9	5.9	5.8	5.8	6.2	5.9	5.9
C. difficile		3.8	5.1	5.1	4.2	4.2	5.1	4.4	4.1

Colonization frequencies and median fecal population counts (Log_{10} CFU/feces) in colonized infants of bacteria detected in the FARMFLORA cohort at sampling times from 3 days to 18 months of age

Population counts of facultative and anaerobic bacteria

The fecal population counts of facultative bacteria in colonized infants were, in general, higher in *E. coli* and other Enterobacteriaceae than in Gram-positive bacteria (Table 6). The population counts of all facultative bacteria declined throughout the study period, most notable for the staphylococci, where the population counts for both CoNS and *S. aureus* decreased already during the first two months of life. The population counts of Enterobacteriaceae other than *E. coli* (non-*E.coli*) decreased more rapidly than those of *E. coli* (Table 6).

Among the anaerobic bacteria, *Bifidobacterium* spp. had the largest populations in the first 4 months but their population counts declined slowly between 4 and 18 months (Table 6). At 18 months, *Bacteroides* spp. was instead the most abundant of the anaerobic bacteria, and their population counts reached their highest level at 4 months and remained high thereafter. Population levels of lactobacilli in colonized infants were highest between 1 and 4 months and declined more than hundredfold between 6 and 12 months of age. Clostridia (spore counts) and *C. difficile* were present at low population levels in colonized infants at all sampling times.

The overall trend for bacterial population counts showed a significant decrease for all facultative bacteria, reflected in an increasing ratio of anaerobic to facultative bacterial counts, from 1 week of age to 18 months of age. The median population counts of anaerobic and facultative bacteria at different sampling times are shown in Figure 5.

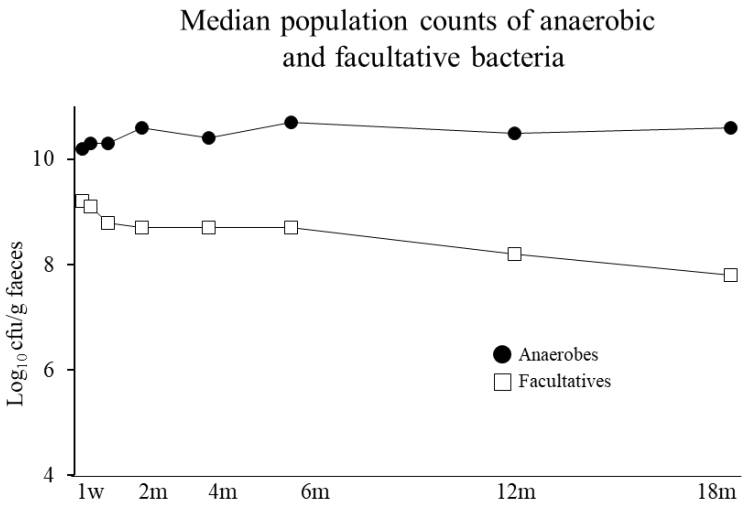

Figure 5: Median fecal population counts Log10 CFU/g feces) in colonized infants of anaerobic and facultative bacteria at sampling times between 1 week and 18 months of age.

Impact of early life exposures on gut microbiota (Paper I and II)

In **Paper I and II**, the gut colonization pattern of facultative and anaerobic bacteria, bacterial population counts in colonized infants and the ratio of anaerobic to facultative bacterial population counts at different sampling times were investigated for associations with early life exposures. In **Paper I**, bacterial variables were analyzed in relation to farm-living and contact with household pets, while in **Paper II**, the exposures studied were presence of older siblings, vaginal or cesarian delivery and antibiotic treatment of the mother during delivery. The results were analyzed unadjusted and adjusted for potential confounders, as specified below. Only findings that were significant in the adjusted analyses were regarded as significant. A summary of major associations between bacterial groups and the exposures studied in **Paper I** and **II** are illustrated in Table 7.

Farm living and household pets in relation to gut microbiota development (paper I)

In **Paper I**, farm living and pets were mutually adjusted for in the statistical analyses, and also adjusted for infants' sex and breastfeeding. Important findings are summarized in Table 7. When we investigated the early gut colonization by facultative and anaerobic bacteria in children growing up in a farming environment compared to controls, there were mostly no clear associations between farm living and colonization rates by most of the facultative and anaerobic bacteria investigated here, with the exception of *C. difficile*, where farmers´ children were much less likely than controls to carry this bacterium at 12 months of age (11% vs 50%). However, farm living was clearly associated with lower population counts of *E. coli* between 1 week and 4 months of age and with an increased ratio of anaerobic to facultative bacterial counts in feces at one week of age (Table 7).

Infants from pet keeping families showed higher colonization rates of both bifidobacteria and *Bacteroides* than other infants. The colonization rate by bifidobacteria was significantly higher in infants with pets at two weeks of age and tended to be somewhat higher up to 4 months, while the increased colonization by *Bacteriodes* was seen up to 6 months of age, and was significant at 4 months. Another bacterial group that in the early period was favored by pet keeping was lactobacilli, which was significantly more frequent at 1 month of age in infants with pets in the family (Table 7). There was also a tendency towards an increased ratio of anaerobic to facultative bacterial counts in feces in pet keeping infants at one week of age, however this finding was not significant after adjustment.

Being firstborn, delivered by cesarian section or exposed to antibiotics during delivery and gut microbiota development (paper II)

In **Paper II** we explored the associations between the presence of older siblings, delivery mode or antibiotics given to the mother during delivery and the same bacterial variables as described above, that is the colonization rates and bacterial population counts of major bacterial groups and the anaerobe/facultative ratio at different sampling times. The three exposures

were clearly interrelated and were mutually adjusted for in the analyses, which were also adjusted for sex and breastfeeding. Important results are summarized in Table 7.

There were several associations between birth order and colonization by different bacterial groups. Firstborn infants had lower colonization rates of bifidobacteria in the first month (Table 7), and a tendency towards less bifidobacterial colonization persisted to at least 4 months of age. They were also less frequently colonized by *E. coli* until two months of age as compared to infants with older siblings. The opposite was seen for colonization by *S. aureus* and *C. difficile*, where being firstborn was instead linked to higher colonization rates by these bacteria. For *S. aureus*, the increased colonization in firstborn infants was significant between 1 and 6 months of age, and for *C. difficile* at 12 months of age (Table 7). Firstborn infants also more frequently harbored Enterobacteriaceae other than *E. coli* and clostridia than infants with older siblings early in life. There were also some differences in bacterial population counts, where firstborn infants had higher population levels of enterococci in the first month, and of *E. coli* at one week, but also at 1 year of age, and at the latter sampling time also the ratio of anaerobic to facultative bacterial counts was decreased in firstborn infants (Table 7).

Table 7: Major findings on colonization rates of bacterial groups, bacterial population counts and anaerobic/facultative ratio in association with exposures studied in Paper I and II.

Colonization rate	Farm	Pets	Firstborn	Cesarean delivery	Antibiotics during delivery
Anaerobic bacteria					
Bifidobacterium		↑ 2w	↓ 1w, 1m		
Bacteroides		↑ 4m			↓ 2w
Lactobacilli		↑ 1m			
Clostridia			↑ 1w	↑ 1m	
C. difficile	↓ 12m		↑ 12m		
Facultative bacteria					
E. coli			↓ 2m	↓ 3d, 1w	
Other *Enterobacteriaceae*			↑ 2m	↑ 2m	
S. aureus			↑ 1–6m		
Population counts					
Facultative bacteria					
E. coli	↓ 1w, 1m,4m		↑ 1w, 12m		↑ 4m
Other *Enterobacteriaceae*			↑ 1w	↑ 4m	
Enterococcus			↑ 1–2w, 1m		
S. aureus					↑ 2w, 4m
Anaerobic/facultative ratio	↑ 1w		↓ 12m		↓ 1w

Major findings for bacterial colonization rates, fecal population counts in colonized infants and anaerobic/facultative ratio in associations with exposures studied in Paper I and II, after adjustment for potential confounders. Farm and pets were adjusted for one another, sex and breastfeeding, while each of the three exposures firstborn, cesarian delivery and antibiotics during delivery were adjusted for the other two, infant sex and breastfeeding

Delivery by cesarean section was associated with lower colonization rates by *E. coli* in the first week of life and instead higher colonization rates of non-*E. coli* Enterobacteriaceae than in vaginally delivered infants at 2 months and of clostridia at one month of age. Also, cesarian delivery was associated with higher population counts of non-*E. coli* in infants colonized by these bacteria in the first months, with significantly increased counts at 4 months of age (Table 7).

Infants exposed to antibiotics during delivery showed delayed colonization by *Bacteroides* spp. (Table 7) and tended to be less frequently colonized by these bacteria still at 1 year of age. Population counts of *S. aureus* were higher in infants exposed to antibiotics during delivery at 1 week, and the population levels of both *S. aureus* and *E. coli* were higher at 4 months in exposed than in unexposed infants colonized by these bacteria. Also, the ratio of anaerobic to facultative bacterial counts were lower at one week of age in infants exposed to antibiotics compared to unexposed infants (Table 7).

Early gut colonization pattern by major groups of culturable gut bacteria and associations with allergy at 3 or 8 years of age in the FARMFLORA cohort (Paper I)

In **Paper I**, we investigated the gut colonization pattern by major bacterial groups during infancy in relation to allergy at 3 and 8 years of age. At 3 years of age, 11/63 children were allergic, whereas 10/48 children were allergic at 8 years of age. The analyses were adjusted for sex, breastfeeding, allergic heredity (at least one allergic parent), farm living and pet ownership, and associations are shown in Table 8.

Table 8: Major findings regarding colonization rates and population counts of different bacterial groups in relation to allergy at 3 and 8 years of age.

	Allergy at 3	Allergy at 8
Colonization rate		
Anaerobic bacteria		
Bifidobacterium	↓ 2m	↓ 1w, 2w,
Bacteroides	↓ 1w	↓ 1w, 2w, 1m
C. difficile	↑ 4m	↑ 6m, 12 m ↓ 2 w
Lactobacilli	↓ 4m	↑ 18m
Facultative bacteria		
CoNS	↑ 12m,18m	
Bacterial population counts		
Anaerobe/facultative ratio		↓ 1w, 2w

Major findings for bacterial colonization rates, fecal population counts in colonized infants and anaerobic/facultative ratio in associations with allergy at 3 and 8 years of age observed in Paper I after adjustment for farm, pets, allergic parent(s), infant sex and breastfeeding

Allergy at age 3 years was associated with lower colonization rates of *Bacteroides* spp at 1 week, and *Bifidobacterium* spp at 2 months (Table 8), but there were clear tendencies towards less colonization by these bacteria up to 2 and 4 months of age, respectively, in children who developed allergy. Also, lactobacilli were less frequently isolated in fecal samples from allergic children at 4 and 6 months, with a significant difference at 4 months of age. In contrast, colonization by *C. difficile* was more common in the allergic group between 4 and 12 months of age, significantly at 4 months, when also clostridia was more prevalent in this group. Further, CoNS was more common at 12 and 18 months of age in children later developing allergy (Table 8). A non-significant association was also seen between a lower ratio of anaerobic to facultative population counts at one week and allergy at 3 years of age. Allergy at 8 years was clearly associated with colonization by certain gut bacteria, and there were strong associations between reduced colonization by especially *Bacteroides*

spp, but also bifidobacteria in the first two months and allergy at age 8 years (Table 8). Also, the anaerobe/facultative ratios were lower in allergic children in the first two weeks of life. Allergy at 8 was associated with lower colonization rates by *C. difficile* in the first week, but the opposite was seen at 6 and 12 months of age when *C. difficile* was significantly more common in children who had developed allergy at 8 years of age. During the first year, lactobacilli tended to be less commonly isolated from subsequently allergic children, however, at 18 months, this was reversed and lactobacilli were significantly more common in children who received an allergy diagnosis at 8 years of age than in non-allergic children (Table 8).

Gut colonization by individual species of lactobacilli in the FARMFLORA cohort (Paper III)

In **Paper III**, the frequency of isolation and population counts of different species of lactobacilli from the fecal samples obtained between 1 week and 18 months of age in the FARMFLORA cohort was studied. Isolates from the fecal samples that had previously been identified as lactobacilli using a group-specific multiplex PCR assay (PCR-G) (**Paper I**), were here further analyzed and typed to the species level using a set of species-specific primers and multiplex PCR assays (see Table 5, methods section). The isolates that could not be identified in these analyses were typed to the species level by whole genome sequencing. In total, the isolates were shown to belong to 17 different species, many of them represented by only one or two isolates (Table 1**, Paper III**). *L. rhamnosus, L. paracasei, L. gasseri, L. acidophilus, L. delbueckii* and *L. plantarum* were the most commonly isolated species.

During the first six months, the three most frequently isolated species were *L. rhamnosus*, *L. paracasei* and *L. gasseri*, all three present in some samples already at 1 week of age. *L. gasseri* and *L. paracasei* increased in isolation rate during the first 1 and 2 months respectively. The isolation rate of the former declined steadily thereafter while colonization by *L. paracasei* was more stable. *L. rhamnosus* reached their highest isolation rates at 4 months of age, whereafter colonization declined.
From 6 months of age, *L. delbrueckii* and *L. acidophilus* were isolated from an increasing number of infants, and also *L. plantarum* were more stably isolated.

L. delbrueckii and *L. acidophilus* were found in a significant portion of all samples at 18 months of age (see **Figure 1 A-B**, **Paper III**).

The three most common species all showed high fecal population levels in the first six months. *L. gasseri* reached both its peak population size and its peak prevalence at 1 month of age, while counts of *L. rhamnosus* were high at most sampling times in the first 6 months. *L. paracasei* population levels peaked at slightly lower levels at 2-6 months of age.
The population sizes of *L. rhamnosus* and *L. paracasei* declined between 6 and 12 months of age while *L. gasseri* population counts decreased continuously already after the first month.
L. delbrueckii, *L. acidophilus* and *L. plantarum* also had low population counts in infants harboring theses species after 6 months of age (see **Figure 1C-D**, **Paper III**)

Gut colonization by individual species of lactobacilli and associations with various life-style factors and exposures (Paper III)

The colonization rates and population levels of different species of lactobacilli were then related to the following exposures or lifestyle factors: delivery mode, antibiotic exposure during delivery, being firstborn, growing up on a farm, contact with household pets, breastfeeding, infant sex and allergic parent(s). Associations were analyzed statistically, both unadjusted and adjusted for potential confounders, as specified below. There were various factors that associated with colonization rate and/or population counts of different species of lactobacilli, and the full results, including associations that were significant in unadjusted and/or adjusted analyses are shown in **Supplemental Tables 6 and 7**, **Paper III.**
In the analyses involving delivery mode, exposure to antibiotics during delivery or being firstborn, these factors were mutually adjusted for since they were clearly interrelated. The analyses were also adjusted for sex and breastfeeding. Here, breastfeeding was defined as exclusive breastfeeding in analyses involving sampling in the first 4 months and as any breastfeeding at later sampling times.

Cesarean delivery seemed to be associated, mostly negatively, with colonization by several species, but only decreased colonization by *L. delbrueckeii* and *L. acidophilus* at 18 months of age remained significant after adjustment. Also *L. plantarum* were less common in cesarean delivered infants at late sampling occasions, as were *L. paracasei* at 4 months, whereas *L. rhamnosus* were increased in cesarean delivered children at 12 months of age, but not after adjustment (see **Figure 2 A-E**, **Paper III**). When comparing infants exposed to antibiotics during delivery to unexposed infants, only decreased colonization by *L. acidophilus* at 18 months of age was significant after adjustment. In unadjusted analyses, *L. paracasei* were significantly less frequently isolated from exposed infants at several sampling times, as were also *L. delbrüeckii* at the two latest time points (see **Figure 2 F-H**, **Paper III**). Firstborn children harbored *L. paracasei* less frequently than children with older siblings, but significantly only at one sampling time and significance was lost after adjustment. Furter, population counts of *L. rhamnosus* were significantly increased in firstborn children at two early sampling times, but only in unadjusted analyses. *L. rhamnosus* counts tended to be increased in colonized firstborn children also from one year of age (see **Figure 2 I-J, Paper III**).

In the statistical analyses involving farm living or household pets, these two exposures and heredity for allergy were mutually adjusted for, since they were interrelated. Also infant sex and breastfeeding were adjusted for in these analyses.

Farm living was associated with more frequent isolation of *L. rhamnosus* at 1 week and 1 month of age, while the population counts of this species in children colonized at 18 months were lower in farmers´ children as compared to controls. *L. gasseri* was significantly lower in children from farming compared to non-farming families but only at a single early sampling time.These associations remained also after adjustment. In addition, *L. plantarum* was more frequent, but *L. delbrueckii* less frequent in farmers children at 12 months of age in the unadjusted analyses (see **Figure 3 A-E Paper III**).

Contact with household pets showed few associations with the isolation of specific species of lactobacilli, except for *L. paracasei*, which was more commonly found in children with pets at 2 months of age but less common in this group at 18 months of age, although only the latter finding was significant

after adjustment. Children in households with pets also had lower fecal population counts of *L. rhamnosus* at 12 months of age than other children, but only in the unadjusted analyses (see **Figure 3F, G**, **Paper III**).

Breastfeeding (exclusive breastfeeding at samplings up to 4 months, any breastfeeding at later sampling times) also seemed to affect colonization by lactobacilli. As can be seen in **Figure 4**, **Paper III**, breastfed infants were more often colonized by *L. rhamnosus* at 1 week and 6 months of age than other infants, and the fecal population counts of *L. rhamnosus* in colonized children were increased at 12 months in children still receiving some breastmilk at that age. The latter association was significant also after adjustment for sex, household pets and delivery mode (see **Figure 4**, **Paper III).** Further, none of the few children still receiving some breastmilk at 18 months harbored *L. acidophilus* by that age, although they more frequently harbored any lactobacilli than other infants (see **Figure 4, Paper III**). Colonization by any lactobacilli was less common in exclusively breastfed than other infants at one month of age, but significance was lost after adjustment. At sampling times after 4 months children still receiving any breastmilk instead tended to be colonized by lactobacilli more frequently than completely weaned children (see **Figure 4, Paper III**).

Infant sex and allergic parent(s) were also investigated for associations with the most frequently isolated species of lactobacilli, in both unadjusted analyses and in analyses adjusted for breastfeeding and farm living, and breastfeeding, farm living, pets in household and sex, respectively. *L. gasseri* was more frequently isolated from girls than from boys at 2 months of age. *L. paracasei* was more common at 1 year and colonization by any lactobacilli was more common at 6 months of age in infants with allergic parent(s) (see **Supplemental Figures 1 and 2, Paper III**).

Associations between individual species of gut colonizing lactobacilli and allergy at 3 or 8 years of age (Paper III)

In **Paper III**, we also investigated associations between gut colonization or fecal population counts of individual species of lactobacilli during infancy and allergy at 3 or 8 years of age. We considered significant results both in

unadjusted analyses, and in analyses adjusted for sex, breastfeeding, allergic heredity, farm living, delivery mode and exposure to antibiotics during delivery.

As seen in **Figure 5**, **Paper III,** allergy at 3 years of age was associated with lower colonization rates of *L. gasseri* at several timepoints in the first six months of life, and the differences at 1 and 2 months of age were significant also after adjustment. Further, children who were allergic at age 3 years were less frequently colonized by *L. rhamnosus* at 1 week, by *L. paracasei* at 4 and 6 months, by *L. plantarum* at 12 months and by *L. delbrueckii* at 18 months of age as compared to children with no allergy at age 3 years, although only the last association remained significant after adjustment. Indeed, none of the children who were allergic at 3 years of age were colonized by *L. delbrueckii* at 18 months or by *L. plantarum* at any time point between 1 week and 18 months of age.

Allergy at 8 years of age was associated with reduced colonization by *L. rhamnosus* at one week and 6 months, by *L. paracasei* at one and 4 months and by *L. delbrueckii* at 18 months of age. The findings on reduced colonization by *L. rhamnosus* at 6 months and by *L. paracasei* at one month of age were significant also after adjustment. None of the children who were allergic at 8 years of age and none of those allergic at 3 years had *L. rhamnosus* in feces at one week of age or harbored *L. delbrueckii* in their microbiota at 18 months of age (see **Figure 5, Paper III**).

DISCUSSION

Allergies have increased in industrialized societies and almost tripled in incidence in Sweden during 1970-1990 (270). One suggested explanation is that decreased exposure to microbial stimuli, due to an increasingly hygienic life style could lead to failing immunological tolerance to harmless substances and thereby an increased risk of allergy. The hygiene hypothesis, first introduced by David Strachan in 1989, was based on observations that children from large families were less likely to develop hay fever (219), at first believed to be the result of repeated infections in children with older siblings. The hypothesis was later expanded to include putative protective effects of a broader range of microbial exposures, e.g. gut colonizing bacteria (220).

Children growing up on dairy farms have low rates of allergy (225, 227, 271), which could possibly relate to differences in the gut microbiota between farmers' and non-farmers' children (138). Here, we followed children growing up on small dairy farms in a rural area in Southwest Sweden and children from the same rural area not living on farms, in the FARMFLORA birth cohort. In this small cohort (N=65) we did not expect to reveal any protective effects of farm living on allergy development. Still, a decreased prevalence of allergy at 3 (255), but not 8 years of age (257) was observed in farmers´ children. The establishment and development of the gut microbiota was investigated in the cohort from 3 days to 18 months of age using quantitative culture and also related to farm living and other early life exposures which have been linked to protection against allergy development in previous studies. Another aim of the study was to explore associations between the bacteria colonizing the infant gut and allergy as diagnosed at 3 and 8 years of age. We were also interested in exploring if the previously observed effects of various lifestyle factors and exposures on the risk of allergy development could possibly be mediated by effects of these factors on the gut microbiota.

Among the facultative bacteria, Coagulase negative staphylococci were the most frequently isolated of the early colonizers, followed by enterococci and *E. coli*. CoNS are typical skin bacteria, which has become more common in the early gut microbiota of infants in industrialized societies, possibly due to lower exposure to more traditional facultative gut bacteria such as enterococci and *E. coli* (93, 98) and in this cohort, CoNS was present in almost all infants

at 1 week already and continued to colonize the majority of children throughout infancy. Also *S. aureus,* another skin bacterium, was a quite frequent colonizer in the first year of life, as previously observed in Swedish infants (95) Bifidobacteria were the most frequently isolated of the anaerobes, and colonized the majority of infants from 1 week and onwards, followed by *Clostridium* spp. *Bacteroides* spp. started to increase somewhat later but almost all infants were colonized by one year of age. The finding that bifidobacteria were the most frequently isolated early anaerobes was also observed when analyzing the fecal samples of the FARMFLORA infants using 16S rRNA gene sequencing, in which bifidobacteria were the most abundant bacteria in the first month but not at 6 months of age (Gio-Batta *et al,* in manuscript). Bifidobacteria, *Clostridium* spp and *Bacteroides* spp are common gut bacteria, and the sequential establishment of these bacteria in the present cohort is consistent with previous studies (93, 98). Lactobacilli colonized around 60% of the infants from 1 month and onwards which is a quite high colonization rate (38, 143).

Gut microbiota maturation is characterized by an increase in numbers and diversity of anaerobic bacteria, and shrinking population counts of facultatives (38, 90, 91). In the FARMFLORA cohort, maturation of the microbiota was observed by decreasing population counts of all facultative bacteria with increasing age and by the gradual increase in the ratio of anaerobic to facultative bacterial population counts. This ratio increased between 1 week and 2 months, whereafter it was relatively stable up to 6 months, followed by an increase from 6 to 18 months of age. As our culture-based methodology did not identify the fastidious and oxygen sensitive anaerobes in the infant gut establishing mostly from around 6 months of age (90), the ratio of anaerobic to facultative bacteria was likely higher at later time points than we could determine here. *Clostridioides difficile* is a bacterium that is common in infants, but in general this bacterium is outcompeted during infancy by an increasingly complex microbiota rich in anaerobes (49). In the FARMFLORA children, the reduced colonization by *C. difficile* from 6-12 months of age was interpreted as a sign of an increasingly mature gut microbiota able to suppress this bacterium.

In general, infants in the FARMFLORA cohort seemed to acquire *Bacteroides*, *E. coli* and possibly *Bifidobacterium* spp. earlier and to more frequently harbor lactobacilli than urban Swedish infants previously studied using the same

culturing protocol (the Swedish ALLERGYFLORA cohort) (98, 143). Colonization by *S. aureus* and *C. difficile*, bacteria that thrive in the gut in the absence of competition of more successful gut colonizers, instead tended to be less common among the FARMFLORA infants (95, 98). It is tempting to speculate that these differences may partly relate to differences in bacterial exposures between rural and urban areas. However, the vast majority of the ALLERGYFLORA infants had at least one allergic parent, as compared to 40% of the FARMFLORA infants, which might have influenced their lifestyle. For instance, few of the ALLERGYFLORA families kept pets. Also, the children in the two cohorts were born several years apart (1998-2000 and 2005-2008, respectively), and practices during child births, like the use of enemas before delivery to prevent fecal soiling, may have changed in Sweden over these years.

However, the comparably high colonization rate by lactobacilli in the FARMFLORA cohort. i.e. approximately 60% colonized between 1 and 18 months of age compared to a peak colonization rate of 45% at 6 months of age in the ALLERGYFLORA children (143), is interesting, since neither delivery related factors (delivery mode, exposure to antibiotics during delivery) **(Paper II)** nor heredity for allergy **(Paper III)** was associated with less colonization by lactobacilli (all species combined). Lactobacilli represent a very heterogenous group of bacteria (56) which are commonly present on plants and in insects and animals and may be enriched in a rural environment. It is possible that infants in more rural areas are exposed to lactobacilli to a larger extent, which may lead to an increased gut colonization by these bacteria. Other studies have observed differences between countries in colonization rates by lactobacilli during infancy, with a higher prevalence in less industrialized societies (133, 134, 272) suggesting that surrounding exposure may contribute to higher colonization rates by lactobacilli.

Even though the colonization rate of lactobacilli in the FARMFLORA children was fairly constant over the study period, the population counts of these bacteria dropped sharply between 6 and 12 months of age in colonized infants, as also observed previously (93, 143). This could relate to the large changes in the gut microbiota shown to occur around and after weaning, when the gut microbiota becomes more mature (90). The median counts of lactobacilli observed at 12-18 months of age largely resembled the counts usually observed in adults (273).

Regarding the results observed for individual species of lactobacilli, we could see that there was a shift in the species isolated before and after 6 months of age. *L. rhamnosus*, *L. gasseri* and *L. paracasei* dominated completely in the first 6 months. This agrees with the study of urban ALLERGYFLORA infants (143) but the isolation rates of all three species were clearly higher in the present study. While colonization by *L. gasseri* and *L.rhamnosus* declined (after 1 and 4 months of age, respectively) colonization by *L. paracasei* was more stable. That these three species actually colonize the infant gut was shown by analyses at the strain level in the study by Ahrné and coworkers (143), where several strains could be repeatedly isolated from the child colonized, indicating "true" colonization. From one year of age, the food-related species *L. acidophilus* and *L. delbrueckeii* became more common and *L. plantarum* was more regularly isolated, also much in accordance with the previous study of Swedish urban children (143), but with higher isolation rates in the FARMFLORA children. We are not aware of other studies besides ours, that have described this shift in species composition during early childhood. However, several studies have identified *L. rhamnosus, L. gasseri* and *L. paracasei* as common colonizers in infancy (101, 107, 274), and also as less common in adults than in newborns (57). Especially *L. gasseri* and *L. rhamnosus* seem to establish preferentially in the less complex infant gut microbiota. The later occurrence of. *delbrüeckii*, *L. acidophilus* and *L. plantarum* may relate to increased intake of foods containing these lactobacilli or increased exposure from other environmental sources from one year of age, but could also relate to a better capacity of these species to establish in a more mature gut microbiota. Regarding the population counts of individual species of lactobacilli, no species retained high fecal population counts after 6 months of age.

Several early life exposures have been associated with later allergy development, some of them also in previous studies of the FARMFLORA cohort. Farm living was negatively associated with allergy at 3 years of age (255) and this was also observed for breastfeeding (256). Another observation was that allergy at age 3 was more common in boys than in girls (255). That children growing up on dairy farms have a reduced risk of later allergy has been shown in many studies (227, 271, 275), and this has also been observed for children in pet-keeping families (231, 234) as well as for children with older siblings (121, 219, 220). In contrast, delivery by cesarian section (239 , 276)

and antibiotic treatment (277) are factors commonly associated with an increased risk of later allergy. As farm living, household pets, being firstborn, delivery by cesarian section and antibiotic exposure during delivery may affect the infant gut microbiota, we investigated possible associations between these exposures and the gut colonization pattern and bacterial population counts of the bacterial groups detected in this study.

To explore the individual effects of each of these exposures we adjusted for factors that were related to one another, and breastfeeding and infant sex were adjusted for in all analyses. Farm residence and pet keeping was negatively associated with having allergic parent(s), but we only adjusted for allergic heredity in analyses involving individual species of lactobacilli, since allergic heredity has previously been associated with colonization by certain species of lactobacilli (247) but otherwise seem to have a limited effect on the infant gut colonization pattern (93). In **Paper I and II** we focused on results that were statistically significant in the adjusted analyses, whereas in **Paper III** we also report associations that were statistically significant in unadjusted analyses, as colonization by different species of lactobacilli included very few colonized infants in many of the subgroups studied. In the sections below, we specify if we discuss an association that was significant only in the unadjusted analysis.

In **Paper I**, we observed that children from farming families had an increased ratio of anaerobic to facultative bacterial counts in feces in the first week after delivery. The population counts of *E. coli* in colonized infants were also lower during the first 4 months in this group than in controls. This may be interpreted as a more diverse anaerobic gut microbiota able to suppress the population counts of facultative bacteria already early in life in farmers' infants. In the study analyzing fecal samples from the FARMFLORA cohort by 16S rRNA gene sequencing, a farming environment was associated with early acquisition of certain anaerobes not targeted in the present study (Gio-Batta et al. in manuscript), which supports our interpretation. To the best of our knowledge, the FARMFLORA studies are the first to demonstrate associations between farm living and gut colonization patterns already in the first weeks after birth. At one year of age, farmers´ children were less frequently colonized by *C. difficile*, a bacterium that is common in the infant gut but successively outcompeted by an increasingly complex anaerobic microbiota (48, 49). This finding suggests a more mature microbiota in one year old farmers´ children, which is consistent with the findings in other studies (138, 271).

Among the individual species of lactobacilli investigated in **Paper III**, *L. rhamnosus* was more commonly found in farmers´ infants than in controls. *L. rhamnosus* has been isolated from cows´milk (278), and one possibility is that these bacteria are transferred to the infants from the parents' hands after handling fresh milk. Although *L. rhamnosus* was more common in farmers´ children in the first year, the population counts of these bacteria were decreased in farmers´ children as compared to controls at 12 (unadjusted analysis) and 18 months, which could be an effect of a more mature gut microbiota with the capability to suppress their population levels in the latter group. *L. delbruckeii* was less frequently isolated in farmers´ children at 12 months, while the opposite was observed for *L. plantarum* (both associations were significant only in unadjusted analyses). *L. plantarum* is common on vegetables and plants (56) which may offer an explanation to why this particular species would be more prevalent on a farm.

In **Paper I**, analyses revealed that infants from pet-keeping families were earlier colonized by *Bifidobacterium* spp than infants without pets. In another study, an increased gut colonization by *Bifidobacterium pseudolongum*, a species commonly found in animals, was observed in pet-keeping families (123), while others instead observed lower rates of *Bifidobacterium* spp in the gut microbiota of infants with household pets (279), or no difference regarding bifidobacteria in relation to this exposure (109). Children from pet keeping families also acquired *Bacteroides* earlier, and they were more frequently colonized by lactobacilli at one month of age than other infants. The 16S rRNA gene sequencing analyses of the fecal samples from the FARMFLORA cohort revealed elevated levels of anaerobes at one week of age in infants from families with pets (Gio-Batta *et al*, in manuscript) supporting that pet keeping may contribute to more anaerobes in the early gut microbiota.

Regarding gut colonization by individual species of lactobacilli **(Paper III)**, contact with household pets seemed to associate with carriage of *L. paracasei,* which was significant in unadjusted analyses at 2 months of age, but this was instead reversed at 18 months of age, when *L. paracasei* was less common in children with pets in the family. These associations were not observed in the study of urban Swedish infants (143), or in the study by Martin and coworkers (107), who instead reported that *L. reuteri*, a species almost absent in the FARMFLORA children, was decreased in children not exposed to household pets. Other studies have not related colonization by individual species of

lactobacilli to pet keeping (124, 280). Possibly, pets in rural areas are more exposed to lactobacilli, for example *L. paracasei* is common in e.g. ensilage and on plants (56), and transfer these bacteria to the infant. Contact with household pets was, similar to farm living, associated (in unadjusted analysis) with low population counts of *L. rhamnosus* at 12 months of age in colonized children, possibly relating to a more mature gut microbiota by that age in children from pet keeping families.

Several associations between the presence of older siblings and the gut colonization pattern were revealed in **Paper II**. Acquisition of *E. coli* was delayed in firstborn infants, and instead they were more frequently colonized by clostridia and *Enterobacteriaceae* other than *E. coli* than infants with older siblings. The latter associations were also observed in another birth cohort study (93) and increased colonization by clostridia in firstborn infants has also been confirmed by others (281, 282). Firstborn infants also acquired bifidobacteria later than those with older siblings, and showed lower relative abundance of bifidobacteria in the first weeks samples in 16S rRNA gene sequencing analyses of the same infants in the FARMFLORA cohort (Gio-Batta *et al*, in manuscript). This agrees with other studies reporting enrichment of *Bifidobacterium* in infants with older siblings (107 , 283, 284). Others have observed a delayed acquisition by *Bacteroides* spp. in firstborn infants (122), which we could not confirm. The putative effects of being firstborn on the very early gut microbiota observed in the present study much resemble the effects of cesarean delivery reported in many studies. One possible explanation may be that firstborn neonates are not exposed to the maternal fecal microbiota during delivery to the same extent as neonates whose mothers have given birth previously. First deliveries are often longer, giving time for hygienic measures to avoid fecal contamination. Colonization by *S. aureus* was increased in firstborn infants. This bacterium has become more common in the infant gut microbiota in industrialized countries, possibly due to delayed establishment of a complex gut microbiota during infancy in increasingly hygienic societies (95, 114). Also, fecal population counts of *E. coli*, other enterobacteria and enterococci were increased in firstborn children early in life. These findings suggest that firstborn infants have a less complex gut microbiota that infants with older siblings, with less capacity to outcompete *S. aureus* or suppress facultative bacterial counts. Delayed gut microbiota maturation in firstborn children is also supported by the observations of a decreased ratio of anaerobic

to facultative population counts and an increased colonization by *C. difficile* in firstborn infants at 12 months of age, and also by the results of others (93, 285).

We observed no clear effects of being firstborn on the colonization by individual species of lactobacilli (**Paper III**). Firstborn infants tended to be less frequently colonized by *L. paracasei*, and the population counts of *L. rhamnosus* were increased in firstborn infants, but these associations lost significance in adjusted analyses. However, the latter finding may be an indication of a less mature gut microbiota in firstborn infants, allowing *L. rhamnosus* to reach high population counts.

Delivery by cesarian section (38, 107, 109, 111) and exposure to intrapartum antibiotics (140) are factors known to affect the gut microbiota. We explored the association between these exposures and the developing gut microbiota in **Paper II**. However, as there were only 10 infants in each group, the possibilities of detecting associations were limited, and there was also an overlap between being firstborn, delivered by cesarian section and exposed to antibiotics during delivery. Still, we did observe some significant associations in relation to these factors, also in adjusted analyses.

Cesarean delivered infants were less frequently colonized by *E. coli*, and instead more often by other Enterobacteriaceae and clostridia, than vaginally delivered infants, in accordance with several studies (38, 110, 286). Other studies have also observed delayed acquisition by *Bacteroides* in cesarian delivered infants (110, 141, 287), and this was observed also in our study, but this finding lost significance after adjusting for, among other factors, antibiotic exposure during delivery, which also delay the acquisition of these bacteria (140). We observed no clear differences in colonization by bifidobacteria in relation to delivery mode in the present study, and in studies reporting delayed bifidobacterial colonization in cesarian delivered infants the effect is often short termed (110, 111). In analyses of the gut microbiota in FARMFLORA infants by 16S rRNA gene sequencing, *Bacteroides* spp were less relative abundant in infants delivered by cesarian section, but no effect was observed for bifidobacteria (Gio-Batta *et al*, in manuscript).

There were no associations between delivery mode and colonization by lactobacilli (as a group) in the present study which is in agreement with some studies (93, 288), while others have observed delayed acquisition of

lactobacilli in cesarean delivered infants, often short-termed, suggesting that lactobacilli are easily acquired from non-maternal sources (107, 112). We did, however, observe increased population counts of lactobacilli in colonized children by 12 months, possibly reflecting a less mature microbiota in cesarian delivered infants at this age. When comparing cesarean and vaginally delivered infants with regard to colonization by different species of lactobacilli (**Paper III**) we observed no clear pattern, and there were also no clear differences observed in the previous study of urban Swedish infants (143) *L. paracasei* tended to be less common, while *L. rhamnosus* was instead increased at some time points in cesarean delivered infants, but only in unadjusted analyses. No differences in relation to delivery mode were observed in the first weeks after delivery so we found no support for transfer of any species during vaginal deliveries, although this is reported in some studies (101, 102)**.** However, both *L. delbrueckii* and *L. acidophilus* were less common in cesarian than vaginally delivered children at 18 months of age, possibly relating to persistent gut microbiota differences between these groups.

Exposure to intrapartum antibiotics was associated with delayed acquisition of *Bacteroides* and bifidobacteria, although the latter association was only significant in unadjusted analyses (**Paper II**). The 16S rRNA gene sequencing analyses largely confirm these results (Gio-Batta *et al*, in manuscript), as do previous studies (140, 289). Also colonization by clostridia (anaerobic spore-formers) seemed negatively affected by antibiotic exposure, whereas acquisition of lactobacilli seemed unaffected, as also observed by others (289). The anerobic to facultative ratio was significantly lower in exposed infants in the first week of life, whereas the population counts of *S. aureus* and *E. coli* were increased, indicating an anaerobic gut microbiota of low complexity. When investigating the influence of antibiotic exposure during delivery on lactobacilli at the species level in **Paper III**, we observed associations similar to those observed in relation to cesarian delivery. *L. paracasei* was less common in infants exposed to antibiotics - this species was not isolated from any of the antibiotic-exposed infants between 4 and 12 months of age, but these associations were only significant in unadjusted analyses. As in cesarean delivered children, *L. delbruekii* and *L. acidophilus* were less common in the antibiotic-exposed children at 18 months of age, the former only in unadjusted by the latter also in the adjusted analyses. Thus, both cesarean delivery and

antibiotic exposure during delivery may have long-term effects on the gut microbiota.

Breastfeeding is known to influence the gut microbiota as demonstrated in a large number of studies (38, 290), and we adjusted for breastfeeding in all analyses. The diversity of the gut microbiota is lower in breastfed infants, the microbiota is dominated by bifidobacteria and other bacteria are present in lower counts (38). Further, prolonged breastfeeding is negatively associated with gut microbiota maturation (90, 290). However, the influence of breastfeeding on colonization by any lactobacilli, or individual species of lactobacilli is less clear (38) and was investigated in **Paper III**. At early sampling times, exclusive breastfeeding was associated with decreased colonization by any lactobacilli, although in unadjusted analysis only, whereas partial breastfeeding instead associated with increased colonization by lactobacilli at later sampling times. Also, the fecal population counts of lactobacilli in colonized infants tended to be higher in breastfed as compared to other infants. *L. rhamnosus*, was the only species which seemed to be favored by breastfeeding, which was also observed in the study of urban Swedish infants (143) and in studies by others (274). However, the associations between breastfeeding and *L. rhamnosus* colonization lost significance after adjustment for infant sex, household pets and delivery mode. Infants still receiving some breastmilk at 12 months of age had higher population counts of *L. rhamnosus* than other children when colonized by these bacteria, which may relate to that breastmilk actually promote the proliferation of *L. rhamnosus* in the gut microbiota, or to a less mature gut microbiota unable to suppress their population counts in not fully weaned one year old children. Another study showed *L. gasseri* to be enriched in breastfed infants (107), which we could not confirm. Regarding other species, *L. paracasei* tended to be less prevalent in breastfed infants in the first two months and at 18 months of age, and colonization by *L. acidophilus* was significantly decreased in children still receiving breastmilk at 18 months of age. The latter finding could relate to less intake of foods containing *L. acidophilus* in not fully weaned children, but also to the gut microbiota composition in these children. Breastmilk is frequently discussed as a source of lactobacilli colonizing infants, and in the present study *L. rhamnosus* was, first week of life, only isolated from exclusively breastfed infants. However, since no or only small numbers of lactobacilli are found in breastmilk (101, 291), it is most likely not

an important source (292). The observations of similar strains of lactobacilli in infant feces and breastmilk (293, 294) may well relate to transfer from the infant to the mother's breast and not the other way around.

In the FARMFLORA cohort, several of the factors and exposures discussed above were or tended to be associated with allergy as diagnosed at 3 and/or 8 years of age, The protective effect of the farming environment on later allergy development (227, 295) was also observed in this cohort at 3 (255), but not 8 years of age (257). Cesarean delivery is frequently reported as a risk factor (236-239), and tended to be associated with allergy at both 3 (255) and 8 years (257), however not significantly. Breastfeeding was associated with a lower risk of allergy at 3 years of age (256), and in unadjusted analysis performed for comparisons between subgroups in **Paper I** partial breastfeeding at one year was associated with lower risk of allergy at 8. However, in larger studies breastfeeding does not associate negatively with allergy development (296, 297)**.** Exposure to antibiotics during delivery was associated with an increased risk of allergy at 8, also observed in comparisons between subgroups in **Paper I**, however only in unadjusted analyses, and observations on the association between exposure to antibiotics during delivery and later allergy vary (298-300).

The composition of the infant gut microbiota has been linked to allergy development in several studies. For instance, a high bacterial diversity in the gut microbiota during the first weeks of life (249, 251) and also having a relatively mature gut microbiota at one year of age (108, 138, 253, 254) is associated with lower risk of subsequent allergy development. One important aim of this thesis work was to explore possible associations between allergy at 3 and 8 years of age with the early gut colonization pattern by major culturable bacteria in the FARMFLORA cohort (**Paper I**) and also with the colonization by individual species of lactobacilli (**Paper III**). In order to explore individual associations between gut bacteria and allergy, we adjusted for potential confounders, where analyses of the colonization by major bacterial groups and allergy were adjusted for farm living, pet keeping, allergic parent(s), infant sex and breastfeeding as described in **Paper I**, while in analyses of individual species of lactobacilli in relation to allergy at 3 and 8 years of age we also adjusted for delivery mode and antibiotic exposure during delivery but not for pet keeping, as specified in **Paper III.**

There were several associations between colonization by anaerobic bacteria and allergy at 3 and 8. Both allergy at 3 and 8 years was associated with a delayed colonization by *Bifidobacterium* and *Bacteroides* spp. This has been observed previously for both *Bifidobacterium* (246, 301) and *Bacteroides* spp (138, 246, 252), while others have not seen these associations for *Bacteroides* but instead connected colonization by *Bacteroides* with an increased risk for allergy (302, 303). In the FARMFLORA cohort, a low ratio of anaerobic to facultative bacteria in the earliest samples showed a strong association with an increased risk for allergy at 8, which was observed also for allergy at 3 years, but the latter association lost significance after adjustment. In the 16S rRNA gene sequencing analyses of the fecal samples, similar results were observed since a low relative abundance of anaerobic bacteria in the first week of life correlated with allergy at 3 and 8 years (Gio-Batta *et al*, in manuscript). Together, the above findings indicate that the very early gut colonization pattern, and especially the anaerobic bacteria may be of importance for allergy protection. The importance of the very early gut microbiota is supported also by findings where a low diversity in the early gut microbiota is linked to an increased risk of atopic eczema (249, 251). There were also differences observed at later time points, where colonization by C. *difficile* at 4 and 6-12 months was associated with an increased risk for allergy at 3 and 8 years, respectively. These findings are in line with other studies that have shown that increased colonization by *C. difficile* is associated with an increased risk of allergy (80, 245). Since *C. difficile* is a bacterium that is usually outcompeted by anaerobic bacteria in a mature microbiota (49), its presence may be interpreted as a marker for a less complex and immature anaerobic gut microbiota, which we believe explains its association with allergy development. A more mature gut microbiota at 1 year of age has been associated with protection against later allergy in several studies (108, 138, 253, 254). We also found that colonization by any lactobacilli at 4 months correlated negatively with allergy at three years of age, and carriage of lactobacilli at 6 months correlated negatively with allergy at 8 years of age, but the latter association lost significance after adjustment. There are some other studies that also have observed negative associations between lactobacillus colonization and later allergy (247), while others have not seen this effect (80, 93, 246).

On the species level, there was a negative association between allergy at 3 years of age and colonization by *L.gasseri* early in life which was significant also after adjustment for confounders. To our knowledge, this is a new finding, although colonization of the mouth by *L. gasseri* appeared protective in a previous study (304). *L rhamnosus* and *L. paracasei* were associated with protection against allergy at age 3 but in unadjusted analyses only, whereas colonization by these species at certain time-points were associated with protection against allergy at 8 years of age also in adjusted analyses. These findings are in accordance with some earlier studies, where a reduced risk of allergy was linked to gut colonization early in life by a group of lactobacilli containing *L. rhamnosus*, *L. paracasei* and *L. casei* (247, 248, 305) or with colonization by *L. paracasei* (306). Finally, we also found that colonization by *L.delbrueckeii* at 18 months was associated with a reduced risk of allergy at 3 and 8 years, where the association with allergy at 3 years was significant also in adjusted analyses. It is possible that children colonized by *L. delbrueckii* at 18 months have a more mature gut microbiota promoting the establishment of this late colonizing species.

The mechanisms behind the supposed protective effect of the gut microbiota against allergy are not known. There are several ways how the gut microbiota may stimulate the developing immune system during infancy. Every time a new bacterium establishes in the gut, it activates the gut associated immune system (158), and this continuous stimulation by new bacteria may be of importance for the development of tolerance against allergens (307). The gut microbiota may also affect the development of regulatory T cells, Tregs, which play an important role in immunological tolerance mechanisms, as germ free mice have fewer and less functional Tregs than conventional mice (195, 196). In different animal models, the administration of e.g. a mixture of commensal gut bacteria, a combination of anaerobic strains of clostridial clusters IV and XIV or Bacteroidales, or a mixture of lactobacilli (*L. gasseri, L. reuteri* and *L. rhamnosus*) have been shown to promote the expansion of Tregs in the colonic mucosa (198-201). Also, e.g. the development of the oral microbiota during infancy has so far been little studied in relation to later allergy development (304).

Another possibility is that bacterial metabolites, like short chain fatty acids, SCFAs, may help the development of tolerance mechanisms, as some SCFAs stimulate the development of Tregs (308, 309). For instance, valeric acid

increases the population of mucosal Tregs in mice (310). In the FARMFLORA cohort, children from farming families had higher levels of SCFAs in feces than controls, and especially high levels of valeric acid at 3 years of age was associated with a reduced risk of eczema (139), as also observed in another Swedish birth cohort (311). Fecal levels of butyric acid have also been negatively associated with later allergy (138).

One aim of this thesis work was to investigate if the previously observed protective effects against allergy provided by a farming environment or the other exposures studied could find an explanation in their effects on the gut colonization pattern. We observed that there were specific colonization patterns already in the first weeks that were associated with a reduced risk of later allergy, especially early acquisition of anaerobic bacteria like bifidobacteria and *Bacteroides* spp, as well as high ratio of anaerobic to facultative bacteria. A farming environment, household pets, older siblings, vaginal delivery and lack of antibiotic exposure during delivery all showed some associations with the early gut microbiota which, in turn, were associated with protection. However, for vaginal delivery such microbiota associations were only observed in the unadjusted analyses, possibly due to the low number of cesarean delivered children. We also observed that decreased colonization by *C. difficile,* likely reflecting a more mature gut microbiota, was associated with lower risk of later allergy. Both farm living and contact with older siblings were associated with decreased colonization by *C. difficile*.

Colonization by any lactobacilli by 4 or 6 months of age also to some degree associated with protection from later allergy, and at the species level *L. gasseri*, *L. rhamnosus* and *L. paracasei* seemed protective in some way. Of all the exposures studied, only contact with pets associated positively with increased colonization by any lactobacilli, and only at earlier sampling times. None of the factors or exposures studied were associated with increased colonization by *L. gasseri*, whereas contact with pets, having older siblings, being delivered vaginally and not exposed to antibiotics during delivery were all to some degree associated with colonization by *L. paracasei*, but never in the analyses adjusted for confounders. Regarding *L. rhamnosus*, a farming environment seemed to promote such colonization.

There are both strengths and limitations to the explorative studies in this thesis. The low number of infants in the FARMFLORA cohort and in each subgroup

reduces the statistical power to detect differences. Also, due to the low number of infants, we did not correct for multiple testing in any of the studies. Thus, all the results reported here for the first time need to be confirmed in larger studies. Strengths of our studies are the longitudinal design and thorough examinations for allergy at three and eight years of age. Another strength was the collection and culturing of fresh fecal samples at several time points over the first 18 months after birth. As for the methods used for characterization of the gut microbiota, the use of quantitative culture has both strengths and limitations The use of bacterial culture allowed us to identify bacteria such as *S. aureus* and *E. coli* to the species level while 16S rRNA gene sequencing only identifies these bacteria to the genus level (85). Culture-based methods cannot detect many of the oxygen sensitive anaerobic bacteria that usually establish after 6 months of age unless advanced culturing methods are used (312). However, our aim was not to cover the full composition of the microbiota, but to focus on bacterial groups common in the infant gut, that are readily detected by culture. The gradual development and maturation of the gut microbiota was instead interpreted from the ratio of anaerobic to facultative bacterial population counts in the fecal samples. The culture technique used here also made it possible to quantify bacterial population counts, whereas traditional 16S rRNA gene sequencing mainly gives information on relative abundance, unless DNA sequencing methods are combined with either enumeration of bacterial cells or qPCR (86, 313).

CONCLUSION AND FUTURE PERSPECTIVES

In this study, we saw associations between various early life exposures and the establishment of gut bacteria during the first year of life in healthy infants in this study, and our observations add information on the developing gut microbiota during the very first months after birth in relation to a farming environment and other lifestyle factors and exposures previously associated negatively or positively with later allergy. We also observed that some specific early colonization patterns and markers of gut microbiota immaturity were associated with allergy at 3 and 8 years of age. An interesting next step would be explore associations between individual species of the anaerobic bacteria other than lactobacilli isolated, i.e. bifidobacteria and *Bacteroides* in relation to the early life exposures studied here.

The associations between the gut colonization and allergy were stronger than those between early life exposures and the gut microbiota, and the lifestyle factors or exposures studied might, to at least some degree, play a part in allergy development through an influence on gut microbiota establishment in infancy. However, especially regarding a farming environment and contact with pets and older siblings, other mechanisms that are not related to the gut microbiota may be more important for their protective effects, and although several of the findings from this study are in line with other, previous studies, larger studies are clearly needed to confirm our results from this small birth cohort.

USE OF GENERATIVE AI

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