Diploma thesis

THE PEDIATRIC GASTROINTESTINAL MICROBIOME ON TRIAL

Establishment of a control cohort for the intestinal microbiome of infants, children, and adolescents

submitted by

Kristin Sharmaine Aldover

in partial fulfillment of the requirements for the degree of

Doktorin der gesamten Heilkunde

(Dr.in med. univ)

at the

Medical University of Graz

executed at the

Department of Pediatric and Adolescent Medicine

Division for Pediatric Hematology-Oncology

University Hospital Graz

under the supervision of

Dr.in med. univ. Dr.in scient. med. Theresa Margarete Bauer

Assoz. Prof. Priv.-Doz. Dr. med. univ. Markus Seidel

Graz, 17.01.2024

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 17.01.2024 Kristin Sharmaine Aldover, m.p.

Acknowledgments

First of all, I want to express my utmost gratitude towards my supervisors Assoz. Prof. Priv.-Doz. Dr. med. univ. Markus Seidel and Dr.in med. univ. Dr. in scient. med. Theresa Margarete Bauer. Fulfilling this project would not be possible without you two and your continuous guidance. I couldn't have asked for better supervisors. Thank you for the encouragement and for all the opportunities that have come along the way. I feel honored to work alongside you. And most of all, thank you for helping my passion for pediatrics to manifest and my interest in research to grow.

Thank you to all the parents and pediatric patients, who were willing to participate in this study. Special thanks go to the teams of the following departments of the University Hospital Graz and the Medical University of Graz: pediatric surgery during the recruitment period, particularly Assoz. Prof. Priv.-Doz. Dr. med. univ. Georg Singer, Dr.in med. univ. Bettina Sofie Hofer and the plaster room; division for pediatric hematology-oncology, especially Marlene Meyer, BScN, MSc for providing further patient data; diagnostic- and research institute for pathology for the analysis, mostly Andrea Thüringer, BMA, Priv.-Doz. Mag. Dr. phil. Karl Kashofer, Marija Durdevic, MSc, and Assoz. Prof. Priv.-Doz. Dr. med. univ. Gregor Gorkiewicz.

Next, I want to mention my appreciation towards my parents, Rodel and Marylou Aldover, and my brother, Patrick Louis Aldover, who also proof-read my thesis. Their unconditional love, their ongoing support, and our incomparable bond brought me to the place where I am now. Thank you for believing in me and for letting me follow my dreams and aspirations in life. You have given me the foundation I need to pursue.

Another person I would like to recognize is my boyfriend, Gregor Paul Wirnsberger, who also assisted me with graphs created in Python for my thesis. His immeasurable patience and understanding make me feel secure in any task I want to fulfill. Thank you for lifting me up and inspiring me to be the best version of myself. You are the one who empowers me to stand for what I believe in.

Last but not least are the true friendships that have accompanied me through all the years. Their genuine hearts, contagious laughter, and serious talks kept me going, especially in times when I needed it the most. Thank you for having my back without hesitation. You will always have a special place in my heart.

Abstract (English)

Introduction

Research on the human gut microbiome has mostly been done on infants under the age of 3 and adults over 18 years of age. The remaining pediatric age population of preschoolers, primary school attendees, and teenagers is lacking in the abundance of data. Furthermore, little is yet known about the composition and effect of the gut microbiome of patients with severe immune cytopenias (SICs) such as autoimmune hemolytic anemia (AIHA), Evans syndrome (ES), and immune thrombocytopenia (ITP) with or without an underlying inborn error of immunity (IEI).

Materials and Methods

We collected stool samples of (otherwise) healthy children, adolescents, and young adults (HC) (n=95) who underwent elective surgery or conservative treatment of fractures in the absence of infection at a single time point. These were assigned to respective age groups (AGs) from 1 to 5: AG1 (0-1 years old), AG2 (2-5 years old), AG3 (6-10 years old), AG4 (11-15 years old) and AG5 (16-25 years old). Further, these were compared to patients with AIHIA/ES (n=11), ITP (n=21) and IEI (n=12). The ages of the participants ranged between 0 and 25 years. Sequencing of the 16S rRNA variable V4 region was utilized for microbial community profiling. The data was compared further using scripts from QIIME 1.8 and QIIME 2.0 workflows.

Results

HC infants aged 0 to 1 year (AG1) (n=12) have a discrepant gut microbiome profile compared to the remaining AGs. AG1 presents a significantly lower alpha diversity ($p<0.05$), high values of Actinobacteria (15.59%, p=0.001%) and Proteobacteria (11.97%), and reduced abundance of Bacteroidetes (38.02%) and Firmicutes (33.55%). The remaining AGs show no significant difference in the age-dependent dynamic of the gut microbiome.

The gut microbiomes of patients with IEIs exhibit a significantly lower alpha diversity than of the other cohorts, SICs and HC (p=0.009). No significant difference in the microbiome composition was found between other SICs and HC. However, three distinct clusters are formed in the Principal Component Analysis.

Conclusion

Expanding the pediatric control cohort is necessary, to estimate age-dependent changes. To attain a better insight into the suspected association of the gut microbiome with immune tolerance or autoimmunity such as in patients with SICs, longitudinal studies could capture the dynamics of the disease course.

Abstract (German)

Einleitung

Ein Großteil der Forschung über das menschliche Darm-Mikrobiom bezieht sich auf Säuglinge unter 3 Jahren und Erwachsene über 18 Jahren. Die Datenlage in Bezug auf die dazwischen liegenden pädiatrischen Altersgruppen, nämlich bei Vorschulkindern, Schulkindern sowie Jugendlichen ist gering. Bislang wurden pädiatrische PatientInnen mit schweren Immunzytopenien (SICs) in Bezug auf das Darm-Mikrobiom nicht eingehend untersucht. Zu diesen gehören Autoimmunhämolytische Anämie (AIHA), Evans Syndrom (ES), Immunthrombozytopenie (ITP) sowie angeborene Immundefekte (IEI), die eine Prädisposition für SICs darstellen.

Materialien und Methoden

Es wurden Stuhlproben von Infekt freien Kindern und Jugendlichen mit Verletzungen oder elektiven Operationen (HC) (n=95) an einem Zeitpunkt gesammelt. Diese wurden in entsprechende Altersgruppen (AG) 1 bis 5 eingeteilt: AG1 (0-1 Jahren), AG2 (2-5 Jahren), AG3 (6-10 Jahren), AG4 (11-15 Jahren) and AG5 (16-25 Jahren). Diese wurden mit Stuhlproben von PatientInnen mit AIHIA/ES (n=11), ITP (n=21) und IEI (n=12) verglichen. Die Altersspanne der PatientInnen lag zwischen 0 und 25 Jahren. Die Analyse des intestinalen Mikrobioms wurde anhand der 16S rRNA Gensequenzierung der variablen V4 Region durchgeführt. Die Daten wurden mit QIIME 1.8 und QIIME 2.0 analysiert.

Ergebnisse

HC Säuglinge zwischen 0 und 1 Jahr (AG1) (n=12) weisen im Vergleich zu den übrigen AGs einen signifikanten Unterschied in der Zusammensetzung des Darm-Mikrobioms auf. Sie haben im Vergleich zu den anderen AGs eine reduzierte Alpha-Diversität ($p<0.05$) und einen erhöhten Anteil an Aktinobakterien (15.59%, p=0.001%). und Proteobakterien (11.97%) und einen geringeren Anteil an Bacteroidetes (38.02%) und Firmicutes (33.55%). Die restlichen AGs zeigten keinen signifikanten Unterschied in der altersabhängigen Dynamik der mikrobiellen Zusammensetzung.

PatientInnen mit IEI haben eine signifikant niedrigere Alpha-Diversität im Vergleich zu den übrigen SICs und HC (p=0.009). Kein signifikanter Unterschied wurde zwischen

PatientInnen mit SIC und HC gefunden. In der Principal Component Analysis wurden jedoch drei Cluster ersichtlich.

Schlussfolgerung

Eine Erweiterung der gesunden pädiatrischen Kohorte ist notwendig, um altersspezifische Änderungen zu ermitteln. Weiters könnten zukünftig Langzeitstudien vor allem bei schweren Immunzytopenien mehr Einblick in das Darm-Mikrobiom schaffen, um den dynamischen Prozess des Krankheitsverlaufes zu untersuchen.

Table of Contents

List of Abbreviations

AIHA	autoimmune hemolytic anemia
AG(s)	age range(s)
AMP	antimicrobial peptides
Bregs	regulatory B cells
CS	caesarean section
CD	Crohn’s disease
CD4+/8+	cluster of differentiation antigen 4 positive/8 positive
DCs	dendritic cells
DNA	deoxyribonucleic acid
DM1/2	type 1/type 2 diabetes
ES	Evans syndrome
FOS	fructo-oligosaccharides
GI(T)	gastrointestinal (tract)
GOS	galacto-oligosaccharides
HC	healthy controls
HMOs	human milk oligosaccharides
IEI	inborn errors of immunity
IFN	interferon
IL	interleukin
ITP	immune thrombocytopenia
LPS	lipopolysaccharides
mLN	mesenteric lymph nodes
PD	phylogenetic diversity

pH	potentia Hydrogenii
(r)RNA	(ribosomal) ribonucleic acid
Treg(s)	$CD4^+$ regulatory T cell(s)
TMAO	Trimethylamine N-oxide
SCFA	short chain fatty acids
(S)Ig	(secretory) immunoglobulin
SIC(s)	severe immune cytopenia(s)
spp.	species pluralis, multiple species
VD	vaginally delivered

List of Figures and Tables

---

1. Introduction

1.1. The pediatric gut microbiome

1.1.1. Definition of the microbiome

The gastrointestinal tract is one of the various sites located in the human body, where microorganisms reside. The term “microbiome” has often been utilized to describe the microorganisms itself. The ensemble of such microbes is classified as the microbiota, while their genetic information is defined as the microbiome. Bacteria, archaea, fungi, microbial eukaryotes as well as viruses and phages contribute to the custom microbial composition. (1) Of 10^{14} bacteria counted in a single human gut, more than 95% are members of four major phyla, namely Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. (2) It needs to be noted, that bacteria are the main focus in the majority of cases, while archaea, fungi, protozoa, and viruses have been studied less in comparison. (3) Amongst other fungi, *Candida spp.*, *Saccharomyces spp.*, *Penicillium spp.*, and *Aspergillus spp.* were identified in the human GIT. (4,5) Methanobacteriales represent the most abundant archaeal order in the human gut. (6) The variable presence of protozoa and their impact on the microbiome, especially on bacteria, is yet to be understood. (7) Yet, microbes in a healthy human gut live in symbiosis, a state where the host and microbes profit from one another. (8)

Additionally, microorganisms in the human gut microbiota can be characterized as autochthonous, which continuously occupy the gut, and allochthonous, which can be seen as temporary visitors and are environmentally acquired. (9) To date a general definition of beneficial microbiota is difficult to apply. The presence of specific microbes may be advantageous in one aspect, such as *Bacteroides spp.* stimulating immune tolerance, which in turn prevents the onset of hypersensitivities. However, negative traits could be associated with them in another setting. Continuing with the same example, *Bacteroides spp.* may promote infection with virulence factors after the onset of peritonitis. (10,11)

Opportunistic pathogens, such as Enterobacteriaceae, of which *Escherichia coli* and *Klebsiella pneumoniae* are mostly common, and Enterococcaceae, with *Enterococcus faecalis* and *Enterococcus faecium* as representatives, are associated with hospitalization and could reside in the gut of infants delivered through CS. Therefore,

infections are more likely to occur depending on the individual and their pre-existing conditions. (12,13)

The main focus of this diploma thesis will lie on the bacterial intestinal microbiota of healthy children. Its structure and development as well its as impact on physiological and pathophysiological mechanisms will be discussed.

1.1.2. Structure and function of the pediatric microbiome

The intestine of the GIT is anatomically built of the small intestine, which is formed by the duodenum, jejunum, and ileum, and the large intestine, portraying the colon and caecum. Each segment is characterized by a distinct environment, for example, caused by secretions and changes in pH. Hence, different members of the microbiota are pronounced in each division according to the setting. When taking the small intestine into account, the phyla Firmicutes and Actinobacteria dominate the duodenum, Gram-positive aerobes and facultative anaerobes such as *Lactobacilli spp., Enterococci spp.,* and *Streptococci spp.* the jejunum and aerobic species in the ileum are predominantly present. By contrast, anaerobic species prevail in the lower intestine. Here in the distal intestine, Firmicutes and Bacteroidetes build the majority of the microbiota. (14)

Functional diversity quantifies the richness and the arrangement of various functions within the microbiota. (15) Alpha diversity characterizes the variety of microorganisms within the environment of interest. On the other hand, beta diversity represents the differences between two individuals, groups, or environments. (16) Greater richness provides a bigger pool of antigens such as flagellin, lipoproteins, lipopolysaccharides (LPS), and peptidoglycan in the host. Thus, these antigens can shape, activate, and educate the innate and adaptive immune systems. (17–19)

Microbiota characterized by members with various phylogenetic traits are associated with greater functional capability. Furthermore, microbiota allow fermentation for easier digestion, production of essential vitamins, and elimination of toxic byproducts. (15) When the colonic microbiome catabolizes carbohydrates and proteins, short-chain fatty acids such as acetate, propionate, and butyrate are created as metabolites during this process. (20) Short-chain fatty acids (SCFAs) play a crucial role in regulating gut homeostasis regarding intestinal permeability, the immune system, inflammatory responses, gut motility, and bile acid metabolism. The concentration of

SCFA reaches its peak in the cecum and proximal colon and lessens up to the distal colon. (21) Intestinal epithelial cells rely on SCFA for nourishment, proliferation, and differentiation. (22) A great repertoire of metabolic approaches leads to better digestion of various nutrients, which may benefit as an additional source of energy. For example, oligosaccharides such as fructo-oligosaccharides and galacto-oligosaccharides cannot be used as substrates for enzymes of the host. This is where the four families of glycosidases, namely glycoside hydrolases, glycosyltransferases, polysaccharide lyases, and carbohydrate esterases and their respective subfamilies, come into play in the presence of microbiota. (15) Another example is xyloglucans, which are components of dietary vegetables and catabolized by an identified polysaccharide utilization locus in *Bacteroides ovatus*, a colonic symbiont. (23,24) Bacteria are capable of releasing neurotransmitters such as γ-aminobutyric acid, which have an impact on the enteric and central nervous systems, immunomodulators like histamine, and other biologically active compounds. (25) Hollister et al. (26) observed that the amino acids cysteine, lysine, methionine, and tyrosine are metabolized in favor of the gut-brain-axis in pediatric gut communities, which in turn are utilized for the production of biogenic amines and neurotransmitters. Therefore leaving an impact on the developing brain and neuroplasticity. (27)

The gut microbiome can wield its influence within microorganisms and between different kingdoms, such as microbes and hosts. Quorum sensors are signaling molecules for similar bacteria, that regulate apoptosis, biofilm growth, homeostasis, spore formation, and virulence. Additionally, these molecules create an impact on the creation of the biofilm and provide microorganisms adaptation methods to the surrounding habitat. On the contrary, bacteria-produced inhibitors against other competing bacterial communities can be released, which include reactive aldehydes, bacteriocins, hydrogen peroxide, and lactic acid. Lactic acid is an end product after the fermentation process of glucose by lactic acid bacteria, which can defend together with hydrogen peroxide against intruders. Pathogens are impaired to thrive and build colonies by lowering local and intracellular pH and damaging bacterial deoxyribonucleic acid (DNA). 3-hydroxypropionaldehyde, better known as Reuterin, is a derivate of glycerol fermentation operated by lactic acid bacteria and prevents non-commensals from cultivating in the gut. (25)

Functional plasticity is the capability of the microbiome or its members to adjust to interfering factors through alteration in gene expression. If the taxonomic community structure is at risk due to disturbances, functional redundancy, which is the ability to execute the same function, enhances functional resilience. Therefore, regaining the initial community state is made possible, whilst strengthening its stability. A connection between functional redundancy and the health of the host can be assumed due to the correlation between taxon stability and the preservation of functional and taxonomic diversity over time. (15)

1.1.3. Development of the gut microbiome

The hypothesis of a sterile *in utero* environment and the first colonization of the newborn happening during labor, either through vaginal delivery (VD) or caesarean section (CS), is currently challenged. (28) Xiao et al. discuss study results proving vertical transmission of microbes occurs between the birth mother and the fetus. (29) Aagaard et al. detected Bacteroidetes, Firmicutes, Fusobacteria, Tenericutes, and Proteobacteria in the placenta of 320 study participants. These characteristics share similarities to the human oral microbiome. (30) Additionally, various studies found bacteria harboring further sites in healthy full-term pregnancies: such as placental villi (31–33), parts of the uterus such as the endometrium (34), the uterine cervix (32) and decidual tissue (35), fetal membranes (31,32), the basal plate (31), amniotic fluid (32,35,36) and meconium (32,36,37). Furthermore, Gomez-Arango et al. suggest that the maternal gut microbiome also has an impact on the microbial composition of the placenta. (38) Rackaityte et al. found Micrococcaceae and *Lactobacillus* in abundance in fetal meconium. The presence of *Micrococcus luteus* favored the development of immune defense in the fetus. (39) Mishra et al. consistently detected live *Lactobacillus* and *Staphylococcus* in fetal tissues. They too are beneficial for developing immune competence and priming immune cells before birth. (40) However, due to the low biomass of microbes in the placenta and the uterus, critics disagree pointing out false-positive results due to contamination and reagents. (41–43)

The formation of an infant's gut microbiota seems to stabilize within the period of the first 3 years of life. Interestingly, the function of their microbial configuration resembles those of adults. (44–46) Based upon changes in alpha diversity and the dynamics of the major phyla, the evolvement of the gut microbiota proceeds in the following phases: the developmental phase, the translational phase, and the stable phase. The

first phase ranges between 3 and 14 months of age and is characterized by successive changes in alpha diversity and in the identified phyla itself. During this period, *Bifidobacterium spp*. appear in high abundance. The translational phase with its duration from 15 to 30 months of age is marked by the ongoing development of Bacteroidetes and Proteobacteria and shifts in alpha diversity. The stable phase, counting from 31 months and above, is then identified, when the current phyla is stationary and a high alpha diversity has been established. In comparison to the previous phases, Firmicutes dominates among the last described. (47) It is to be noted that the duration concerning the final maturation of the gut microbiota could be underestimated according to recent longitudinal studies. (26,48–50)

Over time, the microbial composition changes as it is a dynamic process, driven by ecological processes. Dispersal is a mechanism that promotes diversity in local microbial communities. On the other hand, local diversification, mostly provided through phages, allows the microbiome to quickly adapt through mutation or recombination. Further, environmental selection forms the microbiota by stimulating the growth of particular members under special circumstances. Lastly, ecological drift is in charge of the clearance of species, which do not serve as an advantage in other ecologic settings or are present in low abundance. However, dispersal might prevent this process on species, that were about to be eliminated locally. (51) Over time, the microbial composition changes as it is a dynamic process and is impacted by various factors at different time points.

Additionally, the microbiota can be impacted by various factors at different points of life. These will be further discussed in chapter “1.2. Influencing factors on the microbial composition”

1.1.4. Comparison between adults and children

Hollister et al. (26) suggest that the gut microbiome of healthy, pre-adolescent children is defined by an extensive range of species, its intricate function, and similar capabilities when combining taxa. The complexity of such can be argued by the continuous development of the pediatric gut microbiome. By contrast, the gut microbiome of a healthy adult seems to be relatively stable. Biagi et al. (52) suggest that the balance of the microbial composition in adults remains until the microbiota has “aged” within 75-80 years. *Bacteroidetes* and *Firmicutes* are the prevalent phyla in

healthy children, whilst having a significant plethora of *Firmicutes* and *Actinobacteria* in comparison to adults. Commonly, the Firmicutes to Bacteroidetes ratio is used to draw associations with specific characteristics of the host. (53) As an example, shifts in the Firmicutes and Bacteroidetes ratio are observed in pathologies such as obesity (54) and bowel inflammation (55). *Firmicutes* to *Bacteroidetes* ratio should not be used as a parameter for health due to the lack of consistency in the ratio values for children and adults respectively. (26) A significant discrepancy in this specific ratio has been detected between infants ranging from 3 weeks to 10 months old and adults between the ages of 25 and 45 years, while the comparison between infants and elderly, who are 70 to 90 years of age, shows no difference. (56)

A shift in specific genes involved in vitamin biosynthesis and metabolism is observed between children and adults. (26) Folate is produced by phyla such as Proteobacteria, Firmicutes, Actinobacteria, and Verrucomicrobia in the gut. (57) Genes necessary for de novo synthesis of folate are in abundance during infancy until children attend school. (26,46) Folate is essential for DNA synthesis, replication, and repair. (58) Additionally, folate ensures the survival of $CD4^+$ regulatory T cells, especially in the intestine. This provides another benefit for the immune system. (59) Genes for the dietary utilization of folate are more prominent in adults. (26) Another vitamin noteworthy is vitamin B12. Vitamin B12 is produced by gut microbiota such as *Pseudomonas* and *Klebsiella sp*. (60) and is especially known for the development of neurological function. (61) Furthermore, adults can produce more vitamin B12 than infants. (46) However, a greater expression of genes that support the biosynthesis of Vitamin B12 was found in the gut microbiome of children. (26) The concentration of vitamin B12 reaches its maximum at the age of 7 years old, suggesting its neurological importance. (62) This example highlights that the gut microbiome of healthy children is equipped with anti-inflammatory genetic properties. (63) Furthermore, the metabolism of amino acids cysteine, lysine, tyrosine, and methionine is supported in children. These components are essential for the synthesis of biogenic amines and neurotransmitters, as they play a role in the gut-brain axis. (27) In contrast, pro-inflammatory genes and genes promoting metabolic imbalance are enriched in healthy adults. Examples therefore concern mechanisms during oxidative stress such as LPS production, the tricarboxylic acid cycle, and oxidative phosphorylation. (26,63)

The composition of the gut microbiome seems to correlate with life stages among individuals. Healthy children have similar microbiota and differ from those of adults. It has been observed, that the configuration of the gut microbiome among healthy adults seems to be alike. This phenomenon is assisted by the increase of *Faecalibacterium* spp. and *Bifidobacterium* spp. in children and the accumulation of *Bacteroides* spp. in adults. (26) A change in the composition of these species were commonly noticed in several studies regarding metabolic dysfunction and inflammation. (49,52,64,65) Adults characterized by a reduction in relative health present less *Faecalibacterium* spp. and *Bifidobacterium* spp. and an increase of *Bacteroides* spp. in their gut microbiota. (63)

Converging to a microbiota similar to adults allows more complex features and the ability to metabolize plant-derived polysaccharides, which is beneficial for the host and the microorganisms. (45) The gut microbiome reaches a plateau in relation to aging. It has been observed that the gut microbiome of a healthy adult remains balanced until reaching the senior stage, in which dysfunction and instability of the microbiota arise. (52,66,67)

1.2. Influencing factors on the gut microbial composition

Throughout life, the establishment of the human gut microbiome is characterized by a dynamic process. External and internal factors can contribute to shaping the microbiota depending on the exposition, the host's developmental stage, and health condition. The following chapter highlights parameters influencing the development of the gut microbial composition from newborn age to adolescence.

1.2.1. Birth mode

Delivery

To start with, the type of delivery influences the formation of the child's microbiome for months and could leave an impact on the onset of further diseases.

If the newborn passes the vaginal canal during birth, it encounters the vaginal and the gut microbiome of the mother. The majority of microorganisms in the gut of the baby can be compared to the vaginal microbiome of the birthing mother, such as the presence of *Lactobacillus* and *Prevotella.* Additionally, genera such as Bacteroides, Bifidobacterium, Escherichia, and *Streptococcus spp.* can be found in vaginally

delivered (VD) babies. As a result, birth through the vaginal canal provides a more diverse microbiome for the newborn rather than through CS. The majority of the infants' gut microbiome represents Bifidobacteria, which is associated with health benefits. (68)

By contrast, CS acts as a bypass towards the vaginal microbiota, allowing nosocomial microbes and the microbiome of the mother's skin to colonize the gut of the newborn. Examples therefore are *Propionibacterium*, *Corynebacterium,* and *Streptococcus*. The microbiota of CS delivered express less variety, characterized by the diminished representation of *Bifidobacteria* and *Bacteroides* and an abundance of *Clostridium*, *Staphylococcus,* and members of the Enterobacteriaceae family. (68) Additionally, Enterococci are more present due to the lack of the two previously mentioned species. (28) Thus, the transmission of *Bacteroides spp.* from the mothers would have occurred during vaginal delivery of the infant. Furthermore, in the case of CS-delivered infants, an increase of *Clostridium spp.* implies nosocomial infection. (40–42) Studies have shown that children, who were delivered by CS, were exposed to a higher risk of asthma bronchiale, celiac disease, type 1 diabetes (DM1), Crohn's disease (CD), and obesity in contrast to VD babies. (69–72) Moreover, the onset of allergic diseases is more likely to be promoted by the decrease in total diversity of the gut microbiota during the first month of life. (73) To minimize the risks and to imitate the vaginal environment to an extent degree, vaginal microbial transfer or fecal microbiota transfer could be options during CS delivery. It must be noted that screening of the mother is mandatory to prevent dysbiosis transmission to the newborn. The long-term advantages of these methods are yet to be determined. (74,75)

Gestational age

Preterm babies are classified as such, who are born alive before completing 37 weeks of pregnancy calculated from the gestational age. (76) Alongside CS delivery, possible organ system-associated complications, and the risk of having a low birth weight require prolonged observance at the neonatal intensive care unit. The environment in the neonatal intensive care unit, early administration of antibiotics and other drugs, lack of extended skin-to-skin contact with the mother, and intake of breast milk set a disadvantage in the establishment of the gut microbiome. Under the circumstances of diminished diversity, the immune system cannot be primed and develop as effectively,

which results in perturbations regarding immune defense. (68) Leakage in the mucosal barrier favors microbiota translocation, the possibility of wandering into extraintestinal sites, which could lead to systemic inflammatory response syndrome or even multisystem organ failure. (77,78) As a result, this dysbiotic state could have a short- or long-term impact on an infant's health. (28) Diseases such as necrotizing enterocolitis or sepsis have been linked to the intestinal microbiome of preterm babies. (79) In comparison to full-term infants, facultative anaerobes come in abundance, which includes *Enterococcus*, *Enterobacter*, *Staphylococcus*, and *Lactobacillus*, whereas strict anaerobes, such as *Bifidobacterium*, *Bacteroides,* and *Atopobium*, appear less present in preterm babies. Moreover, the dominating phyla in the gut of preterm newborns are *Proteobacteria* and *Firmicutes*. (68) To counteract the microbial disadvantages, the intake of probiotics or breast milk should be introduced to the newborn.

1.2.2. Feeding method

Breastfeeding

According to the World Health Organization, sole breastfeeding until the age of 6 months and in a supplemental manner until the age of 2 years is recommended. (80) The milk provided by the mother can be grouped into three classifications by order depending on the components: colostrum, ranging from birth to the first five days of lactation; followed by transitional milk until two weeks postpartum; and lastly, mature milk until 4-6 weeks postpartum. (81) The colostrum is asserted to be responsible for the immunologic development of the infant due to the elevated amounts of secretory immunoglobulins and immunomodulatory cytokines. Thus, the concentration of human milk oligosaccharides (HMOs) in colostrum is twice as high as in mature milk. Growth factors such as epidermal growth factor (EGF), transforming growth factor β (TGF-β) and colony-stimulating factor 1 (CSF-1) are more abundant in colostrum compared to mature milk. (82) As an example, with transforming growth factors β 1 and 2, microbial richness, evenness, and diversity can be achieved in the early stages of life, and protection from atopic disease can be pursued. (83)

Depending on various factors, human breast milk consists of the following: lipids, mostly triacylglycerides; carbohydrates like lactose and human milk oligosaccharides; antibodies such as immunoglobulins found as secretory immunoglobulin A (SIgA) and

secretory immunoglobulin G (SIgG); proteins, in the groups of whey, caseins and mucin; non-protein nitrogen, with nucleotides as one of the components; and short chain fatty acids. (82) Furthermore, *Lactobacillus* and *Bifidobacterium spp.* present in breast milk could have an impact on the infant's microbiota formation. (84) The substantial amount of *Bifidobacterium breve*, *Bifidobacterium bifidum,* and *Bifidobacterium longum* are beneficial for the metabolism of human milk oligosaccharides. (44,47,85,86)

Studies have proven HMOs to be beneficial for the infant's health. Moreover, especially the growth of advantageous bacterial colonies, such as *Bifidobacteria*, is stimulated. (68,87) In addition, a lack of pathogens has been observed. (28) Epithelial pathogens mistake some HMOs for receptors due to their structural resemblance. While pathogens attach to these imitations, their adherence to the tissue is prohibited, which in turn allows their elimination. However, the amount of HMOs depends on the presence of the secretor gene in the mother, which is responsible for the production of 2'-fucosyllactose (2'-FL) and other fucosyl-HMOs. If the secretor gene is missing, a lower concentration of HMOs is established. (87)

The onset of a reduced breastfeeding period appears to be coherent with CS. Decreases in the amount of lactation in the first hour of life could have an impact on the composition of the gut microbiota of CS-delivered infants. (88)

Formula

Even though the formula is given as a supplement to human breast milk, the components are not identical which consequently affects the microbiome. Compared to HMOs, a 9:1 ratio of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) imitate a similar effect in activating *Bifidobacterium spp.* regarding the colon microflora. (89) GOS and FOS are possible prebiotic ingredients in the formula. The difference between GOS, FOS, and HMOs lies in structure and function. Bifidobacteria are targeted in a broad manner rather than specific and the defense mechanism towards pathogens is less effective. (90) To regain the benefits of HMOs, uniform supplementation in all formulas is necessary. Effects such as declined morbidity, especially bronchitis, reduction of antibiotic use, and lower risk for atopic diseases have been proven. (87)

Furthermore. the gut microbiome of formula-fed infants is more diverse, is characterized by higher concentrations of propionate and butyrate, and allows early differentiation to a microbiome similar to adults. (28) Within five days after transitioning from breastmilk to formula, the composition of the gut microbiome shifts, the alpha diversity is enhanced, and fecal pH increases. Instead of a microbiome dominated by *Bifidobacterium* spp. and *Lactobacillus* spp., the prevalence after weaning is taken over by *Bacteroidetes* spp. and *Firmicutes* spp. (91) In addition, alterations in the SCFA pattern could indicate shifts in bacterial residents of the colon. This coincides with a higher concentration of fecal SCFA in formula-fed infants rather than in those, who received breastmilk. (92) A more diverse microbiome serves various functionalities such as the utilization of complex carbohydrates and the production of vitamins, which converges to the adult configuration. (45) Therefore cessation of breastmilk has a major impact on the development of the gut flora in comparison to the introduction to solid food. (44)

1.2.3. Dietary intake

Depending on the feeding method, changes in the infant's microbiota might appear when solids are introduced. (68) In breast-fed infants the shift is characterized by an enhanced presence of *Enterobacteria* and *Enterococci*, together with *Bacteroides* spp., Clostridium, and Streptococcus colonies. This change does not apply to formula-fed infants, due to the prior exposition to complex substances and the existing microorganisms listed above. (93) The pediatric microbiome starts to attain similarities to the gut flora with elevated bacterial load, community stability, increased levels of total SCFA, and richness in diversity, which serves as an advantage in metabolizing complex carbohydrates. (44,45,85) Moreover, the start of solid food intake offers a variety of substrates, which in turn promote the expression of metabolic pathways regarding vitamin biosynthesis and xenobiotic substances. (45)

De Filippo et al. compared the microbiome of children living in Boulpon, Burkina Faso and Florence, Italy. They suggest that the weight of dietary habits in influencing the gut flora is greater than climate, ethnicity, geography, hygiene, and sanitation. Infectious diseases have widely been under control by Western developed countries through the improvement of sanitation and the accessibility of antibiotics and vaccines. However, the incidences of hypersensitivities, autoimmune disorders, and inflammatory bowel disease in children and adults are surging. Due to the Western

diet rich in animal fat, sugar, and caloric density, the microbiota adjusts to the restricted nutritional value with the lack of genetic diversity. While the traditional rural African diet was mostly vegetarian, low in fat, and rich in starch and fiber, their microbiota was more diverse. The absence of pathogens such as *Shigella* and *Escherichia* may be justified through the increase of bacteria, which generate SCFA and protect the host from inflammatory and gastrointestinal diseases. (94) In summary, a Westernized lifestyle has a strong impact on the formation of the pediatric gut microbiome. (3) Alongside the Western diet, the risks of developing diseases such as coronary vascular disease, metabolic syndrome, and obesity are increased. (95)

To support this result, a study in Illinois found an association between the consumption of specific foods and the steady state of the gut microbiota among healthy children between 4 and 8 years of age. A diet high in fiber, and less in starch and sweetened beverages, allowed a more consistent configuration of the microorganisms. (10)

Another study classified three enterotypes in Dutch children depending on the dominant genera, which were either *Bacteroides*, *Prevotella,* or *Bifidobacterium*. The advantages of plant-based nutrition, which is characterized by low insulin levels, could only be found in children high in *Bacteroides* and *Prevotella*. Participants with a *Bifidobacterium*-oriented microbiota could not ferment complex carbohydrates in comparison to the opposite. Moreover, genes affiliated with butyrate biosynthesis were more expressed in the Bacteroides- than in the Prevotella-driven enterotype. The positive impact of *Streptococcus spp*. on a plant-based diet has been determined in *Bacteroides* and *Bifidobacterium*-dominated configurations. (50)

Differences in the presence of specific enzymes have been evident in a study with indigenous peoples of the Americas, participants in rural Malawi, and metropolitan areas of the United States due to their diet. Participants from the U.S. presented enzymes, that can break down amino acids and simple sugars, utilize a fat-rich diet with vitamin biosynthesis, and metabolize xenobiotics and bile salt. The latter showed increased amounts of α-amylase, which supports the digestion of starch. An increased presence of the urease gene was detected in these two groups, which diminishes with age. While this enzyme remains low in the U.S. population until adulthood, urease could be vital allowing the microbial biosynthesis of essential and nonessential amino

acids with the resulting by-product ammonia. This suggests the potential benefits of urease for those who consume less protein. (46)

Perturbations during the formative stages of the microbiome, which are in the first two years of life, may influence the host's weight and length growth. The first one is by being involved in the utilization of energy, the process of fat storage, the feeling of satiety, and stimulating systemic inflammation. The latter is regarding β-diversity and microbiome maturity. (96)

Obesity is characterized by increased levels of Firmicutes and diminished values for Bacteroidetes. (97)

1.2.4. Environment

The hygiene hypothesis implies that the exposition of various environmental antigens improves the immune system in their development. Thus, reducing the chance to encounter atopic disorders. (98) Factors such as healthy lifestyle, wealth, past and present exposition to rural settings, and pets seem to contribute to healthy microbiome patterns. (99) Newborns, who lived in overly hygienic circumstances, presented discrepant configuration of the gut flora. However, other studies have observed that it may not have a relevant impact on the development of allergic diseases. (100)

The extent of influence through family members and siblings towards the colonization of the infant's gut is under debate. Sharing the same household results in similar compositions of the gut flora, as has been observed in mothers and fathers. Furthermore, the genetically related are more likely to harbor akin gut microbiota, irrespective of cohabitation. However, this condition does not necessarily differ between monozygotic and dizygotic twins, which indicates another reason outside genetics. Older siblings seem to cause higher amounts of Bifidobacterium to their 1-month-old sibling than infants without siblings. Furthermore, a more diverse and richer microbiome during the early childhood of younger siblings is associated with the presence of older siblings. In contrast, the infantile only child is characterized by an abundance of non-*Escherichia coli* enterobacteria and clostridia and a decreased anaerobe-to-facultative anaerobe ratio. Moreover, household pets do not have a distinct impact on the gut microbiome. (28)

While examining a family of six children from two months to ten years of age in comparison to their parents for about a month, the members of their microbiota remained coherent. However, the quantity of specific populations fluctuated, apparently unaffected by diet, environmental factors, or health. The reason being is the adjustment of the microbiome to individual disturbances such as food preferences. Therefore, cohabiting in the same environment and having a similar diet may be an important force to the gut flora. (101)

1.2.5. Physical and Physiological Stress

An increased load of emotional, physical, or mental pressure may cause a tense response in the human body. Depending on the frequency and duration of the stressor upon the human being, the magnitude of stress and its outcome depends on the individual. Along with the release of catecholamines like adrenaline and noradrenaline, stress enables the hypothalamic-pituitary-adrenal axis. Corticotropin-releasing hormones promote the synthesis of glucocorticoids such as cortisol in the adrenal cortex. However, the GIT and the gut microbiome are susceptible to stress and stress mediators. (95) Increased cortisol levels allow the gut wall to become more porous, which benefits bacterial translocation and in turn affects the microbial configuration. Specific gut microbiota on the other hand can produce proinflammatory cytokines, which can take effect on the hypothalamic-pituitary-adrenal axis. (102)

Examples of stressors found in a European study with children ranging from 4 to 11 years of age are afflictions within the family or in social settings or the experience of at least one impactful negative life event such as parental divorce. Additionally, the pressure to perform at school and stress of being bullied are stated among 11-year-olds internationally. Therefore, frequent snacking and foods high in fat and sugar are consumed for comfort. Compared to adults, adolescents are more likely to develop eating disorders and are prone to stress-eating. Stress levels affect the amount of fruit and vegetables incorporated in the diet inversely proportional in pediatric settings. Gut dysbiosis hinders appetite regulation through decreased SCFA levels, which normally would allow the production of peptide tyrosine tyrosine (PYY) and glucagon-like peptide-1 (GLP-1) in entero-endocrine cells and suppress hunger. (103)

Another risk of chronic stress lies in the development of ulcers and gut dysmotility. Moreover, athletes complain about GI issues such as bloody diarrhea, nausea, and

emesis. Those who perform high-intensity training are more likely to cause tissue hyperthermia and a decrease in blood flow and oxygen supply in the GIT, which affect the microbial composition and the gut barrier. Yet, exercise and the associated lifestyle presented an increase in diversity, reduction of inflammatory markers, and improvement in metabolic markers. Furthermore, regular exercise acts preventive towards type 2 diabetes (DM2), coronary artery disease, peripheral arterial disease, and, obesity. If extreme exercise is performed for longer, the adverse effects outweigh the benefits. Thus, intestinal ischemia could grant endotoxins the possibility to translocate. To counteract the negative aspects, probiotics such as *Lactobacillus* and *Bifidobacterium* spp. can be prescribed. (95)

1.2.6. Antibiotic therapy / intake of probiotics

Antibiotic therapies are prescribed to target either a broad range of bacteria or specific taxa. However, systemic effects can apply to the gastrointestinal tract and the microbiota. (95)

The procedure of CS mostly requires maternal consumption of antibiotics before, during, and after labor, which has been proven to play a role in altering the infant's gut microbiota. An association between postnatal antibiotic intake, a relative increase in *Clostridium leptum,* and lower numbers of *Bifidobacterium spp.* und *Bacteroides* spp. has been found. (104–106) Limited diversity and the lack of *Bifidobacterium spp.* caused by these manipulated circumstances abet consequences later on in life. Therefore these two factors can be seen as biomarkers for disturbances in the development of the microbiome. (28)

To treat early-onset neonatal sepsis, administration of broad-spectrum antibiotics is necessary. Yet, combinations such as amoxicillin and cefotaxime have an impact on the community composition of the gut microbiome, the stability of microbiota development, and antimicrobial resistance gene shift promptly after therapy. Additionally, amoxicillin-clavulanate and gentamicin decrease the diversity within the GI microbiome and leave a prolonged impact on antimicrobial resistance. Thus, the selection of antibiotics needs to be taken into consideration given the possibilities of adverse side-effects on the newborn’s gut microbiome. (107)

For example, macrolides caused a long-lasting disruption in the gut microbiota in a study with children ranging from 2-7 years of age. Alterations in configuration, function,

and antibiotic resistance have been observed. Antibiotic intake in the early stages of life, whether prenatal or postnatal, is associated with long-term consequences on metabolism. For instance, macrolide-associated weight gain has been described in children, who received the antibiotic prenatally. Other effects are promoting insulin resistance, DM2, and the onset of liver disease. Another example would be the link between the intake of cephalosporins and macrolides in early life and the development of asthma. Their less diverse microbiota is additionally characterized by the lack of *Lactobacillus* and/or *Bifidobacterium*, the abundance of *Bacteroides fragilis,* and members of the Proteobacteria. (28,108) Beside beneficial aspects that are associated with *Bacteroides spp.*, this genus plays a role in the emergence of bacteriemias and abscesses. The latter with *E.coli* as a representative is responsible for urinary tract infections, septicemia, and antimicrobial resistance. (109)

Problems towards antibiotic resistance can occur, when chromosomal genes of the microorganism mutate or when horizontal gene transfer proceeds in an environment, for example, gut microbiome, for antibiotic-resistant genes to be transmitted. The collection of such antibiotic-resistance genes in the human GIT is defined as the “gut resistome” and has been noticed in Gram-positive commensal bacteria, especially in Firmicutes. Through mechanisms, such as conjugation, membrane vesicle fusion, transduction, and transformation, horizontal gene transfer is possible. Furthermore, enzymes such as carbapenemases and extended-spectrum beta-lactamases, which are mostly present in *Escherichia coli* and *Klebsiella pneumoniae*, can easily be distributed in Proteobacteria. (13)

To balance out the post-therapeutical effects of antibiotics, recovery-associated bacteria such as *Alistipes*, *Bacteroides*, *Bifidobacterium*, *Coprococcus*, *Desulfovibrio*, *Faecalibacterium*, *Parabacteroides*, *Roseburia*, *Ruminococcus,* and *Subdoligranulum* assist this process. (110)

Unlike antibiotics, probiotics are additional live microorganisms that are advantageous in appropriate amounts to the host’s health. Focusing on the human gut, *Bifidobacterium*, *Lactobacillus,* and *Saccharomyces* are well-known examples, while *Akkermansia*, *Faecalibacterium*, *Roseburia,* and *Propionibacterium* spp. still are under observation and might continue the list. Functions vary among the probiotics, ranging from the adjustment of immune defense and enzyme formation, communication

among other microbiota, and production of antimicrobial compounds and organic acids. As an example, lactic and acetic acids are the primary end products of *Lactobacillus* and *Bifidobacterium*, which can decrease pH levels in the intestinal lumen and prevent the expansion of pathogens. (111) Furthermore, these two genera are known for lowering the risk of diseases such as gastroenteritis, genitourinary tract infections, inflammatory disease, irritable bowel syndrome, and diseases related to compromised immunity. (112) Through a process called cross-feeding, where nutrients can be transferred among microbes, (113) further SCFA can be released in other microbes. (111,114)

In contrast to probiotics are prebiotics, which are beneficial substrates for the host that are selectively used by microbiota. Evidence has been provided towards glucans and fructans and is growing for further molecules, such as starches, human milk, pectin, and polyphenols. (111)

1.2.7. Changes during the onset of an illness

Similarly to the impact of excessive exercise on the GIT, patients who are critically ill present gut ischemia and mucosal damage. The magnitude of beneficial effects of probiotics, prebiotics, or synbiotics towards the clinical outcome of the critically ill is yet to be understood. (95)

During admission to the hospital, intake of broad-spectrum antimicrobials might be necessary. Consequences of chronic illness on nutrition and breathing, of which hypoxia can result, interfere with the gut microbiota. The presence of recovery-associated bacteria diminishes along with the duration of antimicrobial treatment. Therefore, keeping observation on bile acids and fecal metabolites such as SCFAs and sugars is essential to estimate the condition of the gut microbiota. (110)

Compared to healthy children and adults, a deficit in alpha diversity and an altered beta diversity have been observed in critically ill children. Furthermore, symbionts in the gut like *Faecalibacterium* and *Ruminococcus* are less present, while pathogens such as *Enterococcus* and *Staphylococcus* accumulate. The resulting dysbiosis from the ICU stay influences adverse events such as nosocomial infection, neurocognitive outcomes, and undernutrition. (115) Hence, the gut microbiome could be taken into consideration to reach therapeutic goals in patients, who suffer from critical illness. (116)

The phyla Bacteroidetes and Firmicutes are the most prevalent in the healthy. In contrast phyla such as Proteobacteria, Verrucomicrobiota, Actinobacteria, or Fusobacteria are dominant in individuals presenting illness. (1)

Bacteroides fragilis, *Bacteroides vulgatus, and Bacteroides dorei* are associated with abdominal infections, inflammatory responses regarding celiac disease, and other GI disorders and metabolic disease. (117,118)

Infants with colic presented minor enhancements regarding diversity, which remained until two years postnatal. Moreover, *Bifidobacterium* spp. and *Lactobacillus* spp. were underrepresented compared to healthy controls. These changes in the microbiome may promote the onset of colic, characterized by distinct symptoms in the first 6 weeks of the postnatal period. (28)

1.3. Immune tolerance and autoimmunity

1.3.1 Components of the immune system

The innate and the acquired immune system build the defense mechanism at a cellular level. Messengers in the form of cytokines, such as interleukins (IL), interferons (IFN) and tumor necrosis factor, and chemokines are necessary for the assignment of immune cells to the site. Cytokines are responsible for the magnitude of the immune reaction, in which malfunction is associated with autoimmune diseases. (119) For instance, type 1 interferons (IFN-I) like IFN-α, IFN-β, IFN-ε, IFN-ω, and IFN-κ are involved in systemic lupus erythematosus and sclerosis. (120) On the other hand, chemokines promote the passage of immune cells to the site of interest. Dysregulations in chemokines affect malignancies and autoimmune and inflammatory disorders. (121) Uncontrolled stream of those cells aggravates conditions in the lungs, as seen in chronic obstructive pulmonary disease. (122)

Macrophages, granulocytes, mast cells, natural killer cells, dendritic cells (DCs), and the complement system compose the innate immune system. Macrophages can determine pathogen-associated molecular patterns (PAMPs) through receptors such as complement receptors, scavenger receptors, and toll-like receptors (TLR), which lead to phagocytosis of the intruder. (123) Additionally, the complement system, in which proteins cause an enzyme cascade for its activation, is relevant for the opsonizing process and lysis of pathogens. (119) Granulocytes consist of neutrophils, eosinophils, and basophils, which differ in their prior abilities. Neutrophils are the first cells to reach the site of infection through migration after receiving a chemokine signal. Eosinophils are relevant in terminating helminths, and basophils play a role in allergic reactions. Yet, mast cells are the main cells involved in allergies as they contribute histamine and cytokines to the tissue, which in turn promotes the inflammatory response through IgE. In contrast, natural killer cells prohibit the expansion of contaminated cells through cytolysis. (119) DCs are capable of distinguishing and discarding external pathogens (124), paving the way for antigens to mucosa-associated lymphoid tissue (MALT) or towards draining lymph node antigens. (125) Most importantly, DCs connect the innate and the acquired immune systems. During antigen presentation through the major histocompatibility complex, B and T lymphocytes are activated. Now, the acquired immune system supports the innate component with more specific agents. Depending on the selection process in the

thymus, T lymphocytes can turn into cluster of differentiation (CD) cells such as $CD4^+$ or $CD8^+$ T cells. Moreover, $CD4^+$ T cells as T follicular helper cells promote the swap of information into B cells. Memory T and memory B cells are formed upon the first encounter of an antigen or pathogen, allowing the immune system to remember that a specific pathogen is an already known target and to react more quickly at a future encounter. The primary usage of B cells is the secretion of antibodies through the differentiation into plasma cells. Antibodies have an opsonizing and neutralizing function and have an activating effect on the complement system. (119)

The acquired or adaptive immune response ensures central tolerance and peripheral tolerance. During central tolerance, self-reactive T cells are removed through the thymus, (126) while self-reactive B cells are eliminated in the bone marrow. (127) Peripheral tolerance is achieved through peripheral deletion, clonal anergy of autoreactive cells, and the active elimination of autoreactive T cell responses by regulatory T cells (Tregs). (126) Tregs can either develop from the thymus, known as natural Tregs, or from the periphery, known as acquired Tregs. (126) Tregs limit immune responses through modulating the function or the development of DCs, apoptosis through granzymes, and metabolic disruption through the expression of CD25. Additionally, Tregs secrete cytokines such as TGF-β, IL-10, and IL-35, which have an impact on the regulation of effector T cell signaling, IFN-γ levels, the recruitment of further Tregs and the expression of forkhead box P3 (FoxP3). (128) Autoreactive B cells are removed through processes such as receptor editing, clonal deletion, or follicular exclusion as part of clonal anergy. (127) If a lymphocyte is anergic, its functional inactivity occurs after contact with an antigen and it remains in a hyporesponsive state for a certain amount of time. (129) Regulatory B cells (Bregs) are defined as B cells with immune suppressive behavior, as Catalán et al. (130) described. Bregs release immunomodulatory cytokines similar to Tregs, which are TGF-β, IL-10, and IL-35. Bregs can secrete granzymes as well and lead to apoptosis of targeted cells. Immune checkpoints regulate immune responses and ensure self-tolerance. (130) These receptors can either promote immune responses (co-stimulatory immune checkpoints) or prevent immune responses (co-inhibitory immune checkpoints). (131) Co-inhibitory receptors such as programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated protein 4 (CTLA4) (132,133), lymphocyte activation gene 3 (LAG-3), T lymphocyte immunoglobulin domain and mucin domain

3 (TIM-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) are known. (131) PD-1 can be found on monocytes, DCs, natural killer cells, and lymphocytes such as B and T cells. (130) In contrast, CTLA-4 is located on activated T cells ($CD4^+$, $CD8^+$) (134) and can essentially be expressed on Tregs. (135) While TIGIT is located on natural killer cells and T cells ($CD4^+$, $CD8^+$, and Tregs), (136) LAG-3 is found on activated T cells ($CD4^+$, $CD8^+$) and TIM-3 is expressed on T follicular helper cells (CD $CD4^+$). (131) Examples of co-stimulatory receptors are CD2 and inducible T cell co-stimulator (ICOS) on T cells, which represent the Ig receptor superfamily (IgSF), and TNF receptor 9 (TNFR9 or 4-1BB) on DCs, macrophages and B cells, and TNFR4 (or OX40) additionally on antigen presenting cells, endothelial cells and smooth muscle cells, which represent TNF receptor superfamily (TNFRSF). (137)

Vitamins such as vitamin A, from which the metabolite retinoic acid originates, 1,25-dihydroxy-vitamin D_3 (vitamin D_3), and vitamin E, in which the form α-tocopherol is broader understood than other forms, have shown immunoregulatory properties. (138,139) Retinoic acid supports the adaptive immune system through the increased capability of cytotoxicity, proliferation and differentiation of T cells including Tregs, and modulation of DC function. Suppressing effects on B cell proliferation and B cell apoptosis were noted, however, B cell activation was observed as in studies by Ertesvag et al. (140) and Saurer et al.(141) Vitamin D_3 mainly demonstrates inhibiting effects on the adaptive immune system. Vitamin D_3 suppresses T cells, promotes Tregs, and reduces the proliferation of B cells and the differentiation of plasma cells. The innate immune response can be supported by increased monocyte proliferation and the secretion of interleukins and antimicrobial peptides. However, vitamin D_3 can suppress the differentiation and function of DCs. (138) Vitamin E promotes the innate and adaptive immune response. This can be achieved through ensuring cell membrane integrity, signal transduction, and the availability of mediators. Additionally, vitamin E can modulate the function of T cells and suppress inflammatory responses. (139)

1.3.2 Immune evasion (e.g. tolerance towards the healthy gut flora)

Adaptation towards the host's own symbiotic microbiota and recognition of foreign antigens and pathogens have a fundamental impact on the host's innate and acquired immune response. The gut epithelium consists of certain intestinal epithelial cells, of which each differs in their functionality, called Enteroendocrine cells, Enterocytes,

Goblet cells, and Paneth cells. (142) However, these cells commonly provide antimicrobial peptides as a protective measure for the symbiotic gut microbiota. (143) Together with mucin glycoproteins produced by goblet cells, mucus is known to be part of the innate immune response in the gastrointestinal tract. (144) Therefore it protects the tissue from the consequences of attaching antigens, digestive enzymes, and acids, trespassing foreign particles and pathogens into the epithelium. (145)

Furthermore, this “demilitarized zone” builds a division between the host and the gut microbiome itself. (146) TLR, nucleotide-binding oligomerization domain-containing (NOD) protein-like receptors and short chain fatty acids help with detecting microorganisms and their by-products to maintain the barrier and homeostasis of the tissue. (8) Moreover, cytokines produced by the gut microbiota have an impact on Enteroendocrine cells. (147) Metabolites and products from symbionts influence the development and functional tuning of the host immune system. (8)

The homeostasis among commensal gut microbes is maintained by colonization resistance and quorum sensing. (148) Colonization resistance impedes foreign microorganisms from harboring the same niche as commensals and taking advantage of the resources and the space. (149–151) During this process, commensals defend their habitat and the metabolites available for their use. (152) Furthermore, bacteria can consistently estimate the environment through quorum sensing. (153,154) Signaling molecules produced during bacterial replication give feedback on the population density, upon which gene expression can be adapted. Additionally, these chemical signals can alter the abilities of bacteria regarding adherence, motility, and the production of protective compounds. (148)

Depending on the intestinal segment, the formation of the mucus layer varies, thus allowing different locations of the gut microbiota. The single mucus layer in the small intestine is loosely adherent to the epithelium, which guarantees nutrient absorption for the host and allows the secretion of digestive enzymes in the brush border membrane of epithelial cells. Due to this structure, the mucus barrier can be surpassed by bacteria. Therefore antibacterial modulators are necessary to act as a defense mechanism against bacterial invasion and sustainment of epithelial crypts. (145,155) On the contrary, two continuous mucus layers have been found in the distal colon.

Mucosal tolerance towards the commensal human gut microbiome is provided by Tregs and immunosuppressive cytokines such as interleukin-10 (IL10), (156) transforming growth factor β (TGF- β) (157). Mucosa-associated invariant T (MAIT) cells represent a class of innate-like T lymphocytes, which can be found in many tissues. Mucosa-associated invariant T cells are especially abundant in the gut and help the identification of commensal microbes through their produced riboflavin metabolites. (158) Mononuclear phagocytes, such as macrophages and DCs, and $CD4^+$ T cells are involved in the alignment. (159–161) The utilization of immune cell priming allows the systemic immune response to adapt to the commensal microbiome. Primed DCs are prohibited to enter the circulatory system or reach lymphatic organs, to ensure the absence of inflammatory response under physiological circumstances. If the presence of pathogens is recognized, the immune system reacts accordingly for defense. (156)

1.3.3. Causes of autoimmunity and potential triggers

However, autoantibodies can classify the body's tissue, cells, and hormones as foreign. This process can be physiological in natural autoantibodies to sustain homeostasis. Autoimmune diseases occur when the immune tolerance is disrupted which leads to the involvement of autoantibodies, auto-reactive B, and/or T lymphocytes in inflammatory responses. This phenomenon can affect various organs and even damage multiple sites such as in DM1, multiple sclerosis, and systemic lupus erythematosus. (162) Examples of autoimmune diseases primarily affecting one organ system are Hashimoto's disease in the thyroid gland and inflammatory bowel disease (IBD) in the gastrointestinal tract. The latter includes CD, ulcerative colitis (UC), and inflammatory bowel disease unclassified (IBDU). (163) In this case, the bond between tight junctions and epithelial cells disintegrates and results in porous mucosa. (164) This in turn leads to increased permeability and causes tissue inflammation. (165,166)

In most cases, females are more likely to develop autoimmunity in comparison to men. Exceptions are DM1, CD, and UC, where the female-to-male ratio is at 1:1. The genetic predisposition plays a role in the manifestation of autoimmune diseases. Yet, nutrition, the composition of the microbiota, vaccines, and infections as well as external factors such as tobacco smoke and ultraviolet light radiation can trigger pathologic autoimmune responses. (162,167)

1.3.4. Severe Immune Cytopenias

Autoimmune conditions can affect the blood as an organ system and therefore present hematological symptoms. To narrow the topic, the focus will lie on SIC, such as autoimmune hemolytic anemia, Evans syndrome, and immune thrombocytopenia. All mentioned conditions are rare in children. AIHA has an annual incidence of 0.8 to 1.2 cases per 100,000 children in Aquitaine, France (168,169), the annual incidence of ITP registered 1.0 to 6.4 cases per 100,000 children in a British database (170,171) and the annual incidence of ES lies between 6.7 and 19.3 cases per 1,000,000 children of a Danish cohort. (172) As the name suggests, autoantibodies fail to recognize the body's blood cells through antigens, resulting in defective defense mechanisms and health consequences. While red blood cells (RBC) are being attacked in patients with AIHA (173), peripheral platelets are targeted in patients with ITP (174). Originally, ES relies on the simultaneous presence of both conditions. (175) However, recent definitions describe ES as a cytopenia affecting two or all three blood cell lineages caused by immune dysregulation, which now includes autoimmune neutropenia besides AIHA and ITP. (176–178) Before the manifestation of SIC, the immune response could likely be triggered through viral infections or vaccinations. (176,179)

The acknowledgment and concomitant treatment of underlying causes of SICs are essential. Patient and family history should be further investigated regarding infections and malignancies. (176) Especially in pediatric patients, SICs can result from immunological disorders, such as inborn errors of immunity, which may have been overlooked. (180) An association of AIHA and ES with IEI has been found more frequently than of isolated ITP. (181,182)

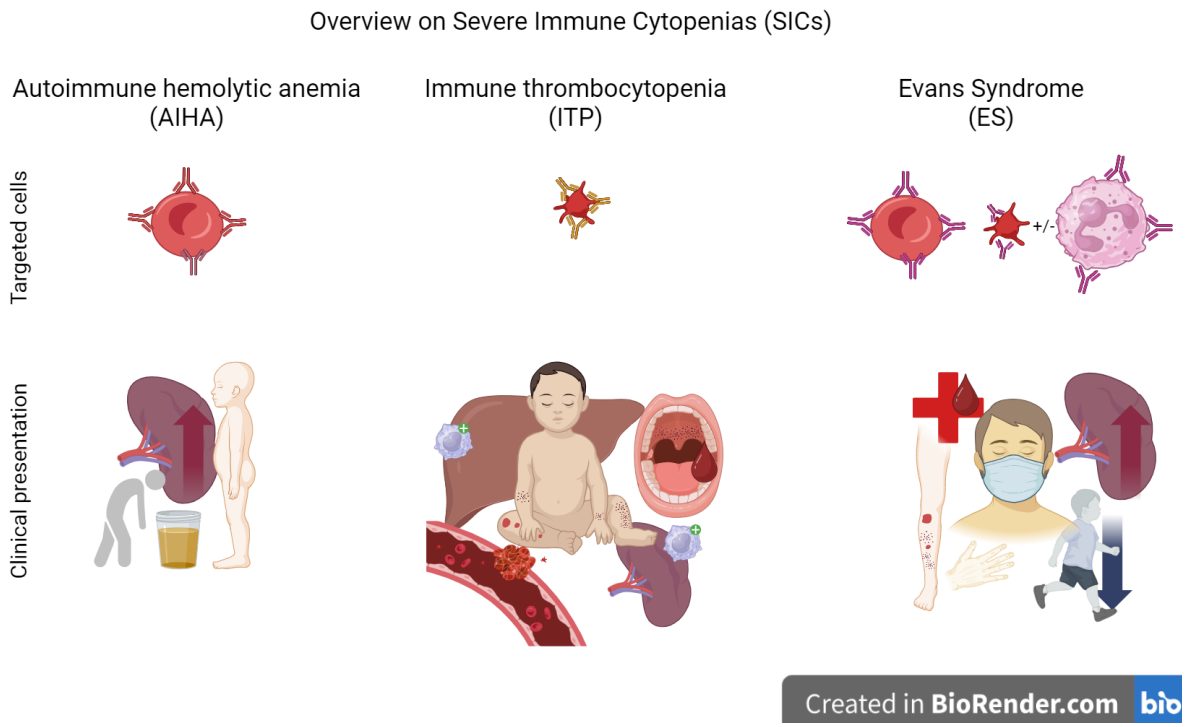

Figure 1. Simplified overview of the characteristics of Severe Immune Cytopenias (SIC). In autoimmune hemolytic anemia (AIHA) antibodies attack red blood cells, while in immune thrombocytopenia (ITP) platelets are targeted. Two blood lineages are affected in Evans syndrome (ES), which can include immune neutropenia. The signs and symptoms in ES are a combination of AIHA and ITP. ***Created with BioRender.com***

Autoimmune hemolytic anemia

AIHA can be distinguished into primary AIHA and secondary AIHA, the latter being the result of an underlying cause or disease. AIHA can occur as warm-reactive AIHA (W-AIHA), cold-agglutinin AIHA (C-AIHA), or paroxysmal cold hemoglobinuria (PCH). However, W-AIHA is more prevalent in children and will be referred to as AIHA throughout this thesis, as it makes up about 90% of all cases. Anemic features such as fatigue, dark-colored urine, jaundice, pallor, and splenomegaly are presented clinically. Yet, fulminant cases are at risk of fatal outcomes. (173,179) Therapeutic measures for AIHA aim for remission with Prednisolone as first-line treatment. (180)

Immune thrombocytopenia

ITP presents isolated thrombocytopenia with a platelet count < 100 x 10^9/L as a primary cause. A differentiation between acute, persistent, and chronic ITP is made. As a result, the patient suffers from mucocutaneous bleeding to various extent in different parts of the body. (179) While the severity of thrombocytopenia can be high, lethal bleeding is seldom. (183) First-line options for treating patients with ITP are intravenous immunoglobulin (IVIG), Dexamethasone, and anti-D-Immunoglobulins,

the latter applies if the Rhesus-factor is positive. Here, clinicians intend to improve the quality of life and lower the risk of hemorrhage. (180)

Evans syndrome

In general, the clinical presentation of ES combines anemic features from W-AIHA and the thrombocytopenic purpura due to ITP. (176,184) Symptoms regarding AIHA also include dyspnea and lack of energy. When immune neutropenia is a feature of ES, recurrent infections may be present in affected patients. (185) However, AIHA displays more of a fundamental role in ES compared to ITP. (176) Thus, 20-25% of pediatric patients with AIHA additionally manifest ITP, while those with ITP rarely develop AIHA. (182) Similar to AIHA, Prednisolone is recommended as first-line therapy in ES. (180)

1.3.5 Inborn Errors of Immunity

IEI with immune dysregulation are risk factors for the onset of autoimmune cytopenia in pediatric patients. (181,186–188) Besides the most frequent category of IEI with predominant antibody deficiencies and potential autoantibody formation, primary immune regulatory disorders (PIRDs) are a subgroup of IEI, in which autoimmune cytopenia can be the first clinical manifestation. (189–191) To date, IEI includes a wide range of 485 diseases, of which a minimum of 430 gene defects have been identified. (192,193) Immune deficits could be expressed in the form of allergies, and inflammatory and autoimmune responses, thus, promoting the vulnerability to infections and malignancies. (186) As an example, AIHA and ITP often appear in patients with Common Variable Immunodeficiency (CVID). (194) The same applies to those with (Severe) Combined Immune Deficiency (SCID, CID), with AIHA being more prevalent as a consequence. Additionally, Autoimmune Lymphoproliferative Syndrome (ALPS) frequently results in AIHA and ES. In contrast, Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) Deficiency and LPS-responsive and beige-like anchor protein (LRBA) Defect are conditions linked to ES. (195,196)

As the term “IEI” covers various diseases with heterogeneous characteristics, a common therapeutic scheme is not applicable. (187) Immunosuppressants seem to benefit patients under IEIs with autoimmune-mediated cytopenia, while others require hematopoietic stem cell transplantation in early stages according to international transplantation guidelines and clinical trials.

Category I	**Immunodeficiences affecting cellular and humoral immunity**
T-B+ Severe Combined Immunodeficiences (SCID)	Janus kinase 3 (JAK3) deficiency, Leucocyte common antigen (CD45) deficiency..
T-B- SCID	Recombination activating gene (RAG) deficiency, Adenylate kinase 2 (AK2) defect,..
CIDs without associated or syndromic features that are generally less profound than SCID	Cluster of differentiation 40 (CD40) deficiency, Interleukin 21 (IL-21) deficiency..
Category II	**Combined immunodeficiences with associated or syndromic features**
Immunodeficiency with Congenital Thrombocytopenia	Wiskott Aldrich syndrome (WAS), WAS protein-interacting protein (WIP) deficiency,..
DNA repair defects other than forms of SCID in category	Ataxia-telangiectasia, Nijmegen breakage syndrome,..
Thymic defects with additional congenital anomalies	DiGeorge 22q11.2 deletion syndrome,..
Immuno-osseous dysplasias	Cartilage hair hypoplasia (CHH), Schimke immuno-osseuous dysplasia,..
Hyper-IgE syndromes (HIES)	Interleukin-6 (IL-6) receptor deficiency,..
Defects of vitamin B12 and folate metabolism	Transcobalamin 2 deficiency,..
Anhidrotic ectodermodysplasia with immunodeficiency (EDA-ID)	EDA-ID due to IκBα gain of function (GOF) mutation,..
Calcium channel defects	Stromal interaction molecule 1 (STIM1) deficiency,..
other defects	Purine nucleoside phosphorylase deficiency,..
Category III	**Predominantly antibody deficiencies**
Severe reduction in all serum immunoglobulin isotypes with profoundly decreased or absent B cells, Agammaglobulinemia	X-linked agammaglobulinemia (XLA), p85 deficiency,..
Severe reduction in at least 2 serum immunoglobulin isotypes with normal or low number of B cells, CVID phenotype	Common variable immune deficiency with no gene defect specified (CVID),..
Severe reduction in serum IgG and IgA with normal/elevated IgM and normal numbers of B cells, Hyper IgM	MutS homolog 6 (MSH6) deficiency,..
Isotype, light chain or functional deficiencies with generally normal numbers of B cells	Kappa chain deficiency, selective Immunglobulin A (IgA) deficiency,..
Category IV	**Diseases of immune dysregulation**
Familial hemophagocytic lymphohistiocytosis (FHL syndromes)	Perforin deficiency (FHL2),.
FHL syndromes with hypopigmentation	Chediak-Higashi syndrome,..
Regulatory T cell defects	Lipopolysaccharide-responsive and beige-like anchor protein (LRBA) deficiency, cytotoxic T-lymphocyte antigen 4 (CTLA4) halpoinsufficiency, immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX),..
Autoimmunity with or without lymphoproliferation	autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy (APECED),..
Immune dysregulation with colitis	Interleukin-10 (IL-10) deficiency, IL-10 receptor (IL-10R) deficiency,..
Autoimmune lymphoproliferative syndrome (ALPS, Canale-Smith syndrome)	ALPS-Fas cell surface death receptor (FAS), ALPS-Caspase10,..
Susceptibility to Epstein-Barr-Virus (EBV) and lymphoproliferative conditions	CD70 deficiency, X-linked magnesium EBV and neoplasia (XMEN),..

Category V	**Congenital defects of phagocyte number or function**
Congenital neutropenias	Elastase deficiency/Severe congenital neutropenia 1, Granulocyte-colony stimulating factor (G-CSF) receptor deficiency,..
Defects of motility	Cystuc fubrosis, Leukocyte adhesion deficiency type 1 (LAD1),..
Defects of respiratory burst	X-linked chronic granulomatous disease (CGD),..
other non-lymphoid defects	GATA2 deficiency, Pulmonary alveolar proteinosis
Category VI	**Defects in intrinsic and innate immunity**
Mendelian susceptibility to myobacterial disease (MSMD)	Janus kinase 1 (JAK1) deficiency, Interferon γ (IFN-γ) receptor 1 deficiency,..
Epidermodysplasia verruciformis (HPV)	Epidermodysplasia verruciformis gene 1 (EVER1) deficiency, Warts. hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome,..
Predisposition to severe viral infection	RNA polymerase III deficiency, Anti-Melanoma Differentiation-associated gene 5 (MDA5) deficiency,..
Herpes simplex encephalitis (HSE)	Toll-like receptor 3 (TLR3) deficincy, Tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) deficiency,..
Predisposition to invasive fungal diseases	Caspase recruitment domain containing protein 9 (CARD9) deficiency,..
Predisposition to mucocutaneous candidiasis	IL-17 receptor A (IL-17RA) deficiency,..
TLR signaling pathway deficiency with bacterial susceptibility	Myeloid differentiation primary response 88 (MyD88) deficiency, TLR7 deficiency,..
Other IEI related to non-hematopoetic tissues	Isolated congenital asplenia (ICA), acute necrotizing encephalopathy,..
Other IEI related to leukocytes	Interferon regulatory factor 4 (IRF4) haploinsufficiency,..
Category VII	**Autoinflammatory disorders**
Type 1 interferonopathies	Adenosine deaminase 2 (ADA2) deficiency, pediatric systemic lupus erythematosus due to Deoxyribonuclease 1-like 3 (DNASE1L3) deficiency,..
Defects affecting the inflammasome	Familial mediterranean fever, Hyper IgD syndrome,..
Non-inflammasome related conditions	TNF receptor-associated periodic syndrome (TRAPS),..
Category VIII	**Complement deficiences**
C1q deficiency due to defects, C2 deficiency, complete C4 deficiency, Factor I deficiency, Thrombomodulin deficiency,..	
Category IX	**Bone marrow failure**
Fanconi Anemia Type A, Ataxia pancytopenia syndrome, Myeodysplasia, infection, restriction of growth, adrenal hypoplasia, genital phenoypes, enteropathy (MIRAGE),..	

Figure 2. Continuation of Classification of Human Inborn Errors of Immunity (IEI) from International Union of Immunological Societies Expert Committee, taken from Tangye, Al-Herz, Bousfiha et al. 2022 (193)

1.4. The correlation between gut dysbiosis and diseases

Under the influence of pathogenic processes, an imbalance of the gut microbiome is the result. Mucosa leakage allows the gut bacteria to reach mesenteric lymph nodes (mLN) and enter the blood circulation. This can cause discrepancies in the composition of cytokines in the gut mucosa and promote inflammatory responses of the mLN. As a response, Th17 and effector T cells get activated, which leads to the migration of neutrophils in the tissue. Thus, local inflammation in the gut can have a systemic impact through the influence on the immune system. (156,197)

1.4.1. Impact on the immune system

Studies proved that germ-free mice with an absence of intestinal microbiota presented immune deficiencies. Their characteristics were a loss of mucous layer, modification in IgA secretion, reduction of proportion and functionality of Peyer's patches, which are lymphoid follicles arranged in groups located in specific areas of the GIT, and insufficient drainage through mLN. (198,199)

For instance, the expression of Angiogenin-4 (Ang4), one of the antimicrobial peptides produced by Paneth cells, is lower in germ-free mice in contrast to the healthy cohort. (200) The presence of Angiogenein-1 (Ang1) prohibits the expansion of Alphaproteobacteria and in turn promotes the growth of Lachnospiraceae. (201) Furthermore, a lack of IgA-secreting plasma cells in the lamina propria, intraepithelial lymphocytes (202), and Tregs (203) is reported in germ-free mice. (124)

As an example, Trompette et al. found that higher levels of SCFA impact the production and function of DCs, macrophages, and their precursors in lung diseases. In their study, we see that a diet rich in fiber correlates with an increased abundance of the phylum Bacteroidetes, which in turn elevates SCFA levels in the blood. (204)

Alterations of the gut microbiota impact the differentiation and the supply of antibodies. IgE levels can be controlled by the diversity of the gut microbiome. (205) Early contact with omnipresent microbes could result in greater diversity and elevate the effectiveness of the immune response towards pathogens. Additionally, IgA deficiencies are characterized by a less diverse gut microbiome, as IgA can impact the formation and function of the gut microbiome. (206)

Illustration of the immune defense

Commensal microbes in the gut are involved in protecting the host from other microorganisms which can be mutualists, parasites, or even pathogens. During colonization resistance, commensals defend their habitat through the production of antimicrobial molecules and metabolites. These substances limit pathogens in their survival and their virulent characteristics. (207) These can enzymatically target the bacterial cell wall or the bacterial inner membrane. (143) Additionally, commensals stimulate the production of epithelial antimicrobial peptides (AMPs) to strengthen tight junctions. (207) This process promotes an intact epithelial barrier against intruders. An

example of epithelial AMPs is the lectin RegIIIγ, which acts bactericidal towards Gram-positive bacteria. (208–210) The effects of AMPs such as RegIIIγ support the "demilitarized zone" in the gut. (146) Furthermore, the effect of commensals towards effector T and B cells serves as another opportunity to combat pathogens. (207)

Translocated commensals and mutualists are executed by macrophages or DCs in the lamina propria to prevent the invasion of tissue. (211,212) DCs sample commensals harbored in the gut, which communicate with B and T cells in mLN and Peyer's patches. (212) IgA^+ B cells travel to the intestinal lamina propria and secrete SIgA to the mucosa. These SIgA are directed specifically towards antigens found on commensals. They influence the gene expression of commensals and prohibit commensals from adhering to the epithelium. (168,207,213,214) Therefore, SIgA contributes to the maintenance of an intact intestinal barrier by prohibiting inflammation caused by bacteria. (214)

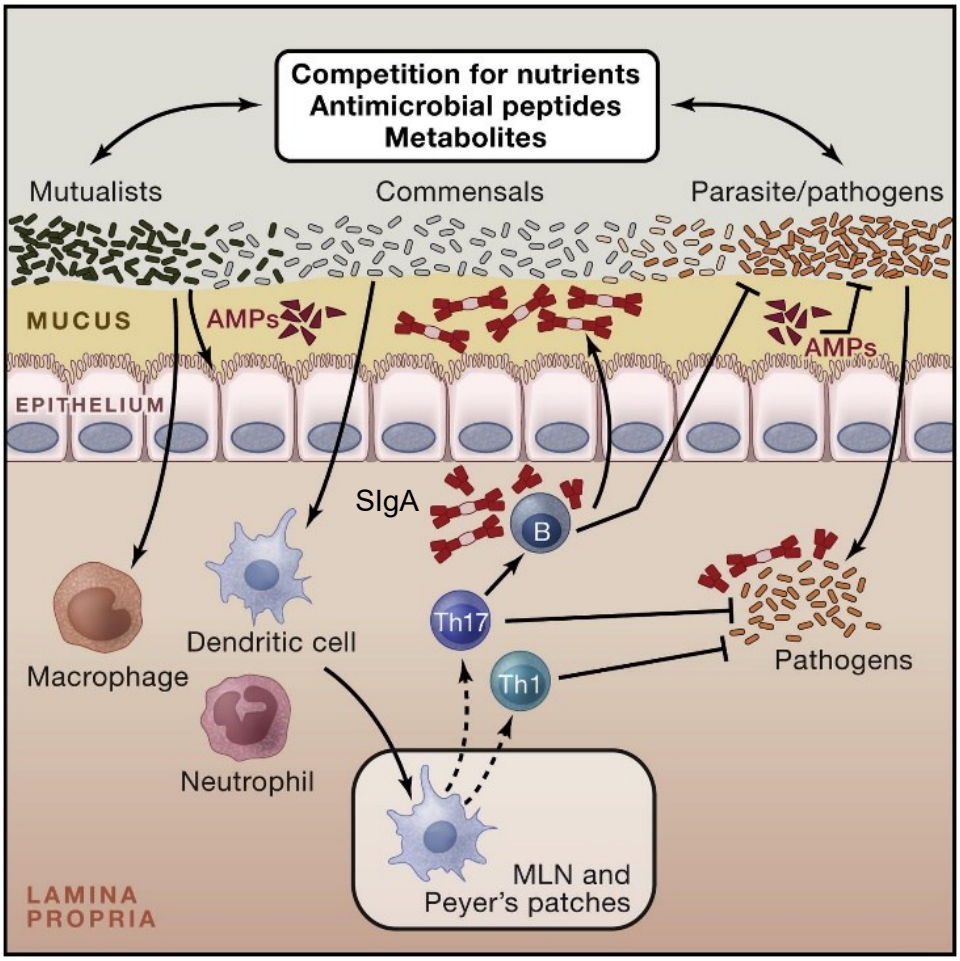

Figure 3. Promotion of Protective Immunity by the Microbiota, taken from Belkaid and Hand, 2014. (207)

Inflammation process

Increased permeability of the gut epithelium and promoted commensal bacterial translocation are caused by gut dysbiosis. (164,165) Polysaccharides produced by *Bacteroides spp.* and mucosa adherent segmented filamentous bacteria stimulate Th1 and Th17 cells to eliminate the translocated bacteria. (215,216) Due to the number of translocated bacteria, this strategy appears to be infective. The majority of bacteria activate TLRs and boost the production of proinflammatory cytokines. As a result, the intestinal tissue is affected by chronic inflammation. (217) Additionally, gut dysbiosis affects the development of the innate immune system such as the functions of neutrophils and DC. This leads to an ineffective defense mechanism against pathogens and a decrease in IFN-I and IL-15. (218,219)

Inflammatory markers in the gut include LPS, SCFA, C-reactive protein (CRP), trimethylamine N-oxide (TMAO), and cytokines such as TNF-α. (220) Increased permeability in the gut epithelium allows LPS to enter systemic circulation, which is hindered in the physiological state. This results in local tissue inflammation as macrophages either enter the site or are activated peripherally. (221) Proinflammatory mediators secreted in intestinal and extraintestinal sites occur due to LPS binding TLR-4 on immune cells. (222) In addition, SCFA such as butyrate reduces the shift of LPS outside the gut epithelium. (223) TMAO is a product of hepatic flavin monooxygenase, of which the substrate was trimethylamine. Trimethylamine is a product of the genera *Clostridium*, *Escherichia,* and *Proteus* utilizing dietary choline, carnitine, and betaine. These components can be found in animal meat. (224,225) Increased serum levels of TMAO are associated with endothelial dysfunction as in vascular inflammation, atherosclerosis, and cardiovascular risk factors like type 2 diabetes and chronic kidney disease. (226–229) Furthermore, elevated TMAO boosts the production of the pro-inflammatory cytokine TNF-α. (230) TNF-α is secreted by macrophages, T-lymphocytes, and natural killer cells. (231) This cytokine plays a role in metabolic disorders such as insulin resistance and glucose intolerance. (232)

Alterations in response due to dysbiosis

Gut microbial imbalance plays an important role in the development of inflammatory bowel diseases (IBD). Decreased richness of the gut microbiome is observed in patients with IBD. (233,234) Pathogens such as Clostridium difficile, Escherichia coli,

and Fusobacterium nucleatum might directly cause IBD. However, the diverse microbial composition of IBD patients challenges the search for a single species responsible for the development of the disease. Functionally changes in the microbiota such as diminished presence of butyrate-producing bacteria and promoted sulfate reduction are found. (235,236) Sulfate-reducing bacteria such as Desulfovibrio and Desulfomicrobium from the class of Deltaproteobacteria were more abundant in patients with IBD. (237,238) These sulfate-reducing bacteria produce hydrogen sulfide, which enhances gut inflammation along with the lack of butyrate in IBD patients. (197)

While multiple sclerosis rarely affects children, the onset can occur before 18 years of age. (239,240) In adults, Chen et al. found a discrepant gut microbiome of MS patients compared to healthy controls. The genera *Blautia*, *Dorea*, *Haemophilus*, *Mycoplana*, and *Pseudomonas* were enriched in MS patients. (241) In children, Horton et al. suggest that increased *Blautia stercoris* and *Christensenellaceae catabacter* and decreased *Odoribacter splanchnicus* have an association with subsequent disease activity in the pediatric cohort. (242) As a comparison, low values of Odoribacter were also found in active systemic lupus disease (243), cystic fibrosis (244), IBD (245), and Crohn's disease (246). This proves once again that the lack of butyrate-producing bacteria promotes the development of gut inflammation.

Vatanen et al. discovered that LPS produced by Bacteroides spp. was more abundant in Finnish and Estonian infants, where early-onset of autoimmune diseases are common, in comparison to the control group of Russian infants. LPS originating from Bacteroides spp. prevent immunostimulatory responses of LPS produced by E.coli. This could affect early immune education and can impact the progression of type 1 diabetes. In contrast, LPS produced by E.coli was more present in the gut microbiome of the control group. (247)

1.4.2. Disparities in selected autoimmune diseases

Inborn errors of immunity and the intestinal microbiome

In a recent review by Hazime et al. (192) and Castagnoli et al. (248), the gut microbiota of patients with 11 IEIs have been examined: SCID, WAS, CVID, SIgAD, HIES, IPEX, APECED, IL10RA, CGD, XIAP and TTCA7 deficiency. Zhang et al. characterized

pediatric patients from 5 months to 2 years with Wiskott-Aldrich-Syndrome (WAS) with lower α-diversity than HC. (249) Jørgensen et al. (250) found decreased α-diversity in patients with CVID and immune dysregulation in contrast to HC, while CVID patients without immune dysregulation had similar α-diversity as HC. Elevated levels of classes Bacilli, Gammaproteobacteria, Clostridia, and *Prevotella spp.* have been detected in patients with CVID, while those of the phyla Actinobacteria, Firmicutes, and *Bacteroides spp.* are low in comparison to HC. *Bifidobacterium* had diminished amounts in patients with CVID, suggesting the loss of associated health benefits (251). The discrepant age range of the study from Jørgensen et al. from 18 to 82 years of age to ours of 0 to 25 years old needs to be taken into consideration.

Fecal Microbiota Transplantation (FMT) is an option for those suffering from *Clostridium difficile* infections with recurring or persistent characteristics and are immunocompromised. (192,252) For example, a pediatric patient with Immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome was treated with FMT to combat severe diarrhea. Afterward, regression of the prior state was observed in the absence of side effects. Subsequently, restoration of the gut microbiome diversity was possible to an extent. However, hematopoietic stem cell transplantation (HSCT) was necessary to attain full recovery. (253)

A gluten-free diet was found to be advantageous in B-cell-deficient mice. The plethora of anaerobic bacteria causing the associated enteropathy was able to be contained. Enrichment of the pathobiont *Streptococcus lutetiensis* could suppress anaerobic bacteria small intestine. (254)

Severe immune cytopenias and the intestinal microbiome

To date, the microbial composition of the gut in SIC patients is yet to be understood. While ITP has relatively more data in this regard, AIHA and ES present a lack thereof, especially in human studies.

In 2021 Dei Zotti et al. introduced a new murine model of AIHA that reflects study results of humans under the same condition. No significant association between the manifestation of the disease and inflammation processes or the gut flora was found in the mouse model. The autoantibody status of mice did not influence the concentration of pro- and anti-inflammatory cytokines. According to their findings, the failed T cell tolerance may be less likely caused by a pathway linked to the gut microbiome. (255)

However, Aplastic anemia (AA) is another example of anemia that could be triggered by infections and inflammation. (256) It is suspected that AA is a T cell mediated autoimmune disease. Patients with AA are characterized with pancytopenia, where two or all three blood cell lineages are affected, and a hypoplastic bone marrow. (257,258) While studies have shown that the gut microbiome could be involved in the onset of AA, the role in AIHA/ES is yet to be discovered. An association with the gut microbiome has been found in autoimmune cytopenias such as ITP and severe congenital neutropenia, whether regarding therapeutic response or altered composition of the gut microbiome (256).

Liu et al. analyzed the relationship between the gut microbiome and the onset of ITP among 94 adults ranging from 16 to 80 years. In their study, patients with ITP differed in increased levels of Bacteroidetes (46.0% vs. 34.3%), decrease in Firmicutes (38.6% vs. 50.9%), and lower percentages of Proteobacteria (11.4% vs. 13.6%) compared to their HC. According to statistical measures, no significant changes in richness and diversity have been observed. However, all 94 patients with ITP were treated with high-dose Dexamethasone, a glucocorticoid, and it is important to note the age range. (259)

By contrast, Wang et al. divided 96 ITP patients depending on receipt of corticosteroids and their sensitivity towards this treatment. As a result, differences among genera and species have been found between those resistant and sensitive to corticosteroids. Yet, the microbiome of the corticoid resistant group is unequal to that of the untreated ITP patients. It is to be kept in mind, that the results are based on ages ranging from 18 up to 68 years of age. However, age groups have not been formed in this study. (174)

A recent study in 25 Chinese pediatric ITP patients was done by Li et al. Study participants were between 11 months and 10 years of age. They detected the following differences between ITP and HC in the phyla Firmicutes (54.3% vs. 45.8%), Actinobacteria (19.8% vs. 40.15%), Bacteroidetes (16.1% vs. 3.4%) and Proteobacteria (8.7% vs. 10.2%). Another observation was the positive correlation between the abundance of Bacteroidetes and disease severity with increased platelet-associated IgG, suggesting a possible impact of the gut microbiome on the development of ITP. (260)

1.5 Aim of the study

The first aim of the study is to recruit a healthy, pediatric cohort and to analyze the composition of their gut microbiota.

The second aim of the study is to compare the pediatric control cohort to children with severe immune cytopenias (SICs) such as autoimmune hemolytic anemia, Evans syndrome and immune thrombocytopenia with or without inborn errors of immunity, which display risk factors for SICs and distinguish possible alterations.

2. Materials and Methods

2.1 Patient recruitment

To recruit a healthy pediatric cohort for this study, patients presenting fractures without an accompanied internal medical condition were recruited in the outpatient ward of the pediatric surgery department at the University Hospital Graz. Patients between 14 and 25 years or the legal guardians of patients under 14 years old were informed about the formation of a healthy pediatric control cohort for the Severe Immune Cytopenia Registry (SIC-Reg) (https://www.sic-reg.org/) and in contrast to patients with inborn errors of immunity. Table 1 displays the number of participating children and adolescents up to 25 years of age. The aim was to collect 3 to 5 participants of the same age and consider a balance in gender, when possible. For analysis purposes, Age Groups (AG) were created based on an age range: AG1 (0-1 years old), AG2 (2-5 years old), AG3 (6-10 years old), AG4 (11-15 years old) and AG5 (16-25 years old). Table 2 illustrates the distribution of study participants of SIC-Reg recruited in pediatric hematology-oncology departments located in Graz, Leoben, and Innsbruck.

Age Group	Range [in years]	male	female	n_HC
1	0 - 1	8 (67%)	4 (33%)	12 (100%)
2	2 - 5	11 (61%)	7 (39%)	18 (100%)
3	6 - 10	11 (42%)	15 (58%)	26 (100%)
4	11 - 15	14 (58%)	10 (42%)	24 (100%)
5	16 - 25	10 (67%)	5 (33%)	15 (100%)
	n =	57	41	95 (100%)

Table 1. Age distribution of healthy controls (HC) (n=95). Age Groups (AGs) are categorized by the age range: e.g. Age Group 1 consists of participants between 0 and 1 years of age. The distribution of male and female healthy controls is displayed in absolute and relative frequency. a.. years, n.. number in total

Age Group	Range [in years]	AIHA/ES (m:f)	ITP (m:f)	IEI (m:f)
1	0 - 1	0 : 2	1 : 1	1 : 0
2	2 - 5	1 : 1	1 : 3	2 : 1
3	6 - 10	0 : 1	2 : 3	2 : 0
4	11 - 15	2 : 3	2 : 3	2 : 1
5	16 - 25	1 : 0	3 : 2	3 : 0
	n =	11	21	12

Table 2. Age distribution of patients with autoimmune hemolytic anemia and Evans Syndrome (AIHA/ES) (n=11), immune thrombocytopenia (ITP) (n=21), and inborn errors of immunity (n=12) in Age Groups (AGs). The layout of males and females are displayed as m:f in the number of participants respectively.

After the approval for consent, a Stratec test tube (Invitek Molecular) was handed out to obtain the stool sample. The sample was two days or one day prior or from the same date when the pediatric patient returned for a check-up at the clinic. We collected 102 samples of healthy children, yet 95 participants were eligible for this study. Three children were treated with antibiotic therapy and the test tubes of four teenagers did not contain stool samples.

The samples are sent to the Diagnostic- and Research Institute for Pathology of the Medical University of Graz and are analyzed respectively. Exclusion criteria were antibiotic intake before 4 weeks, long-term cortisol therapy, acute inflammation or infection, and current or chronic gastrointestinal diseases. Personal information was rendered anonymous through the assignment of an ID ranked upon receipt of the stool sample.

2.2 Matching of study and control population

Before analysis, the controls were assigned to corresponding patients who are participating in SIC-Reg and patients with inborn errors of immunity. During the matching process, proximity to age and the equivalence in sex were kept into consideration.

2.3 DNA extraction

Magnapure Compact Nucleic Acid Isolation Kit I was utilized for this approach. The samples were homogenized on a MagNA Lyser Instrument with the use of MagNA Lyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany). Afterward, the homogenized samples were first treated with 2,5mg/ml Lysozyme (Roth GmbH, Karlsruhe, Germany) for 30 minutes at 37 °C, then were followed by a digestion process with 1mg/ml Proteinase K for 60min at 56°C. Enzymes were inactivated for 10 minutes at 95 °C and to isolate the DNA in the Magnapure Compact instrument, 600 µl of lysate. Picogreen fluorescence was used to measure the concentration of DNA. This method of DNA extraction has been described elsewhere such as in Weber et al. (261)

2.4 16S Amplification

The variable V4 region of the bacterial 16S rRNA gene was chosen for amplification from 20 ng DNA with oligonucleotide primers 16s_515_fwd: TGCCAGCAGCCGCGGTAA and 16s_806_rev: GGACTACCAGGGTATCTAAT.

Regarding the choice of region, its taxonomic classification was solid and it was applicable for community clustering. Bacterial 16S rRNA was amplified with Mastermix 16S Complete PCR Kit (Molzym, Bremen, Germany) as per manufacturer's instructions using 0.4μM final concentration of primers and 57 °C annealing temperature for 25 cycles. The first PCR reaction product followed a second round of PCR with primers fusing the 16S primer sequence to the A and P adapters necessary for Ion Torrent sequencing. Simultaneously, a molecular barcode sequence was added to a multiplex of up to 96 samples. PCR products then ran through agarose gel electrophoresis. Afterwards, the band of the expected length (350nt) was excised from the gel and purified using the QiaQick (Qiagen, Hilden, Germany) gel extraction system. The concentration of DNA in the final PCR product was measured by Picogreen fluorescence. This method of 16s Amplificiation has been described elsewhere such as in Weber et al. (261)

2.5 Sequencing

Amplicons from up to 60 samples were pooled equimolarly and underwent emulsion PCR. With the Ion 530 Chef Kit and the 400bp workflow (all reagents from Thermo Fisher Scientific, MA, USA) the libraries were able to be sequenced on Ion Torrent S5. All samples were sequenced to a depth of approximately 50,000 reads. Sequences were split by barcode and were transferred to the Torrent Suite server. Unmapped binary alignment and map (BAM) files were used as input for bioinformatics, hence the files were smaller, practical to store and efficient to work with. (262)

2.6 Bioinformatics

All sequences were initially trimmed by a sliding window quality filter with a width of 20nt and a cutoff of Q20. Reads shorter than 100 nucleotides and reads mapping to the human genome were removed by utilizing deconseq. (263) The resulting reads were subjected to error correction using the Acacia tool (264), thus leading to error correction of 10-20% of reads. Subsequently, PCR chimeras were removed by usearch algorithm in de-novo and reference based settings. (265) The final sequence files were then analyzed by QIIME 1.8 and QIIME 2.0 workflow scripts. (266) Operational taxonomic unit (OTU) search was performed by using the parallel_pick_open_reference_otus workflow script and the greengenes 13_8 reference database.

2.7 Analysis

All taxons with at least 10 reads were included in the final result.

With Alpha-Rarefaction for operational taxonomic units (OTUs), metrics such as observed species, chao1, Shannon, Simpson, and Faith's phylogenetic diversity (PD) were used to determine the alpha diversity. Community richness can be calculated with chao1 and community diversity can be expressed by Shannon and Simpson. Beta diversity can be displayed through Principal Component Analysis (PCA).

Bar plots were generated in Python to illustrate the taxa composition among all age groups in HC and among the four major groups HC, AIHA/ES, ITP, and IEI.

Linear discriminant analysis (LDA) effect size (LEfSe) method identifies genomic features such as taxa in biological conditions or groups. During this analysis, features differentially abundant in comparison to the remaining examined groups can be found. Assigning characteristic relative abundances of taxa to a specific group allows a differentiation between groups. The LDA score represents the effect size of each differentially abundant feature. (267)

Differential abundance analysis with Microbiome Multivariable Associations with Linear Models (MaAsLin2) was used to find associations of abundant microbial features such as taxa and metadata variables. Metadata variables can include environmental factors, clinical presentation, or covariates. This helps to understand the origins and the occurrence of differences within the groups of interest. (268)

3. Results

Healthy control cohort

As shown in Table 1, we recruited 95 healthy controls, of which 57 were male and 41 were female. They were divided into five age groups (AGs). AG1 (n=12) represented newborns and infants from 0 to 1 years of age, of which 8 were male and 4 were female. The age range for AG2 (n=18) was set between 2 and 5 years old, which consisted of 11 males and 7 females. AG3 (n=26) included children from 6 to 10 years of age, where 11 males and 15 females participated. Pre-teens and teenagers from 11 to 15 years of age can be found in AG4 (n=24), where 14 were male and 10 were female. AG5 consisted of teenagers and young adults from 16 to 25 years of age, where 10 males and 5 females participated.

16S rRNA sequencing with the V4 amplicon and analysis with QIIME 1.8 and QIIME 2.0 reveal a discrepancy among newborns in comparison to older children and teenagers. Table 1 displays the distribution of the sexes and participants sorted according to their age in five age groups (AGs) depending on the age range. During recruitment, the age of healthy children was monitored.

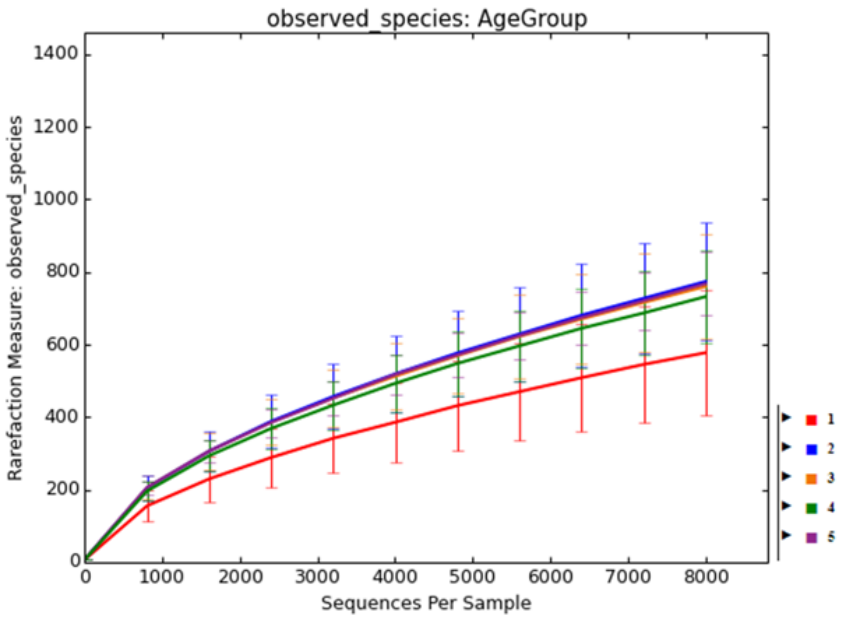

Figure 4. Alpha-Rarefaction with the metric observed species for healthy controls (n= 95). AG1 ranges between 0-1 years old, AG2 from 2-5 years old, AG3 from 6-10 years old, AG4 from 11-15 years old, and AG5 from 16-25 years old. AG1 shows a significant decrease in observed species compared to AG3 (p= 0.04), AG4 (p= 0.02), and AG5 (p= 0.02). The latter AGs present similar numbers of observed species.

No significant differences between male and female participants were found. The community richness calculated shows no significant difference among all age groups (AG). A significant difference in observed species was found between AG1 (577.33 ± 171.28) towards AG3 (759.93 ± 144.52, p= 0.04), AG4 (731.86 ± 126.16, p= 0.02), and AG5 (767.57 ± 85.89, p= 0.02) respectively, as seen in Figure 4. Faith's PD displays that AG1 (30.84 ± 10.75) is significantly discrepant towards AG2 (44.20 ± 8.28, p = 0.02), AG3 (43.86 ± 7.42, p= 0.01), AG4 (42.51 ± 6.74, p= 0.01) and AG5 (45.06 ± 4.15, p= 0.01). Shannon shows a significant discrepancy in community diversity between AG1 (5.82 ± 0.79) and AG2 (6.69 ± 0.58, p= 0.02), AG3 (6.68 ± 0.49, p= 0.01), AG4 (6.56 ± 0.46, p= 0.04) and AG5 (6.71 ± 0.29, p= 0.02) respectively. However, Simpson limits the significant difference between AG1 (0.94 ± 0.03) and AG3 (0.97 ± 0.02, p= 0.02) and AG1 and AG5 (0.97 ± 0.01, p= 0.02).

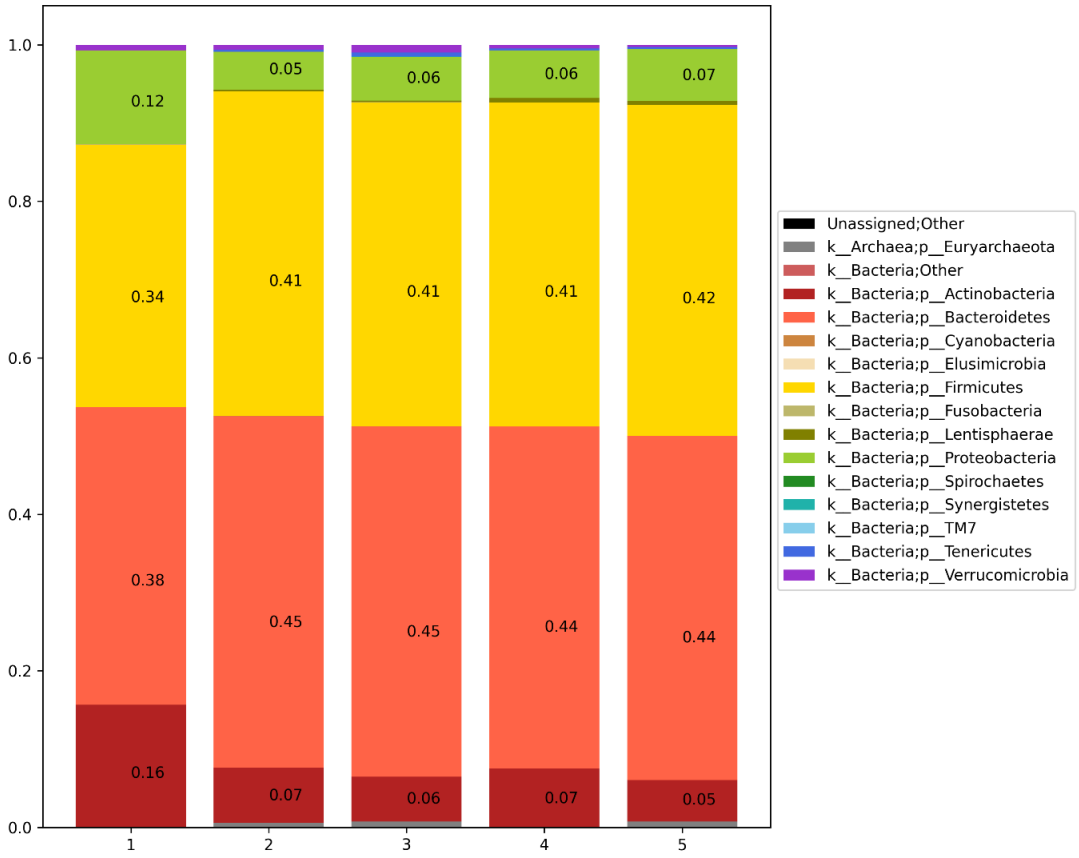

Figure 5. Bar plots at phylum level for healthy controls (n= 95). AG1 ranges between 0-1 years old, AG2 from 2-5 years old, AG3 from 6-10 years old, AG4 from 11-15 years old, and AG5 from 16-25 years old. AG1 shows a significant difference between the remaining AGs, while these are similar at the phylum level. In all five AG, the phyla Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, Lentisphaerae, Proteobacteria, Tenericutes and Verrucomicrobia are represented. AG.. Age Group, k.. kingdom, p.. phylum

Figure 5 shows bar plots at the phylum level. In all five AG, the phyla Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, Lentisphaerae, Proteobacteria, Tenericutes, and Verrucomicrobia are present. Elusimicrobia were detected in AG2 (0.001%), AG3 (< 0.001%), and AG4 (< 0.001%). Low percentages of Synergistetes (0.002%) and Spirochaetes (< 0.001%) were found in AG3. Little amounts of TM7 were detected in AG2 (< 0.001%) and AG5 (< 0.001%).

AG1 was characterized by elevated amounts of Actinobacteria (15.59%) (p = 0.001%) and Proteobacteria (11.97%), decreased Bacteroidetes (38.02%), Firmicutes (33.55%) and Lentisphaerae (0.02%) compared to the remaining AGs. The ratio of Firmicutes/Bacteroidetes weighed consistently on the side of Bacteroidetes throughout all five AGs, however, we noticed that the difference between the two phyla decreased with increasing AG. While the Firmicutes/Bacteroides ratio was at 33.55% / 38.02% in AG1, it was at 42.25% / 43.96% in AG5. Bacilli were most abundant in AG1 (5.77%), while AG2 (1.73%), AG3 (1.64%), AG4 (2.03%), and AG5 (2.31%) showed lower values than AG1. The order Lactobacillales (p = 0.01), *Streptococcus spp.* (p=0.02), *Vagococcus spp.* (p = 0.0006) and *Enterococcus spp.* (p = 0.0001) were significantly abundant in AG1 compared to the remaining AGs. In contrast, the class Clostridia were low in AG1 (27.21%), while the percentages remained stable in the following age groups as in AG2 (38.70%), AG3 (38.45%), AG4 (38.49%), AG5 (38.95%). The family Ruminicoccaceae was low in AG1 (2.71%) and seemed to plateau in the older AGs, as detected in AG2 (7.89%), AG3 (8.15%), AG4 (8.40%) and AG5 (8.60%) (p = 0.0003). *Faecalibacterium spp.* was more abundant in AG2 (6.33%) (p = 0.01), compared to AG1 (2.82%). The percentages decreased gradually from 5.30% in AG3 to 5.06% in AG5. *Ruminococcus spp.* decreased in AG1 (0.92%) and rose in the following AGs up to 4.24% in AG5 (p = 0.01). *Veillonella spp*. was significantly abundant in AG1 (2.13%) (p = 0.0003) and diminished in the remaining AGs under 0.10%. *Megasphaera spp.* was significantly increased in AG1 (0.50%) (p = 0.02) in contrast to the other AGs, which were below 0.30%.

While the highest value of Actinobacteria was detected in AG1 (15.59%), the levels of this phylum were incohesive throughout the greater AGs. Actinobacteria was at 7.02% in AG2 and 7.39% in AG4, while it made up 5.75% in AG3 and 5.30% in AG5. The order of Bifidobacteriales was present in all AGs. However, *Bifidobacterium spp.* was significantly abundant in AG1 (14.08%) (p = 0.003) while values lower than 7% were

presented in other AGs. Regarding the order Coriobacteriales, AG2 showed significantly increased levels of *unclassified Coriobacteriaceae spp.* ($p = 0.08$) and Adlercreutzia ($p = 0.03$).

In contrast, Proteobacteria was more abundant in AG1 (11.90%) and decreased to 4.80% in AG2. However, the percentage increased gradually, to 5.59% in AG3, 6.10% in AG4, and 6.62% in AG5. The class Gammaproteobacteria (6.74%) ($p = 0.03$) was significantly abundant in AG1 compared to other AGs. Gammaproteobacteria dropped to 1.1% in AG2 and slowly decreased with increasing AGs. The family Enterobacteriaceae was abundant in AG1 (4.99%) and decreased with age starting at 0.72% (AG2) up to 0.67% in AG5. In comparison to Betaproteobacteria, half of the value in AG1 was detected in AG2 but elevates with increasing age group. In terms of Betaproteobacteria, *Sutterella spp.* was the dominant genus in all AG. *Neisseria spp.* was present in AG1 (0.02%) and reduced to < 0.001% at AG2 and continuously held its value in the greater AGs. In the remaining age groups, Betaproteobacteria were prominent relatively to their percentages. Alphaproteobacteria and Deltaproteobacteria have been detected in all age groups as well. While Deltaproteobacteria elevated from 0.29% in AG1 to 1.05% in AG5, Alphaproteobacteria did not seem to correlate with AG.

No significant difference in abundance was found among all AGs in the following phyla Lentisphaerae, Tenericutes, Verrucomicrobia, and Cyanobacteria.

The relative abundance of Lentisphaerae increased with age, starting at 0.02% in AG1 and reached 0.53% in AG5. AG2 (0.23%) and AG3 (0.21%) had similar percentages, as well as AG4 (0.57%) and AG5 (0.53%) to one another. The percentages of Lentisphaerae were mostly caused by the presence of the family Victivallaceae.

The minimum of Tenericutes was found in AG1 (0.02%), whereas the maximum among all five groups was detected in AG3 (0.56%). Tenericutes levels in the remaining age groups ranged between 0.23% and 0.26%. The order RF39 made up the majority, followed by ML615J-28. However, the proportion remained equivalent to the distribution at the phylum level.

High percentages of Verrucomicrobia were detected in AG3 (0.98%) and its lowest value in AG5 (0.32%). Verrucomicrobia ranged between 0.72% and 0.70% in AG1 and

AG2 respectively, yet 0.46% was found in AG4. *Akkermansia spp.* was the dominant genus in Verrucomicrobia and was rationed similarly as previously mentioned.

Cyanobacteria was low in AG4 (0.001%) and AG1 (0.003%) compared to AG5 (0.02%), AG3 (0.04%) and AG2 (0.07%). The class 4C0d_2 (p = 0.01) and the genus *unclassified YS2* (p = 0.01) were significantly abundant in AG2.

As shown in Figure 6, AG1 was significantly richer in the genera *Escherichia shigella* (p<0.001, q-value = 0.001) and *Ruminococcus gnavus* (p<0.001, q-value = 0.002) in contrast to the other age groups.

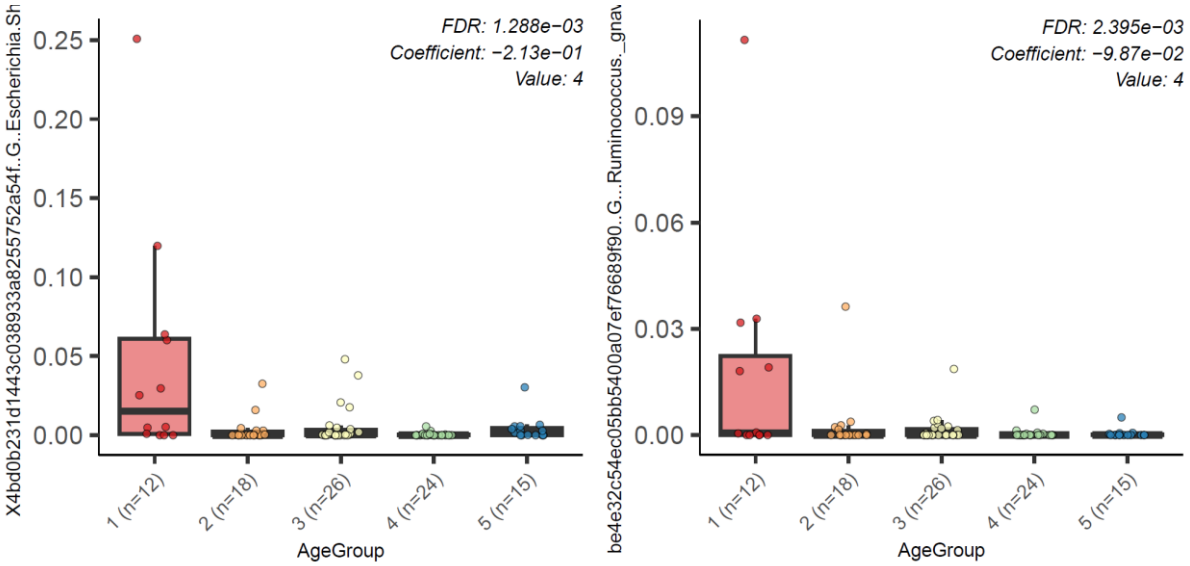

Figure 6. Differential abundance analysis (MaAsLin) displaying the relative abundances of the five age groups among healthy controls (n= 95). AG1 ranges between 0-1 years old, AG2 from 2-5 years old, AG3 from 6-10 years old, AG4 from 11-15 years old, and AG5 from 16-25 years old. AG1 is characterized by increased Shigella (p<0.001, q-value = 0.001) and Ruminococcus gnavus (p<0.001, q-value = 0.002). The remaining AGs do not present a plethora of significantly elevated levels from a specific taxon.

We observed that participants under the age of 6 months (n=2, AG<1) were separated by AG1 and the remaining age groups. We observed the trend of AG1 in detail by analyzing infants from 1 and 2 years old separately. Figure 7A displayed the trend of AG1* with those under 1 year of age. However, two HCs of AG1 were assigned to the same point cloud as AG2, AG3, AG4, and AG5. Figure 7B highlights that 2 out of 8 of the 1-year-old participants fell into the assembly of the greater AGs. Participants ranging from 2 and 25 years old built one cluster, with no distinct partition among the four remaining age groups.

Additionally, Table 3 illustrates the linear discriminant analysis (LDA) score of all five AGs and presents significant abundances between the AGs. In this analysis, LDA scores above 2 are used to show relevant results. Most of the significant differences were observed in AG1 among all AGs. Significant differences were especially found within the phyla Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria.

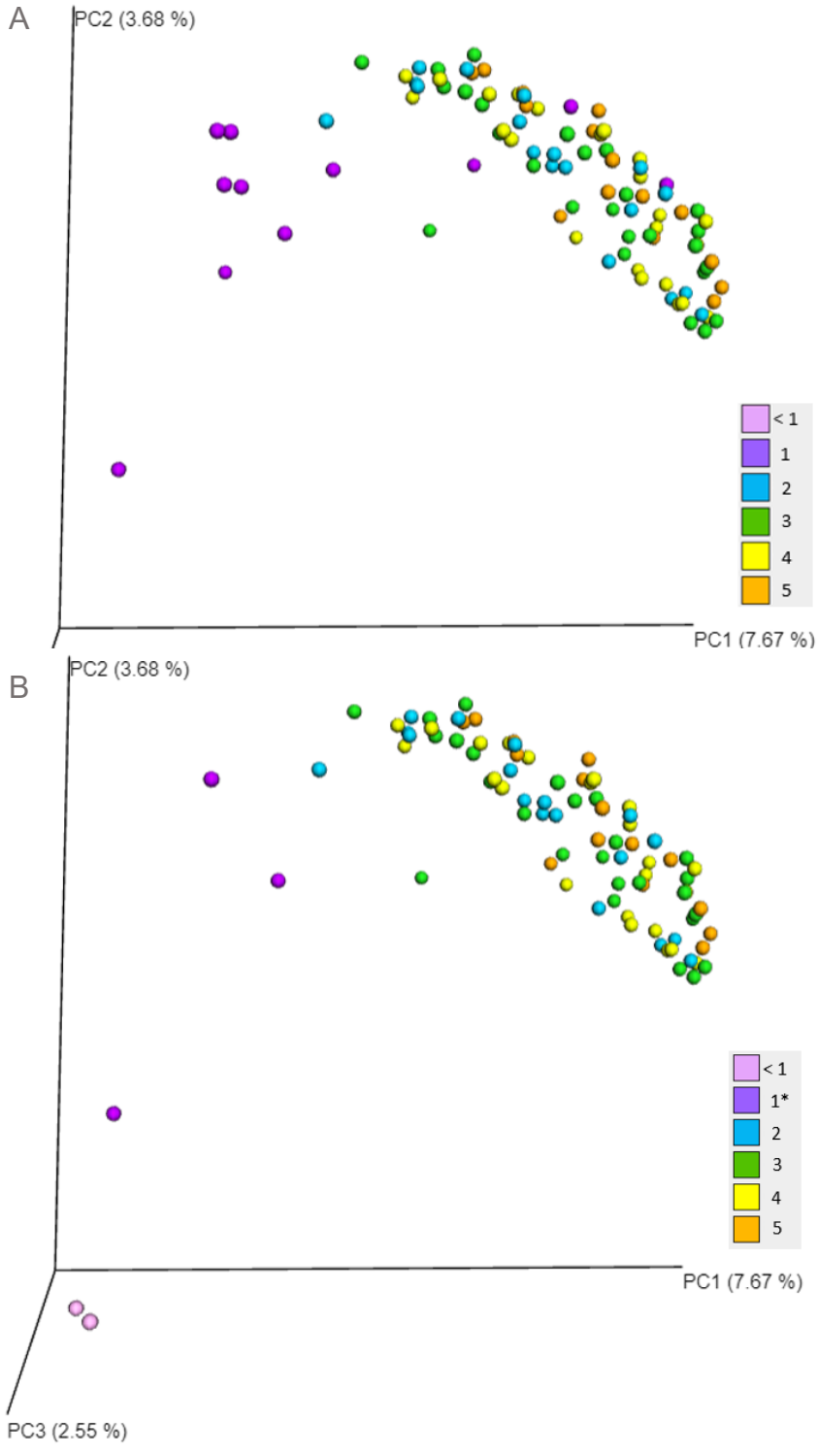

Figure 7. Principal Component Analysis (PCA) of healthy controls (n= 95).

(A) AG<1 consists of participants under the age of 6 months, AG1 ranges between 0-1 years old, AG2 from 2-5 years old, AG3 from 6-10 years old, AG4 from 11-15 years old and AG5 from 16-25 years old. A separation between AG<1 and AG1 towards the remaining age groups is noted. However, two participants are assigned to the same point cloud as the older AGs.

(B) For demonstration purposes, AG1 shows the trend only with those under 1 years old.*

Featured bacteria	mean_max	class_max	LDA-score	p-value
k__Bacteria.p__Actinobacteria	5.181	1	4.67	0.001
k__Bacteria.p__Actinobacteria.c__Actinobacteria	5.163	1	4.66	0.002
k__Bacteria.p__Actinobacteria.c__Actinobacteria.o__Bifidobacteriales.f__Bifidobacteriaceae.g__Bifidobacterium	5.149	1	4.64	0.003
k__Bacteria.p__Actinobacteria.c__Actinobacteria.o__Bifidobacteriales.f__Bifidobacteriaceae	5.149	1	4.64	0.003
k__Bacteria.p__Actinobacteria.c__Actinobacteria.o__Bifidobacteriales	5.149	1	4.64	0.003
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria	4.904	1	4.53	0.004
k__Bacteria.p__Firmicutes.c__Bacilli	4.846	1	4.45	0.022
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales	4.829	1	4.44	0.012
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Enterobacteriales.f__Enterobacteriaceae	4.760	1	4.39	0.002
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Enterobacteriales	4.760	1	4.39	0.002
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Enterobacteriales.f__Enterobacteriaceae.g__unclassifiedEr	4.748	1	4.38	0.002
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae	4.742	1	4.34	0.050
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Streptococcus	4.740	1	4.34	0.028
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Enterococcaceae.g__Vagococcus	0.967	1	4.26	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Veillonellaceae.g__Veillonella	4.512	1	4.18	0.000
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__OtherGammaproteobacteria	0.913	1	3.97	0.001
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__OtherGammaproteobacteria.f__OtherGammaproteobacteri	0.913	1	3.87	0.001
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__OtherGammaproteobacteria.f__OtherGammaproteobacteri	0.913	1	3.85	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Veillonellaceae.g__Megasphaera	3.858	1	3.51	0.022
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__OtherLactobacillales	3.309	1	3.11	0.015
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Enterobacteriales.f__Enterobacteriaceae.g__Trabulsiella	1.948	1	3.08	0.000
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__OtherLactobacillales.g__OtherLactobacillales	3.309	1	3.05	0.015
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Enterococcaceae	3.330	1	3.03	0.000
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Enterococcaceae.g__Enterococcus	3.302	1	3.00	0.000
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Enterobacteriales.f__Enterobacteriaceae.g__OtherEnteroba	2.963	1	2.75	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Epulopiscium	2.793	1	2.64	0.002
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Carnobacteriaceae.g__Granulicatella	2.873	1	2.62	0.021
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Carnobacteriaceae	2.873	1	2.62	0.021
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Faecalibacterium	4.842	2	4.26	0.010
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__OtherClostridiales.g__OtherClostridiales	3.589	2	3.14	0.002
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__OtherClostridiales	3.589	2	3.14	0.002
k__Bacteria.p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales.f__Coriobacteriaceae.g__unclassifiedCoriobacteria	3.416	2	3.08	0.008
k__Bacteria.p__Firmicutes.c__Bacilli.o__Turicibacterales	3.295	2	2.87	0.005
k__Bacteria.p__Firmicutes.c__Bacilli.o__Turicibacterales.f__Turicibacteraceae	3.295	2	2.86	0.005
k__Bacteria.p__Firmicutes.c__Bacilli.o__Turicibacterales.f__Turicibacteraceae.g__Turicibacter	3.295	2	2.85	0.005
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Anaerotruncus	1.815	2	2.74	0.022
k__Bacteria.p__Cyanobacteria.c__4C0d_2.o__YS2	2.538	2	2.69	0.012
k__Bacteria.p__Cyanobacteria.c__4C0d_2.o__YS2.f__unclassifiedYS2.g__unclassifiedYS2	2.538	2	2.63	0.012
k__Bacteria.p__Cyanobacteria.c__4C0d_2.o__YS2.f__unclassifiedYS2	2.538	2	2.60	0.012
k__Bacteria.p__Cyanobacteria.c__4C0d_2	2.538	2	2.60	0.012
k__Bacteria.p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales.f__Coriobacteriaceae.g__Adlercreutzia	2.431	2	2.49	0.032
k__Bacteria.p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Holdemania	2.410	2	2.40	0.000
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Barnesiellaceae_	4.308	3	3.84	0.013
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Barnesiellaceae_.g__unclassified_Barnesiellaceae_	4.308	3	3.84	0.013
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Christensenellaceae	3.497	3	3.31	0.004
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Christensenellaceae.g__unclassifiedChristensenellaceae	3.490	3	3.31	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__OtherRuminococcaceae	3.402	3	3.18	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Anaerostipes	2.658	3	2.42	0.002
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__unclassifiedClostridiales	4.705	4	4.17	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__unclassifiedClostridiales.g__unclassifiedClostridiales	4.705	4	4.17	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Veillonellaceae.g__Dialister	4.342	4	4.01	0.046
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Mogibacteriaceae_	2.463	4	2.85	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Mogibacteriaceae_.g__unclassified_Mogibacteriaceae_	2.447	4	2.73	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Lachnobacterium	2.783	4	2.59	0.004
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae	5.281	5	4.76	0.000
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__unclassifiedRuminococcaceae	4.970	5	4.52	0.000
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Ruminococcus	4.605	5	4.21	0.002
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Odoribacteraceae_	4.425	5	4.00	0.001
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae	4.335	5	3.98	0.000
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae.g__unclassifiedRikenellaceae	4.335	5	3.98	0.000
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Odoribacteraceae_.g__Butyricimonas	4.063	5	3.73	0.038
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Odoribacteraceae_.g__Odoribacter	4.177	5	3.66	0.013
k__Bacteria.p__Proteobacteria.c__Deltaproteobacteria	3.982	5	3.51	0.005
k__Bacteria.p__Proteobacteria.c__Deltaproteobacteria.o__Desulfovibrionales	3.982	5	3.51	0.005
k__Bacteria.p__Proteobacteria.c__Deltaproteobacteria.o__Desulfovibrionales.f__Desulfovibrionaceae	3.982	5	3.51	0.005
k__Bacteria.p__Proteobacteria.c__Deltaproteobacteria.o__Desulfovibrionales.f__Desulfovibrionaceae.g__Bilophila	3.902	5	3.36	0.006

Table 3. LEfSE-Table of healthy controls (n=95) among age groups. The table represents the highest mean (mean_max) found in the Age Group (AG) (class_max) with an LDA score (LDA>2) and p-value (p<0.05). AG1 ranges between 0-1 years old, AG2 from 2-5 years old, AG3 from 6-10 years old, AG4 from 11-15 years old, and AG5 from 16-25 years old. *k_ kingdom, p_ phyla, c_ class, o_ order, f_ family, g_ genus.*

Patient cohort

HC were then compared to participating patients in the Severe Immune Cytopenia Registry at study inclusion (timepoint 1). These include autoimmune hemolytic anemia and Evans Syndrome (AIHA/ES or AIHAES) (n=11), immune thrombocytopenia (ITP) (n=21), and inborn errors of immunity (IEI) (n=12), as seen in Table 2.

Alpha diversity was significantly greater in HC than in patients overall (p=0.01), as seen in Figure 8. Significant differences were found between HC and patients with IEI (Shannon H=9.06, p=0.54; observed species H= 6.76, p=0.009; Faith's PD H=7.40, p=0.007). HC and patients with ITP seemed to have similar alpha diversities, while those with IEI and AIHAES shared similar ranges. Significant differences were found between patients with IEI and ITP (H= 5.57, p=0.02) in Shannon, but not in Faith's PD or observed species. No significant values were found between other SICs and HC and among SICs.

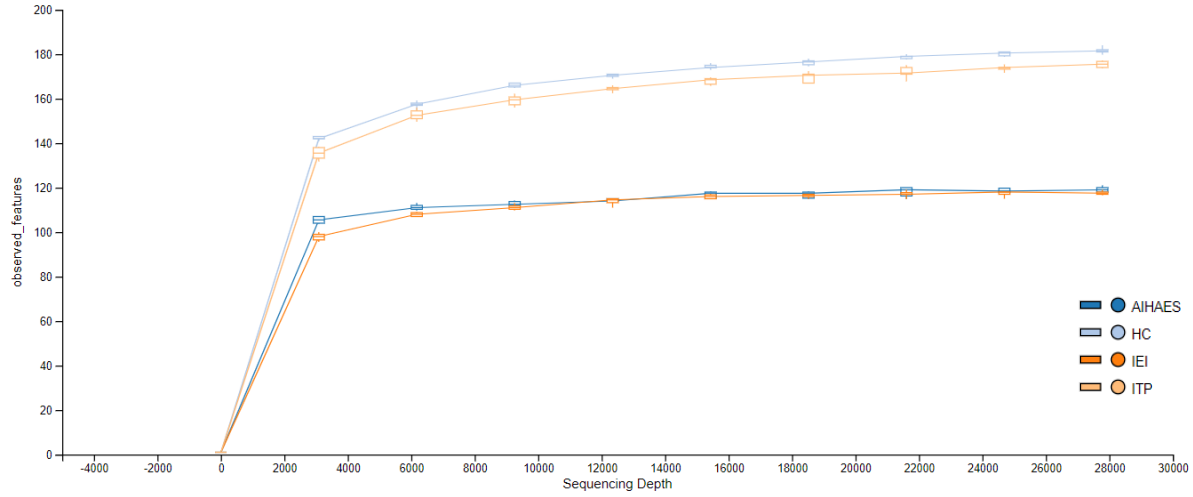

Figure 8. Alpha rarefaction of the four groups at study inclusion: healthy controls (HC), autoimmune hemolytic anemia and Evans syndrome (AIHAES), immune thrombocytopenia (ITP), and inborn errors of immunity (IEI). According to sequencing depth in the x-axis and observed features on the y-axis.

Beta diversity showed no significant differences in the microbiome composition in HC versus patients (p>0.05). The PCA (weighted unifrac) showed no distinct formation of clusters that distinguishes the four groups respectively, as seen in Figure 9. However, a formation of three clusters was observed. Two clusters appeared heterogeneous, mostly dominated by HC. The other cluster was dominated by IEI. Given the relatively high probability of the existence of an underlying monogenic IEI

(181,186,189,269,270), one could hypothesize that the AIHA/ES patients whose microbiome data clustered with IEI were in fact undetected / undiagnosed IEI patients. However, this assumption could neither be confirmed nor excluded due to, *i)*, the lack of genetic data in the sic-reg study and, *ii)*, pending correlation of the results of microbiome analyses with deep immune phenotyping such as high-resolution FACS analyses and immune functional tests. During the study period of the microbiome analyses presented in this thesis, no patient with AIHA/ES was newly diagnosed with or reclassified as IEI.

The following comparison of the microbiome composition among all groups was statistically not significant in adjusted p-values. Thus, the calculation of relative abundances was not possible.

However, we noticed that participants with IEI featured discrepant proportions of phyla in contrast to the other groups, as seen in Figure 10. The phyla Firmicutes (48.942%) made up the majority of their microbiome. The class Bacilli were more abundant in IEI (11.16%) than in AIHA/ES (4.30%), HC (2.53%), and ITP (2.28%). Pronounced levels of *Lactobacillus spp.* were detected in IEI (7.0%) compared to AIHA/ES (0.3%), HC (0.1%) and ITP (0.04%). Further, increased Lactococcus spp. have been found in IEI (0.4%) rather than in AIHA/ES (0.05%), HC, and ITP (0.04%). Additionally, heightened values of *Enterococcus spp.* were found in patients with IEI (3.0%). HC (0.05%), AIHA/ES (0.03%), and ITP (0.01%) presented less Enterococcus spp. Patients with IEI (1.6%) had more *Staphylococcus spp.* than HC (0.03%), AIHA/ES (0.006%) and IEI (0.003%). The class Clostridia was more abundant in AIHA/ES (39.27%) compared to the remaining groups ranging between 36.18% and 36.55%. *Faecalibacterium spp.* was elevated in patients with ITP (6.0%), followed by AIHA/ES (5.1%) and HC (4.8%). In comparison, *Faecalibacterium spp.* were decreased in IEI (2.9%). However, *Clostridium spp.* (1.9%) and *Veillonella spp.* (1.5%) were enhanced in IEI.

The gut microbiome of ITP was dominated by Bacteroidetes (46.05%), which mostly consisted of *Bacteroides spp.* (32.4%). Similar characteristics applied to HC as Bacteroidetes (43.60%) were more prevalent compared to other phyla and consisted of comparable ratios of *Bacteroides spp.* (28.7%). AIHA/ES showed fewer Bacteroidetes (38.55%) than ITP, of which the ratio to *Bacteroides spp.* (30.1%) was reduced. In comparison, Bacteroidetes (30.82%) were less abundant in IEI.

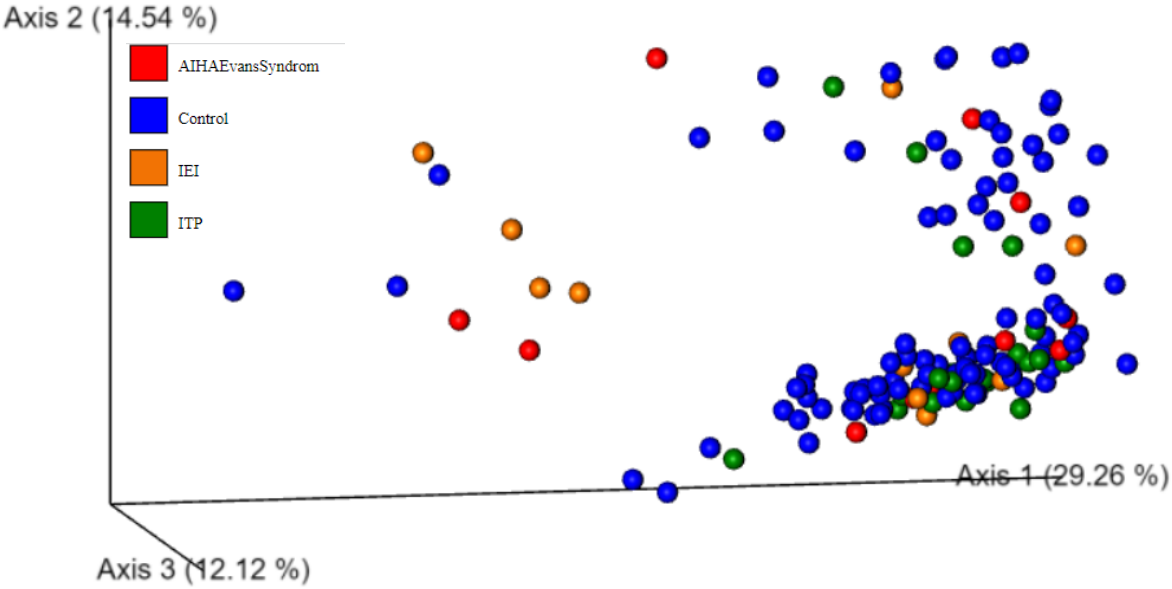

Figure 9. PCA of all four major groups at study inclusion: healthy controls (HC) (=95), autoimmune hemolytic anemia and Evans syndrome (AIHAES), immune thrombocytopenia (ITP) (n=21), and inborn errors of immunity (IEI) (n=12).

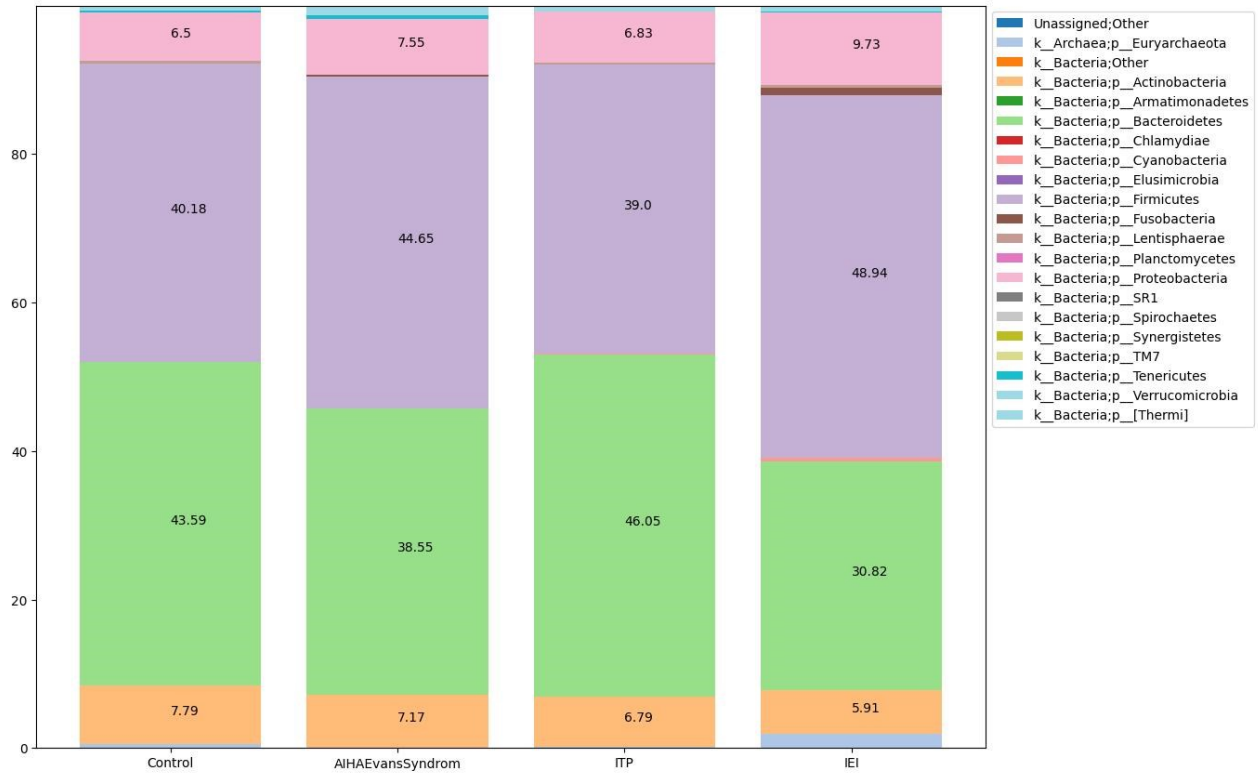

Figure 10. Bar plots of the four groups at study inclusion: healthy controls (HC) (=95), autoimmune hemolytic anemia and Evans syndrome (AIHAES), immune thrombocytopenia (ITP) (n=21), and inborn errors of immunity (IEI) (n=12).

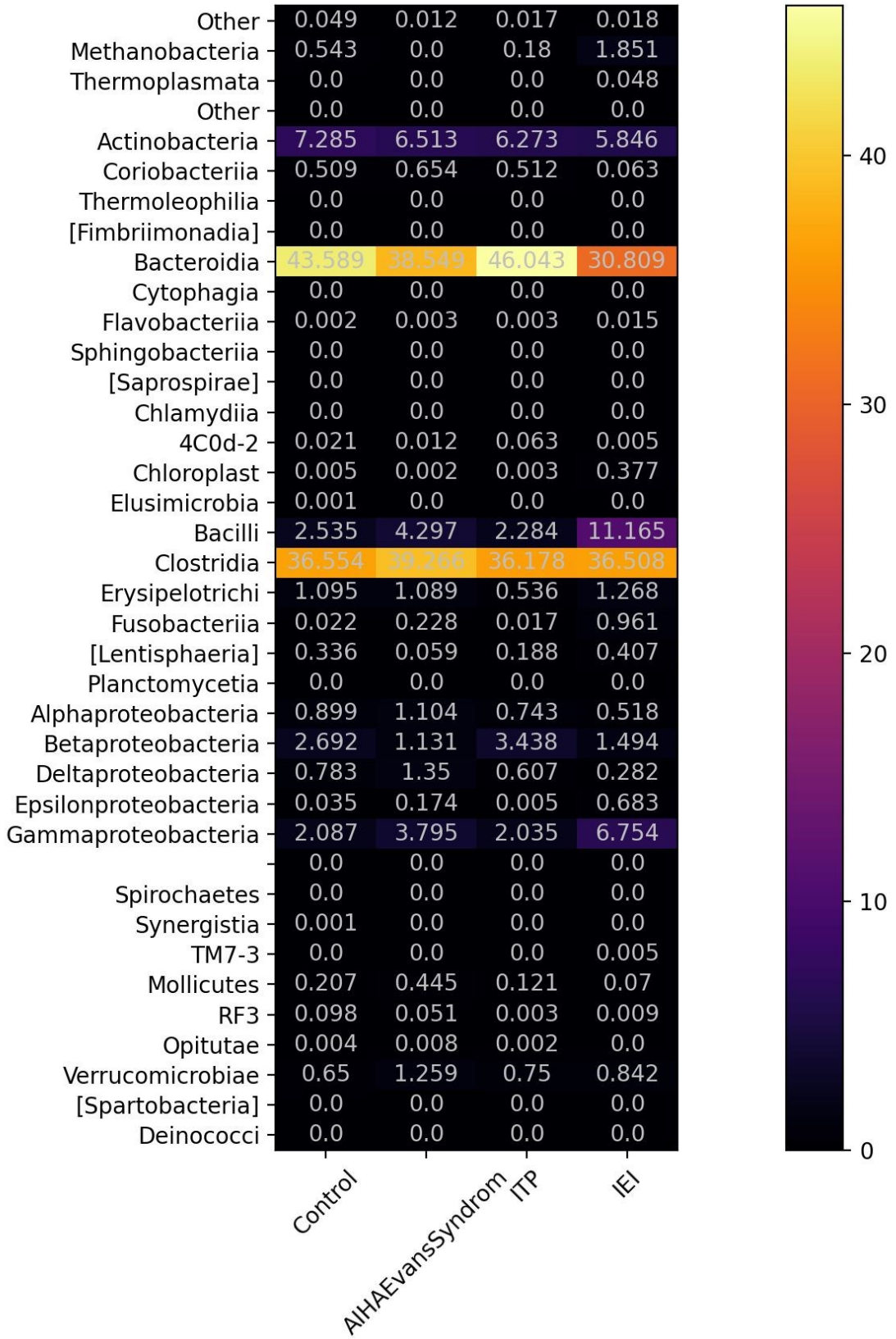

Table 4. Heatmap of the gut microbial composition at the class level of the four major groups: healthy controls (HC) (=95), autoimmune hemolytic anemia and Evans syndrome (AIHAES), immune thrombocytopenia (ITP) (n=21), and inborn errors of immunity (IEI) (n=12).

Lower values of *Bacteroides spp.* (16.9%) were detected in IEI in contrast to the other groups.

Furthermore, Proteobacteria (9.73%), Fusobacteria (0.961%), Cyanobacteria (0.383%), and Lentisphaerae (0.407%) were prevalent in patients with IEI. The distribution of Proteobacteria classes Alpha-, Beta-, Delta-, Epsilon- and Gammaproteobacteria were distinct, as seen in Table 4. The highest percentage of Gammaproteobacteria was found in IEI (6.754%). Representatives therefore were the species *Pseudomonas spp.* (0.599%), *Aggregatibacter spp.* (0.070%), *Citrobacter spp.* (0.029%), *Serratia spp.* (0.057%) and *Proteus spp.* (0.024%). While Gammaproteobacteria were dominant in patients with AIHA/ES (3.795%), Betaproteobacteria were more prevalent in those with ITP (3.438%). *Sutterella spp.* were more abundant in ITP (3.194%) compared to HC (3.020%), AIHA/ES (1.145%), and IEI (0.865%). Yet, in the same Betaproteobacteria class, the arrangement was changed for *Neisseria spp*. as levels were higher in IEI (0.334%), rather than AIHA/ES (0.022%), HC (0.004%) and ITP (0%). HC resembled ITP considering the ratio of Proteobacteria classes with the peak in Betaproteobacteria (2.692% (HC): 3.438% (ITP)) followed by Gammaproteobacteria (2.087%: 2.035%), Alphaproteobacteria (0.899%: 0.743%), Deltaproteobacteria (0.783%: 0.607%) and Epsilonbacteria (0.04%: 0.005%). Similarly, less Epsilonbacteria were found in AIHA/ES (0.035%). However, the lowest Proteobacteria class in patients with IEI was Deltaproteobacteria (0.282%). A thorough layout of the taxonomic classes showed that Bacilli (11.165%), Gammaproteobacteria (6.754%), Epsilonbacteria (0.683%), and Chloroplast (0.377%) were more prevalent in patients with IEI. *Campylobacter spp.* were abundant in IEI (0.860%), followed by AIHA/ES (0.077%), and were low in HC (0.036%) and ITP (0.003%). However, *Succinivibrio spp.* were elevated in ITP (0.661%) than in HC (0.001%), AIHA/ES, and IEI (0%). *Fusobacterium spp.* were enhanced in IEI (1.931%) rather than in AIHA/ES (0.050%), HC (0.018%) and ITP (0.013%). In addition to Fusobacteria, *Leptotrichia spp.* were more common in IEI (0.307%) compared to AIHA/ES (0.048%), HC (0.003%), and ITP (0.001%). Cyanobacteria were more common in patients with ITP (0.066%) and IEI (0.383%), compared to AIHA and ES (0.014%) and HC (0.026%). Similarities to HC were found in the percentages of Lentisphaerae between those with IEI, as HC presents 0.336% and the latter 0.407%.

Tenericutes were detected in the four groups remaining under 0.5%. The highest rate was found in patients with AIHA and ES (0.496%), ITP (0.124%) and IEI (0.079%). The prevalence of class Mollicutes was elevated in AIHA and ES (0.445%). Verrucomicrobia were common in AIHA (1.266%), followed by IEI (0.843%) and ITP (0.752%). In contrast, in HC 0.304% Tenericutes and 0.654% Verrucomicrobia were present. A representative genus of Verrucomicrobia was Akkermansia spp., which were prevalent in AIHA/ES (1.5%) and ITP (0.9%). However, less common in HC (0.7%) and IEI (0.63%).

Actinobacteria were increased in HC (7.794%) and AIHA and ES (7.164%), followed by ITP (6.785%) and IEI (5.909%). *Bifidobacterium spp.* made up the majority of HC (7.5%), ITP (6.1%), and IEI (5.8%). While *Bifidobacterium spp.* were decreased in AIHA/ES (5.5%), *Rothia spp.* (0.303%), *Actinomyces spp.* (0.18%), *Atopobium spp.* (0.125%) had higher values in IEI. In contrast, HC, ITP, and AIHA/Evans presented percentages lower than 0.05%. Yet, *Collinsella spp.* were little represented in IEI (0.038%) compared to ITP (0.345%), HC (0.285%) and AIHA/ES (0.209%).

Phyla in low abundance were TM7 (0.005%), which was found in IEI, and Elusimicrobia and Synergistetes (0.001%), which were detected in HC.

4. Discussion

Because the evolution process of the gut microbiota is dynamic, it can be altered by different perinatal conditions from the host itself through SIgA and from external parameters such as the mode of delivery and lifestyle. This is why it is even more important to review neglected age groups in this scientific question. (271–273) In this study, our focus lies on age-related differences in the gut microbiome in healthy infants and children and between these healthy controls and patients with various types of immune cytopenia with or without a known underlying IEI.

The majority of research on the pediatric intestinal microbiome has mostly studied infants under the age of 3 years and adolescents over the age of 18 years and above. (3) Until 2019 Riva et al. were one of a few, who examined this topic with children ranging between 9 and 16 years old. (274) Therefore, we attempted to understand the composition and dynamic changes throughout different age groups (AG) ranging from 0 to 25 years old. We expected gradual changes in the microbiota composition among the age groups. This was not the case in every AG. The deviant behavior of AG1 was verified with the results of the unweighted PCA and the taxa summaries. We see hints that could support the statements of Palmer et al. and Koenig et al. They suggest that similarities to the gut microbiome of adults can be found at the end mark at the age of 1 (86) and full resemblance to adults is achieved by the age of 2.5 years (45). According to the unweighted PCA, 2/7 (28%) of the 1-year-olds (1.1 and 1.4 years old) were grouped with AG 3 and higher in the unweighted PCA. Infants at the age of 2.5 did not appear to continue the trend subsequently but rather were found in the same point cloud as AG3, AG4, and AG5. However, the other distance matrices for PCA such as weighted unifrac, Bray Curtis, and Pearson show the formation of at least three clusters among the participants. Depending on the distance matrix, the clustering needs to be readjusted. This leaves us with the question, which factors may have influenced the clusters.

The presence of *Escherichia shigella* in AG1 does not indicate Shigellosis, as acute infection, inflammation, and chronic gastrointestinal diseases were part of the exclusion criteria in HC. Further, Escherichia shigella is a facultative pathogen, which may cause symptoms in immunocompromised patients. (275,276) Füeßl (275) suggests that Escherichia shigella could be associated with metabolic liver diseases

and DM2 in adults. According to several studies, *Ruminococcus gnavus* is present in the early stages of life, and associations between diet were observed. (277) Tannock et al. (278) have described that diet may have an impact on the abundance of *Ruminococcus gnavus* in 2-month-old infants. In babies fed with breast milk and goat milk, Lachnospiraceae were limited to the species *Ruminococcus gnavus*. In contrast, those fed with cow milk had a greater diversity of Lachnospiraceae. Further, Mennella et al. (279) observed an increase in *Ruminococcus gnavus* that infants intolerant to cow milk. These infants were fed with isocaloric extensive protein hydrolysate formula, while others were fed with cows' milk formula. As the number of our study participants per age group (n_HC=19±7) is relatively low, a broad generalization of our findings is not possible and should be investigated in the future.

When comparing our results to the European results from the review by Deering et. al (280), the dominant phyla Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia are present in both parties. However, a significant disproportion of Firmicutes (67.7%) to Bacteroidetes (21.1%) is noticeable in European studies. In 3 out of 4 studies, the study subjects were infants between 2 and 3 years of age. (281–283) While the other analyzed the composition of 9 to 16-year-olds. (274) As studies on 4- to 8-year-olds are limited, the age gap makes it difficult to directly correlate our results.

Although we observed trends of microbiome alterations in patients with AIHA and ES similar to patients with IEI, no clear or general association was found between the damaged immune tolerance linked to autoimmunity and detectable gut dysbiosis in our study. We could not verify this hypothesis in ITP. A general statement upon this matter on AIHA/ES and IEI is invalid because of the low number of study participants. Moreover, the deviant behavior of results of microbiome analyses in patients with IEI might be explained by the heterogenous IEI patient cohort. In their case, timepoint 1 could not be determined as "untreated"; IEI patients were recruited for observation at random time points, or their microbiome was analyzed for clinical purposes. This resulted into including patients with severe disease courses, a few under parenteral diet, under immunosuppressive therapy, or even post hematopoietic stem cell transplantation (HSCT). Therefore this selection may have imposed an impact on the gut microbiome composition of IEI patients and represent a bias. However, it is inherent in the clinical course of patients diagnosed with severe IEI and thus a

determining factor for the composition of the cohort of patients with IEI, that they will be hard or impossible to be included in a study as treatment-naïve individuals. Nevertheless, the IEI cohort appears highly interesting to study further, for instance longitudinally, using sequential microbiome analyses, as the behavior among this heterogenous cohort could differ and give additional insight into potential diagnostic and therapeutic approaches.

The comparison of the gut microbiome composition among the four groups showed no significant difference. This could be due to the small sample size of study participants in the groups AIHA/ES, ITP, and IEI. As our sample was taken at inclusion (timepoint 1), the dynamic during the disease course is not considered. Longitudinal observations could give further insight into this subject. There is a lack of knowledge considering birth mode, duration of the breastfeeding period, dietary habits, potential stressors, and the environment surrounding our study participants individually. These are factors that influence the gut microbiome at an early age. The assessment of those influences could have given an explanation for the insignificant results or of the cluster formations. In this study, personal information such as date of birth, sex, current diagnosis, past medical history, and therapeutic interventions were gathered. Additional facts like BMI and the number of siblings or pets are not noted. Expanding data acquisition could be helpful for further investigation.

By our knowledge, we are the first to analyze additional IEI such as LPS-responsive and beige-like anchor protein (LRBA)-Defect (n=1) and Nijmegen-Breakage-Syndrome (n=2). Further, we studied a patient with Common Variable Immune Disease (CVID) with Granulomatous Lymphocytic Interstitial Lung Disease (GLILD) and Irritable Bowel Disease (IBD). We included conditions such as sickle cell anaemia and cytopenia as observational patients. The state prior to hematopoietic stem-cell transplantation (HCST) could be analyzed in a patient with chronic granulomatous disease. Thus, one participant with ES after HSCT and another diagnosed ES with Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4)-Deficiency were involved in this study. With its complex and heterogeneous characteristics, it is important to keep in mind that the diseases included in our study representing IEI are single case studies. This makes it difficult to represent the disease group accurately as a whole. As an example, the gut microbiome composition shifts towards Firmicutes in patients with WIP defect and LRBA defect, who were both, for a long time, under parenteral nutrition

or enteral tube/formula diet. Bacteroidetes are more dominant in Nijmegen-Breakage-Syndrome, while the Firmicutes: Bacteroidetes ratio in CVID appears even. However, the validity of these findings is limited due to the number of samples per subtype. A broader range of participants in studies regarding the gut microbiome could help to understand the different dynamics of IEI.

Immunological studies such as from Fischer et al. (186) have shown that IEI is more likely associated with AIHAES rather than ITP. The relative risk of AIHAES in patients with IEI is at 830, followed by the relative risks of cytopenias at 120 and of ITP at 60. In our study, alpha rarefaction suggests a similarity of AIHA/ES and IEI in alpha diversity. However, the results of Differential Abundance Analysis show no related plethora in AIHAES and IEI. Further studies with a larger sample size could investigate if the gut microbiome of AIHAES and IEI harbor comparable species.

As Deering et al. (280) stated, the chosen 16S rRNA region to amplify can affect the results. In terms of pediatric HC, 42 studies with more than 2,000 children were included in their review. Out of nine possible regions (V1 – V9) (284), about 39% used the V6 region. We used the V4 region. The most conserved regions V4, V5, and V6 participate during the translation process and are necessary for the interaction with the 23S rRNA. The results for phyla and classes are more precise either with V4, V5, or V6, but at genus and species level V2 and V3 are more reliable. (284) With this in mind, the used amplicon in other studies regarding AIHA/Evans, ITP, and IEI needs to be taken into account as well. Additionally, the choice for the gastrointestinal segment of interest determines the presence of specific phyla during analysis. (285) As the distal gut harbors a highly selective gut flora (281,286), our results represent this section of our study participants and are not directly applicable to other parts of the gastrointestinal tract.

Most of our study participants are of Caucasian descent. Therefore, future studies should examine the pediatric gut microbiome of non-Caucasians in contrast to our predominantly white healthy control cohort. Hollister et al. stated that ethnicity and race made a small, yet statistically significant impact on the gut community richness. However, no statistically significant influence of ethnicity and race on community composition was observed in their study. (26) Additionally, the participants of our study are mainly either living in Austrian metropolises such as Graz (Styria) or Innsbruck

(Tyrol) or in smaller towns in proximity to the city centers of the two federal states mentioned. The gut microbiome of children living in rural areas of the federal states should be compared to our pediatric urban cohort as well.

Limitations in collecting pediatric stool samples may occur due to ethical principles and practical reasons. (287) Obtaining samples from children with the consent of their parental guardian or their own remains a challenge, especially with teenagers. Despite the consent for participation in the study, a few test tubes did not contain stool samples. These study participants and their samples were excluded from the analysis. Another reason that hindered some potential candidates was the thought of submitting stool samples. An adapted approach to explaining the topic of gut microbiome analysis could be beneficial for a better understanding and compliance.

5. Conclusion

Analysis of the gut microbiota revealed a distinction in composition between healthy newborns aged 0 to 12 months old and healthy participants ranging from 2 and 25 years of age in our study. No significant difference was detected among the remaining four Age Groups. As several factors have an early impact on the composition of the gut microbiome, further analysis of a broad pediatric cohort is required.

Further, the comparison of the gut microbiome of HC to patients with AIHA/ES, ITP, and IEI showed a significantly lower alpha diversity in IEI compared to HC. This might be due to IEI patients being heterogenous in their disease course and upon the receipt of possible gut-altering therapies as part of the observation. These include parental diet, immunosuppressive therapy and post hematopoietic stem cell transplantation. Principal Component Analysis illustrates no distinct separation of the four groups to one another, but a formation of three clusters. The formed clusters exhibited no significant similarity and would require extensive analysis or a larger sample size to draw definitive conclusions. No significant difference in the gut microbiome composition was found. As the disease course changes over time, longitudinal studies of these pediatric patients might give more insight into the dynamics of the gut microbiome and its potential role in immune tolerance. Further investigations concerning this topic are needed.

6. Bibliography

1. Allaband C, McDonald D, Vázquez-Baeza Y, Minich JJ, Tripathi A, Brenner DA, et al. Microbiome 101: Studying, Analyzing, and Interpreting Gut Microbiome Data for Clinicians. Clin Gastroenterol Hepatol. 2019 Jan;17(2):218–30.

2. Landman C, Quévrain E. [Gut microbiota: Description, role and pathophysiologic implications]. Rev Med Interne. 2016 Jun;37(6):418–23.

3. Derrien M, Alvarez AS, de Vos WM. The Gut Microbiota in the First Decade of Life. Trends Microbiol. 2019 Dec;27(12):997–1010.

4. Hallen-Adams HE, Suhr MJ. Fungi in the healthy human gastrointestinal tract. Virulence. 2017 Apr 3;8(3):352–8.

5. Suhr MJ, Hallen-Adams HE. The human gut mycobiome: pitfalls and potentials--a mycologist's perspective. Mycologia. 2015 Dec;107(6):1057–73.

6. Gaci N, Borrel G, Tottey W, O'Toole PW, Brugère JF. Archaea and the human gut: new beginning of an old story. World J Gastroenterol. 2014 Nov 21;20(43):16062–78.

7. Chabé M, Lokmer A, Ségurel L. Gut Protozoa: Friends or Foes of the Human Gut Microbiota? Trends Parasitol. 2017 Dec;33(12):925–34.

8. Belkaid Y, Harrison OJ. Homeostatic Immunity and the Microbiota. Immunity. 2017 Apr 18;46(4):562–76.

9. Ventura M, Turroni F, Canchaya C, Vaughan EE, O'Toole PW, van Sinderen D. Microbial diversity in the human intestine and novel insights from metagenomics. Front Biosci (Landmark Ed). 2009 Jan 1;14:3214–21.

10. Berding K, Holscher HD, Arthur AE, Donovan SM. Fecal microbiome composition and stability in 4- to 8-year old children is associated with dietary patterns and nutrient intake. J Nutr Biochem. 2018 Jun;56:165–74.

11. Wexler HM. Bacteroides: the good, the bad, and the nitty-gritty. Clin Microbiol Rev. 2007 Oct;20(4):593–621.

12. Shao Y, Forster SC, Tsaliki E, Vervier K, Strang A, Simpson N, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. Nature. 2019 Oct;574(7776):117–21.

13. McInnes RS, McCallum GE, Lamberte LE, van Schaik W. Horizontal transfer of antibiotic resistance genes in the human gut microbiome. Curr Opin Microbiol. 2020 Feb;53:35–43.

14. Adak A, Khan MR. An insight into gut microbiota and its functionalities. Cell Mol Life Sci. 2019 Feb;76(3):473–93.

15. Heintz-Buschart A, Wilmes P. Human Gut Microbiome: Function Matters. Trends Microbiol. 2018 Jul;26(7):563–74.

16. Lahti L, Shetty S, Ernst G. Orchestrating Microbiome Analysis [Internet]. 2022. Available from: https://microbiome.github.io/OMA/microbiome-diversity.html

17. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem. 1999 Jun 18;274(25):17406–9.

18. Wang Q, Dziarski R, Kirschning CJ, Muzio M, Gupta D. Micrococci and peptidoglycan activate TLR2-->MyD88-->IRAK-->TRAF-->NIK-->IKK-->NF-kappaB signal transduction pathway that induces transcription of interleukin-8. Infect Immun. 2001 Apr;69(4):2270–6.

19. Patten DA, Collett A. Exploring the immunomodulatory potential of microbial-associated molecular patterns derived from the enteric bacterial microbiota. Microbiology (Reading). 2013 Aug;159(Pt 8):1535–44.

20. Macfarlane GT, Macfarlane S. Bacteria, colonic fermentation, and gastrointestinal health. J AOAC Int. 2012 Feb;95(1):50–60.

21. Murugesan S, Nirmalkar K, Hoyo-Vadillo C, García-Espitia M, Ramírez-Sánchez D, García-Mena J. Gut microbiome production of short-chain fatty acids and obesity in children. Eur J Clin Microbiol Infect Dis. 2018 Apr;37(4):621–5.

22. Martin-Gallausiaux C, Marinelli L, Blottière HM, Larraufie P, Lapaque N. SCFA: mechanisms and functional importance in the gut. Proc Nutr Soc. 2021 Feb;80(1):37–49.

23. Shreiner AB, Kao JY, Young VB. The gut microbiome in health and in disease. Curr Opin Gastroenterol. 2015 Jan;31(1):69–75.

24. Larsbrink J, Rogers TE, Hemsworth GR, McKee LS, Tauzin AS, Spadiut O, et al. A discrete genetic locus confers xyloglucan metabolism in select human gut Bacteroidetes. Nature. 2014 Feb 27;506(7489):498–502.

25. Ruan W, Engevik MA, Spinler JK, Versalovic J. Healthy Human Gastrointestinal Microbiome: Composition and Function After a Decade of Exploration. Dig Dis Sci. 2020 Mar;65(3):695–705.

26. Hollister EB, Riehle K, Luna RA, Weidler EM, Rubio-Gonzales M, Mistretta TA, et al. Structure and function of the healthy pre-adolescent pediatric gut microbiome. Microbiome. 2015 Aug 26;3:36.

27. Douglas-Escobar M, Elliott E, Neu J. Effect of intestinal microbial ecology on the developing brain. JAMA Pediatr. 2013 Apr;167(4):374–9.

28. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiol Mol Biol Rev. 2017 Dec;81(4).

29. Xiao L, Zhao F. Microbial transmission, colonisation and succession: from pregnancy to infancy. Gut. 2023 Apr;72(4):772–86.

30. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. Sci Transl Med. 2014 May 21;6(237):237ra65.

31. Parnell LA, Briggs CM, Cao B, Delannoy-Bruno O, Schrieffer AE, Mysorekar IU. Microbial communities in placentas from term normal pregnancy exhibit spatially variable profiles. Sci Rep. 2017 Sep 11;7(1):11200.

32. Liu CJ, Liang X, Niu ZY, Jin Q, Zeng XQ, Wang WX, et al. Is the delivery mode a critical factor for the microbial communities in the meconium? EBioMedicine. 2019 Nov;49:354–63.

33. Tuominen H, Collado MC, Rautava J, Syrjänen S, Rautava S. Composition and maternal origin of the neonatal oral cavity microbiota. J Oral Microbiol. 2019;11(1):1663084.

34. Leoni C, Ceci O, Manzari C, Fosso B, Volpicella M, Ferrari A, et al. Human Endometrial Microbiota at Term of Normal Pregnancies. Genes (Basel). 2019 Nov 26;10(12).

35. Zhu L, Luo F, Hu W, Han Y, Wang Y, Zheng H, et al. Bacterial Communities in the Womb During Healthy Pregnancy. Front Microbiol. 2018;9:2163.

36. Stinson LF, Boyce MC, Payne MS, Keelan JA. The Not-so-Sterile Womb: Evidence That the Human Fetus Is Exposed to Bacteria Prior to Birth. Front Microbiol. 2019;10:1124.

37. Younge N, McCann JR, Ballard J, Plunkett C, Akhtar S, Araújo-Pérez F, et al. Fetal exposure to the maternal microbiota in humans and mice. JCI Insight. 2019 Oct 3;4(19):127806.

38. Gomez-Arango LF, Barrett HL, McIntyre HD, Callaway LK, Morrison M, Nitert MD. Contributions of the maternal oral and gut microbiome to placental microbial colonization in overweight and obese pregnant women. Sci Rep. 2017 Jun 6;7(1):2860.

39. Rackaityte E, Halkias J, Fukui EM, Mendoza VF, Hayzelden C, Crawford ED, et al. Viable bacterial colonization is highly limited in the human intestine in utero. Nat Med. 2020 Apr;26(4):599–607.

40. Mishra A, Lai GC, Yao LJ, Aung TT, Shental N, Rotter-Maskowitz A, et al. Microbial exposure during early human development primes fetal immune cells. Cell. 2021 Jun 24;184(13):3394-3409.e20.

41. Lauder AP, Roche AM, Sherrill-Mix S, Bailey A, Laughlin AL, Bittinger K, et al. Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. Microbiome. 2016 Jun 23;4(1):29.

42. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol. 2014 Nov 12;12:87.

43. Perez-Muñoz ME, Arrieta MC, Ramer-Tait AE, Walter J. A critical assessment of the ‘sterile womb’ and ‘in utero colonization’ hypotheses: implications for research on the pioneer infant microbiome. Microbiome. 2017 Apr 28;5(1):48.

44. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. Cell Host Microbe. 2015 May 13;17(5):690–703.

45. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, et al. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A. 2011 Mar 15;108 Suppl 1(Suppl 1):4578–85.

46. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012 May 9;486(7402):222–7.

47. Stewart CJ, Ajami NJ, O'Brien JL, Hutchinson DS, Smith DP, Wong MC, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. Nature. 2018 Oct;562(7728):583–8.

48. Cheng J, Ringel-Kulka T, Heikamp-de Jong I, Ringel Y, Carroll I, de Vos WM, et al. Discordant temporal development of bacterial phyla and the emergence of core in the fecal microbiota of young children. ISME J. 2016 Apr;10(4):1002–14.

49. Ringel-Kulka T, Cheng J, Ringel Y, Salojärvi J, Carroll I, Palva A, et al. Intestinal microbiota in healthy U.S. young children and adults--a high throughput microarray analysis. PLoS One. 2013;8(5):e64315.

50. Zhong H, Penders J, Shi Z, Ren H, Cai K, Fang C, et al. Impact of early events and lifestyle on the gut microbiota and metabolic phenotypes in young school-age children. Microbiome. 2019 Jan 4;7(1):2.

51. Lyte M, Vulchanova L, Brown DR. Stress at the intestinal surface: catecholamines and mucosa-bacteria interactions. Cell Tissue Res. 2011 Jan;343(1):23–32.

52. Biagi E, Nylund L, Candela M, Ostan R, Bucci L, Pini E, et al. Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PLoS One. 2010 May 17;5(5):e10667.

53. Frioux C, Ansorge R, Özkurt E, Ghassemi Nedjad C, Fritscher J, Quince C, et al. Enterosignatures define common bacterial guilds in the human gut microbiome. Cell Host Microbe. 2023 Jul 12;31(7):1111-1125.e6.

54. Magne F, Gotteland M, Gauthier L, Zazueta A, Pesoa S, Navarrete P, et al. The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? Nutrients. 2020 May 19;12(5).

55. Stojanov S, Berlec A, Štrukelj B. The Influence of Probiotics on the Firmicutes/Bacteroidetes Ratio in the Treatment of Obesity and Inflammatory Bowel disease. Microorganisms. 2020 Nov 1;8(11).

56. Mariat D, Firmesse O, Levenez F, Guimarães V, Sokol H, Doré J, et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol. 2009 Jun 9;9:123.

57. Mölzer C, Wilson HM, Kuffova L, Forrester JV. A Role for Folate in Microbiome-Linked Control of Autoimmunity. J Immunol Res. 2021;2021:9998200.

58. Stover PJ. Physiology of folate and vitamin B12 in health and disease. Nutr Rev. 2004 Jun;62(6 Pt 2):S3-12; discussion S13.

59. Kunisawa J, Hashimoto E, Ishikawa I, Kiyono H. A pivotal role of vitamin B9 in the maintenance of regulatory T cells in vitro and in vivo. PLoS One. 2012;7(2):e32094.

60. Albert MJ, Mathan VI, Baker SJ. Vitamin B12 synthesis by human small intestinal bacteria. Nature. 1980 Feb 1;283(5749):781–2.

61. Marques TM, Wall R, Ross RP, Fitzgerald GF, Ryan CA, Stanton C. Programming infant gut microbiota: influence of dietary and environmental factors. Curr Opin Biotechnol. 2010 Apr;21(2):149–56.

62. Monsen ALB, Refsum H, Markestad T, Ueland PM. Cobalamin status and its biochemical markers methylmalonic acid and homocysteine in different age groups from 4 days to 19 years. Clin Chem. 2003 Dec;49(12):2067–75.

63. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013 Aug 29;500(7464):541–6.

64. Agans R, Rigsbee L, Kenche H, Michail S, Khamis HJ, Paliy O. Distal gut microbiota of adolescent children is different from that of adults. FEMS Microbiol Ecol. 2011 Aug;77(2):404–12.

65. Woodmansey EJ. Intestinal bacteria and ageing. J Appl Microbiol. 2007 May;102(5):1178–86.

66. Tiihonen K, Ouwehand AC, Rautonen N. Human intestinal microbiota and healthy ageing. Ageing Res Rev. 2010 Apr;9(2):107–16.

67. Lan Y, Kriete A, Rosen GL. Selecting age-related functional characteristics in the human gut microbiome. Microbiome. 2013 Jan 9;1(1):2.

68. Kumbhare SV, Patangia DVV, Patil RH, Shouche YS, Patil NP. Factors influencing the gut microbiome in children: from infancy to childhood. J Biosci. 2019 Jun;44(2).

69. Black M, Bhattacharya S, Philip S, Norman JE, McLernon DJ. Planned Cesarean Delivery at Term and Adverse Outcomes in Childhood Health. JAMA. 2015 Dec 1;314(21):2271–9.

70. Adlercreutz EH, Wingren CJ, Vincente RP, Merlo J, Agardh D. Perinatal risk factors increase the risk of being affected by both type 1 diabetes and coeliac disease. Acta Paediatr. 2015 Feb;104(2):178–84.

71. Kowalska-Duplaga K, Gosiewski T, Kapusta P, Sroka-Oleksiak A, Wędrychowicz A, Pieczarkowski S, et al. Differences in the intestinal microbiome of healthy children and patients with newly diagnosed Crohn's disease. Sci Rep. 2019 Dec 11;9(1):18880.

72. Kuhle S, Tong OS, Woolcott CG. Association between caesarean section and childhood obesity: a systematic review and meta-analysis. Obes Rev. 2015 Apr;16(4):295–303.

73. Abrahamsson TR, Jakobsson HE, Andersson AF, Björkstén B, Engstrand L, Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. Clin Exp Allergy. 2014 Jun;44(6):842–50.

74. Dominguez-Bello MG, De Jesus-Laboy KM, Shen N, Cox LM, Amir A, Gonzalez A, et al. Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. Nat Med. 2016 Mar;22(3):250–3.

75. van Best N, Dominguez-Bello MG, Hornef MW, Jašarević E, Korpela K, Lawley TD. Should we modulate the neonatal microbiome and what should be the goal? Microbiome. 2022 May 10;10(1):74.

76. UNICEF/Ayene. Preterm birth. 2018.

77. Berg RD. Bacterial translocation from the gastrointestinal tract. Adv Exp Med Biol. 1999;473:11–30.

78. Mai V, Torrazza RM, Ukhanova M, Wang X, Sun Y, Li N, et al. Distortions in development of intestinal microbiota associated with late onset sepsis in preterm infants. PLoS One. 2013;8(1):e52876.

79. Korpela K, Blakstad EW, Moltu SJ, Strømmen K, Nakstad B, Rønnestad AE, et al. Intestinal microbiota development and gestational age in preterm neonates. Sci Rep. 2018 Feb 6;8(1):2453.

80. Infant and Young Child Feeding: Model Chapter for Textbooks for Medical Students and Allied Health Professionals. Geneva: World Health Organization; 2009.

81. Cacho NT, Lawrence RM. Innate Immunity and Breast Milk. Front Immunol. 2017;8:584.

82. Andreas NJ, Kampmann B, Mehring Le-Doare K. Human breast milk: A review on its composition and bioactivity. Early Hum Dev. 2015 Nov;91(11):629–35.

83. Sitarik AR, Bobbitt KR, Havstad SL, Fujimura KE, Levin AM, Zoratti EM, et al. Breast Milk Transforming Growth Factor β Is Associated With Neonatal Gut Microbial Composition. J Pediatr Gastroenterol Nutr. 2017 Sep;65(3):e60–7.

84. Solís G, de Los Reyes-Gavilan CG, Fernández N, Margolles A, Gueimonde M. Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. Anaerobe. 2010 Jun;16(3):307–10.

85. Laursen MF, Andersen LBB, Michaelsen KF, Mølgaard C, Trolle E, Bahl MI, et al. Infant Gut Microbiota Development Is Driven by Transition to Family Foods Independent of Maternal Obesity. mSphere. 2016 Feb;1(1).

86. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol. 2007 Jul;5(7):e177.

87. Vandenplas Y, Carnielli VP, Ksiazyk J, Luna MS, Migacheva N, Mosselmans JM, et al. Factors affecting early-life intestinal microbiota development. Nutrition. 2020 Oct;78:110812.

88. Bai DL, Wu KM, Tarrant M. Association between intrapartum interventions and breastfeeding duration. J Midwifery Womens Health. 2013 Feb;58(1):25–32.

89. Knol J, Scholtens P, Kafka C, Steenbakkers J, Gro S, Helm K, et al. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. J Pediatr Gastroenterol Nutr. 2005 Jan;40(1):36–42.

90. Sela DA, Mills DA. Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. Trends Microbiol. 2010 Jul;18(7):298–307.

91. Davis MY, Zhang H, Brannan LE, Carman RJ, Boone JH. Rapid change of fecal microbiome and disappearance of Clostridium difficile in a colonized infant after transition from breast milk to cow milk. Microbiome. 2016 Oct 7;4(1):53.

92. Mountzouris KC, McCartney AL, Gibson GR. Intestinal microflora of human infants and current trends for its nutritional modulation. Br J Nutr. 2002 May;87(5):405–20.

93. Stark PL, Lee A. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J Med Microbiol. 1982 May;15(2):189–203.

94. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A. 2010 Aug 17;107(33):14691–6.

95. Cresci GA, Bawden E. Gut Microbiome: What We Do and Don't Know. Nutr Clin Pract. 2015 Dec;30(6):734–46.

96. Ronan V, Yeasin R, Claud EC. Childhood Development and the Microbiome-The Intestinal Microbiota in Maintenance of Health and Development of Disease During Childhood Development. Gastroenterology. 2021 Jan;160(2):495–506.

97. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006 Dec 21;444(7122):1022–3.

98. Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D, et al. Cohabiting family members share microbiota with one another and with their dogs. Elife. 2013 Apr 16;2:e00458.

99. Gacesa R, Kurilshikov A, Vich Vila A, Sinha T, Klaassen MAY, Bolte LA, et al. Environmental factors shaping the gut microbiome in a Dutch population. Nature. 2022 Apr 1;604(7907):732–9.

100. Ege MJ. The Hygiene Hypothesis in the Age of the Microbiome. Ann Am Thorac Soc. 2017 Nov;14(Supplement_5):S348–53.

101. Schloss PD, Iverson KD, Petrosino JF, Schloss SJ. The dynamics of a family's gut microbiota reveal variations on a theme. Microbiome. 2014;2:25.

102. Vogel SC, Brito NH, Callaghan BL. Early Life Stress and the Development of the Infant Gut Microbiota: Implications for Mental Health and Neurocognitive Development. Curr Psychiatry Rep. 2020 Sep 12;22(11):61.

103. Michels N. Biological underpinnings from psychosocial stress towards appetite and obesity during youth: research implications towards metagenomics, epigenomics and metabolomics. Nutrition Research Reviews. 2019/07/12 ed. 2019;32(2):282–93.

104. Yap GC, Chee KK, Hong PY, Lay C, Satria CD, Sumadiono, et al. Evaluation of stool microbiota signatures in two cohorts of Asian (Singapore and Indonesia) newborns at risk of atopy. BMC Microbiol. 2011 Aug 26;11:193.

105. Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 2008 Nov 18;6(11):e280.

106. Penders J, Vink C, Driessen C, London N, Thijs C, Stobberingh EE. Quantification of Bifidobacterium spp., Escherichia coli and Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. FEMS Microbiol Lett. 2005 Feb 1;243(1):141–7.

107. Reyman M, van Houten MA, Watson RL, Chu MLJN, Arp K, de Waal WJ, et al. Effects of early-life antibiotics on the developing infant gut microbiome and resistome: a randomized trial. Nature Communications. 2022 Feb 16;13(1):893.

108. Korpela K, Salonen A, Virta LJ, Kekkonen RA, Forslund K, Bork P, et al. Intestinal microbiome is related to lifetime antibiotic use in Finnish pre-school children. Nat Commun. 2016 Jan 26;7:10410.

109. McDonnell L, Gilkes A, Ashworth M, Rowland V, Harries TH, Armstrong D, et al. Association between antibiotics and gut microbiome dysbiosis in children: systematic review and meta-analysis. Gut Microbes. 2021 Dec;13(1):1–18.

110. Kean IRL, Wagner J, Wijeyesekera A, De Goffau M, Thurston S, Clark JA, et al. Profiling gut microbiota and bile acid metabolism in critically ill children. Sci Rep. 2022 Jun 21;12(1):10432.

111. Sanders ME, Merenstein DJ, Reid G, Gibson GR, Rastall RA. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. Nat Rev Gastroenterol Hepatol. 2019 Oct;16(10):605–16.

112. Nowak A, Paliwoda A, Błasiak J. Anti-proliferative, pro-apoptotic and anti-oxidative activity of Lactobacillus and Bifidobacterium strains: A review of mechanisms and therapeutic perspectives. Crit Rev Food Sci Nutr. 2019;59(21):3456–67.

113. Fritts Ryan K., McCully Alexandra L., McKinlay James B. Extracellular Metabolism Sets the Table for Microbial Cross-Feeding. Microbiology and Molecular Biology Reviews. 2021 Jan 13;85(1):e00135-20.

114. Oniszczuk A, Oniszczuk T, Gancarz M, Szymańska J. Role of Gut Microbiota, Probiotics and Prebiotics in the Cardiovascular Diseases. Molecules. 2021 Feb 22;26(4).

115. Rogers MB, Firek B, Shi M, Yeh A, Brower-Sinning R, Aveson V, et al. Disruption of the microbiota across multiple body sites in critically ill children. Microbiome. 2016 Dec 29;4(1):66.

116. Miniet AA, Grunwell JR, Coopersmith CM. The microbiome and the immune system in critical illness. Curr Opin Crit Care. 2021 Apr 1;27(2):157–63.

117. De Palma G, Nadal I, Medina M, Donat E, Ribes-Koninckx C, Calabuig M, et al. Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. BMC Microbiol. 2010 Feb 24;10:63.

118. Davis-Richardson AG, Triplett EW. A model for the role of gut bacteria in the development of autoimmunity for type 1 diabetes. Diabetologia. 2015 Jul;58(7):1386–93.

119. Lambring CB, Siraj S, Patel K, Sankpal UT, Mathew S, Basha R. Impact of the Microbiome on the Immune System. Crit Rev Immunol. 2019;39(5):313–28.

120. Hall JC, Rosen A. Type I interferons: crucial participants in disease amplification in autoimmunity. Nat Rev Rheumatol. 2010 Jan;6(1):40–9.

121. Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. FEBS J. 2018 Aug;285(16):2944–71.

122. Vishweswaraiah S, Thimraj TA, George L, Krishnarao CS, Lokesh KS, Siddaiah JB, et al. Putative Systemic Biomarkers of Biomass Smoke-Induced Chronic Obstructive Pulmonary Disease among Women in a Rural South Indian Population. Dis Markers. 2018;2018:4949175.

123. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. Int J Mol Sci. 2017 Dec 29;19(1).

124. Shi N, Li N, Duan X, Niu H. Interaction between the gut microbiome and mucosal immune system. Mil Med Res. 2017;4:14.

125. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol. 2001 Apr;2(4):361–7.

126. Buckner JH, Ziegler SF. Regulating the immune system: the induction of regulatory T cells in the periphery. Arthritis Res Ther. 2004;6(5):215–22.

127. Lee S, Ko Y, Kim TJ. Homeostasis and regulation of autoreactive B cells. Cell Mol Immunol. 2020 Jun;17(6):561–9.

128. Kawai K, Uchiyama M, Hester J, Wood K, Issa F. Regulatory T cells for tolerance. Hum Immunol. 2018 May;79(5):294–303.

129. Schwartz RH. T cell anergy. Annu Rev Immunol. 2003;21:305–34.

130. Catalán D, Mansilla MA, Ferrier A, Soto L, Oleinika K, Aguillón JC, et al. Immunosuppressive Mechanisms of Regulatory B Cells. Front Immunol. 2021;12:611795.

131. Alemohammad H, Najafzadeh B, Asadzadeh Z, Baghbanzadeh A, Ghorbaninezhad F, Najafzadeh A, et al. The importance of immune checkpoints in immune monitoring: A future paradigm shift in the treatment of cancer. Biomedicine & Pharmacotherapy. 2022 Feb 1;146:112516.

132. Paluch C, Santos AM, Anzilotti C, Cornall RJ, Davis SJ. Immune Checkpoints as Therapeutic Targets in Autoimmunity. Front Immunol. 2018;9:2306.

133. Chikuma S. CTLA-4, an Essential Immune-Checkpoint for T-Cell Activation. Curr Top Microbiol Immunol. 2017;410:99–126.

134. Hosseini A, Gharibi T, Marofi F, Babaloo Z, Baradaran B. CTLA-4: From mechanism to autoimmune therapy. Int Immunopharmacol. 2020 Mar;80:106221.

135. Sobhani N, Tardiel-Cyril DR, Davtyan A, Generali D, Roudi R, Li Y. CTLA-4 in Regulatory T Cells for Cancer Immunotherapy. Cancers (Basel). 2021 Mar 22;13(6).

136. Harjunpää H, Guillerey C. TIGIT as an emerging immune checkpoint. Clinical and Experimental Immunology. 2020 May 1;200(2):108–19.

137. Jeong S, Park SH. Co-Stimulatory Receptors in Cancers and Their Implications for Cancer Immunotherapy. Immune Netw. 2020 Feb;20(1):e3.

138. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol. 2008 Sep;8(9):685–98.

139. Lewis ED, Meydani SN, Wu D. Regulatory role of vitamin E in the immune system and inflammation. IUBMB Life. 2019 Apr;71(4):487–94.

140. Ertesvag A, Aasheim HC, Naderi S, Blomhoff HK. Vitamin A potentiates CpG-mediated memory B-cell proliferation and differentiation: involvement of early activation of p38MAPK. Blood. 2007 May 1;109(9):3865–72.

141. Saurer L, McCullough KC, Summerfield A. In vitro induction of mucosa-type dendritic cells by all-trans retinoic acid. J Immunol. 2007 Sep 15;179(6):3504–14.

142. Takiishi T, Fenero CIM, Câmara NOS. Intestinal barrier and gut microbiota: Shaping our immune responses throughout life. Tissue Barriers. 2017 Oct 2;5(4):e1373208.

143. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol. 2010 Mar;10(3):159–69.

144. McGuckin MA, Lindén SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. Nat Rev Microbiol. 2011 Apr;9(4):265–78.

145. Herath M, Hosie S, Bornstein JC, Franks AE, Hill-Yardin EL. The Role of the Gastrointestinal Mucus System in Intestinal Homeostasis: Implications for Neurological Disorders. Front Cell Infect Microbiol. 2020;10:248.

146. Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, et al. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science. 2011 Oct 14;334(6053):255–8.

147. Thaiss CA, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. Nature. 2016 Jul 7;535(7610):65–74.

148. Wiertsema SP, van Bergenhenegouwen J, Garssen J, Knippels LMJ. The Interplay between the Gut Microbiome and the Immune System in the Context of Infectious Diseases throughout Life and the Role of Nutrition in Optimizing Treatment Strategies. Nutrients. 2021 Mar 9;13(3).

149. Libertucci J, Young VB. The role of the microbiota in infectious diseases. Nat Microbiol. 2019 Jan;4(1):35–45.

150. Lawley TD, Walker AW. Intestinal colonization resistance. Immunology. 2013 Jan;138(1):1–11.

151. Chiu L, Bazin T, Truchetet ME, Schaeverbeke T, Delhaes L, Pradeu T. Protective Microbiota: From Localized to Long-Reaching Co-Immunity. Front Immunol. 2017;8:1678.

152. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol. 2013 Jul;14(7):685–90.

153. Lazar V, Ditu LM, Pircalabioru GG, Gheorghe I, Curutiu C, Holban AM, et al. Aspects of Gut Microbiota and Immune System Interactions in Infectious Diseases, Immunopathology, and Cancer. Front Immunol. 2018;9:1830.

154. Thompson JA, Oliveira RA, Xavier KB. Chemical conversations in the gut microbiota. Gut Microbes. 2016;7(2):163–70.

155. Johansson MEV, Hansson GC. Microbiology. Keeping bacteria at a distance. Science. 2011 Oct 14;334(6053):182–3.

156. Gopalakrishnan V, Helmink BA, Spencer CN, Reuben A, Wargo JA. The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy. Cancer Cell. 2018 Apr 9;33(4):570–80.

157. Ohtsuka Y, Sanderson IR. Transforming growth factor-beta: an important cytokine in the mucosal immune response. Curr Opin Gastroenterol. 2000 Nov;16(6):541–5.

158. Wang X, Liang M, Song P, Guan W, Shen X. Mucosal-associated invariant T cells in digestive tract: Local guardians or destroyers? Immunology. 2023 Oct;170(2):167–79.

159. Manicassamy S, Reizis B, Ravindran R, Nakaya H, Salazar-Gonzalez RM, Wang YC, et al. Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. Science. 2010 Aug 13;329(5993):849–53.

160. Geuking MB, Cahenzli J, Lawson MAE, Ng DCK, Slack E, Hapfelmeier S, et al. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity. 2011 May 27;34(5):794–806.

161. Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 2015 Sep;33(9):496–503.

162. Wang L, Wang FS, Gershwin ME. Human autoimmune diseases: a comprehensive update. J Intern Med. 2015 Oct;278(4):369–95.

163. Thurgate LE, Lemberg DA, Day AS, Leach ST. An Overview of Inflammatory Bowel Disease Unclassified in Children. Inflamm Intest Dis. 2019 Aug;4(3):97–103.

164. Ahmad R, Sorrell MF, Batra SK, Dhawan P, Singh AB. Gut permeability and mucosal inflammation: bad, good or context dependent. Mucosal Immunol. 2017 Mar;10(2):307–17.

165. Fasano A, Shea-Donohue T. Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. Nat Clin Pract Gastroenterol Hepatol. 2005 Sep;2(9):416–22.

166. Kinashi Y, Hase K. Partners in Leaky Gut Syndrome: Intestinal Dysbiosis and Autoimmunity. Front Immunol. 2021;12:673708.

167. Pisetsky DS. Pathogenesis of autoimmune disease. Nat Rev Nephrol. 2023 Aug;19(8):509–24.

168. Aladjidi N, Jutand MA, Beaubois C, Fernandes H, Jeanpetit J, Coureau G, et al. Reliable assessment of the incidence of childhood autoimmune hemolytic anemia. Pediatr Blood Cancer. 2017 Dec;64(12).

169. Ware RE, O'Brien S, Armsby C. Autoimmune hemolytic anemia (AIHA) in children: Classification, clinical features, and diagnosis. In: UpToDate [Internet]. 2023 [cited 2023 Mar 10]. Available from: https://www-1uptodate-1com-1fzjl90500107.han.medunigraz.at/contents/autoimmune-hemolytic-anemia-aiha-in-children-classification-clinical-features-and-diagnosis?search=aiha&source=search_result&selectedTitle=1~150&usage_type=default&display_rank=1#H2

170. Terrell DR, Beebe LA, Vesely SK, Neas BR, Segal JB, George JN. The incidence of immune thrombocytopenic purpura in children and adults: A critical review of published reports. Am J Hematol. 2010 Mar;85(3):174–80.

171. Bussel JB, O'Brien S, Armsby C. Immune thrombocytopenia (ITP) in children: Clinical features and diagnosis. In: UpToDate [Internet]. 2023 [cited 2023 Mar 10]. Available from: https://www-1uptodate-1com-1fzjl90500107.han.medunigraz.at/contents/immune-thrombocytopenia-itp-in-children-clinical-features-and-diagnosis?search=itp&source=search_result&selectedTitle=3~150&usage_type=default&display_rank=3#H3

172. Mannering N, Hansen DL, Frederiksen H. Evans syndrome in children below 13 years of age - A nationwide population-based cohort study. PLoS One. 2020;15(4):e0231284.

173. Voulgaridou A, Kalfa TA. Autoimmune Hemolytic Anemia in the Pediatric Setting. J Clin Med. 2021 Jan 9;10(2).

174. Wang Y, Liu F, Zhang G, Su Y, Sun X, Chen Q, et al. Gut microbiome alterations and its link to corticosteroid resistance in immune thrombocytopenia. Sci China Life Sci. 2021 May;64(5):766–83.

175. EVANS RS, TAKAHASHI K, DUANE RT, PAYNE R, LIU C. Primary thrombocytopenic purpura and acquired hemolytic anemia; evidence for a common etiology. AMA Arch Intern Med. 1951 Jan;87(1):48–65.

176. Miano M. How I manage Evans Syndrome and AIHA cases in children. Br J Haematol. 2016 Feb;172(4):524–34.

177. Rivalta B, Zama D, Pancaldi G, Facchini E, Cantarini ME, Miniaci A, et al. Evans Syndrome in Childhood: Long Term Follow-Up and the Evolution in Primary Immunodeficiency or Rheumatological Disease. Front Pediatr. 2019;7:304.

178. Grimes AB, Kim TO, Kirk SE, Flanagan J, Lambert MP, Grace RF, et al. Refractory autoimmune cytopenias in pediatric Evans syndrome with underlying systemic immune dysregulation. Eur J Haematol. 2021 Jun;106(6):783–7.

179. Teachey DT, Lambert MP. Diagnosis and management of autoimmune cytopenias in childhood. Pediatr Clin North Am. 2013 Dec;60(6):1489–511.

180. Seidel MG. Treatment of immune-mediated cytopenias in patients with primary immunodeficiencies and immune regulatory disorders (PIRDs). Hematology Am Soc Hematol Educ Program. 2020 Dec 4;2020(1):673–9.

181. Aladjidi N, Leverger G, Leblanc T, Picat MQ, Michel G, Bertrand Y, et al. New insights into childhood autoimmune hemolytic anemia: a French national observational study of 265 children. Haematologica. 2011 May;96(5):655–63.

182. Savaşan S, Warrier I, Ravindranath Y. The spectrum of Evans' syndrome. Arch Dis Child. 1997 Sep;77(3):245–8.

183. Neunert CE, Buchanan GR, Imbach P, Bolton-Maggs PHB, Bennett CM, Neufeld EJ, et al. Severe hemorrhage in children with newly diagnosed immune thrombocytopenic purpura. Blood. 2008 Nov 15;112(10):4003–8.

184. Jaime-Pérez JC, Aguilar-Calderón PE, Salazar-Cavazos L, Gómez-Almaguer D. Evans syndrome: clinical perspectives, biological insights and treatment modalities. J Blood Med. 2018;9:171–84.

185. Shaikh H, Mewawalla P. Evans Syndrome. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2023.

186. Fischer A, Provot J, Jais JP, Alcais A, Mahlaoui N. Autoimmune and inflammatory manifestations occur frequently in patients with primary immunodeficiencies. J Allergy Clin Immunol. 2017 Nov;140(5):1388-1393.e8.

187. Seidel MG. Autoimmune and other cytopenias in primary immunodeficiencies: pathomechanisms, novel differential diagnoses, and treatment. Blood. 2014 Oct 9;124(15):2337–44.

188. Zama D, Conti F, Moratti M, Cantarini ME, Facchini E, Rivalta B, et al. Immune cytopenias as a continuum in inborn errors of immunity: An in-depth clinical and immunological exploration. Immun Inflamm Dis. 2021 Jun;9(2):583–94.

189. Schiavo E, Martini B, Attardi E, Consonni F, Ciullini Mannurita S, Coniglio ML, et al. Autoimmune Cytopenias and Dysregulated Immunophenotype Act as Warning Signs of Inborn Errors of Immunity: Results From a Prospective Study. Front Immunol. 2021;12:790455.

190. Chan AY, Torgerson TR. Primary immune regulatory disorders: a growing universe of immune dysregulation. Curr Opin Allergy Clin Immunol. 2020 Dec;20(6):582–90.

191. Thalhammer J, Kindle G, Nieters A, Rusch S, Seppänen MRJ, Fischer A, et al. Initial presenting manifestations in 16,486 patients with inborn errors of immunity include infections and noninfectious manifestations. J Allergy Clin Immunol. 2021 Nov;148(5):1332-1341.e5.

192. Hazime R, Eddehbi FE, El Mojadili S, Lakhouaja N, Souli I, Salami A, et al. Inborn errors of immunity and related microbiome. Front Immunol. 2022;13:982772.

193. Tangye SG, Al-Herz W, Bousfiha A, Cunningham-Rundles C, Franco JL, Holland SM, et al. Human Inborn Errors of Immunity: 2022 Update on the Classification from the International Union of Immunological Societies Expert Committee. J Clin Immunol. 2022 Oct;42(7):1473–507.

194. Michel M, Chanet V, Galicier L, Ruivard M, Levy Y, Hermine O, et al. Autoimmune thrombocytopenic purpura and common variable immunodeficiency: analysis of 21 cases and review of the literature. Medicine (Baltimore). 2004 Jul;83(4):254–63.

195. Audia S, Grienay N, Mounier M, Michel M, Bonnotte B. Evans' Syndrome: From Diagnosis to Treatment. J Clin Med. 2020 Nov 27;9(12).

196. Notarangelo LD. Primary immunodeficiencies (PIDs) presenting with cytopenias. Hematology Am Soc Hematol Educ Program. 2009;139–43.

197. Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E. Dysbiosis and the immune system. Nat Rev Immunol. 2017 Apr;17(4):219–32.

198. Johansson MEV, Jakobsson HE, Holmén-Larsson J, Schütte A, Ermund A, Rodríguez-Piñeiro AM, et al. Normalization of Host Intestinal Mucus Layers Requires Long-Term Microbial Colonization. Cell Host Microbe. 2015 Nov 11;18(5):582–92.

199. Spiljar M, Merkler D, Trajkovski M. The Immune System Bridges the Gut Microbiota with Systemic Energy Homeostasis: Focus on TLRs, Mucosal Barrier, and SCFAs. Front Immunol. 2017;8:1353.

200. Hooper LV, Stappenbeck TS, Hong CV, Gordon JI. Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol. 2003 Mar;4(3):269–73.

201. Sun D, Bai R, Zhou W, Yao Z, Liu Y, Tang S, et al. Angiogenin maintains gut microbe homeostasis by balancing α-Proteobacteria and Lachnospiraceae. Gut. 2021 Apr;70(4):666–76.

202. Crabbé PA, Bazin H, Eyssen H, Heremans JF. The normal microbial flora as a major stimulus for proliferation of plasma cells synthesizing IgA in the gut. The germ-free intestinal tract. Int Arch Allergy Appl Immunol. 1968;34(4):362–75.

203. Ostman S, Rask C, Wold AE, Hultkrantz S, Telemo E. Impaired regulatory T cell function in germ-free mice. Eur J Immunol. 2006 Sep;36(9):2336–46.

204. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med. 2014 Feb;20(2):159–66.

205. Cahenzli J, Köller Y, Wyss M, Geuking MB, McCoy KD. Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. Cell Host Microbe. 2013 Nov 13;14(5):559–70.

206. Catanzaro JR, Strauss JD, Bielecka A, Porto AF, Lobo FM, Urban A, et al. IgA-deficient humans exhibit gut microbiota dysbiosis despite secretion of compensatory IgM. Sci Rep. 2019 Sep 19;9(1):13574.

207. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell. 2014 Mar 27;157(1):121–41.

208. Brandl K, Plitas G, Schnabl B, DeMatteo RP, Pamer EG. MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection. J Exp Med. 2007 Aug 6;204(8):1891–900.

209. Ismail AS, Severson KM, Vaishnava S, Behrendt CL, Yu X, Benjamin JL, et al. Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. Proc Natl Acad Sci U S A. 2011 May 24;108(21):8743–8.

210. Mukherjee S, Partch CL, Lehotzky RE, Whitham CV, Chu H, Bevins CL, et al. Regulation of C-type lectin antimicrobial activity by a flexible N-terminal prosegment. J Biol Chem. 2009 Feb 20;284(8):4881–8.

211. Kelsall B. Recent progress in understanding the phenotype and function of intestinal dendritic cells and macrophages. Mucosal Immunol. 2008 Nov;1(6):460–9.

212. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science. 2004 Mar 12;303(5664):1662–5.

213. Peterson DA, McNulty NP, Guruge JL, Gordon JI. IgA response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host Microbe. 2007 Nov 15;2(5):328–39.

214. Boullier S, Tanguy M, Kadaoui KA, Caubet C, Sansonetti P, Corthésy B, et al. Secretory IgA-mediated neutralization of Shigella flexneri prevents intestinal tissue destruction by down-regulating inflammatory circuits. J Immunol. 2009 Nov 1;183(9):5879–85.

215. Mazmanian SK, Kasper DL. The love-hate relationship between bacterial polysaccharides and the host immune system. Nat Rev Immunol. 2006 Nov;6(11):849–58.

216. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009 Oct 30;139(3):485–98.

217. Karczewski J, Poniedziałek B, Adamski Z, Rzymski P. The effects of the microbiota on the host immune system. Autoimmunity. 2014 Dec;47(8):494–504.

218. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med. 2010 Feb;16(2):228–31.

219. Ganal SC, Sanos SL, Kallfass C, Oberle K, Johner C, Kirschning C, et al. Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. Immunity. 2012 Jul 27;37(1):171–86.

220. Al Bander Z, Nitert MD, Mousa A, Naderpoor N. The Gut Microbiota and Inflammation: An Overview. Int J Environ Res Public Health. 2020 Oct 19;17(20).

221. Ghosh SS, Wang J, Yannie PJ, Ghosh S. Intestinal Barrier Dysfunction, LPS Translocation, and Disease Development. J Endocr Soc. 2020 Feb 1;4(2):bvz039.

222. Janssen AWF, Kersten S. Potential mediators linking gut bacteria to metabolic health: a critical view. J Physiol. 2017 Jan 15;595(2):477–87.

223. Hartstra AV, Bouter KEC, Bäckhed F, Nieuwdorp M. Insights into the role of the microbiome in obesity and type 2 diabetes. Diabetes Care. 2015 Jan;38(1):159–65.

224. Janeiro MH, Ramírez MJ, Milagro FI, Martínez JA, Solas M. Implication of Trimethylamine N-Oxide (TMAO) in Disease: Potential Biomarker or New Therapeutic Target. Nutrients. 2018 Oct 1;10(10).

225. Fennema D, Phillips IR, Shephard EA. Trimethylamine and Trimethylamine N-Oxide, a Flavin-Containing Monooxygenase 3 (FMO3)-Mediated Host-Microbiome Metabolic Axis Implicated in Health and Disease. Drug Metab Dispos. 2016 Nov;44(11):1839–50.

226. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med. 2013 May;19(5):576–85.

227. Ma G, Pan B, Chen Y, Guo C, Zhao M, Zheng L, et al. Trimethylamine N-oxide in atherogenesis: impairing endothelial self-repair capacity and enhancing monocyte adhesion. Biosci Rep. 2017 Apr 30;37(2):BSR20160244.

228. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011 Apr 7;472(7341):57–63.

229. Al-Obaide MAI, Singh R, Datta P, Rewers-Felkins KA, Salguero MV, Al-Obaidi I, et al. Gut Microbiota-Dependent Trimethylamine-N-oxide and Serum Biomarkers in Patients with T2DM and Advanced CKD. J Clin Med. 2017 Sep 19;6(9).

230. Rohrmann S, Linseisen J, Allenspach M, von Eckardstein A, Müller D. Plasma Concentrations of Trimethylamine-N-oxide Are Directly Associated with Dairy Food Consumption and Low-Grade Inflammation in a German Adult Population. J Nutr. 2016 Feb;146(2):283–9.

231. Jang DI, Lee AH, Shin HY, Song HR, Park JH, Kang TB, et al. The Role of Tumor Necrosis Factor Alpha (TNF-α) in Autoimmune Disease and Current TNF-α Inhibitors in Therapeutics. Int J Mol Sci. 2021 Mar 8;22(5).

232. Srikanthan K, Feyh A, Visweshwar H, Shapiro JI, Sodhi K. Systematic Review of Metabolic Syndrome Biomarkers: A Panel for Early Detection, Management, and Risk Stratification in the West Virginian Population. Int J Med Sci. 2016;13(1):25–38.

233. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A. 2007 Aug 21;104(34):13780–5.

234. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe. 2014 Mar 12;15(3):382–92.

235. Peng L, Li ZR, Green RS, Holzman IR, Lin J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J Nutr. 2009 Sep;139(9):1619–25.

236. Dordević D, Jančíková S, Vítězová M, Kushkevych I. Hydrogen sulfide toxicity in the gut environment: Meta-analysis of sulfate-reducing and lactic acid bacteria in inflammatory processes. J Adv Res. 2021 Jan;27:55–69.

237. Kushkevych I, Castro Sangrador J, Dordević D, Rozehnalová M, Černý M, Fafula R, et al. Evaluation of Physiological Parameters of Intestinal Sulfate-Reducing Bacteria Isolated from Patients Suffering from IBD and Healthy People. J Clin Med. 2020 Jun 19;9(6).

238. Kushkevych I, Dordević D, Vítězová M. Possible synergy effect of hydrogen sulfide and acetate produced by sulfate-reducing bacteria on inflammatory bowel disease development. J Adv Res. 2021 Jan;27:71–8.

239. Jakimovski D, Awan S, Eckert SP, Farooq O, Weinstock-Guttman B. Multiple Sclerosis in Children: Differential Diagnosis, Prognosis, and Disease-Modifying Treatment. CNS Drugs. 2022 Jan;36(1):45–59.

240. Brenton JN, Kammeyer R, Gluck L, Schreiner T, Makhani N. Multiple Sclerosis in Children: Current and Emerging Concepts. Semin Neurol. 2020 Apr;40(2):192–200.

241. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Paz Soldan MM, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. Sci Rep. 2016 Jun 27;6:28484.

242. Horton MK, McCauley K, Fadrosh D, Fujimura K, Graves J, Ness J, et al. Gut microbiome is associated with multiple sclerosis activity in children. Ann Clin Transl Neurol. 2021 Sep;8(9):1867–83.

243. Luo XM, Edwards MR, Mu Q, Yu Y, Vieson MD, Reilly CM, et al. Gut Microbiota in Human Systemic Lupus Erythematosus and a Mouse Model of Lupus. Appl Environ Microbiol. 2018 Feb 15;84(4).

244. Burke DG, Fouhy F, Harrison MJ, Rea MC, Cotter PD, O'Sullivan O, et al. The altered gut microbiota in adults with cystic fibrosis. BMC Microbiol. 2017 Mar 9;17(1):58.

245. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol. 2012 Apr 16;13(9):R79.

246. Lewis JD, Chen EZ, Baldassano RN, Otley AR, Griffiths AM, Lee D, et al. Inflammation, Antibiotics, and Diet as Environmental Stressors of the Gut Microbiome in Pediatric Crohn's Disease. Cell Host Microbe. 2015 Oct 14;18(4):489–500.

247. Vatanen T, Kostic AD, d'Hennezel E, Siljander H, Franzosa EA, Yassour M, et al. Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. Cell. 2016 Jun 2;165(6):1551.

248. Castagnoli R, Pala F, Bosticardo M, Licari A, Delmonte OM, Villa A, et al. Gut Microbiota-Host Interactions in Inborn Errors of Immunity. Int J Mol Sci. 2021 Jan 31;22(3).

249. Zhang L, Li YY, Tang X, Zhao X. Faecal microbial dysbiosis in children with Wiskott-Aldrich syndrome. Scand J Immunol. 2020 Jan;91(1):e12805.

250. Jørgensen SF, Trøseid M, Kummen M, Anmarkrud JA, Michelsen AE, Osnes LT, et al. Altered gut microbiota profile in common variable immunodeficiency associates with levels of lipopolysaccharide and markers of systemic immune activation. Mucosal Immunol. 2016 Nov;9(6):1455–65.

251. Ventura M, Turroni F, Lugli GA, van Sinderen D. Bifidobacteria and humans: our special friends, from ecological to genomics perspectives. J Sci Food Agric. 2014 Jan 30;94(2):163–8.

252. Kelly CR, Ihunnah C, Fischer M, Khoruts A, Surawicz C, Afzali A, et al. Fecal microbiota transplant for treatment of Clostridium difficile infection in immunocompromised patients. Am J Gastroenterol. 2014 Jul;109(7):1065–71.

253. Wu W, Shen N, Luo L, Deng Z, Chen J, Tao Y, et al. Fecal microbiota transplantation before hematopoietic stem cell transplantation in a pediatric case of chronic diarrhea with a FOXP3 mutation. Pediatr Neonatol. 2021 Mar;62(2):172–80.

254. Mohammed AD, Hall N, Chatzistamou I, Jolly A, Kubinak JL. Gluten-free diet exposure prohibits pathobiont expansion and gluten sensitive enteropathy in B cell deficient JH-/- mice. PLoS One. 2022;17(3):e0264977.

255. Dei Zotti F, Qiu A, La Carpia F, Moriconi C, Hudson KE. A New Murine Model of Primary Autoimmune Hemolytic Anemia (AIHA). Front Immunol. 2021;12:752330.

256. Fattizzo B, Cavallaro F, Folino F, Barcellini W. Recent insights into the role of the microbiome in malignant and benign hematologic diseases. Crit Rev Oncol Hematol. 2021 Apr;160:103289.

257. Furlong E, Carter T. Aplastic anaemia: Current concepts in diagnosis and management. J Paediatr Child Health. 2020 Jul;56(7):1023–8.

258. Xiao Y, Zhao S, Li B. Aplastic anemia is related to alterations in T cell receptor signaling. Stem Cell Investig. 2017;4:85.

259. Liu C, Cheng L, Ji L, Li F, Zhan Y, Wu B, et al. Intestinal microbiota dysbiosis play a role in pathogenesis of patients with primary immune thrombocytopenia. Thromb Res. 2020 Jun;190:11–9.

260. Li X, Zhang M, He L, Zhou J, Shen P, Dai W, et al. Gut microbiota alterations in children and their relationship with primary immune thrombocytopenia. Front Pediatr. 2023;11:1213607.

261. Weber T, Tatzl E, Kashofer K, Holter M, Trajanoski S, Berghold A, et al. Fibromyalgia-associated hyperalgesia is related to psychopathological alterations but not to gut microbiome changes. PLoS One. 2022;17(9):e0274026.

262. Zymo Research. What are SAM & BAM files? Understanding the SAM & BAM file format [Internet]. 2021. Available from: https://www.zymoresearch.com/blogs/blog/what-are-sam-and-bam-files

263. Schmieder R, Edwards R. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. PLoS One. 2011 Mar 9;6(3):e17288.

264. Bragg L, Stone G, Imelfort M, Hugenholtz P, Tyson GW. Fast, accurate error-correction of amplicon pyrosequences using Acacia. Nat Methods. 2012 Apr 27;9(5):425–6.

265. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010 Oct 1;26(19):2460–1.

266. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010 May;7(5):335–6.

267. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011 Jun 24;12(6):R60.

268. Mallick H, Rahnavard A, McIver LJ, Ma S, Zhang Y, Nguyen LH, et al. Multivariable association discovery in population-scale meta-omics studies. PLoS Comput Biol. 2021 Nov;17(11):e1009442.

269. Hadjadj J, Aladjidi N, Fernandes H, Leverger G, Magérus-Chatinet A, Mazerolles F, et al. Pediatric Evans syndrome is associated with a high frequency of potentially damaging variants in immune genes. Blood. 2019 Jul 4;134(1):9–21.

270. Besnard C, Levy E, Aladjidi N, Stolzenberg MC, Magerus-Chatinet A, Alibeu O, et al. Pediatric-onset Evans syndrome: Heterogeneous presentation and high frequency of monogenic disorders including LRBA and CTLA4 mutations. Clin Immunol. 2018 Mar;188:52–7.

271. Korpela K, de Vos WM. Early life colonization of the human gut: microbes matter everywhere. Curr Opin Microbiol. 2018 Aug;44:70–8.

272. Planer JD, Peng Y, Kau AL, Blanton LV, Ndao IM, Tarr PI, et al. Development of the gut microbiota and mucosal IgA responses in twins and gnotobiotic mice. Nature. 2016 Jun 9;534(7606):263–6.

273. Pabst O, Cerovic V, Hornef M. Secretory IgA in the Coordination of Establishment and Maintenance of the Microbiota. Trends Immunol. 2016 May;37(5):287–96.

274. Riva A, Borgo F, Lassandro C, Verduci E, Morace G, Borghi E, et al. Pediatric obesity is associated with an altered gut microbiota and discordant shifts in Firmicutes populations. Environ Microbiol. 2017 Jan;19(1):95–105.

275. Füeßl HS. Intestinales Mikrobiom und metabolische Erkrankungen. Gastro-News. 2021 Aug 1;8(4):26–7.

276. Buchta M, Höper DW, Sönnichsen A. Infektionen durch fakultativ pathogene Bakterien. In: Buchta M, Höper DW, Sönnichsen A, editors. Das Zweite StEx: Basiswissen Klinische Medizin für Examen und Praxis [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2004. p. 251–4. Available from: https://doi.org/10.1007/978-3-642-18569-4_76

277. Crost EH, Coletto E, Bell A, Juge N. Ruminococcus gnavus: friend or foe for human health. FEMS Microbiol Rev. 2023 Mar 10;47(2).

278. Tannock GW, Lawley B, Munro K, Gowri Pathmanathan S, Zhou SJ, Makrides M, et al. Comparison of the compositions of the stool microbiotas of infants fed goat milk formula, cow milk-based formula, or breast milk. Appl Environ Microbiol. 2013 May;79(9):3040–8.

279. Mennella JA, Li Y, Bittinger K, Friedman ES, Zhao C, Li H, et al. The Macronutrient Composition of Infant Formula Produces Differences in Gut Microbiota Maturation That Associate with Weight Gain Velocity and Weight Status. Nutrients. 2022 Mar 15;14(6).

280. Deering KE, Devine A, O'Sullivan TA, Lo J, Boyce MC, Christophersen CT. Characterizing the Composition of the Pediatric Gut Microbiome: A Systematic Review. Nutrients. 2019 Dec 19;12(1).

281. Avershina E, Storrø O, Øien T, Johnsen R, Pope P, Rudi K. Major faecal microbiota shifts in composition and diversity with age in a geographically restricted cohort of mothers and their children. FEMS Microbiol Ecol. 2014 Jan;87(1):280–90.

282. Jakobsson HE, Abrahamsson TR, Jenmalm MC, Harris K, Quince C, Jernberg C, et al. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut. 2014 Apr;63(4):559–66.

283. Yassour M, Vatanen T, Siljander H, Hämäläinen AM, Härkönen T, Ryhänen SJ, et al. Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. Sci Transl Med. 2016 Jun 15;8(343):343ra81.

284. Bukin YS, Galachyants YP, Morozov IV, Bukin SV, Zakharenko AS, Zemskaya TI. The effect of 16S rRNA region choice on bacterial community metabarcoding results. Sci Data. 2019 Feb 5;6:190007.

285. Magurran AE. Measuring biological diversity. Curr Biol. 2021 Oct 11;31(19):R1174–7.

286. Marchesi JR. Human distal gut microbiome. Environ Microbiol. 2011 Dec;13(12):3088–102.

287. Joseph PD, Craig JC, Caldwell PHY. Clinical trials in children. Br J Clin Pharmacol. 2015 Mar;79(3):357–69.