Dissertation

Changes of intestinal microbiota composition and diversity in very low birthweight infants related to strategies of NEC prophylaxis

Veränderungen von Komposition und Diversität des intestinalen Mikrobioms bei Frühgeborenen unter 1.500g Geburtsgewicht in Zusammenhang mit Strategien zur NEC Prophylaxe

submitted by

Dr.med.univ.
Stefan KURATH-KOLLER

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Division of Neonatology and Pediatric Cardiology
Department of Paediatrics and Adolescence Medicine

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Univ. Prof. Dr. med.univ. Bernhard Resch

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used and permission to reproduce was obtained from the copyright holder when necessary. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz“.

Certain parts of this thesis have been published in renowned journals before the preparation of this thesis was finally completed. Due to the publication policies of some journals, it was necessary to transfer the copyright to the journal’s publishers. To avoid copyright infringement and self-plagiarism, I applied for the right to incorporate materials, including figures and tables, from my articles into this thesis. The journal’s publishers, which hold the copyright on, granted back the right to include the articles within my dissertation, provided that proper credit is given to the original source.

This is a list of my published articles, which are partly included into this thesis.

1. Kurath-Koller, S., et al. *Changes of intestinal microbiota composition and diversity in very low birth weight infants related to strategies of NEC prophylaxis: protocol for an observational multicentre pilot study.* Pilot Feasibility Stud, 2017. **3**: p. 52.

2. Kurath-Koller S, Neumann C, Moissl-Eichinger C, et al. *Hospital Regimens Including Probiotics Guide the Individual Development of the Gut Microbiome of Very Low Birth Weight Infants in the First Two Weeks of Life.* Nutrients. 2020;12(5):1256.

Graz, March 2021 Stefan Kurath-Koller

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Abstract

Background

At the Division of Neonatology, Department of Pediatrics, Medical University Graz, a unique regimen for prophylaxis of necrotizing enterocolitis (NEC) in preterm infants <1.500g birth weight is used. This regimen includes the combined use of antibiotics, mycostatics and probiotics, as well as a structured feeding regimen. The incidence of NEC in preterm infants treated by this regimen has been shown to be as low as 0.7% when treatment was initiated at the first day of life, compared to international incidence rates reported to be about 5.1%. We hypothesized that differences in hospital regimens influence diversity and composition of intestinal microbiota development. Thus, intestinal microbial diversity and abundance as well as development of the intestinal microbiome within the first two weeks of life were analyzed from stool samples.

Methods

The doctoral thesis was carried out as a prospective controlled triplecenter cohort study in preterm infants with a birth weight <1.500g (i.e. very low birth weight, VLBW). Partizipating centers were located in south eastern Austria (NICU, Department of Pediatrics, Medical University Graz; NICU, Department of Pediatrics, General Hospital of Klagenfurt; NICU, Department of Pediatrics, General Hospital of Leoben). At the Division of Neonatology, Department of Pediatrics, General Hospital of Klagenfurt a different probiotic supplement containing different strains and a different feeding strategy are used. Furthermore, oral antibiotics are not routinely used. At the Department of Pediatrics, General Hospital of Leoben no probiotic supplements are used but antibiotics, mycostatics and feeding regimens adhere to the Graz protocol. Seven stool samples from each of the 54 included infants, 18 from each center, collected every other day throughout the first two weeks of life, were analyzed by 16S rRNA gene analysis. All 378 samples were sequenced at the Core Facility for Molecular Biology at the Medical University of Graz. Data was processed and interpreted with the help of biostatisticians and statisticians, as well as specialists of the Interactive Microbiome Research Unit Graz.

Results

Regarding gestational age, birth weight, APGAR scores and oxygen demand, no

statistical differences were found between the three groups. Overall, 2.029 different taxa were detected, showing a predominance of the probiotic genera *Lactobacillus* and *Bifidobacterium*, and further comprising *Enterococcus* and *Staphylococcus*. Meconium was found to harbor bacterial DNA. With the use of probiotic supplementation, an earlier increase in bacterial load was found. Although predominating in samples from infants who were supplemented with probiotics, *Lactobacillus* and *Bifidobacterium* contributed only marginally to the fecal microbiome if no probiotics supplementation was administered. However, some infants did not respond to probiotics supplementation. By the end of the second week of life microbial diversity in samples of all partizipating centers showed a similar pattern. Microbial samples from all centers clustered significantly, yet varied from each other. No adverse effects attributable to probiotic administration or hospital regimen were documented.

Conclusion

The administration of probiotics supplementation in very low birth weight preterm infants in our study seemed to be well tolerated. Furthermore, it seems to initiate an earlier increase in bacterial load with a predominance of probiotic strains, which might be essential for the prevention of neonatal morbidities and NEC. Meconium samples were not found to be free of bacterial DNA. Oral antibiotics did not influence the development of the stool microbiome in a negative way, and did not interfere with probiotics colonization despite combined use. Hospital regimens lead to center-specific and distinct clustering as a microbial hallmark.

Zusammenfassung

Hintergrund

An der Abteilung für Neonatologie der Universitätsklinik für Kinder- und Jugendheilkunde Graz erhalten Frühgeborene <1.500g Geburtsgewicht eine spezielle Prophylaxe zur Verhinderung einer nekrotisierenden Enterokolitis (NEC). Dieses Prophylaxe-Regime beinhaltet die Kombination eines Antibiotikums, Probiotikums und Mykostatikums, sowie ein strukturiertes Ernährungsregime. Es konnte gezeigt werden, dass die Inzidenz der NEC unter Anwendung dieser Prophylaxe bei 0.7% lag, und damit deutlich niedriger als der internationale Durchschnitt (5.1%). Unsere Hypothese war, dass unterschiedliche Krankenhausregime im Hinblick auf die Verhinderung einer NEC die Diversität und Zusammensetzung des sich entwickelnden intestinalen Mikrobioms beeinflussen. Um dies zu überprüfen analysierten wir Stuhlproben von Frühgeborenen <1.500g während der ersten zwei Lebenswochen.

Methoden

Wir führten eine prospektive Triple-Center Kohortenstudie an Frühgeborenen <1.500g Geburtsgewicht (very low birth weight - VLBW) durch. Die teilnehmenden Zentren (Abteilung für Neonatologie, Universitätsklinik für Kinder- und Jugendheilkunde Graz; Abteilung für Neonatologie des LKH Klagenfurt; Abteilung für Neonatologie des LKH Leoben) waren im südöstlichen Raum Österreichs lokalisiert. Im Vergleich zur NEC Prophylaxe in Graz, erhielten Frühgeborene an der Abteilung für Neonatologie des LKH Klagenfurt ein anderes probiotisches Präparat als die Grazer Kinder, und Antibiotika wurden nur bei Bedarf verabreicht. An der Abteilung für Kinder- und Jugendheilkunde des LKH Leoben wurde kein probiotisches Präparat verwendet, die Gabe von Antibiotikum und Mykostatikum erfolgte jedoch in gleicher Weise wie am Zentrum in Graz. Sieben Stuhlproben, entnommen an jedem zweiten Tag und beginnend mit der ersten Portion Mekonium, wurden von allen 54 inkludierten Kindern gesammelt und mittels 16S-rRNA Genanalyse untersucht. Alle 378 Proben wurden an der Core Facility für Molekularbiologie der Medizinischen Universität Graz sequenziert. Die daraus resultierenden Daten wurden in Zusammenarbeit mit Statistikern und Biostatistikern, sowie Experten der interaktiven Mikrobiom-Forschungsgruppe Graz ausgewertet und interpretiert.

Resultate

Im Hinblick auf Gestationsalter, Geburtsgewicht, APGAR Scores und Sauerstoffbedarf unterschieden sich die drei Gruppen nicht signifikant voneinander. Insgesamt konnten 2.029 verschiedene Taxa nachgewiesen, und eine Prädominanz der probiotischen Genera *Lactobacillus* und *Bifidobacterium* gefunden werden. Weiters waren *Enterococcus* und *Staphylococcus* häufig vertreten. Mekoniumproben enthielten bereits bakterielle DNA. Unter Anwendung einer Probiotika Supplementation konnte ein früher Anstieg der Bakterienzahl detektiert werden. Während *Lactobacillus* und *Bifidobacterium* in Stuhlproben von Kindern welche eine Probiotika Supplementation erhielten deutlich überwogen, waren diese in Stuhlproben von Kindern ohne Probiotika Supplementation kaum vorhanden. Einige Kinder zeigten kein Ansprechen auf die Probiotika Supplementation. Am Ende der zweiten Lebenswoche näherte sich die Diversität des Stuhlmikrobioms an allen Zentren einander an. Dennoch zeigten Stuhlproben aller Zentren ein zentrumsspezifisches Clustering. Im Rahmen der Studie konnten keine Nebenwirkungen im Zusammenhang mit Probiotika Supplementation oder Krankenhausregimen festgestellt werden.

Konklusion

Die Verabreichung einer Probiotika Supplementation im Rahmen unserer Studie zeigte eine gute Toleranz bei Frühgeborenen mit einem Geburtsgewicht unter 1.500g. Des Weiteren scheint diese einen früheren Anstieg der Bakterienzahl im Stuhl und ein Überwiegen der durch die Probiotika Supplementation zugeführten Keime zu bedingen. Dies könnte essentiell für die Prävention einer NEC und anderer neonataler Komorbiditäten sein. Mekonium enthielt bereits bakterielle DNA. Die konkomitante Verabreichung von Antibiotika zeigte keinen Einfluß auf die supplementierten probiotischen Stämme und beeinflußte das Stuhlmikrobiom nicht negativ. Krankenhausregime zur NEC Prophylaxe führen zu zentrumsspezifischem Clustering als mikrobielles Markenzeichen des Stuhlmikrobioms.

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List of Abbreviations

ASV	Amplicon Sequence Variant
BLG	Bifidobacterium Lactobacillus Geobacillus
BMI	Body Mass Index
BMI	Breast Milk
BPD	Bronchopulmonary Dysplasia
BW	Birth Weight
CFU	Colony Forming Unit
CMV	Cytomegalovirus
CPR	Cardiopulmonary Resuscitation
DANN	Desoyribonucleic Acid
ENA	European Nucleotide Archive
EOS	Early Onset Sepsis
fFN	fetal Fibronectin
GA	Gestational Age
GI	Gastrointestinal
HMO	Human Milk Oligosaccharide
I/PVH	Intraventricular/Periventricular Hemmorrhage
IFN	Interferone
IgA	Immunoglobulin A
IL	Interleukin
LKH	Landeskrankenhaus
LOHS	Lenghts of Hospital Stay
LOS	Late Onset Sepsis
LPI	Late Preterm Infant
MAMP	Microbial Associated Molecular Pattern
NCHS	National Center for Health Statistics
NEC	Necrotizing Enterocolitis
NF-κβ	Nuclear Factor Kappa Beta
NICU	Neonatal Intensive Care Unit
NPO	Non per os
O2	Oxygen
PCoA	Principle Coordinates Analysis
PCR	Polymerase Chain Reaction
PIT	Point in Time
PROM	Premature Rupture of Membranes
PTB	Preterm Birth
qPCR	quantitative Polymerase Chain Reaction
RDS	Respiratory Distress Syndrome
RNA	Ribonucleic Acid
ROP	Retinopathy of prematurity
SD	Standard Deviation
SOP	Standardized Operating Proedure

TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
TPN	Total Parenteral Nutrition
UN	United Nations
VLBW	Very Low Birth Weight
WHO	World Health Organisation

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1 INTRODUCTION

1.1 NEONATOLOGY AND PRETERM BIRTH

Preterm birth is defined as birth before having completed 37 weeks of gestation and is the leading cause mortality in the neonatal period [1]. About 15 million infants are born prematurely every year [2]. Annually, about one million infant deaths can be attributed to complications from preterm birth. The overall rate of preterm births is increasing and may mainly be attributed to an increasing number of, mostly late, preterm births [3]. Preterm birth is also linked to increased morbidities and mortality. Over the past 15 years neonatal research focused on prematurity, leading to improvements in research and clinical care. Several specific policies and guidelines were provided for this special infant cohort.

Newborn infants can be classified with regard to gestational age or birth weight. This classification is broadly used for clinical and research purposes and has undergone various modifications over the past decades (Table 1) [4, 5].

TABLE 1: DEFINITION OF NEONATES AT BIRTH BASED ON BIRTH WEIGHT AND GESTATIONAL AGE. ADAPTED FROM [3].

GESTATIONAL AGE	BIRTH WEIGHT
Preterm: <37 completed weeks Extreme preterm: <28 weeks Very preterm: 28+0 until 31+6 weeks Moderate preterm: 32+0 until 33+6 weeks	Low Birth Weight: < 2.500g Very Low Birth Weight: < 1.500g Extremely Low Birth Weight: < 1.000g Micro premie: < 750g
Late preterm: 34+0 until 36+6 weeks	Small for GA: > 2SD below mean BW
Term: 37+0 until 41+6 weeks Early term: 37+0 until 38+6 weeks Full term: 39+0 until 40+6 weeks Late term: 40+6 until 41+6 weeks	Large for GA: > 2SD above mean BW
Post term: > 42+0 weeks	

Leg.: Data derives from open access publication [3].

1.1.1. Global burden

Late preterm infants (LPIs), i.e. birth between 34 and 37 completed weeks of gestation, seem to majorly contribute to preterm births worldwide. However, although World Health Organization (WHO) and United Nations (UN) agencies have developed standardized global indicators to optimize reporting, collection, and international comparisons of data on conditions and diseases, availability of data remains limited. For example, data on trends of LPIs in developing countries remain mostly speculative. This may be attributed to various factors as lacking uniform definitions, inaccuracies in gestational age assessment, incomplete data collection and low reporting rates. Therefore, extrapolation of data, mostly from developed countries needs to be performed. For example, the United States preterm birth rate increased from 9.93% in 2017 to 10.02% in 2018, according to 2019 National Vital Statistics report [8]. Between 2007 and 2014 the preterm birth rate declined to 10.44%. Lately, preterm births, especially of LPIs, have increased throughout the past 4 years. Given absolute numbers LPIs represented 276.000 total births in 2018 in the United States. Other countries report slightly lower LPI birth rates (2006 to 2014): 5.5% in the United Kingdom, 4.8% in Canada, 3.8% in Norway, 3.6% in Denmark and Sweden, 3.3% in Finland [1, 6].

WHO estimates the global preterm birth rate to be 11% of all births (range: 5–18%). The south eastern Asian region, south Asian region, and sub-Saharan African region contribute the majority of preterm infants in the developing world with nine million preterm born infants in the sub-Saharan African and south Asian region, resembling 60% of worldwide preterm births [1]. Overall, the vast majority of preterm births occur in resource poor settings.

1.1.2. Aetiology and risk factors

Multiple factors contribute to and pose a risk of preterm birth (Table 2), with spontaneous preterm labor and spontaneous rupture of membranes (PROM) being responsible for up to 75% of late preterm births. Table 3 gives major risk factors for preterm birth and associated percentage. Further risk factors comprise a prior preterm delivery, multiples, cervical shortening, maternal stress, infection and inflammation, fetal anomalies, and gestational hypertension (preeclampsia or eclampsia). Increasing

rates of cesarean section and birth induction contribute to a shift towards premature birth, a phenomenon that was discovered as of the millennium [7]. From US studies it is known that maternal age, ≥13 years of education, multiparty, or previous birth of an infant with ≥4000g birth weight are risk factors of late preterm birth [8]. However, assisted reproductive therapy, increased maternal age and multiples are interrelated. Twin birth is associated with an almost ten-fold increased risk of prematurity. Amounts of in vitro fertilizations have almost doubled between 2000 and 2013 in the United States [9].

TABLE 2: RISK FACTORS FOR PRETERM BIRTH. ADAPTED FROM [10].

PRIOR OB/GYN HISTORY	**PRIOR PTB, PRIOR CERVICAL SURGERY, MULTIPLE D&E´S, UTERINE ANOMALIES**
Maternal demographics	<17 or >35 years of age, lower educational level, lower socioeconomic status, single marital status, short interpregnancy interval, social factors (e.g. access to medical care, physical abuse,…)
NUTRITIONAL STATUS/PHYSICAL ACTIVITY	BMI <19 or prepregnancy weight <50kg, poor nutritional status, long working hours, hard physical labor
CURRENT MATERNAL/PREGNANCY CHARACTERISTICS	Conception by assisted reproductive techniques, multiple gestation, fetal disorder, vaginal bleeding, poly-/oligohydramnios, maternal medical conditions, maternal abdominal surgery during pregnancy, psychological issues, adverse behaviours (e.g. nicotine, alcohol, drugs, …), infections, short cervical length, positive fFNbetween 22 – 34 weeks, uterine contractions

Leg.: PTB = preterm birth, D&E = dilation and evacuation, BMI = body mass index, fFN = fetal fibronectin.

TABLE 3: PROPORTION OF PRETERM BIRTH BY ETIOLOGY. ADAPTED FROM [10].

ETIOLOGY	FREQUENCY (%)
SPONTANEOUS PRETERM LABOR	30 – 50
PPROM	5 – 40
MULTIPLE GESTATION	10 – 30
PREECLAMPSIA/ECLAMPSIA	12
ANTEPARTUM BLEEDING	6 – 9
FETAL GROWTH RESTRICTION	2 – 4
OTHER	8 – 9

Leg.: PPROM = premature rupture of membranes.

1.1.3. Mortality

The risk of death is indirectly proportional to birth weight and gestational age. The annual analysis from the National Center for Health Statistics (NCHS), linking all births and deaths within the first year of life in the United States, revealed that a birth weight less than 500g is associated with mortality rates of up to 85% [11].

Factors influencing preterm mortality rates comprise degree of prematurity, maternal ethnicity, level of neonatal care and congenital anomalies.

Mortality in preterm infants correlates with gestational age and birth weight, with lower values each associated with poor survival [11, 12]. Thus, extremely premature infants and extremely low birth weight infants have the greatest risk of death. For example, in 2005 infants with a birth weight below 1.000 g accounted for 0.8 percent of births in the United States, while they accounted for 55 percent of all infant deaths [11]. Mortality rates based upon gestational age and birth weight are given in table 4.

TABLE 4: INFANT MORTALITY RATES PER 1000 LIVE BIRTHS IN THE UNITED STATES BASED UPON BIRTH WEIGHT AND GESTATIONAL AGE. ADAPTED FROM [11]

BIRTH WEIGHT (G)	MORTALITY RATE	GESTATIONAL AGE (W)	MORTALITY RATE
>2.500	2.1	40	1.75
2.000 – 2.499	9.9	37 – 39	2.41
1.500 – 1.999	24.7	34 – 36	7.23
1.250 – 1.499	39.9	32 – 33	16.02
1.000 – 1.249	61.7	28 – 31	35.7
750 - 999	124.6	<28	374.74
500 - 749	394.3		
<500	853		

Leg.: g=grams; w= weeks; mortality rates are given per 1.000 live births. Data derives from open access publication [11].

Major causes leading to death or severe neurosensory impairment in extremely low birth weight infants (i.e. < 1.000g) and very low birth weight infants (i.e. < 1.500g), include infection (e.g. meningitis, sepsis, and necrotizing enterocolitis), ventricular hemorrhage, bronchopulmonary dysplasia (BPD), brain injury, and severe retinopathy of prematurity (ROP) [13, 14]. Furthermore, cardiopulmonary resuscitation (CPR) in the delivery room is associated with increased risk of mortality or severe neurodevelopmental impairment [13].

Mortality rates from prematurity are greater in low- and moderate-income countries. A systematic review including pooled data from Latin America, Africa, and Asia reported that preterm infants (i.e. less than 37 weeks gestational age) had a 6.8-fold increase in neonatal death compared with term infants [15].

The **limit of viability** currently is considered as infants born at or below 25 weeks gestation. Overall, more than two-thirds of infant deaths occur throughout the first 28 days of life, i.e. the newborn period.

From **gender aspects**, male preterm infants have a higher mortality rate compared to female infants [16, 17]. They are also more likely to have major morbidities (e.g. BPD, retinopathy of prematurity - ROP, NEC, late-onset sepsis) and adverse neurologic outcome.

Among **ethnic groups**, mortality rates of preterm infants vary. However, highest mortality rates are found in black infants [11]. Table 5 gives infant mortality rates for very low birth weight (VLBW) preterm infants and gestational age <32 weeks based upon maternal ethnicity.

TABLE 5: INFANT MORTALITY RATES PER 1.000 LIVE BIRTHS FOR PRETERM INFANTS WITH BIRTH WEIGHT <1.500 G (I.E. VERY LOW BIRTH WEIGHT [VLBW] INFANTS) AND GESTATIONAL AGE <32 WEEKS BASED UPON MATERNAL ETHNICITY.

ETHNIC GROUP	MORTALITY IN <1500G BW	MORTALITY IN <32W GA
WHITE	231.9	168.4
BLACK	274	216.2
ASIAN / PACIFIC ISLANDER	222.7	173.2
OVERALL	244.5	182.5

Leg.: g=grams; w= weeks, BW=birth weight, GA=gestational age. Adapted from www.uptodate.com (accessed May 2020).

The level of neonatal care impacts on mortality rate by changes in care over time (e.g. new therapeutic interventions, altered management strategies etc.). Also, hospital resources and caregivers´ expertise, as well as evidence-based practice impacts mortality.

Over the past decades, neonatal care has improved a lot. For example, the use of surfactant and antenatal steroids has markedly improved outcome in infant respiratory distress syndrome, resulting in decreased mortality [18-21]. Extremely preterm infants at the limit of viability still have a high mortality rate. However, improvements in neonatal care and management seem to have increased survival in this particular age group. As centers with high numbers of preterm infants cared for have lowest mortality rates, transportation of women at risk for marked preterm delivery to a hospital with a level III neonatal intensive care unit should be performed antenatally. Survival rates for extremely preterm infants are higher in high volume centers [22-24], especially with regard to neuromotor and neurosensory outcome [25-27].

1.2. DEVELOPMENT OF THE INTESTINAL TRACT

The gastrointestinal tract, reaching from the mouth until the anus, harbors several different but specific acid conditions along anatomical regions, such as the esophagus, stomach, small intestine, colon, and rectum [28]. Development of the gastrointestinal tract, structurally and functionally, plays a crucial role in human development originating throughout the fetal period. Diverse dietary inputs, as well as foreign antigens, are introduced into the body via the gastrointestinal tract [29]. Not all functions of the intestinal tract are established at birth. For example, the epithelial barrier function or the intestinal immune system continue to develop after birth over several months to years [29]. Twenty-five days into gestation, the so-called foregut and midgut are formed from the yolk sac´s dorsal section [30]. By five weeks post conceptional age the stomach develops and the midgut increases in length and finally herniates into the vitelline sac. Further the midgut complexly rotates and finds its final position in the abdominal cavity by 10 to 12 weeks post conceptional age [30]. By 20 weeks post conceptional age all major tissue components of a mature intestine, including regional-specific tissue features (e.g. gastric pits, glands / crypts or villi) are present [29]. It has been shown in the literature that the intestinal microbiome affects the structural, as well as the functional development of the intestinal tract [31-33]. For example, in germ free mice intestinal lymphoid tissue development was impaired and antibody production decreased [31]. It is hypothesized that intestinal microbiota impacting on the development of the intestinal tract are present even before birth [34]. However, bacterial DNA may be due to contamination of reactants or materials used. Thus, this topic remains object to discussion.

The former commonly believed assumption that the intestinal tract is colonized just at birth and remains sterile until then had to be rejected as bacterial DNA was identified even in amniotic fluid, the placenta, and meconium, and the placenta [35, 36]. The advent of next generation sequencing, using 16S rRNA gene-based profiling or whole DNA sequencing, led to new insights in this field. Furthermore, live bacterial culture diagnostics, sampling placental tissue or amniotic fluid, show limited results [35]. A distinct placental microbiome pattern has been described, potentially harboring many commensal bacterial species (e.g. *Firmicutes*, *Proteobacteria*, or *Bacteriodetes*) [37]. To what extent the placental microbiome (if any) affects the colonization of the infants´

intestinal tract remains uncertain. Throughout fetal life, large amounts of amniotic fluid are swallowed, potentially exposing the intestinal mucosa to certain bacteria. This could, in turn, impact the colonization of the infants´ intestinal tract [38]. Microbial DNA detected in meconium aid this hypothesis [39, 40].

In case of an early exposure, appropriate microbial populations need to be selected and the fetus must be exposed to such so that the microbiome can affect intestinal tract development already in utero. Considering the fetus being submerged in amniotic fluid, this seems the obvious medium. Amniotic fluid is primarily composed of fetal urine, and microbial composition varies throughout gestation [41]. Additional secretions contributing to amniotic fluid are lung liquid, buccal secretions, and to some part transmembrane flow. Furthermore, amniotic fluid contains immune modulating proteins, hormones and growth regulators [42], and potentially also microbial components [43]. To date, selection mechanisms favoring particular microbes over others in amniotic fluid remain mostly undiscovered. Data would support interactions between innate and learned immunity, and environmental factors (including pH, oxygen levels, or carbon sources).

One route via which the developing intestinal tract could be exposed to microbes/microbial products from within the amniotic fluid is swallowing. Swallowing of amniotic fluid begins as early as 10 weeks gestational age [42], coinciding with the beginning of esophageal innervation, which typically occurs by 13 weeks of gestation. Throughout the final 12 weeks of pregnancy, the human fetus swallows about 1 L of amniotic fluid per day [44, 45]. Therefore, swallowing amniotic fluid provides passage to the infants gut for bacteria and their products, such as glycoproteins, RNA or DNA.

As derived from data of infants suffering from congenital gut abnormalities (e.g. gastroschisis or intestinal atresia) fetal gut development affects intrauterine growth despite nutrition is obtained through the placenta [45]. Birth weight is reduced in infants suffering from proximal intestinal atresia when compared to those suffering from distal atresia [45, 46]. Also, infants born at term who suffer from intestinal atresia show significant reduction in birthweight when compared to those born prematurely [45]. Animal studies demonstrated a trophic effect for amniotic fluid [47] depicting that the mechanism of swallowing essentially contributes to normal intestinal development throughout fetal life [48]. Example given, fetal sheep that underwent esophageal

ligation develop reduced external muscle layer diameter in the stomach, duodenum and proximal parts of the small intestine. Also they show changed intestinal villus length and altered epithelial cell migration rates. In rabbits who underwent esophageal ligation, infusing amniotic fluid post ligation caused an almost normal development of the intestinal tract when compared to rabbits who were not provided amniotic fluid [47]. All of the above mentioned observations outline the importance for intestinal development of swallowing amniotic fluid in fetal life.

To date, to what extent swallowing amniotic fluid and its´ specific components might impact on intestinal development cannot be quantified. In a murine model, orally administered *Enterococcus fecium* could be isolated from meconium of pregnant mice, but not from meconium of controls [49]. Transmission of maternal microbes to the amniotic fluid and the placenta was also described [43, 49]. However, up to date, intrauterine microbe transmission from mother to fetus resulting in active colonization, as well as these microbes´ impact on intestinal development, still lacks certain evidence and remains substance to heated debate.

1.3. THE INTESTINAL MICROBIOME IN NEONATES

The origins of the infants` intestinal microbiome lie within maternal microbiota transmission during fetal life. Recent literature suggests potential for a distinct microbiome being present even within meconium or placenta [37, 50]. However, concerns about contamination of samples (e.g. from materials used or environment) could not yet be cleared, and this topic remains substance to heated discussion.

The infant's individual microbial intestinal colonization was found to be significantly influenced by birth mode [38, 51-54]. Patterns of microbial intestinal colonization within the first week of life seem to affect the individual´s future intestinal microbiota composition [29-31, 54]. In infants born at term, the intestinal microbiome rapidly maturates within the first year of life until reaching a more steady state throughout the fourth year of life [33]. Maturation of the intestinal microbiome, however, is delayed in preterm infants. The initially colonizing microbiota seem to affect the adult individuals´ intestinal microbiome. The latter is likely also influenced by genetics, intestinal

development, dietary factors and environmental factors [32, 33, 53, 55]. Whether the infant is breastfed or receives formula milk influences and alters intestinal colonization patterns in early life. Human milk benefits the infant by harboring lactoferrin or secretory IgA among several other components, providing effects against potentially pathogenic microbes and inflammation, and furthermore stimulate *Bifidobacterium* growth [56-58]. Again, live bacteria are present in breast milk [59]. The host and the individual intestinal microbiome form a symbiosis. Microbiota help utilize nutrients by increasing digestive capacity and harvesting nutrients from food. Vitamin biosynthesis is supported by the intestinal microbiota, as is production of several other metabolites [32, 33, 60, 61]. Additionally, the intestinal microbiome is able to limit nutrient resource availability to pathogens, mainly limiting metabolic resources and physical space available to pathogens [61, 62]. Furthermore, intestinal microbiota aid in development of intestinal barrier function, cell integrity or immune function.

In term born infants, *Lactobacillus, Enterobacter, Streptococcus, Staphyococcus*, and *Escherichia coli* are the first bacteria to colonize the intestinal tract. Consuming oxygen, these species enable subsequent colonization of anaerobic bacteria as *Clostridia, Bifidobacterium,* and various other Firmicutes [33].

There seems to be a biphasic pattern in the development of the intestinal microbiome of neonates. Two major factors influence the microbial development, the mode of childbirth and the type of feeding. While the mode of childbirth (vaginally vs. cesarean section) equally affects term and preterm infants, the type of feeding (breast vs formula) differently affects term and preterm infants. Breast feeding leads to higher numbers of *Bifidobacteria* and *Bacteroides*. Formula feeding leads to higher numbers of *streptococci, staphylococci* and *lactobacilli*. [63]

The single most important factor contributing to microbiome development in preterm infants appears to be the gestational age [64]. Birth mode, the use of antibiotics, feeding, and gestational age all alter the pace of developmental progression but the sequence of development seems to follow an orderly fashion [64]. It was postulated by La Rosa et al [64] that gut bacterial communities have a non-random assembly punctuated by microbial population abruptions. *Bacilli, Gammaproteobacteria*, and *Clostridia* were found to represent 92% of all bacteria [64].

1.4. INTESTINAL MICROBIOTA IN PRETERM INFANTS

In preterm born infants the intestinal microbiome develops differently. This may in part be caused by receiving enteral nutrition, fortified milk, way earlier than nature intended. Furthermore, the microbiome development is influenced by indwelling supportive devices as nasogastric tubes, and by the immaturity of the immune system [65]. Compared to term born infants, preterm infants´ gut microbiota show reduced levels of anaerobes (e.g. *Bifidobacterium* or Bacteroides) and higher levels of facultative anaerobes [66]. Also, increased amounts of potentially pathogenic bacteria (e.g. *Escherichia coli, Klebsiella*, or *Staphylococcus*) can be found (Table 6) [67].

TABLE 6: COMPARISON OF THE MICROBIOME OF TERM AND PRETERM INFANTS. ADAPTED FROM [68].

TERM INFANTS	PRETERM INFANTS
CLOSTRIDIA	↑ facultative anaerobes
BIFIDOBACTERIUM	↓ *Bifidobacterium*
OTHER FIRMICUTES	↑ *Staphylococcus*
	↑ *E. coli, Klebsiella* & other *Enterobacteriaceae*
	↓ Bacteroidetes

Leg.: ↑ indicates increased, ↓ indicates decreased. Data derives from open access publication [68].

The preterm infants´ intestinal microbiota are reduced in diversity with an increase in colonization with pathogenic organisms [36, 54]. Also, the intestinal microbiome is less stable in preterm infants compared to term born infants. Transformation of the intestinal microbial pattern towards a pattern present in adults is delayed in pretmaturely born infants [32, 53, 69, 70]. Facultative anaerobes (e.g. *Enterobacter, Enterococcus*, or *Lactobacillus*) and *Staphylococcus* numerally dominate, while non-facultative anaerobes (e.g. Bacteroides*, Bifidobacterium*, or *Atopobium*) are reduced [69-71]. By the end of the first week of life, healthy term born infants who are breastfed, in contrast

to preterm infants, are colonized by *Bifidobacterium* species [71]. Intestinal colonization pattern seems to differ, depending on gestational age [71] and current data suggest that genetic factors also impact on the intestinal microbiome development [36]. Abrupt population changes mark the evolution of the intestinal microbiome in preterm infants [64]. Qualitative and quantitative immune function are compromised in preterm infants, mostly related to continuing development of the infants´ immune system, which may influence alterations in microbial diversity found in preterm infants. As the epithelial gut barrier function is still insufficient in preterm born infants, they are predisposed to pathogen invasion. The latter may trigger exaggerated inflammatory responses which may enhance disease processes such as NEC [38, 72]. The aforementioned immune dysfunction in preterm infants combined with a decreased microbial diversity and a predominance of pathogenic bacteria form a state of dysbiosis [53, 73, 74].

1.5. FACTORS AFFECTING THE MICROBIOME

Diversity and abundance of the intestinal microbiome progress through several developmental stages beginning even before birth. Several interrelated factors have been identified which impact on composition of the human gut microbiome (figure 1). Among these are diet [75, 76], host genetics [75-77], the physiology of the colonization site [78], age [79, 80], antibiotic usage [75, 76, 80], mode of birth [75, 76, 81], birth environment (e.g. NICU setting) [81] and whether human milk or formula are used [75, 81].

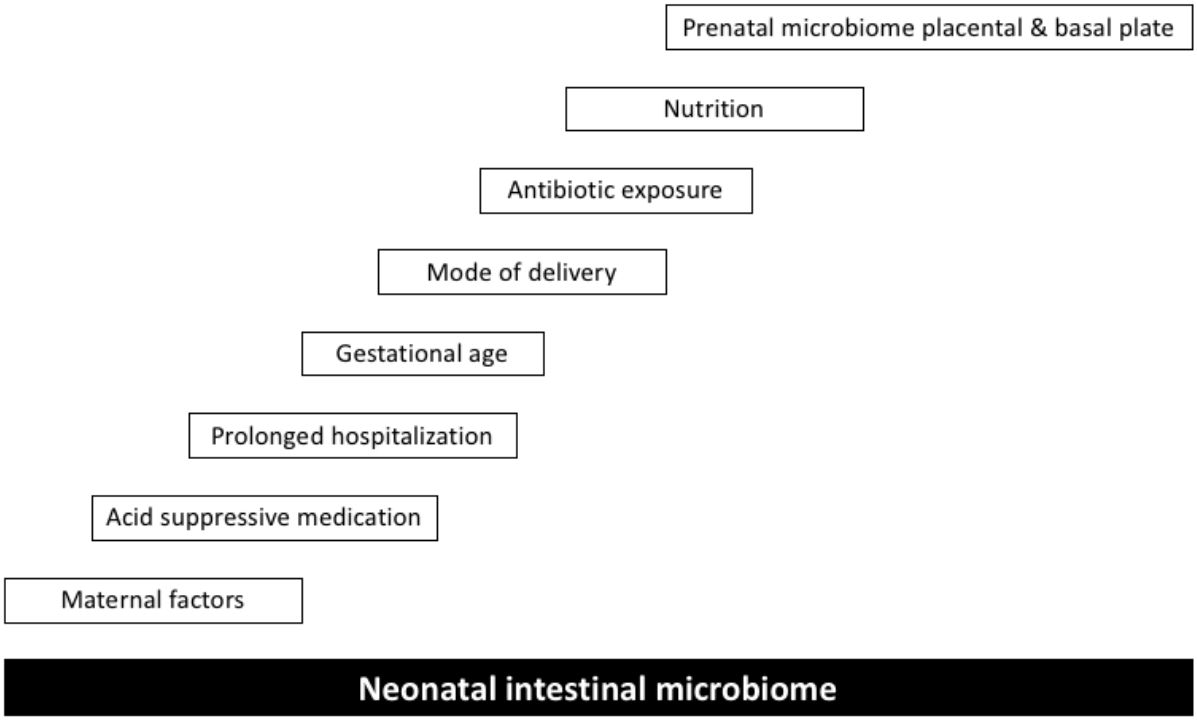
Furthermore, technical factors affect composition of intestinal microbiota. Example given, in culture-based techniques, bias may arise from oxygen-sensitivity, competition between fast- and slow-growing bacteria, or the recalcitrance of some bacterial species to culture media. Therefore, with current culture-based methods it is possible to successfully isolate 70% of present intestinal microbiota within a certain specimen, but no more [82].

The latter usually use array technologies or high throughput sequencing to analyze nucleic acids extracted from the sample. However, in these techniques variation due to processing (e.g. kits for preparation of DNA prior to analysis) or computer based

analyses is frequently observed. Several commercially available kits including the "QIAamp DNA Stool Mini Kit" (QIAgen, Hilden, Germany) [43], The "PowerLyzer PowerSoil DNA Isolation Kit" (MoBio, Carlsbad, CA, USA) [83, 84], "PowerMag® Soil DNA Isolation Kit" (MO-BIO Laboratories, Inc., Carlsberg, CA, USA) [85], the "MOBIO PowerSoil DNA Isolation Kit" (MOBIO) [86], and the "Fast DNA SPIN Kit" (MP BIO, Santa Ana, CA, USA) [82, 87] are broadly used.

Despite usage of commercially available DNA preparation kits, potential biases are introduced by sample processing methods. Among others, these include differences in amounts of material needed to start with (e.g. 100 mg to 200 mg) [83, 84]. Sample storage prior to processing, in regard of duration and temperature, may vary (e.g. storage at +4 ◦C and processing within 24 hours [86] or 72 hours [88]; immediate placement on dry-ice just after collection and storage at −80 ◦C [83] or snap-freeze in liquid nitrogen). Thereby sample composition may be biased as well. Using different 16S-rRNA gene hypervariable regions (e.g. V5 − V3 [86]; V1 and V3 [87]; V1 − V3 [43]; V3 − V6 [89]; V2 − V4, V6 + V7 − V8 and V9 [90] and V4, which represents the most frequently amplified region [83-85]) contribute to inter-study variation. These potential sources of bias are important to consider when interpreting different studies on intestinal microbiota.

FIGURE 1: FACTORS IMPACTING THE NEONATAL INTESTINAL MICROBIOME. ADAPTED FROM [34].



Leg.: Data derives from open access publication [34].

1.5.1. Influencing factor: birth mode

Birth mode strongly influences the establishment of GI microbiota in infants by exposure to the maternal microbial community. In spontaneous birth, infants are exposed to microbes colonizing the mother's birth canal. Vaginally delivered infants´ microbiota composition shows microbial pattern similar to their own mother [30, 91]. In infants born via cesarean section, no significant overlap between maternal and infantile microbiota has been observed [30, 91]. Environmental factors (i.e. healthcare workers, infants sharing the room, delivery or surgical equipment, surrounding air) seem to exert greater impact on the individual infants´ microbiome in infants delivered via cesarean section [78, 88]. However, in infants born via cesarean section following a period of labor showed microbial patterns which more closely resembled those of spontaneously born counterparts. When lacking any period of labor, microbiota resembled that of the mothers´ skin [86]. Microbial disruption in early life and influence of cesarean section on microbial colonization may lead to long-term metabolic consequences in these infants [92-94]. Furthermore, based on 2.500 infants, the LISA-Study [95] reported

atopic disease being more likely to develop throughout the first two years of life when infants were born via cesarean section.

Intestinal acquisition of *Lactobacillus* may be utilized to explain the effect of birth mode on gut microbiota development, as *Lactobacillus* is highly specific to the maternal vagina with high abundance [86]. Vaginally born infants microbiome contains *Lactobacillus*, whereas infants born by C-section do not [81, 93]. It has been shown that this difference concerning intestinal Lactobacillus abundance persists throughout the first six months of life and then slowly equalizes and finally vanishes by three years of age [81].

An association of birth mode within an individuals´ microbiome concerning the levels of Bacteroides and Clostridium genera (e.g. *Bacteroides fragilis* or *Clostridium difficile*) has also been shown [30, 31, 51, 83, 88, 91, 96]. The KOALA Birth Cohort study performed in the Netherlands with a high number of included infants (*n* = 1.032) used real-time quantitative PCR assays analyzing stool specimens taken at one month of age with regard to bacterial species [31]. Infants born vaginally (*n* = 826) showed increased numbers of *B. fragilis* while numbers of *C. difficile* were decreased when compared to infants born via cesarean section [31]. There origin of *C. difficile* seemed not to be maternal but could rather be linked to environmental factors (detection on hands and in fecal samples from healthy healthcare workers) [31, 97, 98]. As *C. difficile* has been considered a nosocomial microorganism [99] and has not been found in vaginal swabs of women taken prior to delivery [100, 101], it seems expectable to find increased levels of *C. difficile* in hospitalized infants delivered via cesarean section [31].

In preterm infants, however, the effect of the mode of birth on gastrointestinal microbiota development seems to not to be substantial [66, 84].

Summarizing all the above, current literature is indicative of infants delivered via Cesarean section trend to show the following findings:

- decreased number of anaerobes (e.g. *Bacteroidetes*)
- reduced diversity [30, 51, 88]

- delayed colonization [83]
- prone to atopic diseases [51] and metabolic disorders [93]

However, diversity in ethnicity or geographical background as well as different analytical methods must be considered when interpreting these studies.

1.5.2. Influencing factor: nutrition

First food, i.e. milk (either maternal or formula), impacts on the development of early intestinal microbiota [32, 102]. This effect is due to delivering essential nutrients for bacterial growth [102], immunomodulatory molecules [103], and, in case of breast milk, microbes capable of colonization [104]. Supporting the impact of food on the development of intestinal flora, similar microbial composition has been shown in colostrum and meconium samples from breast fed infants [43]. Even shared bacterial DNA has been identified in breast fed infants and their stool samples [105], with a more pronounced relationship between the infant mothers` breast milk and skin surrounding the maternal areola, than with any random mothers [106]. rDNA sequences of *Bifidobacterium longum* were simultaneously detected in infants` stool, maternal blood, maternal stool and their own mothers` breast milk (collected within four weeks after birth) [105]. These findings may be attributed to vertical transfer of microbiota through breast milk [106].

A more diverse microbiome was found in infants receiving formula milk, when compared to infants receiving breast milk [87, 107]. As described in the literature [108], relatively small amounts of formula supplementation result in microbial patterns similar to those of infants exclusively fed formula milk. In stool samples from exclusively breast fed vs exclusively formula fed infants, five bacterial species were present in all stool samples (i.e. *Bifidobacterium longum*, *Streptococcus salivarius*, *Streptococcus pseudopneumoniae*, *Strepotococcus lactarius*, and *Lactobacillus gasseri*) at the age of four weeks. Since the presence of these species seemed to be independent from feeding regimen, the authors argued that these species constitute common commensal bacteria in infants at four weeks of age. However, in breast fed infants *Streptococcus salivarius*, and *Streptococcus lactarius* showed lower abundances, while *Bifidobacterium longum*, *Streptococcus pseudopneumoniae*, and *Lactobacillus gasseri* were found to have greater abundance, when compared to formula fed infants.

These data support that different feeding patterns (formula vs mothers milk) alter common commensal bacteria relative abundance.

Infants receiving formula milk show a relatively stable, however diverse intestinal microbiome containing higher levels of facultative as well as strict anaerobes, than do infants fed with mothers milk [109-111]. In turn, the microbiome in stool specimens from infants fed mothers milk shows less complexity, contains more aerobes, and changes more dramatically throughout the first year of life [110, 111]. However, these differences in intestinal microbiota get lost when solid foods are introduced, initiating conversion towards an adult microbiome pattern [78, 102]. Diversification of gut microbiota is reduced when continuing breast milk feeding after introduction of solid foods into the diet [106]. However, underlying mechanisms leading to this suppression remain to be determined.

As lactation progresses, the composition of nutrients contained in human milk changes. Breast milk contains not only nutrients, but also hormones [109], growth factors [109], microbiota [106, 112], immunoglobulins [109], and enzymes [109]. In maternal breast milk of preterm born infants, the protein content is higher compared to breast milk from mothers who gave birth at term [113, 114]. Thereby the breast milk composition accounts for a greater protein demands to support growth in premature infants. However, amounts of protein content decline throughout lactation [115] with milk volume output being negatively associated with protein content [116].

In breast milk of term born infants` mothers *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Corynebacterium*, *Peptostreptococcus*, and *Escherichia* species were found [105]. The origin of *Escherichia* species probably lies in breast milk, as abundance, and hence the possibility for bacterial transfer, of *Escherichia* species from skin, vagina or other maternal sites is low [86]. The microbial composition of breast milk varies over time and with lifestyles (e.g. higher microbial diversity in rural vs urban women) [106].

Human milk oligosaccharides (HMOs) account for one third of breast milk components and are referred to as prebiotics, shown to promote growth of specific bacteria (*Bifidobacteria* species [117, 118], Bacteroidetes). However, they were not found to promote growth of pathogenic bacteria (e.g. *Enterobacteriaceae* [118]). *Bifidobacteria* predominate in breast- as well as formula fed infants [108, 109], although with less

frequent appearance in the latter group [107, 109, 119-121]. Infants acquire a large spectrum of *Bifidobacteria* from their mother. However, those strains able to degrade individual specific HMOs found in human milk will predominate [117, 121, 122]. Further factors positively influencing the growth of *Bifidobacteria* are a high lactose content or sialylated and fucosylated oligosaccharides [87].

Maternal stress and hormones seem to influence the breast milk microbiome more directly than mode of delivery [123]. The microbiome pattern of breast milk samples from mothers who underwent non-elective cesarean section and mothers who underwent vaginal delivery are quite similar, while the pattern differes in breast milk samples from mothers who underwent elective cesarean section.

1.5.3. Influencing factor: antibiotics

Infants born via cesarean section [31], as well as preterm born infants [124] receive antibiotics more frequently than infants born per vias naturales and at term. Perinatal exposure to antibiotics may increase the risk for diseases with an onset later in life (e.g. asthma [125], obesity [126], chronic inflammatory bowel disease [127], as well as allergies and inflammatory diseases [128]). Furthermore, exposure to antibiotics during the prenatal, perinatal, and postnatal periods might delay microbial development and maturation throughout the first year of life [129].

Antimicrobial prophylaxis administered intrapartum, to prevent fetal or neonatal infection and early onset sepsis in the newborn, when the mothers´ genitourinary tract is colonized by group B *streptococcus* resembles the main source of antibiotic exposure within newborns [90, 130, 131]. Mainly, penicillin, ampicillin, or ampicillin plus erythromycin are used [132]. Intrapartum antimicrobial prophylaxis was found to cause lower absolute levels of Actinobacteria and Bacteroidetes [130], as well as *Bifidobacteriaceae* [130, 133, 134], while Firmicutes [130] and Proteobacteria [133] phyla increased in numbers.

Antibiotic exposure delays intestinal colonization of beneficial bacteria and reduces the intestinal microbiomes´ diversity. Both are hypothesized to resemble predisposing factors to NEC [38, 135-137]. Duration of antibiotic therapy directly impacts on risk to

develop NEC in newborn infants [138], and prolonged use of antibiotics was found to be associated with an increased risk of NEC in uninfected infants [139].

1.5.4. Influencing factor: environment

The extra uterine environments influence colonization pattern and evolution of the infants` intestinal microbiota. A higher susceptibility to environmental factors has been found in infants delivered via cesarean section [117, 140]. Preterm infants develop an intestinal microbiome reflecting that of the Neonatal Intensive Care Unit (NICU) [32]. This may be due to long time exposure to this environment combined with intestinal immaturity [32].

Even though environmental microbes have been isolated from infant fecal samples, routes of transfer remain difficult to verify [141, 142]. Cross-transmission between patients and dissemination within an NICU have been reported [142]. Observational studies revealed that different geographical background (including different location of hospitals) result in differences of the infants` microbiome [110, 141]. Within the “PiPS” trial (double-blinded, randomized, placebo-controlled, including 1.310 preterm infants 23 to 30 weeks GA), carried out in southeast UK, probiotic supplementation using *Bifidobacterium breve* to prevent sepsis and NEC was researched. The authors were able to show cross-colonisation to be significantly associated with environmental factors [143].

The hospital environment, as well as handling or feeding, might all enhance microbial transmission to newborn infants [141]. However, detailed information regarding the underlying mechanisms of transmission are still lacking.

1.5.5. Influencing factor: oxygen

Preterm born infants` intestinal microbiome tends to contain pathogenic microorganisms (e.g. *Klebsiella pneumoniae* and *Clostridium difficile*) more frequently than term born infants`microbiomes [66, 118, 144]. Facultative anaerobes dominate [145] while levels of anaerobes are low [119, 146], as described above. Furthermore,

microbial diversity is decreased [118, 144] and short chain fatty acid levels are low in preterm infants [66].

The “aerobic intestine” of newborn infants at birth [147] supports growth of facultative anaerobes (e.g. *Streptococcus*, *Enterobacteriaceae*, or *Enterococcus*) [66, 78, 111, 146, 147]. Oxygen consumption in the intestine leads to creation of an anaerobic environment over time, and thereby facilitates the establishment of obligate anaerobes (e.g. Clostridium, *Bifidobacterium*, *Veillonella*, Bacteroides, *Eubacterium*, or *Ruminococcus* species) [66, 78, 111, 146, 147]. Additionally, many of the initial facultative anaerobic colonizers show pathogenic potential [111, 147].

Oxygen levels of term vs preterm born infants differ [148]. However, sufficient evidence to support the hypothesis that oxygen levels contribute to the differences of the preterm infants` intestinal microbiome remains to be established. It seems probable that the aforementioned alterations in oxygen levels within the gut of prematurely born vs term born infants might originate in diverse approach to oxygen supplementation at neonatal intensive care units, such as continuous positive airway pressure ventilation. Health related complications may be linked to intestinal oxygen levels in prematurely born infants enhancing growth of facultative anaerobes within the preterm gut [34].

1.5.6. Influencing factor: therapeutic agents

Acid suppression therapy, e.g. H_2 blockers, impacts on the preterm infants´ intestinal microbiome, by enhancing Proteobacteria and limiting diversity. Thereby, acid suppression might predispose preterm infants to NEC [149]. The use of H_2 blockers in preterm infants was found to be associated with an increased risk to death, sepsis, or necrotizing enterocolitis in hospitalized preterm infants with a birth weight below 1.500g [150].

Oral supplementation of probiotics was found to prevent severe episodes of necrotizing enterocolitis and all-cause mortality in preterm infants [151]. In 2016, Denkel et al. found that a reduction of necrotizing enterocolitis and mortality among preterm infants could be linked to oral supplementation with dual-strain probiotics [152]. The use of probiotics was found to shorten the length of hospital stay. The effectiveness of probiotics might be enhanced when administered in breast milk or a

combination of breast milk and formula, over a time period of less than six weeks at a dosage of less than 109 CFU (colony forming units) per day, and using preparations including multiple strains [153]. However, due to inhomogeneous study design, the use of different probiotic strains for supplementation, and differing feeding regimens applied in several trials regarding this topic, evidence on probiotics use remains difficult to interpret [154]. As NEC seems to be linked to dysbiosis, probiotics and other medications altering the infants` intestinal microbiome seem to constitute promising therapies for the future [155]. Which probiotic strain to use seems an important question. Thus far, this question remains unanswered. However, sole supplementation of *Bifidobacterium breve* did not demonstrate any beneficial effects [156]. Information on possible disadvantages from probiotics supplementation remains scarce in the literature. In preterm infants, immature intestinal barrier function may increase the risk for microbial translocation from the intestinal tract into the systemic or lymphatic circulation [157].

However, as preterm infants resemble a particularly vulnerable cohort, the use of live bacteria remains controversial.

1.6. THE INTESTINAL MICROBIOME AND INFLAMMATION

For the intestinal mucosa to recognize bacteria pattern recognition receptors are needed. Among these, toll like receptors (TLRs) are studied most extensively. TLRs recognize microbial associated molecular patterns (MAMPs) [158]. MAMPs in turn activate specific TLRs and thereby activate nuclear factor kappa-beta (NF-κβ) and its inflammatory pathway [38], propagating apoptosis and cytokine induction (e.g. IL-1, IL-6, IL-8, TNF-α, INF-1) [38]. Intestinal colonization patterns aid TLR expression, and dysbiotic patterns may trigger inappropriate responses. Large amounts of MAMPs are encountered by the newborn infants´ intestine just after birth, constituting the first large scale activation of TLRs [159]. Both pathogenic as well as commensal intestinal bacteria contain MAMPs. Commensal bacteria usually do not express key virulence factors, but may cause proinflammatory effects in case of impaired host conditions [160]. A fine balance must be kept by TLRs between executing inflammatory response

to pathogenic microbes and maintaining homeostasis and important intestinal functions (e.g. cell growth/proliferation, regulation of barrier function, cytoprotection, and antimicrobial peptide secretion) [154]. Lipopolysaccharides from Gram-negative bacteria activate TLR-4 [161], which, in the premature infant, is likely to increase cytokine-mediated inflammatory response. In preterm infants TLR-4 is upregulated in the intestine [162, 163]. Among the inflammatory cascade interleukin-8 (IL-8), one of the most prominent cytokines, increases neutrophil chemotaxis, resulting in inflammation and leading to tissue injury and reduced epithelial repair, and eventually necrotizing enterocolitis [164, 165]. Whether a deficiency in expression of NF-κβ inhibitors predisposes preterm infants to an exaggerated inflammatory response or not remains controversial [166, 167]. Commensal bacteria do not trigger inflammation and aid the intestine to tolerate the constantly present stimulation by microbes and MAMPs by downregulation of the NF-κβ pathway (blocking degradation of inhibitors) and preventing recognition by TLRs. Also, the low-level stimulation of TLR-4 by the commensal flora seems beneficial for intestinal homeostasis [168, 169].

1.7. NECROTIZING ENTEROCOLITIS

Considered the most common severe, potentially life-threatening, intestinal emergency in newborn infants, Necrotizing enterocolitis (NEC) was first described in 1965 [170]. NEC typically affects prematurely born infants, while infants born at term are rarely affected. In NICUs NEC has an overall prevalence of 1-5% increasing to 7-11% in VLBW infants [171, 172]. Both incidences are indirectly proportional with birth weight and gestational age, as do fatality rates, putting infants with a birth weight <1.500g at highest risk. Multiple factors have been associated with the development of NEC. While prematurity resulting in intestinal immaturity seems to be the most important one, others (e.g. nutrition, or altered bacterial flora) are discussed controversely [171, 172].

1.7.1. Pathogenesis

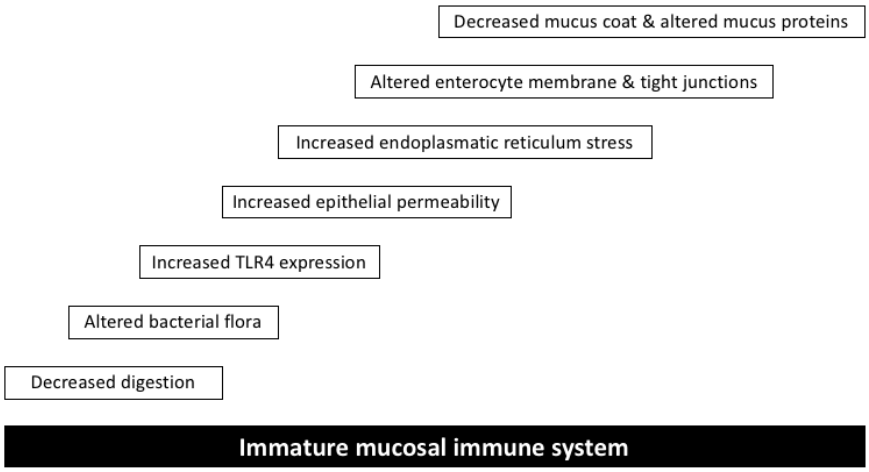
As far as currently known, enteral feeding, intestinal integrity and pathogens and ischemic events are held responsible for NEC development [28, 173-175].

Nutrition seems to play a key role, as NEC rarely occurs before enteral feedings are proposed, and appears to a much lesser degree in infants receiving mothers milk [176]. Infants in whom enteral feeds are introduced aggressively seem prone to develop NEC [173]. Premature infants show decreased digestion and intestinal nutrient absorption [177], as well as reduced intestinal motility [178].

Loss of mucosal integrity may possibly result in NEC. This lack of integrity may be caused by several different factors, including prematurity of the intestinal tract, and a cellular response causing necrosis. In the immature intestine, numbers of goblet cells (mucus production) are decreased [179], tight junctions are immature [180], and mesenterial microvascular tone is increased [181]. Although a variety of bacterial and viral organisms could be found in cultures, no distinct pathogen could reproducibly be identified.

Translocation of bacteria through the intestinal mucosa, however, activates TLR-4 on endothelia of intestinal blood vessels, thereby reducing blood flow and leading to intestinal ischemia and necrosis [182]. Among other factors (e.g. platelet activating factor, macrophages, or elevated baseline levels of cellular endoplasmic reticulum stress in premature infants´ intestine [183-186]) increased TLR-4 signaling predisposes the premature infants´ intestine to necrotizing enterocolitis (figure 2).

FIGURE 2: FACTORS PREDISPOSING THE IMMATURE INTESTINE TO NEC. ADAPTED FROM [34].



Leg.: Data derives from open access publication [34].

1.7.2. Clinical manifestations

Necrotizing enterocolitis generally develops by two to three weeks of age with various clinical symptoms, while radiographic signs may be obvious (in severe cases) or rather subtle [187]. Onset of symptoms may appear sudden and intense, with severe illness (bowel perforation, peritonitis, shock) and high mortality. However, initial symptoms may as well present subtle and nonspecific (e.g. lethargy or temperature instability; see table 7). Abdominal distention, gastric retention, and bloody stools (25% of affected patients) represent classical gastrointestinal findings in NEC. At any point, NEC may progress rapidly. Table 8 shows gastrointestinal and systemic clinical findings.

TABLE 7: INITIAL AND SYSTEMIC SYMPTOMS OF NEC.

INITIAL SYMPTOMS	SYSTEMIC SYMPTOMS
VOMITING	Apnea
DIARRHEA	Lethargy
DELAYED GASTRIC EMPTYING	Decreased peripheral perfusion
ABDOMINAL DISTENSION, TENDERNESS OR BOTH	Shock
ILEUS OR DECREASED BOWEL SOUNDS	Cardiovascular collapse
ABDOMINAL WALL ERYTHEMA	Bleeding diathesis
HEMATOCHEZIA	

TABLE 8: GASTROINTESTINAL AND SYSTEMIC CLINICAL FINDINGS IN NEC.

GASTROINTESTINAL SIGNS	SYSTEMIC SIGNS
INCREASED ABDOMINAL GIRTH	Respiratory failure
VISIBLE INTESTINAL LOOPS	Decreased peripheral perfusion
OBVIOUS ABDOMINAL DISTENTION AND DECREASED BOWEL SOUNDS	Circulatory collapse
CHANGE IN STOOL PATTERN	
HEMATOCHEZIA	
PALPABLE ABDOMINAL MASS	
ERYTHEMA OF THE ABDOMINAL WALL	

1.7.3. Diagnosis

Pneumatosis intestinalis (i.e. air within the bowel wall) on abdominal fluoroscopic images is pathognomonic for NEC, and allows for diagnosing the disease when combined with clinical symptoms [188]. Complete blood count and abdominal sonography yield supportive findings [189]. Table 9 gives radiographic and laboratory findings in NEC. NEC can be staged using “Bell´s Staging Criteria score”, which combines clinical (i.e. systemic and intestinal) and radiographic signs and symptoms, and is widely used in clinical practice.

TABLE 9: RADIOLOGIC AND LABORATORY FINDINGS IN NEC.

RADIOLOGIC FINDINGS	LABORATORY FINDINGS
A**BNORMAL GAS PATTERN, DILATED INTESTINAL LOOPS, THICKENED BOWEL WALL, FIXED AND DILATED INTESTINAL LOOP PERSISTING OVER SEVERAL EXAMINATIONS (X-RAY)**	Moderate to profound neutropenia (absolute neutrophil count [ANC] < 1.500/µL)
SCARCE OR ABSENT INTESTINAL GAS OR DIFFUSE DISTENTION CHANGING OVER TIME (X-RAY)	Acute decrease in hematocrit (bleeding or developing consumptive coagulopathy); elevated hemoglobin and hematocrit (hemoconcentration; accumulation of extravascular fluid)
PNEUMATOSIS INTESTINALIS	Thrombocytopenia
ABDOMINAL AIR	Blood cultures usually negative
PORTAL GAS	Hyponatremia
INTRAPERITONEAL FLUID	Low serum bicarbonate
ABDOMINAL SONOGRAPHY: IDENTIFY AREAS OF ABSCESS / WALLED-OFF PERFORATION, ASCITES	

1.7.4. Treatment

Whether NEC is suspected clinically or already proven, therapy must be rapidly initiated in order to decrease morbidity and mortality. The mainstay in therapy is supportive care and prevention of further gastrointestinal injury. Oral feedings must be discontinued immediately and substituted by parenteral nutrition. Nasogastric tubes are used for gastric decompression. Intravenous fluids and broad-spectrum antibiotics are used. Furthermore, as is the case in our institution, some centers administer probiotics in order to reverse possible intestinal dysbiosis. Therapy should be guided by Bell´s stage scoring.

Bell stages IA and IB (i.e. suspected NEC)

- Discontinuation of enteral feeding, begin parenteral nutrition and start antibiotics over three days
- Intravenous fluid supplementation

Bell stages IIA and IIB (i.e. definite NEC)

- Respiratory support and katecholamine support as needed
- Fluid resuscitation
- Total parenteral nutrition and antibiotics over 14 days
- Surgical consultation is recommended

Bell stage IIIA (i.e. advanced NEC)

- Total parenteral nutrition over 14 days
- Fluid resuscitation
- Katecholamine support
- Respiratory support
- Surgical consultation and surgical intervention

For hemorrhagic necrosis or intestinal perforation surgical treatment seems imperative. Further surgical indications include abdominal wall erythema, portal vein pneumatosis, as well as worsening clinical condition. If a surgical approach is necessary, it is recommended to perform histological examination of resected intestinal specimens on a routine basis.

1.7.5. Prognosis

If pneumatosis intestinalis is present, medical management was reported to fail in up to 40% of patients with a mortality of up to 30% [190]. Premature infants suffering from NEC and requiring surgery, as well as those showing concomitant bacteremia, pose a high-risk group for impaired growth and neurodevelopmental outcome [191, 192]. Short bowel syndrome may appear post operatively.

1.7.6. Economics

Across the United States of America, the total estimated healthcare costs inflicted by NEC amounts $500 million to $1 billion per year [193]. For Austria, such estimations do not exist. However, extrapolating US estimations to the Austrian population, NEC might account for health care costs of about €10 million to €20 million annually.

1.7.7. Prophylaxis

The risk for NEC was shown to be reduced in exclusively breastfed newborns [176, 194]. Early feeding with swift increase in feeding volumes raised concern regarding a possible increase in NEC risk [173, 175]. Safe feeding regimens in regard of NEC risk remain to be determined. It has been shown that prophylactic use of enteral antibiotics was able to reduce the risk for NEC [195]. However, the development of resistant bacteria remains a major concern. The use of probiotics reduces NEC risk with enteral probiotics supplementation reducing the risk for severe NEC (i.e. ≥ Bell´s stage II) [35, 196-203]. At our institution in Graz, a unique prophylaxis regimen combining enteral antibiotics, probiotics and antimycotics with predominating feeding breast milk is used with great success [204].

1.8. INTESTINAL MICROBIOTA AND NEC

Premature birth and intestinal colonization are major risk factors for NEC [205]. Therefore, in case that prematurity is inevitable, the preterm infants´ intestinal colonization constitutes the major target to modify NEC risk. Until now intestinal colonization patterns and preterm infants´ intestinal microbiome composition and diversity in NEC cases remain inconsistent. Thus far, some papers report on a distinct composition of the gut microbiome in prematurely born infants immediately prior and at onset of NEC [38, 52, 53, 62, 206], while others do not [207, 208]. A first shift away from the healthy infants´ intestinal microbiome appears as early as three weeks prior to NEC onset [62]. Around two weeks prior to NEC onset, *Proteobacteria* were found to be increased in numbers while Bacterioidetes abundance was decreased [53]. Differences within the intestinal microbiome persist until NEC onset with lower diversity compared to preterm infants not suffering from NEC [206]. Onset of NEC before or later than 22 days of life seems to define a discriminating age limit regarding abundance of gut microbiota [146]. Thus, a variety of specific microbiota potentially associated with disease onset might exist depending on the infants´ age.

Several studies demonstrated that fundamental differences in the microbiome of neonates with and without NEC exist [144, 146, 209-212]. However, no single causative species could be identified, as speciation is dependent on the quality and length of the sequence and primers, and that it is not possible to distinguish between life and dead bacteria. That, and heterogeneous study populations complicate comparison of present studies.

Despite these limitations, current studies reveal the following information:

Infants diagnosed with NEC showed significantly decreased diversity compared to infants without NEC. Infants diagnosed with NEC showed increased colonization of *Gammaproteobacteria,* but decreased levels of other bacterial species [144].

Infants diagnosed with NEC show increased colonization of Proteobacteria and decreased levels of Firmicutes 72h to one week prior clinical diagnosis of NEC [209].

Firmicutes dominating the intestinal microbiome 4 to 9 days post partum seem to be associated with NEC. Ten to 16 days post partum, samples from infants diagnosed

with NEC were dominated by Proteobacteria. Interestingly, infants showing dysbiosis with high levels of Firmicutes seem to develop NEC earlier. *Propionibacterium* was absent in all infants diagnosed with NEC. All infants diagnosed with NEC showed either Firmicutes or Proteobacteria dysbiosis. However, in 25% of controls the same phenotype could be found as well [210]. Proteobacteria might be associated with increased risk of NEC, as validated in a 2017 meta-analysis of 14 studies on intestinal dysbiosis in preterm infants diagnosed with NEC by Pammi et al. [212].

2 OBJECTIVES

2.1 AIM OF THE STUDY

The aim of this study was to evaluate the influence on diversity and composition of fecal microbiota caused by different therapeutic and preventive approaches to VLBW infants concerning NEC prophylaxis. The intestinal microbiome is likely influenced by probiotics, antibiotics, and antifungal agents as well as introduced feedings. Thus, due to use of different hospital regimens, diversity and abundance of gut microbiota and the development of the gut microbiome throughout the first weeks of life should show particular differences among included infants. Furthermore, we hypothesize that specific regimens used at different centers might lead to center specific clustering of microbiota composition.

3 METHODS

We performed a prospective controlled cohort study on VLBW preterm infants (i.e. infants` birth weight <1.500g) cared for at three neonatal care centers in southeastern Austria implying different hospital regimens for prophylaxis of NEC and feeding regimens. Table 10 shows regimens of NEC prophylaxis at the three participating centers.

TABLE 10: NEC PROPHYLAXIS REGIMENS AT THE NICU OF THE MEDICAL UNIVERSITY GRAZ COMPARED TO THE REGIMENS AT THE GENERAL HOSPITAL OF KLAGENFURT AND LEOBEN.

	NICU GRAZ	NICU KLAGENFURT	NICU LEOBEN
PROBIOTICS	*Lactobacillus rhamnosus* 1g = $1x10^9$ CFU/d p.o. split into 2 doses	*Bifidobacterium infantis* $2x10^9$ CFU/d and *Lactobacillus acidophilus* $2x10^9$ CFU/d in combination p.o.	None
ANTIBIOTICS	Gentamycin 7 mg/kg every 12 hours p.o.	None	Gentamycin 7 mg/kg every 12 hours p.o.
ANTIFUNGAL AGENTS	Nystatin 10.000 U/kg every 6 hours p.o.	Fluconazol 6 mg/kg i.v. every 72 hours (<1.000 g birth weight)	Nystatin 10.000 U/kg every 6 hours p.o.
FEEDING	Pooled or pasteurized BM; subsequent transition to mothers´ BM or preterm formula (hydrolyzed in case of birth weight <1.000g)	Pasteurized BM (no pooled BM) or preterm formula	Pooled or pasteurized BM; subsequent transition to mothers´ BM or preterm formula (hydrolyzed in case of birth weight <1.000g)

Legend: BM = breast milk; p.o. = per os; CFU = colony forming unit; U = units; d = day; g = gram; kg = kilogram; mg = milligram. Figure excerpted from [213].

Participating centers were the Division of Neonatology, Department of Pediatrics, General Hospital of Klagenfurt, the Division of Neonatology, Department of Pediatrics, General Hospital of Leoben, and the Division of Neonatology, Department of Pediatrics, Medical University Graz. All three centers are located in the southeastern region of Austria. Different regimens with regard to prophylaxis of necrotizing enterocolitis are shown in table 10. Each regimen is considered standard care at the respective center. Standardized feeding regimens are used at every center. All feeding regimens included introduction of mothers´ milk early with gradual increase in feeding amounts to establish infants´ food tolerability within days. Also, uniformly the amounts of fluid (either enterally or parenterally) are tailored to birth weight (i.e. <1.000g birth weight receive 100 ml/kg/d, 1.001-1.499g receive 90 ml/kg/d, 1.500g-2.499g receive 80 ml/kg/d, >2.500g receive 70 ml/kg/d). Amounts of each feed are again tailored to birth weight, with infants weighing more than 1.500g at birth receiving 5 ml on the first day

after birth, and infants weighing more than 2.500g at birth receiving 10 ml per feed on the first day of life. Maternal cytomegalovirus (CMV) IgG positivity is considered a potential risk in infants weighing less than 1.800g at birth. Thus, in these infants pasteurized breast milk is used. In case of insufficient maternal breast milk supply, pooled donor milk was used only at the center in Graz, while formula milk was provided at every center. Human milk fortifiers were administered to infants < 1.500g birth weight. However, milk fortifiers were not used prior the infant tolerated at least 50% of feeding amounts orally.

3.1 PATIENTS

3.1.1 Inclusion criteria

Preterm infants weighing <1.500g at birth and treated at the neonatal intensive care units of the partizipating centers. Recruitment phase lasted between October 2015, and October 2017. Prior to participation, written informed consent was obtained from the parents.

3.1.2 Exclusion criteria

We excluded infants who died at birth or within two weeks of life. Further, we excluded patients diagnosed with genetic syndromes or disease, congenital anomalies, or meconium ileus.

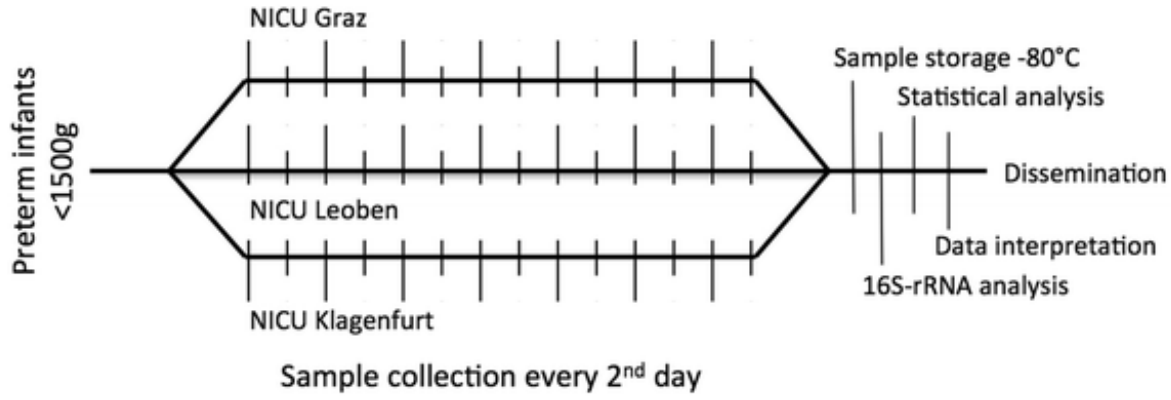
3.1.3 Dropout

Certainly, upon request by the parents, withdrawal from the study was possible at any time. Parents informed consent included a passage clearly outlining this opting out possibility (any time and without any reason). Consequently, these infants would be immediately discharged from the study and all associated data would not be analysed. Parents were all assured that a decision towards withdrawal from the study would not influence of impact further care and medical treatment of their children at any point.

3.2 STUDY PROTOCOL

Overall, 60 VLBW preterm infants, cared for at the neonatal intensive care units of the three participating centers Graz, Leoben and Klagenfurt (i.e. 20 infants each) were planned to be included. Fecal samples were collected every second day throughout the first two weeks of life, equaling seven samples of each included infant. No stool softeners were used in included infants. Figure 3 shows the study protocol. Samples were then split into two separate sterile tubes, with one of them remaining stored for backup analysis and the other one being analysed for the study purpose. All samples were stored at -80°C, at which the microbiome of the samples is known to be effectively contained without alteration of bacterial diversity or abundance over long periods of time. Before inclusion of infants and first stool sampling, we performed feasibility testing on the DNA isolation method. This feasibility testing was successfully concluded using six test samples from infants weighing <1.500g at birth. These six infants, however, were not included in the final study. The detailed study protocol was published in *Pilot and Feasibility Studies* [214] and can be accessed from there.

FIGURE 3: GRAPHICAL OVERVIEW ON THE STUDY PROTOCOL. FROM [214].



Leg.: Vertical lines illustrate dates of sample collection; timeline is given in days. Figure excerpted from [214]

3.3 METHODOLOGY

Recruitment of infants took place at the abovementioned centers. Parental informed consent was acquired prior to inclusion in the study by one local study coordinator on site. Parents were informed on the purpose of the study, procedural measurements to obtain stool samples, as well as potential advantages or disadvantages for their infant when enrolled in the study. Furthermore, parents were assured that the infants would not get harmed or receive inferior treatment when enrolled in the study. Parents signed a written informed consent form, as did the local study coordinator on site. Parental written informed consent was mandatory prior enrollment in the study. The parental informed consent form is added in the appendix of this manuscript. In total, seven fecal samples were taken from each enrolled infant, partitioned into two sterile containers. Fecal samples were taken from spontaneously produced stools throughout the first two weeks of life. First samples were taken from meconium (i.e. infants first stool). No stimulation of defecation or any other procedure to produce stools deviating from standard care were performed throughout the study. To collect the feces we used sterile single use spatulas and sterile single use fecal sample containers (CryoTube™ Vials, Thermo Fischer Scientific, Denmark). Stool sample containers had to be freeze resistant up to -80°C. A maximum of 1ml of stool per sample was taken. Samples were assigned a running number preceded with a letter indicating the center the sample was taken at. E.g. G-001 for the first sample from the center at Graz. We used a standardized stool sample protocol-sheet to keep track of sampling and monitor study progress. All samples were immediately stored at -80°C. Inside the freeze device we kept sample containers inside cardboard boxes (Fiberboard Cryo Box, Thermo Scientific™, Denmark) for damage protection. To deliver samples from the centers at Klagenfurt and Leoben to our center at Graz we incorporated a professional delivery service, a temperature protocol, and stored all samples on dry ice during the transport. Once collection of all samples at every center was completed, we facilitated 16S-rRNA-analysis of all samples at once to ensure comparable results and minimizing procedural bias.

3.3.1 Standard Operating Procedures

To guarantee equal performance and study-quality at all three centers we implied Standard Operating Procedures (SOP) tailored for each center. SOPs are shown below. For the purpose of originality, SOPs are presented in original German language in the appendix section of the manuscript.

3.3.2 Data collection

Throughout the duration of the study we collected all the below mentioned data on enrolled infants. All data were noted in an excel sheet and, additionally, on paper for backup purposes.

3.3.2.1 Maternal and pregnancy associated data

We collected data on mothers' age, former pregnancies in number and whether it was a life birth or still birth, number of siblings to the patient, whether it was a multiple pregnancy, existence of a mother-child-passport (i.e. an official document by the Austrian government, printed as a standardized booklet to document pre-/peri- and postnatal findings; detailed information can be found on https://www.help.gv.at/Portal.Node/hlpd/public/content/8/Seite.082201.html). Furthermore, we collected data on pregnancy-associated complications, whether the mother received steroids to induce lung maturation of the infant, or tocolysis.

3.3.2.2 Perinatal data

We collected data regarding complications at birth, birth-mode (cesarean section vs vaginal birth), APGAR-scores, and umbilical cord pH.

3.3.2.3 Neonatal data

Infants` data were recorded in terms of sex, birth-weight, whether they were small-for-gestational age (SGA; i.e. birth-weight <10th percentile for gestational age), duration of hospital stay, duration they were cared for at the NICU, duration of oxygen-demand, neonatal morbidities (i.e. NEC, early or late onset sepsis, bronchopulmonary dysplasia,

infant respiratory distress syndrome, retinopathy of prematurity, intraventricular hemorrhage, periventricular hemorrhage, periventricular leucomalacia, or spontaneous intestinal perforation), whether they received caffeine or surfactant, as well as demand of inotropic medication and other cardiovascular supportive therapy.

3.3.2.4 Data on specific medications

All medications were recorded with regard to dosage, active pharmaceutical ingredient and oral vs parenteral application. Special focus was based on antibiotics, antifungal agents, probiotics and parenteral vs enteral nutrition.

3.3.3 Microbiome analysis

After unfreezing fecal samples, DNA was extracted using the MagnaPure LC DNA Isolation Kit III by Roche (Corefacility Molecular Biology, ZMF, Medical University of Graz). We applied V4 primers 515F and R926 [215] to amplify hypervariable regions. Further sequencing was performed using Illumina MiSeq with v3 600 chemistry [216]. PCR was run in triplicates and pooled subsequently. Library preparation and amplicon sequencing were performed at the Core Facility Molecular Biology at the Center for Medical Research at the Medical University Graz, Austria.

To determine the number of bacterial 16S rRNA gene copies, a SYBR based qPCR with primer pair Bac331F and RBac797 was performed [217, 218]. Reagents were pipetted using “Hamilton Starlet” and run on a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Overall, we processed 16 negative controls and considered them for subsequent analysis.

Raw reads were made publicly available at the European Nucleotide Archive (ENA) and can be accessed using BioProject No. PRJEB37883.

Raw read processing, bioinformatics and biostatistical analyses were performed using QIIME2 [219], as described previously [220]. Reads in negative controls were interpreted either contaminated (possibly external in origin; either reagent or environment), or possible cross-contamination leaking from samples with high DNA content. Thus, reads per Amplicon Sequence Variant (ASV) per batch were calculated as means and ASVs were deleted in case [(reads in batch NC)/(mean sample reads

per batch)]*100 > 20 held true. Quality checking of this approach was performed using "R" package decontam [221], which yielded highly similar outcomes.

Feature tables and taxonomy files obtained were incorporated into further analysis. To calculate and construct alpha-diversity indices and Principle Coordinates Analysis (PCoA) plots based on Bray-Curtis dissimilarities, and also bubble plots and violin plots of Shannon indices, richness and evenness we used "Calypso" [222] and "R" [223], implying the package ggplot2 [224]. To test differences in the alpha-diversity indices between the groups we used "Qiime2" [219] and Adonis analysis in "Calypso". Alpha- or beta-diversities were used to display fecal microbiome differences between participating centers. We calculated alpha-diversities separately for each sample and tested for significant differences between participating centers. Richness and evenness, contributing to alpha-diversity, were observed separately. Beta-diversities between the centers were displayed using PCoA plots based on unweighted Bray-Curtis dissimilarities.

3.3.4 Time schedule

Originally this project was scheduled for a total duration of 24 months. At the center in Graz, around 100 infants with a birth-weight <1.500g are cared for per anno. At the center in Klagenfurt, 40 to 60 infants with a birth-weight <1.500g are cared for per anno, and at the center in Leoben around 30 infants with a birth-weight <1.500g are cared for per anno. Therefore, we calculated the active recruitment and data collection phase to be completed within 18 months. Due to delayed recruitment at the study center in Leoben, due to less VLBW infants born and cared for than were estimated, the active recruitment and data collection phase lasted 24 instead of 18 months, from October 2015 to October 2017. Stool sample analysis was planned to take place throughout the following 5 months. However, this was also delayed due to high traffic at the analyzing facility. Finally, statistical and biostatistical analysis of data from 16S-rRNA analysis were performed and data were prepared for presentation at meetings and publication in scientific papers. Figures 4 and 5 show intended and actual timelines.

FIGURE 4: INTENDED TIMELINE OF THE PROJECT.

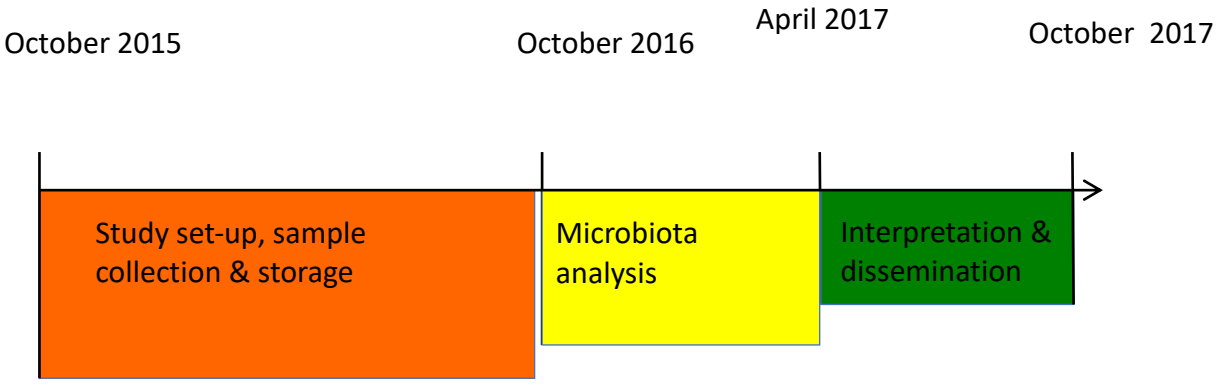
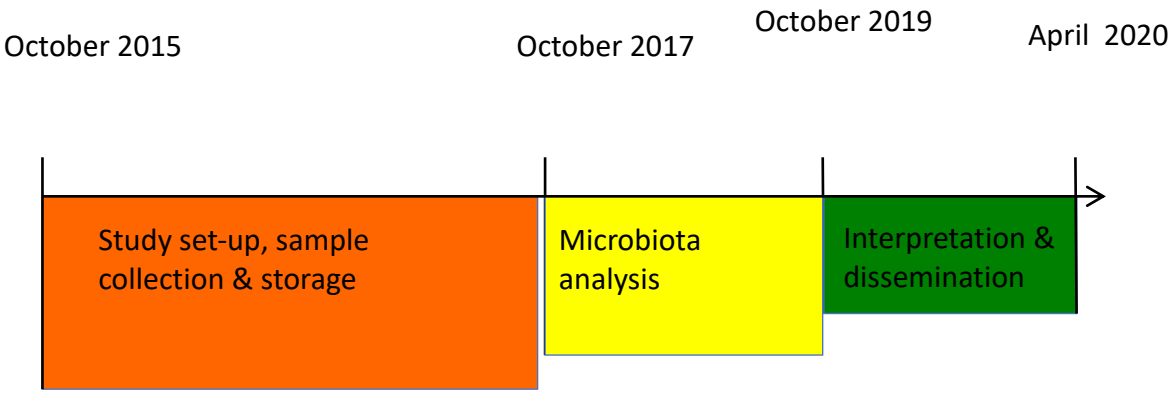


FIGURE 5: ACTUAL TIMELINE OF THE PROJECT.



3.3.5 Ethical considerations

The ethics committee of the Medical University Graz and the ethics committee of Kärnten, both covering ethical regulations at the participating centers, approved the study (application number 27-366 ex 14/15). Parental written informed consent was mandatory prior to infant recruitment. Routine clinical care remained unchanged throughout the entire study participation. We adhered to Good Scientific Practice guidelines. Written informed consent forms are presented in the appendix.

3.3.6 Participating personal

Dr. Stefan Kurath-Koller, principal investigator. Responsible for patient recruitment at center in Graz, and for coordinating patient recruitment at the centers in Klagenfurt and Leoben. Responsible to coordinate and continuously collect and update data, interpretation of data, and dissemination of project findings. Wrote the ethics committee request including a study protocol, organized sample archiving and calculated costs of the project. Responsible to get all materials needed, e.g. sterile sample containers, storage boxes, sterile microspatulas. Wrote standardized operating procedures tailored to each investigation site. Responsible to organize and plan temperature controlled freeze-storage delivery from centers Klagenfurt and Leoben to Graz.

Prof. Dr. Bernhard Resch, Division of Neonatology, Department of Pediatrics, Medical University Graz. Assisted coordination of the project and supported recruitment at the center in Graz. As main supervisor, he provided supervision of doctoral thesis and supported with implementation of the project.

Prof. PD Dr. Gregor Gorkiewicz, Department of Pathology of the Medical University of Graz. Provided knowledge and facility access for microbiota analysis and data interpretation. Co-supervisor of the doctoral thesis.

Prof. Dr. Christine Moissl-Eichinger, Division for Interactive Microbiome Research, Department of Internal Medicine, Medical University of Graz. Assisted with planning and coordination of sample analysis. She provided expertise in the field of microbiome research, supported man- and brainpower of the Interactive Microbiome Research group, utilizing her laboratories and expert personnel who performed analysis and interpretation of results. Essentially contributed to interpretation of the results. Co-supervisor of the doctoral thesis.

Charlotte Neumann, Msc, Division for Interactive Microbiome Research, Department of Internal Medicine, Medical University of Graz. Performed qPCR analyses and processed microbiome raw-reads. She performed microbiome analysis, statistical analyses, visualized the microbiome data, and, together with SKK, CME and BR, interpreted the results.

Dr. Raimund Kraschl, Division of Neonatology, Department of Pediatrics, General Hospital of Klagenfurt. Responsible for recruitment of infants and patient management at the study center in Klagenfurt. Furthermore, he assisted with coordination of specimen collection and freeze-storage at the study center in Klagenfurt.

Dr. Claudia Kanduth, Division of Neonatology, Department of Pediatrics, General Hospital of Klagenfurt. Responsible for patient management and recruitment, and coordination of sample collection and storage at the study center in Klagenfurt.

Dr. Barbara Hopfer, Division of Neonatology, Department of Pediatrics, General Hospital of Leoben. Responsible for patient management and recruitment. Assisted with coordination of sample collection and storage at the study center in Leoben.

3.3.7 Costs and funding

Performing this study, gathering all the necessary material and equipment, as well as performing 16S rRNA gene analysis of stool samples bares certain amounts of costs, and to gain funding was a major task. Table 11 shows an overview of costs.

Throughout the study, the following equipment and expanses had to be met:

Sterile stool sample containers

To collect samples of stool and guarantee storage without contamination. Containers needed to be freeze-proof to store them at -80°C without damage. We used TPP® Cryotubes (2.0 ml, SIGMA Aldrich, Merck KGaA, Darmstadt, Germany).

Sterile spatula

To position the stool samples into the above mentioned storage containers avoiding contamination we needed sterile spatula. Since the possibility of sterilization was not given at every partizipating center, we used disposable sterile spatula, SteriWare® Microspatula (2.0 ml, Sampling Systems Ltd., 4 & 5 Forge Mills Park, Coleshill, Warwickshire, B46 1JH, United Kingdom).

Microbiome analysis

16S rRNA gene analysis of stool samples to detect microbial diversity and abundance was performed by the Core Facility Molecular Biology, Medical University Graz, headed by Ingeborg Klymiuk and by the Interactive Microbiome Research group, Medical University Graz, headed by Christine Moissl-Eichinger. 16S amplicon based analysis to detect Bacteria and Archaea was performed as described above.

Travel costs

Travels to the study centers Klagenfurt and Leoben were necessary in regard of presentation of the study and introduction as well as instruction of the personnel, and supervision and coordination.

Freeze storage

Samples must be stored at -80°C until analysis. To ensure safe storage a deep-freezing storage device is required.

Delivery service

Delivery service is required to transport samples from the study centers at Klagenfurt and Leoben to the center in Graz for storage until processing. Transport is performed using dry ice and a temperature protocol to ensure safe and stable conditions throughout transport and prevent alteration of microbiota composition and diversity.

TABLE 11: OVERVIEW OF COSTS, OVERALL AND PER EACH EXPANSE.

ITEM	COST PER ITEM	ESTIMATED NUMBER OF ITEMS	ESTIMATED TOTAL COST
STERILE SAMPLE CONTAINERS	5€	560	2.800€
STERILE SPATULA	0.9€	600	540€
MICROBIOME ANALYSIS			
	65€	280	18.200€
	55€	280	15.400€
BIOSTATISTICAL / BIOINFORMATICAL DATA ANALYSIS AND PROCESSING			
	1.080€	once for all	1.080€
	1.080€	once for all	1.080€
TRAVEL COSTS	0,42€ / km	300km per visit, 6 visits intended	756€
FREEZER	5.500€	1	5.500€
DHL DELIVERY SERVICE	100€	1	100€
GENERAL PROJECT COSTS (COMPULSATORY 5% OF INTERIM TOTAL)	2.291€	**Total Interim**	**45.826€**
		Total	**48.117€**

Funding

The project received funding support from the initiative for preterm infants “Kleine Helden”.

4 RESULTS

Overall, we included 54 preterm very low birth weight infants, 18 from centers in Klagenfurt and Leoben, and 20 from the center in Graz. Two infants from the center in Graz had to be excluded due to incomplete sample collection. Overall, we collected 383 samples from 54 included infants (18 from each center). Metadata are given in table 12. Statistical assessment of the patient metadata showed that the recruited groups were statistically similar in regard to gestational age (GA), sex, birth weight (BW), APGAR scores and oxygen demand (p>0.049). However, statistically significant differences were found between the three groups in regard to lengths of hospital stay (Graz mean 72 days (min 25; max 126), Leoben 58 days (min 24; max 92), Klagenfurt 68.5 days (min 51; max 87), p 0.04*), with longest hospital stay in Graz and shortest in Leoben.

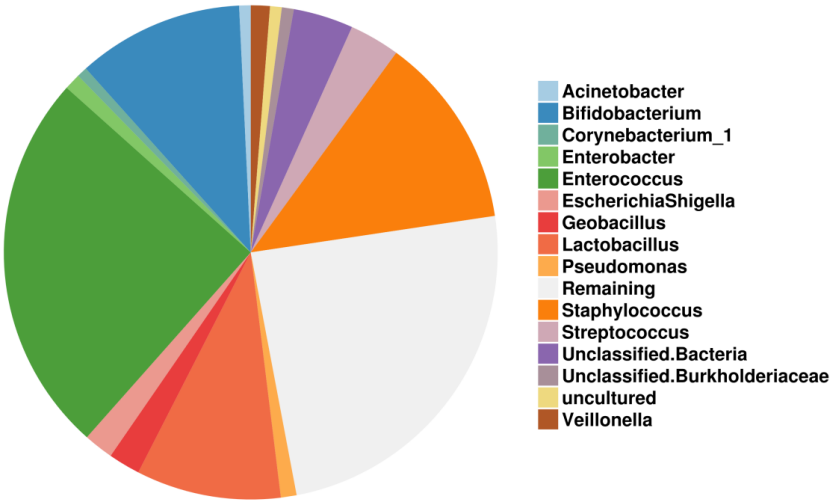
TABLE 12: DEMOGRAPHIC CHARACTERISTICS OF THE STUDY POPULATION PER CENTER.

	GRAZ	LEOBEN	KLAGENFURT	P-VALUE
INFANTS (N)	18	18	18	
M/F	10/8	12/6	12/6	0.61
BW (G)	1,030 (550; 1,495)	1,185 (640; 1495	972.5 (560; 1,485)	0.19
GA (W+D)	27+4 (24+3; 34+0)	28+6 (23+1; 33+0)	27+6 (23+4; 29+5)	0.43
APGAR 1	6 (4; 9)	8 (3; 9)	7 (1; 8)	0.12
APGAR 5	8 (5; 10)	9 (1; 10)	9 (5; 10)	0.27
APGAR 10	9 (6; 10)	9 (5; 10)	10 (7; 10)	0.59
VENTILATION (D)	37 (0; 96)	20 (0; 74)	33 (7; 72)	0.18
O2 DEMAND	17/18	14/18	18/18	0.10
C-SECTION	16/18	14/18	12/18	0.46
LOHS (D)	72 (25; 126)	58 (24; 92)	68.5 (51; 87)	**0.04***
MAT AGE (A)	32 (20; 41)	29 (26; 33)	29 (26; 32)	0.06
MULTIPLES	13/18	8/18	7/18	0.71
NAPH	7.3 (7.17; 7.39)	7.24 (7.0; 7.42)	7.26 (7.09; 7.38)	0.05
BM	18/18	0/18	0/18	1.69
EOS	5/18	7/18	6/18	0.72
LOS	2/18	1/18	0/18	0.38
I/PVH	5/18	4/18	3/18	0.78
RDS	14/18	9/18	12/18	0.32
ROP	6/18	2/18	4/18	0.33
NEC	1/18	0/18	1/18	0.61

Leg.: data are given as median (min; max) or n/total; a = years, d = days, w = weeks, m = male, f = female, n = number, g = gram, BW = birth weight, GA = gestational age, O2 = oxygen, LOHS = lengths of hospital stay, Mat age = maternal age, BM = breast milk, EOS = early onset sepsis, LOS = late onset sepsis, I/PVH = intraventricular/periventricular hemorrhage, RDS = respiratory distress syndrome, ROP = retinopathy of prematurity, NEC = necrotizing enterocolitis.

Overall, we processed 383 samples and 16 negative controls, i.e. over 30 million reads, for analysis. After removing contaminating reads (see “Microbiome analysis” in the materials and methods section), 2,029 different taxa were detected, with *Staphylococcus* and *Enterococcus,* as well as probiotic genera *Bifidobacterium* and *Lactobacillus* predominating (figure 6).

FIGURE 6: PIE CHART OF THE 15 MOST ABUNDANT MICROBIAL GENERA PRESENT IN PREMATURE INFANTS´ STOOL FROM POINT IN TIME (PIT) 1 TO PIT 7.



Leg: Figure excerpted in part from [213].

The amount of microbial 16S rRNA genes obtained using quantitative PCR (qPCR) differed in regard to study center and sampling point in time (PIT; figure 7; table 13). Meconium samples showed low bacterial load (PIT1, 1.56 (1; 3); mean: microbial cells/g stool sample, corrected by rrndb information). Throughout following time points bacterial load increased.

TABLE 13: QPCR DATA: COPY NUMBERS OF BACTERIAL 16S RRNA GENES PER SAMPLE.

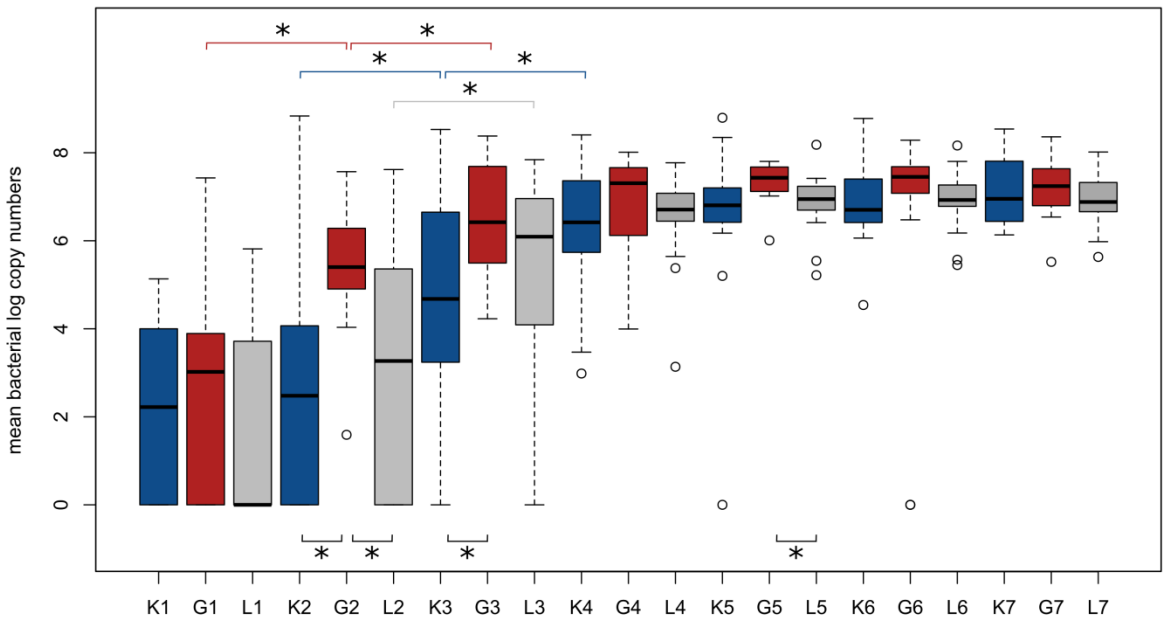
INFANT	PIT1	PIT2	PIT3	PIT4	PIT5	PIT6	PIT7
K14	1.83E+03	0.00E+00	3.84E+03	1.72E+06	1.59E+07	2.60E+06	3.81E+06
K13	0.00E+00	6.12E+02	3.05E+03	9.92E+03	5.87E+06	1.32E+07	1.65E+06
K15	0.00E+00	0.00E+00	1.37E+03	2.95E+03	1.49E+06	7.93E+06	4.44E+06
K16	1.36E+05	6.85E+08	1.43E+08	1.80E+08	6.23E+08	1.82E+08	5.26E+07
K11	0.00E+00	1.17E+04	0.00E+00	5.15E+03	9.35E+06	5.37E+06	2.76E+06
K17	1.82E+02	0.00E+00	4.48E+04	9.90E+05	0.00E+00	1.28E+06	5.92E+06
K18	4.53E+04	0.00E+00	1.74E+03	2.09E+07	1.94E+06	1.18E+07	1.05E+07
K12	2.37E+04	1.47E+03	2.02E+02	9.62E+02	1.59E+05	3.47E+04	2.27E+06
K10	0.00E+00	0.00E+00	1.30E+07	1.34E+07	1.13E+07	3.89E+06	5.24E+07
K09	1.00E+04	4.72E+02	5.08E+04	2.31E+07	1.13E+07	4.75E+06	8.37E+07
K08	1.52E+02	0.00E+00	5.54E+04	5.45E+05	5.27E+06	2.24E+06	1.63E+06
K07	2.96E+03	1.82E+03	4.46E+06	2.29E+06	2.63E+06	7.72E+07	6.44E+07
K06	1.43E+03	1.92E+02	1.02E+07	3.00E+06	2.37E+07	2.53E+07	7.91E+07
K05	0.00E+00	8.30E+06	0.00E+00	2.54E+08	2.23E+08	6.01E+08	3.46E+08
K03	0.00E+00	0.00E+00	2.87E+03	5.35E+07	6.96E+06	4.33E+07	4.28E+07
K04	3.42E+04	6.35E+04	9.96E+05	1.15E+06	5.15E+06	4.08E+06	7.31E+07
K02	0.00E+00	1.13E+07	3.38E+08	1.46E+08	9.40E+07	3.19E+06	7.66E+06
K01	2.16E+01	1.82E+02	5.06E+05	5.76E+06	5.10E+06	1.15E+06	1.36E+06
G04	2.68E+07	2.20E+06	2.64E+06	2.29E+07	5.45E+07	0.00E+00	3.46E+06
G02	5.70E+03	1.68E+06	3.91E+05	2.03E+07	1.25E+07	1.20E+07	6.27E+06
G01	0.00E+00	4.61E+05	4.67E+05	1.28E+07	1.18E+07	2.34E+07	5.33E+06
G03	1.49E+03	1.08E+04	2.04E+05	1.75E+05	1.32E+07	6.13E+07	2.13E+07
G05	7.90E+02	2.84E+07	1.84E+08	5.26E+07	3.79E+07	8.40E+07	2.30E+08
G12	5.40E+03	2.38E+05	2.40E+08	2.72E+07	5.28E+07	1.33E+08	1.20E+08
G11	0.00E+00	2.46E+07	8.12E+07	3.99E+07	2.84E+07	4.61E+07	4.33E+07
G14	0.00E+00	2.35E+05	1.77E+08	8.36E+07	1.50E+07	3.42E+07	3.65E+07
G15	1.05E+03	1.58E+05	4.05E+05	4.28E+05	3.24E+07	1.55E+07	2.31E+07
G17	7.61E+03	3.51E+04	5.74E+07	1.03E+08	4.74E+07	1.93E+08	5.75E+07
G13	1.93E+04	3.71E+07	4.17E+07	9.58E+07	6.36E+07	1.45E+07	6.64E+07
G09	8.02E+03	1.50E+05	2.19E+05	2.18E+05	2.39E+07	4.05E+07	1.76E+07
G10	1.47E+05	2.29E+07	3.57E+07	5.71E+07	2.57E+07	4.82E+07	1.63E+07
G08	0.00E+00	2.27E+04	1.69E+04	2.31E+07	4.00E+07	0.00E+00	1.12E+07
G20	0.00E+00	4.30E+04	2.49E+05	9.89E+03	1.04E+07	4.17E+07	3.31E+05
G07	0.00E+00	2.68E+05	1.46E+06	4.08E+06	1.44E+07	2.99E+06	1.73E+07
G18	0.00E+00	3.87E+01	1.05E+05	5.09E+04	1.02E+06	5.26E+06	4.79E+06
G06	0.00E+00	2.52E+05	1.33E+07	7.73E+06	6.09E+07	1.91E+07	9.08E+06
L01	0.00E+00	7.27E+02	2.64E+05	3.69E+06	8.85E+06	6.35E+07	1.04E+08
L04	0.00E+00	1.86E+04	3.78E+02	8.73E+05	5.20E+06	6.53E+06	7.62E+06
L05	1.13E+03	7.08E+06	1.77E+07	1.02E+07	1.53E+07	1.30E+07	6.64E+06
L06	6.54E+05	7.81E+06	4.13E+06	2.79E+06	6.25E+06	6.07E+06	8.46E+06
L07	0.00E+00	0.00E+00	6.71E+06	9.62E+06	5.71E+06	1.50E+06	1.38E+07
L08	1.04E+03	0.00E+00	4.57E+02	1.37E+03	3.23E+06	7.54E+06	3.65E+06
L09	0.00E+00	8.87E+02	2.74E+02	4.40E+05	1.65E+05	2.81E+05	4.57E+06
L10	0.00E+00	0.00E+00	5.39E+05	2.39E+05	2.59E+06	3.71E+05	4.29E+05
L11	0.00E+00	3.88E+03	0.00E+00	7.66E+06	3.50E+05	1.72E+07	9.54E+05

L12	5.21E+03	0.00E+00	6.39E+04	3.39E+06	9.91E+06	9.49E+06	3.08E+06
L13	0.00E+00	0.00E+00	6.70E+06	3.83E+06	1.85E+07	1.89E+07	3.93E+07
L14	3.74E+05	1.58E+06	9.09E+06	1.20E+07	8.86E+06	6.85E+06	3.72E+07
L15	7.30E+04	8.12E+02	6.96E+07	3.14E+06	1.15E+07	1.85E+07	2.11E+07
L16	0.00E+00	2.24E+04	2.84E+06	1.74E+07	4.97E+06	3.31E+06	6.25E+06
L17	0.00E+00	2.29E+05	1.97E+07	5.91E+07	1.52E+08	1.46E+08	4.64E+07
L18	1.39E+04	4.15E+07	3.42E+07	1.56E+07	2.09E+07	5.26E+07	1.14E+07

In Graz, statistically significant increases in bacterial load were observed in samples from time points one, two and three (indicated by asterisks in figure 7), whereas in samples from Klagenfurt and Leoben a significant increase in bacterial load occured at later time points (PIT 2 and PIT 3).

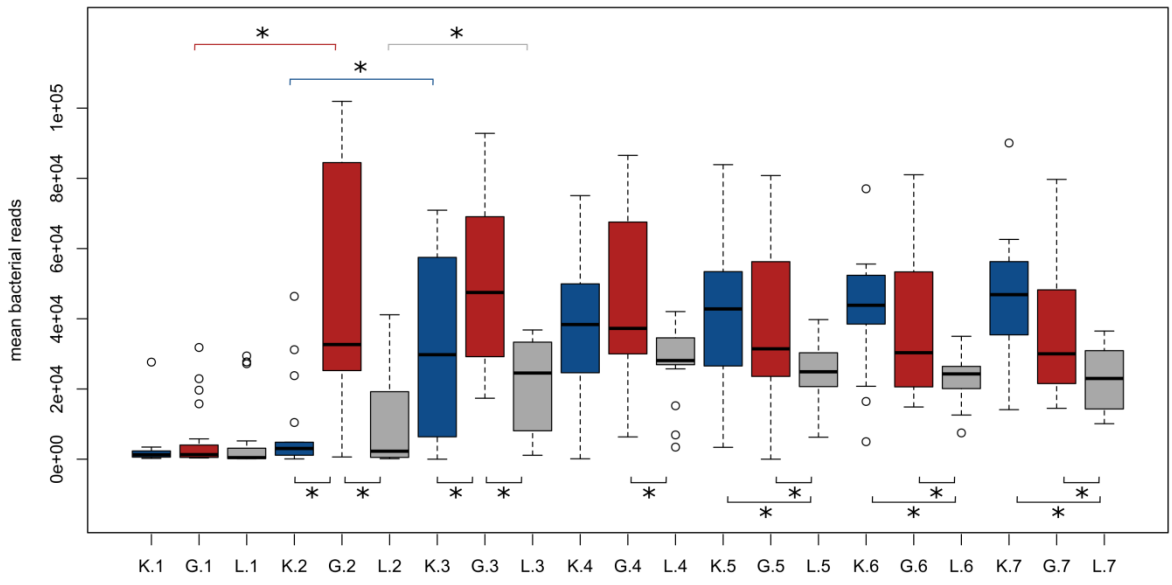
After PIT 4 (8.04 (7; 15)), the bacterial load stabilized at around 3e+07 copies for samples from all centers.

FIGURE 7: 16S RRNA GENE COPIES (MEAN, SD AND OVERALL VARIANCE) AS RECEIVED FROM THE SAMPLES FROM DIFFERENT CENTERS. K, G, L REFERS TO KLAGENFURT, GRAZ, AND LEOBEN, RESPECTIVELY. THE LAST DIGIT INDICATES THE TIME POINT. K.1 THUS REFERS TO SAMPLE NUMBER 1 FROM THE KLAGENFURT CENTER.



When looking at amplicon read numbers revealed by NGS, we found a similar pattern (figure 8). However, samples from Leoben revealed a significantly lower number of reads in samples after PIT 5 ($p < 0.025$), compared to samples from the centers Klagenfurt and Graz. In addition, at the center in Leoben, (no administration of probiotics) mean read numbers were lower comprising 19.338 sequences per sample, when compared to the centers Graz (n=37.800) and Klagenfurt (n=29.401).

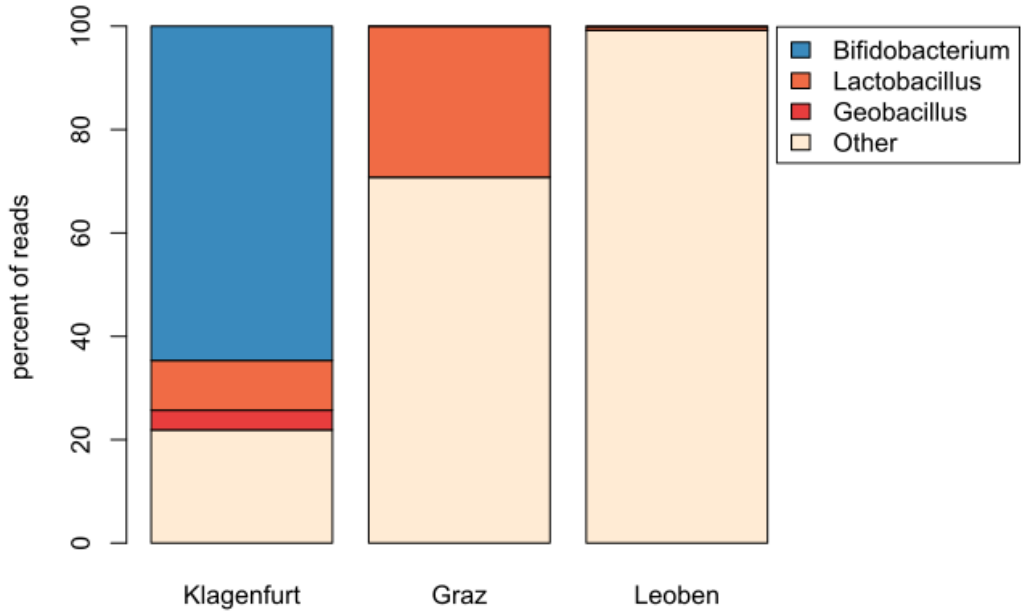
FIGURE 8: ABSOLUTE READ COUNTS (MEAN, SD AND OVERALL VARIANCE) AS RECEIVED FROM THE SAMPLES FROM DIFFERENT CENTERS. K, G, L REFERS TO KLAGENFURT, GRAZ, AND LEOBEN, RESPECTIVELY. THE LAST DIGIT INDICATES THE TIME POINT. K.1 THUS REFERS TO SAMPLE NUMBER 1 FROM THE KLAGENFURT COHORT.



4.1 MICROBIAL SIGNATURES ARE DOMINATED BY ADMINISTERED PROBIOTIC GENERA AND GEOBACILLUS STEAROTHERMOPHILUS

High absolute and relative abundances of probiotic genera were found associated with intake of probiotics in samples from centers Klagenfurt and Graz (figure 9). About 75% of all reads from Klagenfurt samples comprised either *Bifidobacterium* (64.7%) or *Lactobacillus* (9.6%) signatures. Samples from the center in Graz revealed the genus *Lactobacillus* in 29.2% of obtained reads. Notably, when *Lactobacillus* and *Bifidobacterium* were not administered as probiotics, their contribution to the microbiome was low (Leoben: 0.677% *Lactobacillus* and 0.088% *Bifidobacterium*; Graz: 0.005% *Bifidobacterium*). ASVs affiliated to the *Geobacillus* genus represented 3.81% of all bacterial reads in samples from the center Klagenfurt, but were not seen in samples from Graz or Leoben.

FIGURE 9: RELATIVE ABUNDANCE OF SIGNATURES FROM *BIFIDOBACTERIUM, LACTOBACILLUS, GEOBACILLUS* AND OTHER GENERA IN SAMPLES FROM THE DIFFERENT CENTERS.



Leg: Figure excerpted in part from [213].

The longitudinal proportion of *Lactobacillus* signatures is depicted in figure 10a. In samples from Graz, the highest proportion of *Lactobacillus* reads was observed already in time points two and three (up to 100%), but was found to vary strongly

between infants. In infant stool samples from later time points, total read numbers as well as relative abundances of *Lactobacillus* signatures (table 14) per sample decreased significantly (PIT 4 to PIT 5, p 0.030 (absolute abundances), p 0.014 (relative abundances, table 15). In samples from Klagenfurt, *Lactobacillus* reads reached the highest percentages just at PIT 5 and PIT 6, however, with significantly lower read numbers compared to samples from Graz. As indicated already by figure 9, hardly any *Lactobacillus* signatures were detected in samples derived from Leoben, except for one infant (L-15), which showed a high read proportion of this taxon (95.25 and 93.3%) in the first two samples.

The proportion of *Bifidobacterium* and *Geobacillus* signatures, which were almost exclusively present in samples from Klagenfurt infants, are shown separately in figure 10b und 10c. Abundance of *Bifidobacterium* signatures was higher compared *Lactobacillus*, independent from PIT, even though concentration of probiotic strains was identical (table 10). Bifidobacterial signatures were increasing over time (table 16), with a significant increase between PIT 2 and PIT 3 (p 0.024) (figure 10b, table 15). Some samples shows *Bifidobacteria* comprising up to 97% of reads, displaying their extremely high abundance.

In samples from Klagenfurt, *Geobacillus* signatures showed a slight increase over time, however overall they remained lower, comprising less than 4%, compared to *Lactobacillus* or *Bifidobacterium* signatures.

FIGURE 10: MEAN RELATIVE ABUNDANCE OF *LACTOBACILLUS, BIFIDOBACTERIUM* AND *GEOBACILLUS* SIGNATURES. A) RELATIVE ABUNDANCE OF *LACTOBACILLUS* IN SAMPLES FROM ALL CENTERS (K: KLAGENFURT, G: GRAZ, L: LEOBEN) ACCORDING TO TIME POINT (X-AXIS). B) RELATIVE ABUNDANCE OF *BIFIDOBACTERIUM* C) AND *GEOBACILLUS* SIGNATURES IN KLAGENFURT SAMPLES. SIGNIFICANT DIFFERENCES ARE INDICATED BY STARS.

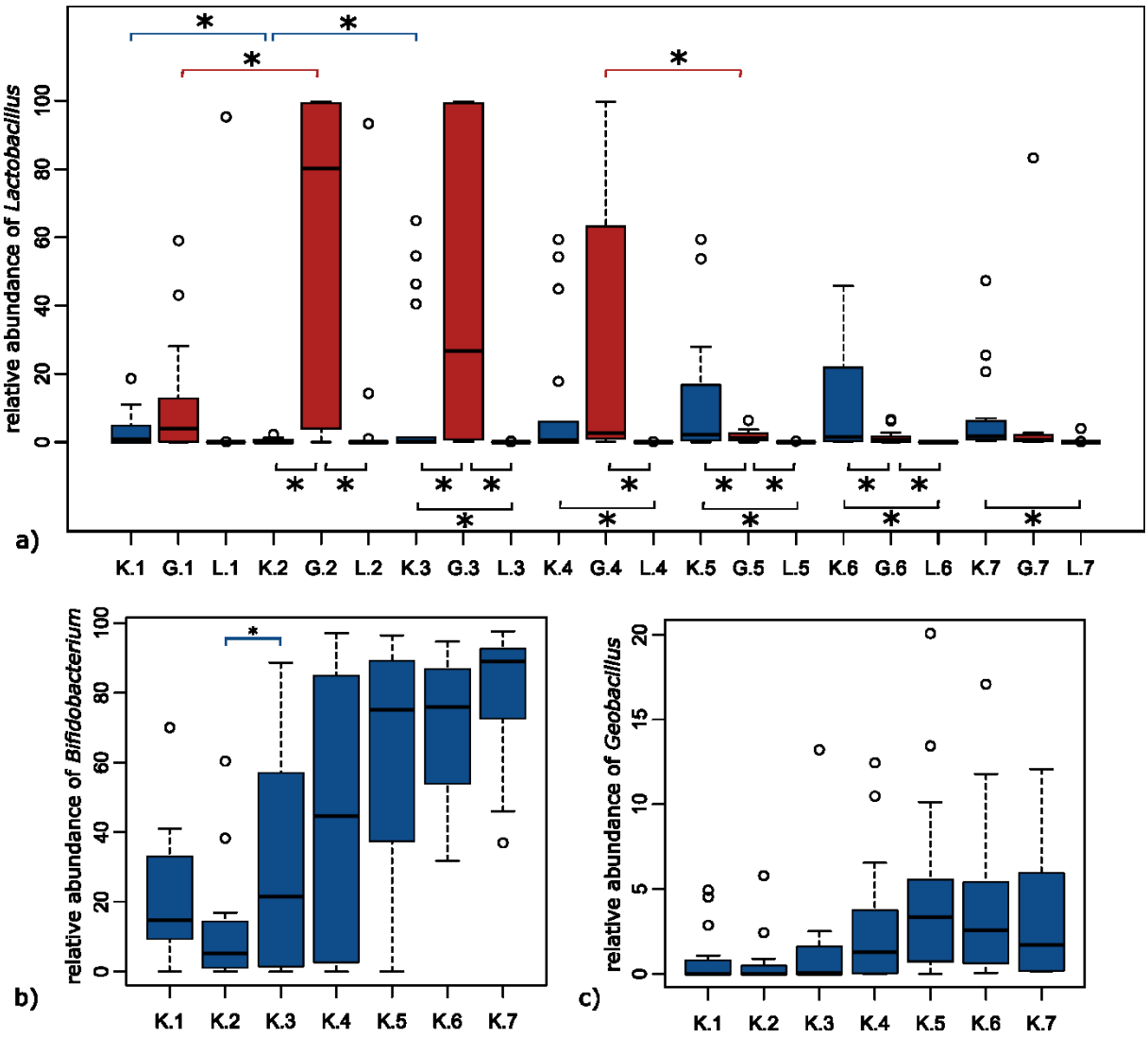


TABLE 14: TOTAL READ NUMBERS OF LACTOBACILLUS IN GRAZ AND KLAGENFURT PER PIT, NON-RESPONDERS ARE HIGHLIGHTED WITH AN ASTERISK (*).

	PIT1		PIT2		PIT3		PIT4		PIT5		PIT6		PIT7		MAX		MEAN	
	total	**%**	**total**	**%**	**total**	**%**	**total**	**%**	**total**	**%**	**total**	**%**	**total**	**%**	**total**	**%**	**total**	**%**
G04	50	2.48	82,897	99.22	16,847	26.68	1,293	2.00	1,417	2.32	251	0.31	82	0.20	82,897	99.22	14,691	19.03
G02	447	7.73	89,252	99.64	92,532	99.71	15,111	28.08	957	1.83	0	0.00	286	0.59	92,532	99.71	28,369	33.94
G01	45	3.45	101,515	99.60	49,208	55.36	3,630	5.19	605	0.75	3,597	6.24	469	0.59	101,515	99.60	22,724	24.45
G03	58	4.50	60	0.84	67,252	99.63	81,433	99.76	2,027	3.60	856	1.26	437	0.74	81,433	99.76	21,732	30.05
G05*	806	43.01	2,161	3.02	62	0.09	597	0.74	219	0.63	29	0.09	220	0.46	2,161	43.01	585	6.86
G12	157	0.80	53,615	56.08	239	0.30	112	0.17	464	0.65	979	1.35	187	0.30	53,615	56.08	7,965	8.52
G11	1,351	59.00	2,504	3.25	367	0.48	1,719	2.54	1,322	2.47	261	0.49	1,489	2.54	2,504	59.00	1,288	10.11
G14	17	3.94	85,173	99.74	158	0.22	2,469	4.04	1,221	1.96	275	0.66	830	2.01	85,173	99.74	12,878	16.08
G15	224	16.62	98,992	99.58	49,446	99.70	85,601	98.92	376	1.29	337	2.18	53	0.23	98,992	99.70	33,576	45.50
G17*	5	0.39	403	63.27	106	0.35	693	2.62	816	3.46	54	0.25	206	1.42	816	63.27	326	10.25
G13*	0	0.00	1,107	4.84	925	3.87	355	0.99	0	0.00	1,094	6.64	422	1.51	1,107	6.64	558	2.55
G09	114	23.55	40,728	99.29	17,417	99.41	36,919	99.10	187	0.55	772	2.80	321	1.56	40,728	99.41	13,780	46.61
G10*	0	0.00	384	1.60	322	1.08	55	0.53	392	2.51	237	1.59	457	2.11	457	2.51	264	1.35
G08	0	0.00	26,630	94.13	20,997	73.32	107	1.68	0	0.00	54	0.21	15	0.07	26,630	94.13	6,829	24.20
G20	19	3.97	29,140	98.93	29,095	97.57	19,402	98.01	150	0.57	229	0.67	26,779	83.23	29,140	98.93	14,973	54.71
G07	44	8.89	32,184	98.55	27,028	99.39	1,544	4.57	188	1.07	56	0.25	65	0.32	32,184	99.39	8,730	30.43
G18	0	0.00	0	0.00	47,009	99.00	35,161	99.40	1,338	6.37	166	0.97	85	0.42	47,009	99.40	11,966	29.45
G06*	144	28.07	2,192	7.54	402	1.35	376	1.12	100	0.35	94	0.46	702	2.68	2,192	28.07	573	5.94
K14	17	0.61	37	0.99	17	0.08	20,156	44.88	2,967	5.50	3,588	7.81	184	0.29	20,156	44.88	3,852.29	8.60
K13	26	1.03	0	0.00	42	0.16	12	0.02	7,396	27.85	130	0.23	437	0.92	7,396	27.85	1,149.00	4.32
K15	69	4.79	0	0.00	0	0.00	1,122	4.11	24,311	59.37	39	0.19	428	0.82	24,311	59.37	3,709.86	9.90
K16*	83	0.30	26	0.62	22	0.88	0	0.00	698	16.59	31	0.62	161	1.14	698	16.59	145.86	2.88
K11*	96	18.60	31	0.10	0	0.00	0	0.00	137	0.30	4,660	8.64	571	3.35	4,660	18.60	785.00	4.43
K17*	0	0.00	0	0.00	81	0.11	383	0.80	0	0.00	5,205	12.97	351	0.58	5,205	12.97	860.00	2.07
K18	21	3.58	0	0.00	125	0.63	67	0.19	27	0.06	12,211	29.25	1603	5.20	12,211	29.25	2,007.71	5.56
K12	17	0.49	51	0.49	0	0.00	418	5.92	588	0.70	593	1.13	19251	47.30	19,251	47.30	2,988.29	8.01
K10	66	4.86	4	1.22	13,182	40.45	1,071	4.35	2,049	5.50	17,793	44.47	2813	6.08	17,793	44.47	5,282.57	15.27
K09	28	4.43	45	0.19	31,367	54.57	25,411	54.28	364	0.64	274	0.36	455	1.28	31,367	54.57	8,277.71	16.54
K08	0	0.00	65	2.20	104	0.16	236	0.31	15,486	23.94	265	0.48	1592	1.77	15,486	23.94	2,535.43	4.12
K07	0	0.00	27	0.86	15,586	46.34	9,007	17.80	1,407	3.39	21,305	45.77	2230	3.96	21,305	46.34	7,080.29	16.87
K06	0	0.00	0	0.00	21,062	64.89	1,123	3.10	102	0.23	81	0.22	2547	7.01	21,062	64.89	3,559.29	10.78
K05	0	0.00	0	0.00	0	0.00	0	0.00	1,814	53.65	5,836	35.49	4940	25.46	5,836	53.65	1,798.57	16.37
K03	66	11.09	8	0.48	0	0.00	22,813	59.37	294	0.55	8,424	21.71	453	0.79	22,813	59.37	4,579.71	13.43
K04	12	5.22	0	0.00	266	0.49	78	0.14	272	0.66	93	0.24	8812	20.63	8,812	20.63	1,361.86	3.91
K02*	0	0.00	0	0.00	0	0.00	0	0.00	238	0.93	855	1.85	428	0.83	855	1.85	217.29	0.52
K01*	23	1.09	0	0.00	782	1.31	91	0.15	2,847	6.13	344	0.68	852	1.66	2,847	6.13	705.57	1.57

TABLE 15: P-VALUES OF ABSOLUTE BACTERIAL READ COUNTS AND RELATIVE ABUNDANCES OF LACTOBACILLUS, BIFIDOBACTERIUM AND GEOBACILLUS READS COMPARED BETWEEN CENTERS AND CONSECUTIVE TIME POINTS.

	ABSOLUTE BACTERIAL READ COUNTS	RELATIVE ABUNDANCE OF *LACTOBACILLUS* READS	RELATIVE ABUNDANCE OF *BIFIDOBACTERIUM* READS	RELATIVE ABUNDANCE OF *GEOBACILLUS* READS
G.1, G.2	**<0.001**	**<0.001**	**-**	**-**
G.2, G.3	0.950	0.393	-	-
G.3, G.4	0.788	0.264	-	-
G.4, G.5	0.288	**0.014**	-	-
G.5, G.6	0.846	0.715	-	-
G.6, G.7	0.975	0.380	-	-
K.1, K.2	0.137	**0.031**	0.085	0.665
K.2, K.3	**0.002**	**0.048**	**0.024**	0.443
K.3, K.4	0.551	0.909	0.276	0.174
K.4, K.5	0.598	0.927	0.126	0.232
K.5, K.6	0.706	0.954	0.507	0.766
K.6, K.7	0.632	0.342	0.081	0.650
L.1, L.2	0.196	0.921	-	-
L.2, L.3	**0.036**	0.262	**-**	**-**
L.3, L.4	0.075	0.455	-	-
L.4, L.5	0.435	0.621	-	-
L.5, L.6	0.506	0.331	-	-
L.6, L.7	0.657	0.305	-	-
K.1, G.1	0.260	0.064	-	-
K.1, L.1	0.377	0.692	-	-
G.1, L.1	0.902	0.399	-	-
K.2, G.2	**<0.001**	**<0.001**	**-**	**-**
K.2, L.2	0.533	0.292	-	-
G.2, L.2	**<0.001**	**<0.001**	**-**	**-**
K.3, G.3	**0.041**	**0.008**	**-**	**-**
K.3, L.3	0.115	**0.041**	-	-
G.3, L.3	**<0.001**	**<0.001**	**-**	**-**
K.4, G.4	0.164	0.112	-	-
K.4, L.4	0.134	**0.034**	-	-
G.4, L.4	**0.005**	**0.009**	**-**	**-**
K.5, G.5	0.856	**0.039**	-	-
K.5, L.5	**0.012**	**0.018**	**-**	**-**
G.5, L.5	**0.025**	**<0.001**	**-**	**-**
K.6, G.6	0.422	**0.016**	-	-
K.6, L.6	**<0.001**	**0.007**	**-**	**-**
G.6, L.6	**0.017**	**0.006**	**-**	**-**
K.7, G.7	0.197	0.775	-	-
K.7, L.7	**<0.001**	**0.028**	**-**	**-**
G.7, L.7	**0.006**	0.256	**-**	**-**

Leg: Comparion of the course of patients within one center (e.g. G.1-G.7) applying Friedmann´s Test followed by Wilcoxon with Bonferroni correction. Pairwise comparison of centers (e.g. K.1 vs G.1) applying a Bonferroni correction.

TABLE 16: TOTAL READ NUMBERS OF BIFIDOBACTERIUM IN KLAGENFURT.

	PIT1	PIT2	PIT3	PIT4	PIT5	PIT6	PIT7	MAX	MEAN
K01	344	285	45,06	60,30	37,81	33,27	37,11	60,30	30,60
K02	159	31	34	412	22,76	42,65	46,38	46,38	16,06
K03	189	207	27	14,85	41,94	30,17	55,72	55,72	20,44
K04	91	35	37,58	24,61	25,78	12,92	32,93	37,58	19,13
K05	115	31	0	11	985	10,05	14,31	14,31	3,645
K06	779	112	11,08	30,74	19,26	32,91	26,38	32,91	17,32
K07	43	18	17,35	34,70	34,36	25,10	52,09	52,09	23,38
K08	76	154	55,14	72,12	46,37	45,48	81,57	81,57	42,99
K09	111	152	24,26	20,68	51,89	72,93	22,98	72,93	27,57
K10	133	198	18,57	22,01	29,97	15,13	41,00	41,00	18,14
K11	150	171	298	128	43,28	46,80	15,46	46,80	15,18
K12	50	279	288	195	80,96	42,04	18,72	80,96	20,36
K13	312	180	2,036	29	18,00	51,35	42,40	51,35	16,33
K14	259	631	415	24,01	49,50	36,39	59,76	59,76	24,42
K15	591	32	841	264	15,05	14,90	49,24	49,24	11,56
K16	19	284	222	5,397	1,578	1,583	12,32	12,32	3,058
K17	70	110	62,82	46,46	0	29,70	56,79	62,82	27,99
K18	411	239	307	36	292	15,89	11,39	15,89	4,083

Leg.: K01 – K18 = patients 01 to 18 from the Klagenfurt cohort.

4.2 THE TIME POINT OF PROBIOTICS ONSET DIFFERS BASED ON PROBIOTICS TYPE AND INDIVIDUAL PARAMETERS

The relative abundances of both probiotic genera (*Lactobacillus* and *Bifidobacterium*) displayed great variability with respect to individual infants and time point (figure 10). In detail, we observed that samples from certain infants (e.g. infants G05, G06, G10, G13, G17 from Graz, or infants K01, K02, K11, K16, K17 from Klagenfurt; table 14, highlighted with an asterisk (*)) showed significantly lower abundances of *Lactobacillus* signatures throughout the study period.

Based on abundances, we grouped all infants into high-read counts and low-read counts of *Lactobacillus* species with a cut-off of 1.000 absolute read counts (table 17). In both centers, groups differed significantly regarding maximum und mean read

numbers but not regarding minimum read numbers. Notably, such a differing response could not be observed for *Bifidobacterium*.

TABLE 17: MINIMUM, MAXIMUM AND MEAN ABSOLUTE ABUNDANCE OF LACTOBACILLUS SIGNATURES ARE GIVEN FOR GRAZ AND KLAGENFURT SAMPLES, GROUPED INTO HIGH- AND LOW-READ COUNTS.

	GRAZ			KLAGENFURT		
READ	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**
HIGH	1.127.5	29.379.75	12.794.55	1.149	8.278	3.706.39
LOW	232.5	512.38	404.85	146	860	542.8
P-VALUE	0.28	**<0.001**	**<0.001**	0.14	**<0.001**	0.0001

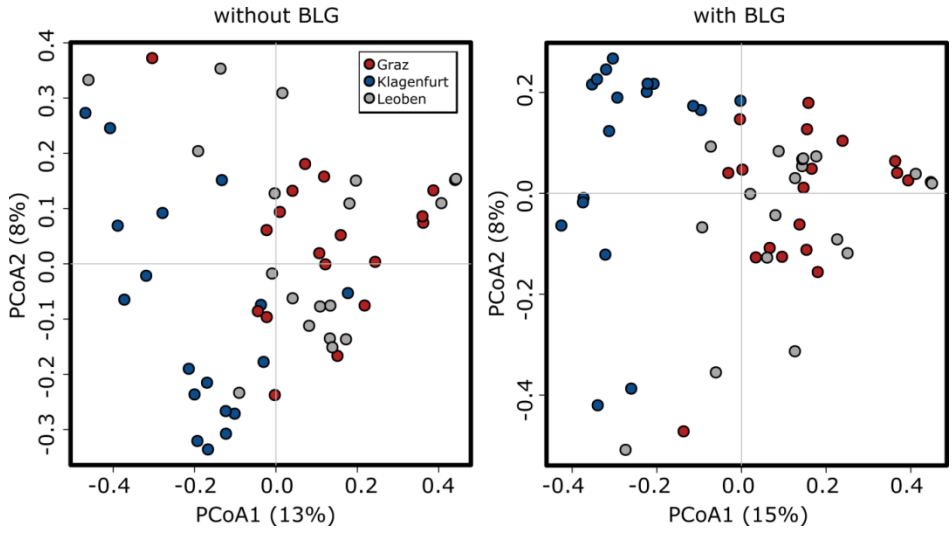
In a next step, we analyzed the differences of preventive therapy with two probiotic strains at the center Klagenfurt (*Lactobacillus* and *Bifidobacterium*) *versus* one probiotic strain at the center Graz (*Lactobacillus*). Overall, the time point of response onset to probiotic therapy was significantly later in infants from Klagenfurt (figure 10). Although in Klagenfurt the concentration of introduced probiotic bacteria was four times higher (in total $4x10^9$ CFU per day; table 10) and no antibiotics were administered, quantitative data (figure 7) do not indicate a higher bacterial load in samples from the center Klagenfurt when compared to the centers Graz or Leoben.

4.3 THE MICROBIOMES OF INFANTS` MECONIUM FROM THE THREE CENTERS DIFFER IN DIVERSITY BUT NOT IN COMPOSITION

We further investigated the differences of fecal microbiomes of infants` stool samples taken at the first day of life. Although the overall amounts of reads retrieved from all meconium samples at PIT 1 (1.56 (1; 3)) were not significantly different, Shannon Index analysis revealed significant differences in microbiome diversity (p 0.012), with samples from Klagenfurt showing the highest microbial diversity (see below).

PCoA analyses (figure 11) indicated a strong and significant (p≤0.03) grouping according to the specific center, with Klagenfurt again clustering strongly apart from the other two centers (p<0.001, table 18). To circumvent potential bias caused by highly abundant probiotic genera (*Bifidobacterium*, *Lactobacillus* and *Geobacillus)*, which could have masked the effects of taxa present with lower abundance, in a second dataset we excluded all ASVs affiliated to probiotic genera ("without BLG", table 19). In addition, this dataset revealed significant differences of the infants' meconium microbiome composition at PIT 1 between Klagenfurt and the other two centers (p<0.001). However, differences between the centers Leoben and Graz disappeared (p 0.272).

FIGURE 11: PCOA PLOTS SHOWING THE BETA DIVERSITIES OF MICROBIOME SAMPLES FROM DIFFERENT CENTERS. A) WITHOUT BLG B) WITH BLG INCLUDED.



Explanation: Principal Coordinates Analysis (PCoA) is a method to explore and to visualize similarities or dissimilarities of data. PCoA tries to find the main axes through a matrix and allows for individual and/or group differences to be visualized. The figure shows beta diversity (i.e. variation of microbial communities between samples) of stool samples. On the left side center specific diversity of stool samples is visualized with samples from Graz in red, samples from Klagenfurt in blue and samples from Leoben in grey. With BLG (*Bifidobacterium, Lactobacillus, Geobacillus*) included, microbial diversity of samples tends to cluster for each center (left plot). Samples from Klagenfurt distinctly cluster apart from the other two centers. Without BLG included Klagenfurt samples again cluster apart from those from Leoben and Graz (right plot). However, the cluster-formation appears differently shaped.

TABLE 18: R^2 AND P-VALUES FOR CHANGES IN DIVERSITY WEIGHTED BY BRAY-CURTIS BETWEEN THE CENTERS WITH AND WITHOUT BLG (BIFIDOBACTERIUM, LACTOBACILLUS, GEOBACILLUS) AT PIT 1. R^2 VALUES GIVE EXPLAINED VARIATION / TOTAL VARIATION.

CENTERS	WITH BLG		WITHOUT BLG	
	R^2	p-value	R^2	p-value
ALL 3	0.131	<0.001	0.0868	<0.001
K-G	0.14	<0.001	0.0918	<0.001
K-L	0.117	<0.001	0.0717	<0.001
G-L	0.0423	0.0243	0.0309	0.268

K-G = center Klagenfurt vs center Graz, K-L = center Klagenfurt vs center Leoben, G-L = center Graz vs center Leoben; BLG = Bifidobacterium, Lactobacillus, Geobacillus.

4.4 SPECIFIC REGIMENS OF NEC PROPHYLAXIS AFFECT THE DEVELOPMENT OF THE MICROBIOME WITHIN THE FIRST TWO TO THREE WEEKS OF LIFE

We calculated alpha diversities of the samples in order to compare diversity, richness and evenness with respect to sample number and center, with and without BLG (figure 12, table 20). Explanations on alpha diversity, richness and evenness are provided in the material and methods part. The highest alpha diversities were observed for samples from Klagenfurt, and significant differences amongst centers were observed throughout PIT 1 to PIT 5 (figure 13). As given in figures 12 and 13, richness and evenness decreased at all centers over time. This effect was even more significant with *Bifidobacterium, Lactobacillus and Geobacillus* (BLG) included, compared to sample analysis without BLG.

FIGURE 12: SHANNON INDICES, RICHNESS AND EVENNESS OF SAMPLES PER PIT AND CENTER, WITH AND WITHOUT BIFIDOBACTERIUM, LACTOBACILLUS, GEOBACILLUS (BLG).

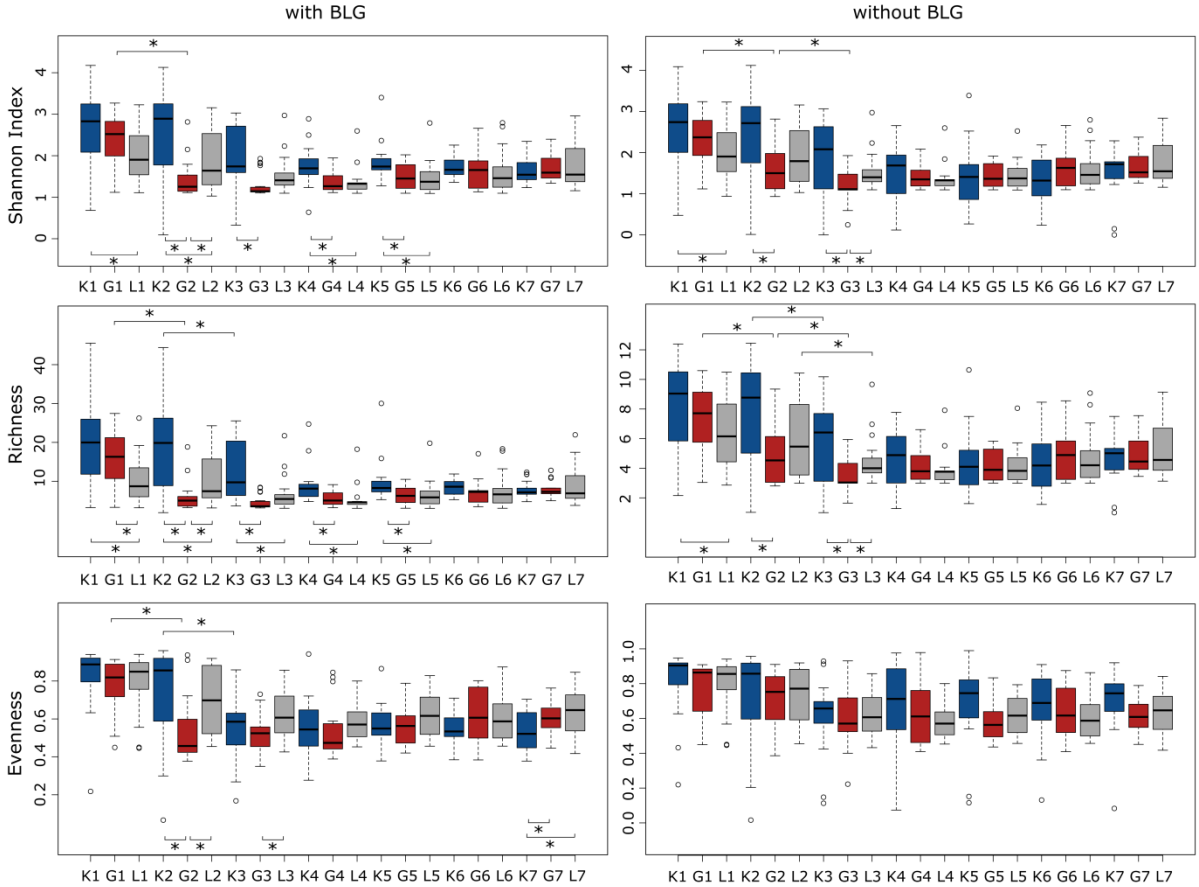
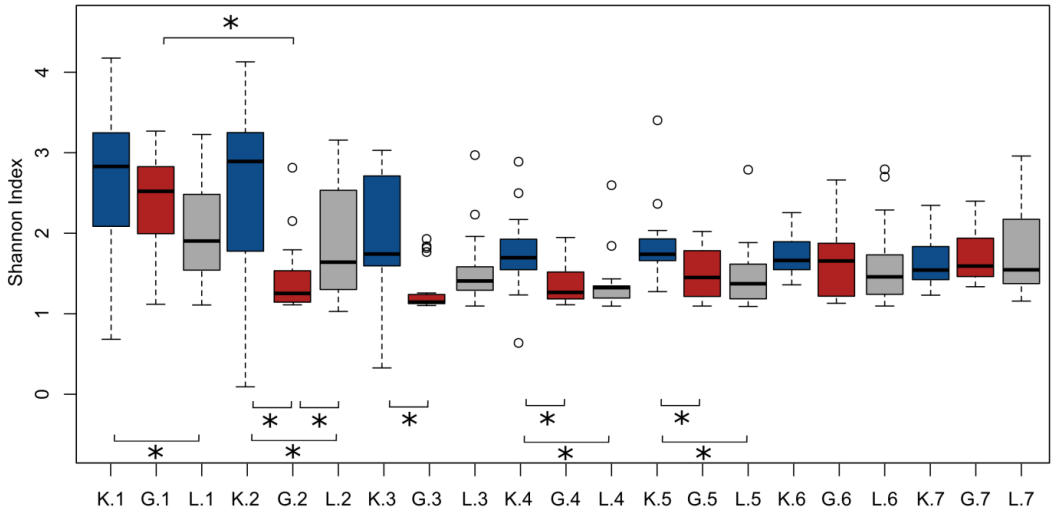


FIGURE 13: SHANNON INDICES FOR CENTERS GRAZ (RED), KLAGENFURT (BLUE) AND LEOBEN (GREY) AT PIT 1 TO PIT 7 (DATASET WITH BLG INCLUDED). SIGNIFICANT DIFFERENCES ARE INDICATED BY STARS.



Shannon index resembles a diversity index, which is a mathematical measure of species diversity in stool samples. Diversity indices provide more information about stool microbial composition than species richness (i.e., the number of species present). Furthermore, they take the relative abundances of different species into account. Shannon index provides important information about rarity and commonness of species.

TABLE 19: P-VALUES FOR SHANNON INDEX, RICHNESS AND EVENNESS WITH AND WITHOUT BLG, COMPARED BETWEEN CENTERS AT EACH PIT AND WITHIN ONE CENTER COMPARING CONSECUTIVE PITS.

	SHANNON		**RICHNESS**		**EVENNESS**	
	withBLG	noBLG	withBLG	noBLG	withBLG	noBLG
G1, G2	**<0.001**	**0.001**	**<0.001**	**0.001**	**<0.001**	0.221
G2, G3	0.290	0.016	0.177	0.015	0.661	0.060
G3, G4	0.345	0.080	0.088	0.103	0.705	0.721
G4, G5	0.219	0.953	0.225	0.949	0.524	0.395
G5, G6	0.278	0.224	0.415	0.215	0.270	0.305

G6, G7	0.643	0.658	0.391	0.738	0.820	0.707
K1, K2	0.636	0.802	0.879	0.726	0.226	0.312
K2, K3	0.061	0.074	**0.026**	**0.045**	**0.018**	0.162
K3, K4	0.380	0.299	0.131	0.240	0.928	0.483
K4, K5	0.564	0.734	0.626	0.657	0.692	0.877
K5, K6	0.373	0.976	0.335	0.854	0.502	0.891
K6, K7	0.448	0.668	0.490	0.682	0.609	0.611
L1, L2	0.605	0.793	0.946	0.731	0.115	0.163
L2, L3	0.114	0.074	0.082	**0.049**	0.157	0.065
L3, L4	0.220	0.223	0.289	0.210	0.262	0.247
L4, L5	0.405	0.444	0.372	0.431	0.273	0.316
L5, L6	0.302	0.251	0.353	0.251	0.595	0.571
L6, L7	0.491	0.491	0.450	0.517	0.350	0.338
K1, G1	0.182	0.390	0.106	0.331	0.367	0.502
K1, L1	**0.006**	**0.045**	**0.002**	**0.039**	0.542	0.849
G1, L1	0.057	0.113	**0.021**	0.168	0.771	0.578
K2, G2	**<0.001**	**0.006**	**<0.001**	**0.003**	**0.010**	0.780
K2, L2	**0.031**	0.089	**0.005**	0.075	0.656	0.956
G2, L2	**0.031**	0.126	**0.023**	0.118	**0.006**	0.752
K3, G3	**0.006**	**0.042**	**0.001**	**0.011**	0.520	0.993
K3, L3	0.090	0.365	**0.017**	0.177	0.144	0.689
G3, L3	0.061	**0.029**	0.065	**0.049**	**0.007**	0.589
K4, G4	**0.013**	0.787	**0.013**	0.406	0.656	0.591
K4, L4	**0.017**	0.605	**0.018**	0.206	0.454	0.253
G4, L4	0.934	0.644	0.801	0.499	0.186	0.418

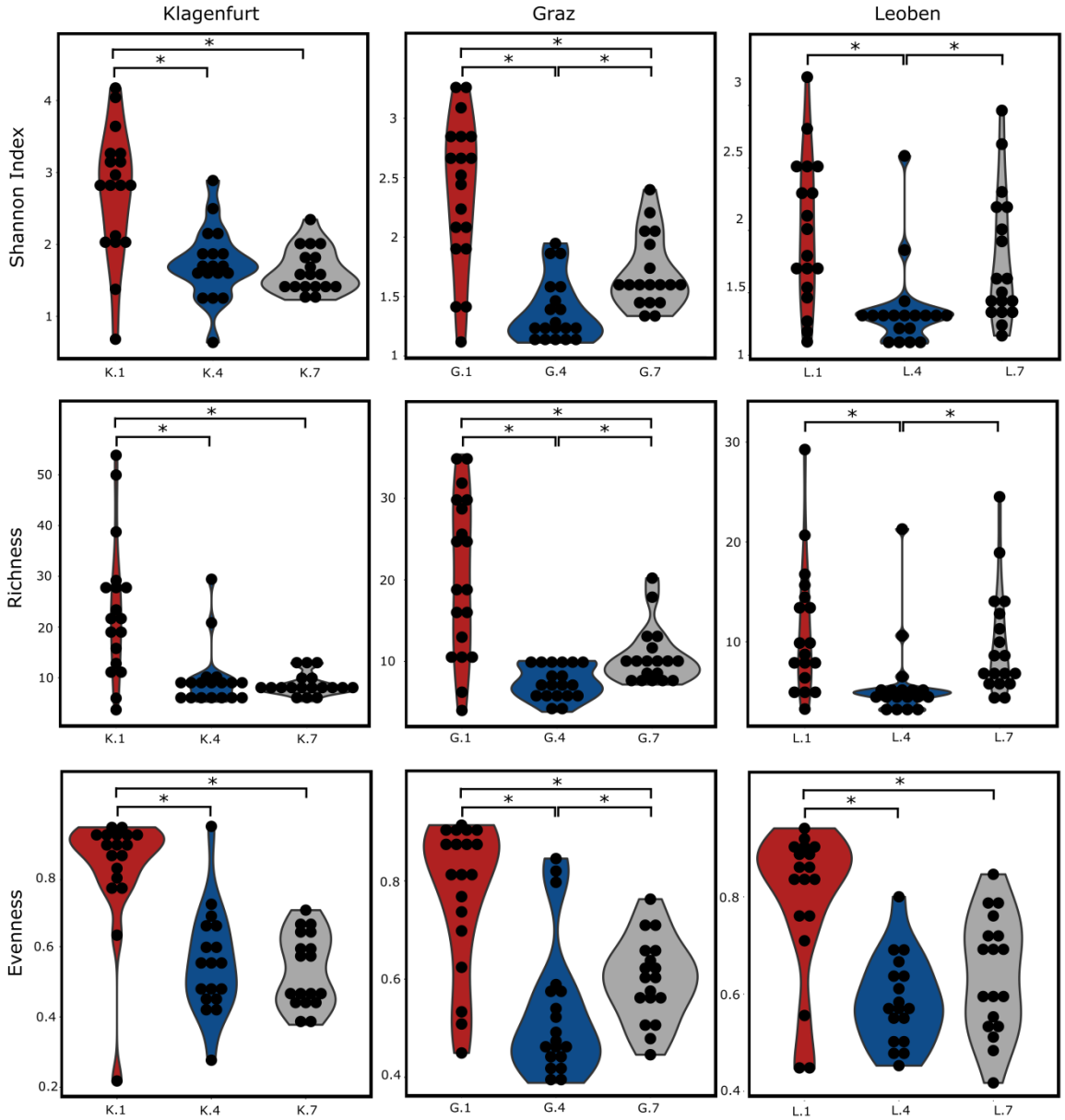
K5, G5	**0.020**	0.835	**0.028**	0.791	0.756	0.133
K5, L5	**0.022**	0.703	**0.047**	0.915	0.162	0.357
G5, L5	0.846	0.746	0.981	0.818	0.101	0.317
K6, G6	0.537	0.319	0.218	0.633	0.143	0.492
K6, L6	0.597	0.225	0.605	0.506	0.139	0.219
G6, L6	0.997	0.730	0.677	0.808	0.900	0.519
K7, G7	0.575	0.341	0.835	0.683	**0.049**	0.094
K7, L7	0.404	0.152	0.399	0.324	**0.008**	0.254
G7, L7	0.660	0.432	0.474	0.472	0.243	0.482
K1, K4	**<0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**	0.071
K4, K7	0.492	0.963	0.428	0.933	0.638	0.641
K1, K7	**<0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**	0.104
G1, G4	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.010**
G4, G7	**0.002**	**0.046**	**0.001**	0.076	0.094	0.803
G1, G7	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**
L1, L4	**0.001**	**0.001**	**0.004**	**0.001**	**<0.001**	**<0.001**
L4, L7	**0.013**	**0.014**	**0.015**	**0.014**	0.134	0.163
L1, L7	0.233	0.144	0.404	0.144	**0.002**	**0.001**

Significant differences in Shannon index (explanation see text), richness and evenness between stool microbiota from different timepoints compared for each center are given as p-values. BLG = ….

Inter individual fecall microbiome richness and diversity showed greater variance at PIT 1, but decreased over time in datasets with and without BLG included (figure 14 and 15). In general, at all centers a similar diversity of fecall microbiota was found by two weeks of life (i.e. at PIT 6 and PIT 7). Prior development, however, was different. Overall, the development in regard of microbiome diversity was similar for centers Graz and Leoben, showing some dipping during the study period (PIT 4, figure 16). The

lower similarity of diversity at PIT 4 may be caused by antibiotics administration (figure 16). In general, in the dataset without BLG, variances are higher and samples show notably lower richness (figure 11).

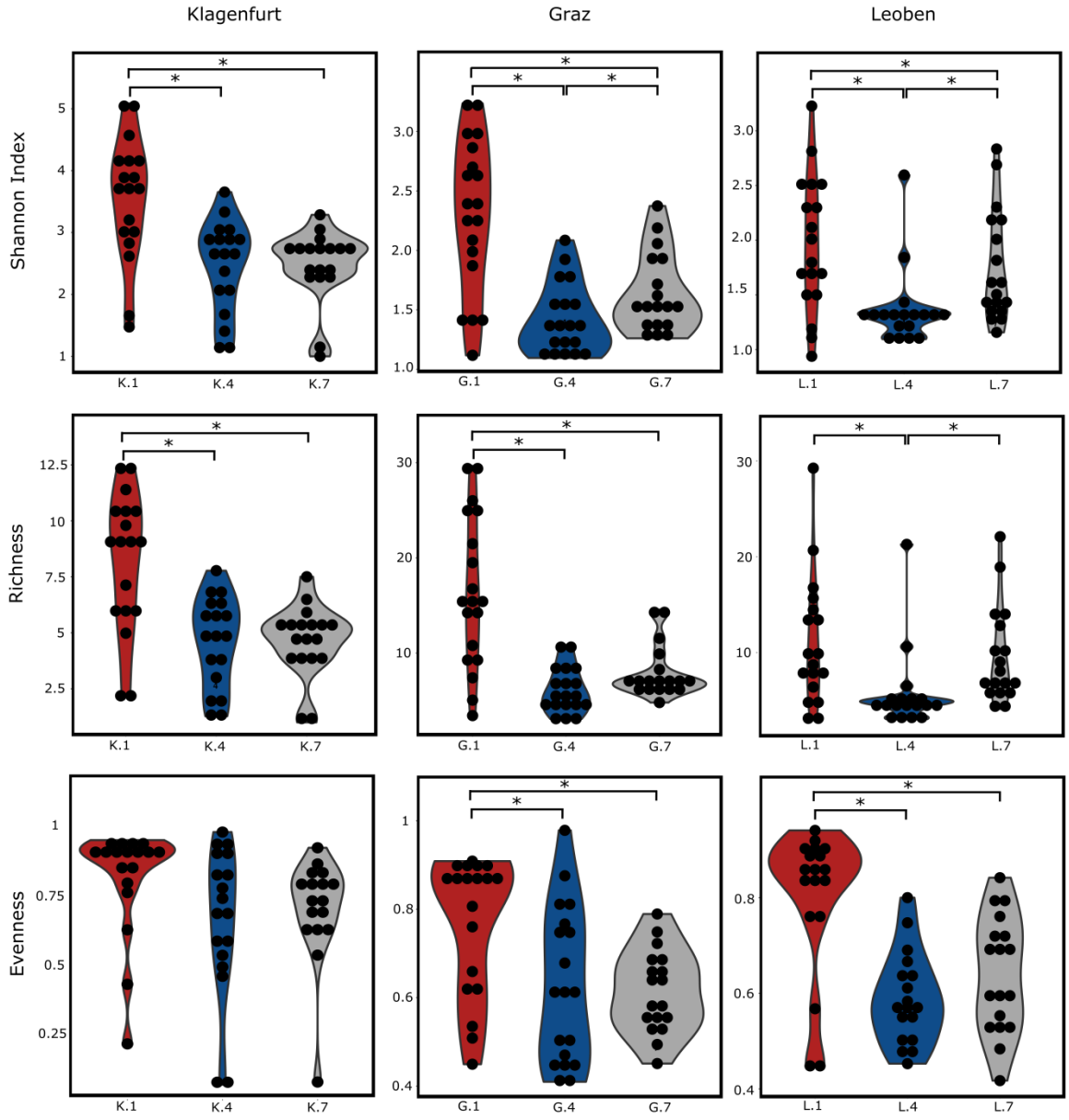
FIGURE 14: VIOLIN PLOTS OF SHANNON INDEX, RICHNESS AND EVENNESS AT PIT 1, PIT 4 AND PIT 7 SHOWN FOR SAMPLES FROM ALL THREE CENTERS SEPARATELY (DATASET WITH BLG INCLUDED).



Explanation: Violin plots represent a method of plotting numeric data. They are considered to be a combination of the box plot with a kernel density plot. The advantage of a violin plot over box plots is that it does not only show statistical values like in a box plot (e.g. median or interquartile range), it also shows the entire distribution of the data. The latter is of special interest when data distribution shows more than one peak. The figure gives Shannon index (explanation see text), richness and evenness as violin plots at time points 1 (red), 4 (blue) and 7 (grey) for each center (Klagenfurt left, Graz middle,

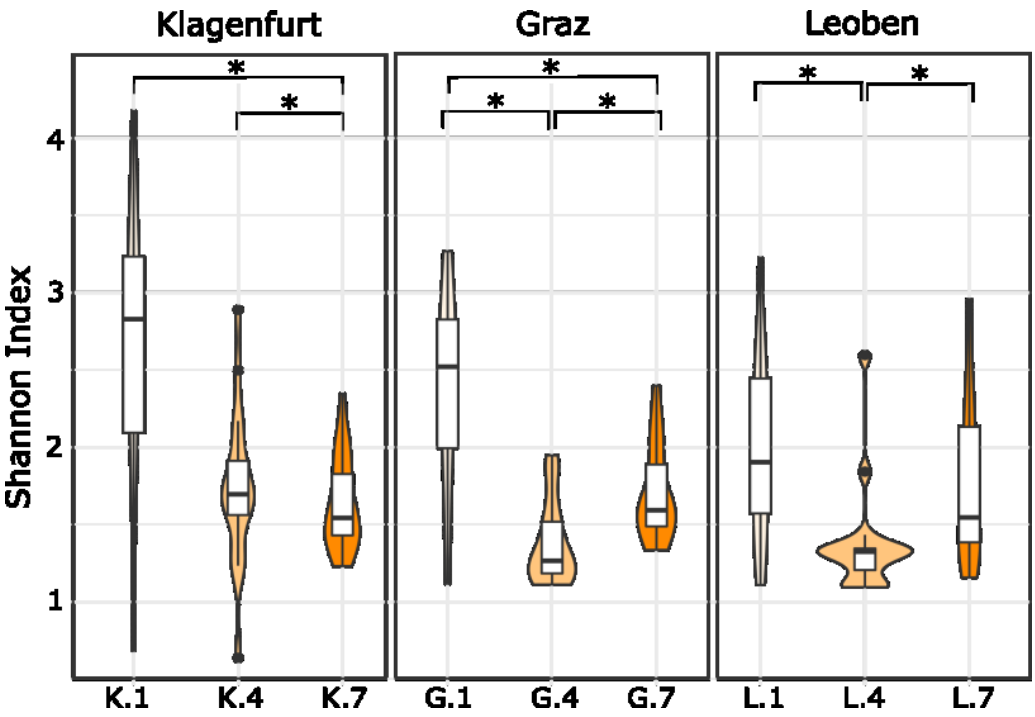
Leoben right column). Diversity decreases at all centers up to PIT 7, as do richness and evenness. At PIT 7 a less diverse and less abundant stool microbiome is established.

FIGURE 15: VIOLIN PLOTS OF SHANNON INDEX, RICHNESS AND EVENNESS AT PIT 1, PIT 4 AND PIT 7 SHOWN FOR SAMPLES FROM ALL THREE CENTERS SEPARATELY (DATASET WITHOUT BLG).



Explanation: The figure gives Shannon index (explanation see text), richness and evenness as violin plots (explanation see figure 19) at time points 1 (red), 4 (blue) and 7 (grey) for each center (Klagenfurt left, Graz middle, Leoben right column). Diversity decreases at all centers up to PIT 7, as do richness and evenness. At PIT 7 a less diverse and less abundant stool microbiome is established.

FIGURE 16: VIOLIN PLOTS FOR SHANNON INDICES (ALPHA DIVERSITY) AT PIT 1, PIT 4 AND PIT 7 SHOWN FOR SAMPLES FROM ALL THREE CENTERS SEPARATELY (DATASET WITH BLG INCLUDED).

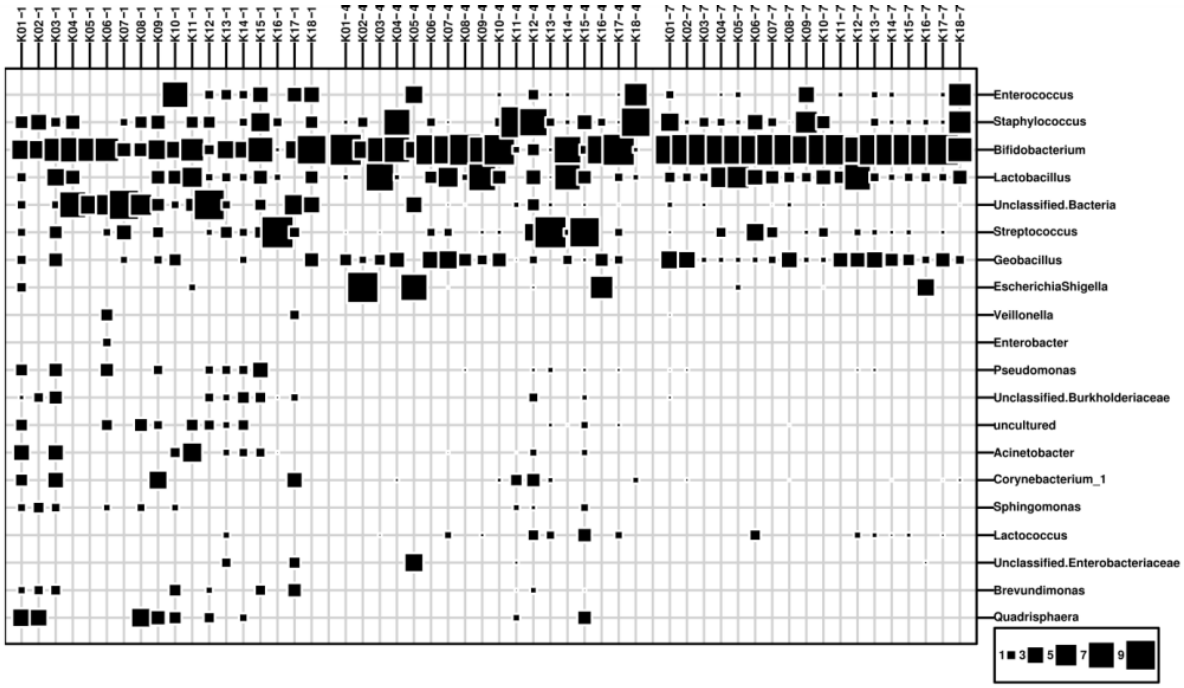


In samples from Klagenfurt, diversity and richness showed highest values from meconium samples at PIT 1 when compared to the other centers, with all three parameters (Shannon index, richness, evenness) decreasing significantly over time (figure 13). In samples from Graz, all three diversity indicators decreased over a short period of time and then increased again, with diversity at PIT 1 (1.56 (1; 3)), PIT 4 (8.04 (7; 15)) and PIT 7 (14.50 (13; 21)) differing significantly from one another (figures 14 and 15). In samples from the center in Leoben, no significant differences, neither for diversity nor for richness, were found comparing PIT 1 and PIT 7. However, Shannon Index was found to be significantly lower at PIT 4 (figure 14). Evenness of the samples did not differ notably between PITs or centers with or without BLG included. The only significant differences found were decreased evenness in infants from the center in Graz at PIT 2 when compared to the other centers (K.2, G.2 p 0.01; G.2, L.2 p 0.006) and decreased evenness at PIT 7 in infants from the center in Klagenfurt (K.7,G.7 p 0.049; K.7,L.7 p 0.009) within the dataset with BLG included.

Bubble plot analysis, based on the 20 most abundant genera, visualizes the decrease in diversity, richness and evenness in samples from Klagenfurt, taken at PIT 1, PIT 4

and PIT 7: less abundant genera, which resemble critical compounds at the beginning, get lost during the first days and weeks of life (e.g. *Pseudomonas, Acinetobacter*). Thus, the resulting microbiome was predominated by fewer genera with higher abundance (figure 17).

FIGURE 17: BUBBLE PLOT OF THE 20 MOST ABUNDANT MICROBIAL GENERA IN SAMPLES FROM KLAGENFURT AT PIT1, PIT4 AND PIT7 (DATASET WITH BLG INCLUDED).



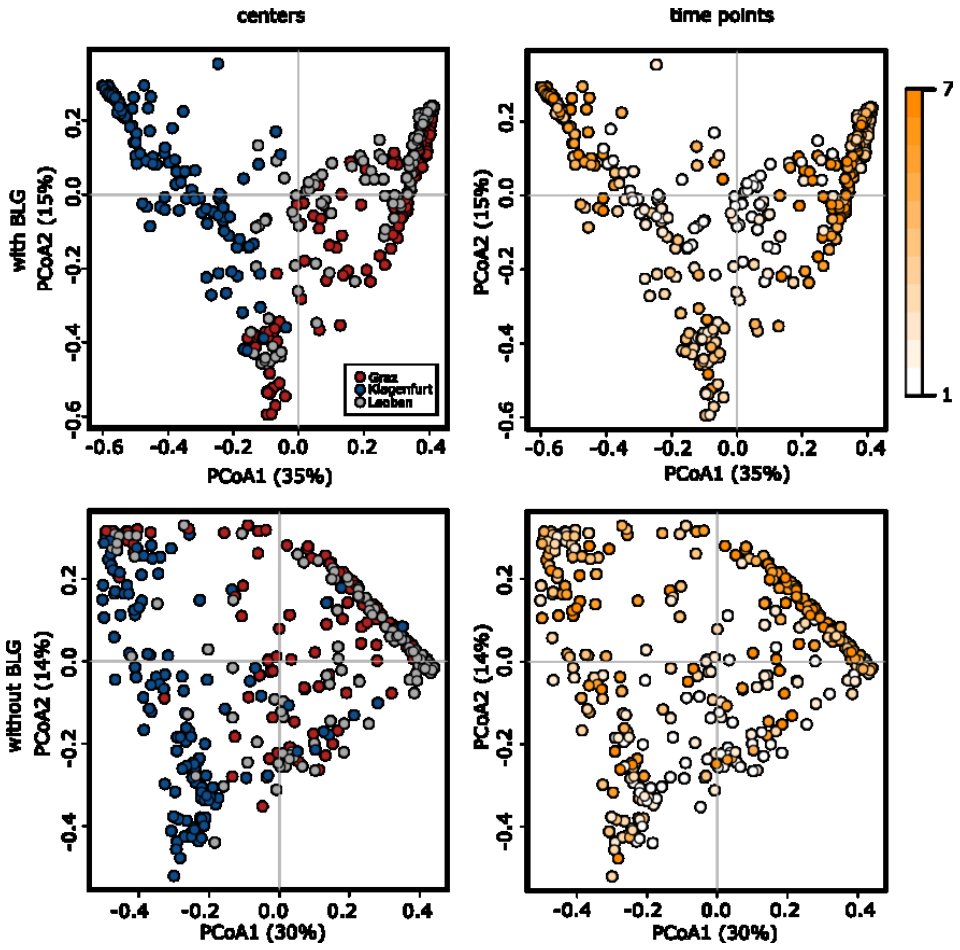
Leg: Bold bubbles depict abundance of genera (y-axis) at different time points and centers (x-axis). Increasing size of bubbles depict higher abundance (right lower corner legend).

Furthermore, to identify factors potentially shaping VLBW infants` intestinal microbiome, we performed beta diversity analyses based on unweighted Bray-Curtis distances on ASV level.

We used the dataset which included BLG, with samples form center-specific clusters ($p<0.001$). The center-specific cluster for samples from Klagenfurt was found clearly apart from the center-specific clusters Leoben and Graz. This clear separation was found to be mainly due to the probiotic strains, as a removal of BLG resulted in higher similarity between clusters (figure 18). Nevertheless, even beyond probiotics,

clustering remained statistically significant. P-values for significant differences between the groups are listed in table 20.

FIGURE 18: CHANGES IN DIVERSITY OVER TIME IN SAMPLES FROM THE THREE PARTIZIPATING CENTERS WITH AND WITHOUT BLG INCLUDED.



Principal Coordinates Analysis (PCoA) is a method to explore and to visualize similarities or dissimilarities of data. PCOA tries to find the main axes through a matrix and allows for individual and/or group differences to be visualized. The figure shows diversity of stool samples. On the left side center specific diversity of stool samples is visualized with samples from Graz in red, samples from Klagenfurt in blue and samples from Leoben in grey. With BLG (Bifidobacterium, Lactobacillus, Geobacillus) included, microbial diversity of samples clusters for each center (top left plot). Without BLG included the centers Klagenfurt and Leoben show less but still obvious cluster formation while Graz does less so (lower left plot).
To the right side of the figure microbial diversity of stool samples with regard to timepoints is visualized, with PIT 1 being illustrated in white and PIT 7 in orange. Colour intensity increases directly proportional to timepoints. The center specific clustering remains over time with BLG included, as illustrated in the top right plot. Without BLG included center specific cluster formation over time seems decreased (lower right plot). Figure excerpted from [213].

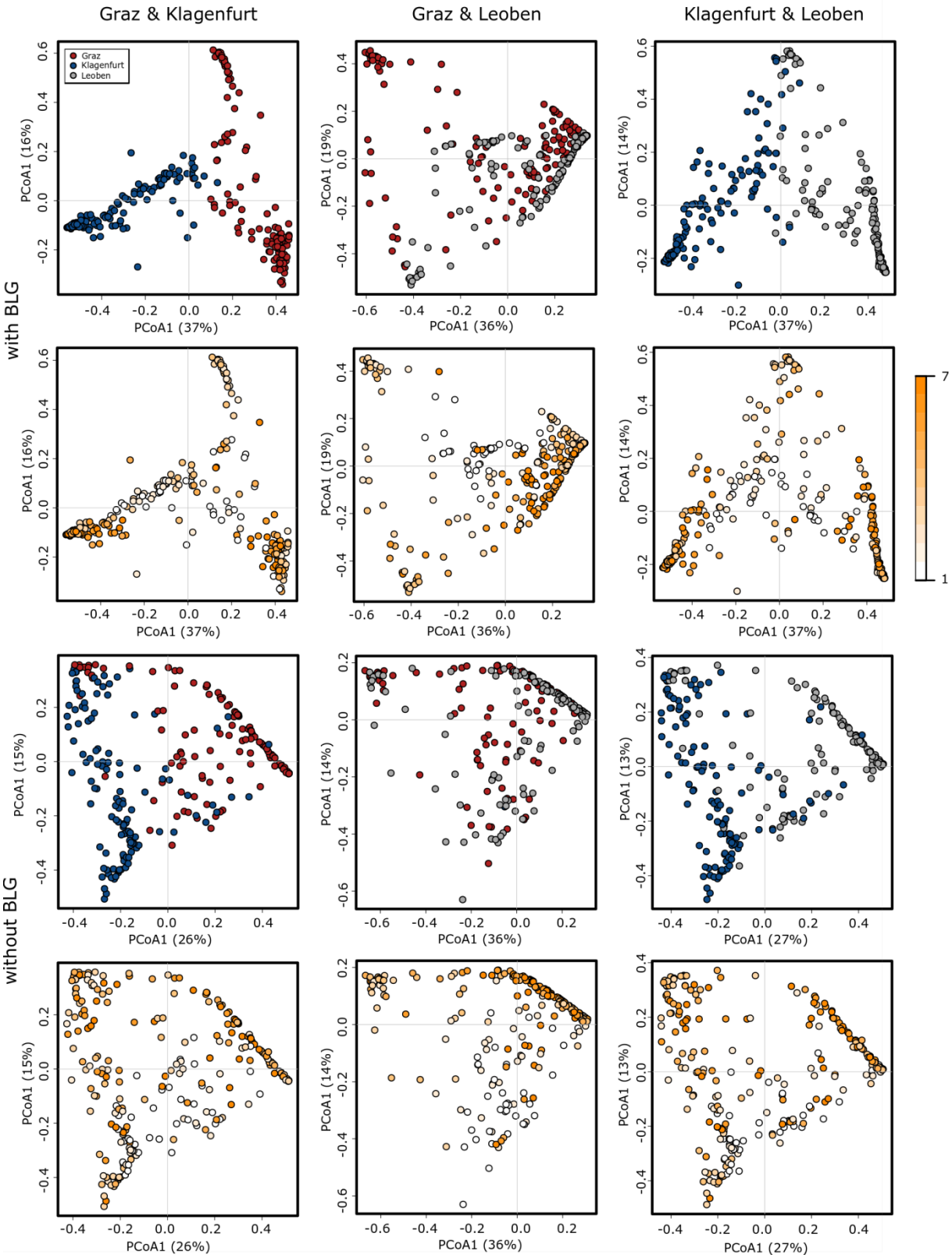
TABLE 20: R^2 AND P-VALUES FOR CHANGES IN DIVERSITY WEIGHTED BY BRAY-CURTIS BETWEEN THE CENTERS WITH AND WITHOUT BLG (BIFIDOBACTERIUM, LACTOBACILLUS, GEOBACILLUS) AT PIT 1. R^2 VALUES GIVE EXPLAINED VARIATION / TOTAL VARIATION

CENTERS	WITH BLG		WITHOUT BLG	
	R^2	p-value	R^2	p-value
ALL 3	0.29	<0.001	0.144	<0.001
K-G	0.298	<0.001	0.147	<0.001
K-L	0.288	<0.001	0.139	<0.001
G-L	0.068	<0.001	0.0103	0.018

Leg.: K-G compares centers Klagenfurt and Graz, K-L compares centers Klagenfurt and Leoben, G-L compares centers Graz and Leoben.

Noteworthy, there appeared to be a timely association regarding cluster formation, since samples taken at earlier PITs clustered more (independent from center), while they diverged more when taken at later PITs. This could also be seen as a trend within the dataset without BLG (figure 16). Figure 19 shows PCoA plots on these datasets with two centers compared at the same time.

FIGURE 19: PCOA PLOTS SHOWING DIVERSITY BETWEEN TWO CENTERS, EACH WITH AND WITHOUT BLG. PLOTS ARE EITHER COLORED FOR CENTER OR FOR TIME POINT.

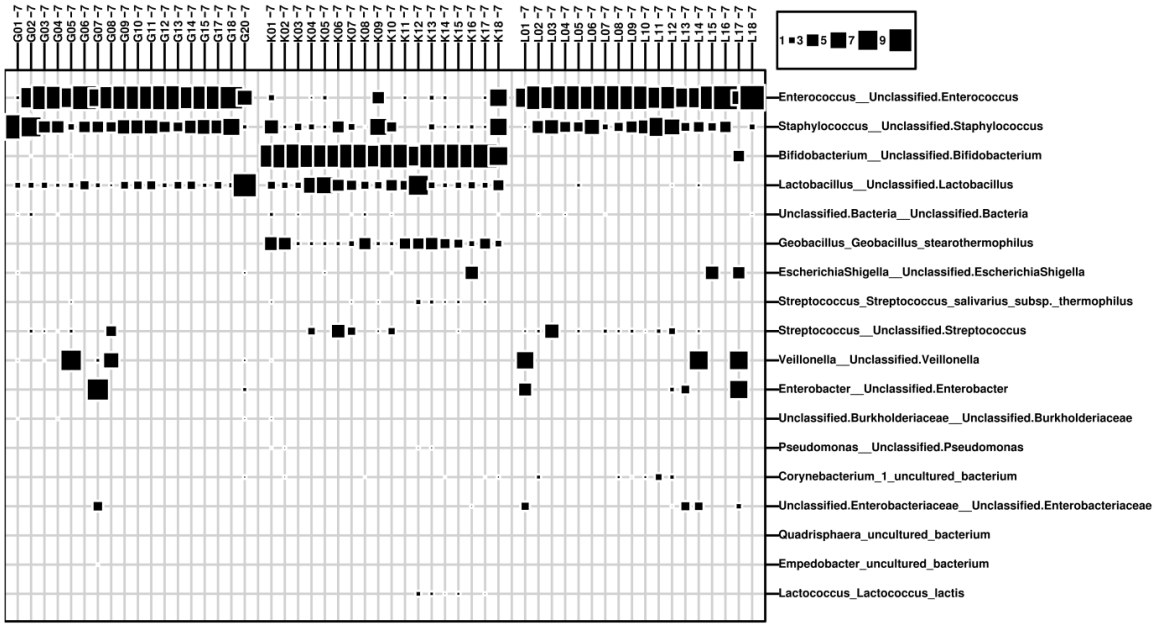


Principal Coordinates Analysis (PCoA) is a method to explore and to visualize similarities or dissimilarities of data. PCOA tries to find the main axes through a matrix and allows for individual and/or group differences to be visualized. The figure shows diversity of stool samples` microbiota of two centers compared to each other. Top two rows give comparisons of diversity with BLG (Bifidobacterium, Lactobacillus, Geobacillus) included, while the lower two rows give comparison without BLG included. Plots in the top row show samples from Klagenfurt (blue) clustering significantly apart from samples from Leoben (grey) and Graz (red). Plots in the second row give microbial diversity of stool samples with regard to timepoints is visualized, with PIT 1 being illustrated in white and PIT 7 in orange. Colour intensity increases directly proportional to timepoints. Here, again, distinct cluster formation can be seen with samples from Klagenfurt clustering apart from the other centers, especially visualized for PIT 7. Plots in row three and four give microbial diversity without BLG included. Again, samples from Klagenfurt cluster apart from those from Leoben and Graz. Cluster formation is given as per specific center (row three with Klagenfurt in blue, Leoben in grey, and Graz in red) and with regard to timepoints (row four), with PIT 1 being illustrated in white and PIT 7 in orange. Colour intensity increases directly proportional to timepoints. However, compared to microbial diversity with BLG included, the cluster-formation in rows three and four appears differently shaped.

4.5 REGIMENS LEAD TO DIFFERENCES IN TWO WEEKS FECAL MICROBIOMES REGARDING PROBIOTIC GENERA BUT ALSO OTHER SPECIES

We analyzed the preterm gut associated taxa at PIT 7 (14.50 (13; 21)), in order to understand the differences of the advanced microbiomes at each partizipating center (figure 20). We identified signatures of five genera, which were predominating. Namely *Enterococcus*, *Staphylococcus*, *Bifidobacterium*, *Lactobacillus* and *Geobacillus*, whereas probiotic strains *Lactobacillus* and *Bifidobacterium* do not represent natural residents of the preterm gut [225]. We can confirm these findings, as no *Bifidobacterium* signatures (except for sample L17-7) were present in samples from the centers in Graz and Leoben. No signatures of *Lactobacillus* were found in specimens from Leoben.

FIGURE 20: BUBBLE PLOT OF THE EIGHTEEN MOST ABUNDANT MICROBIAL SPECIES AT PIT 7 WITH BLG INCLUDED IN THE THREE CENTERS GRAZ, KLAGENFURT AND LEOBEN.



Leg: Bold bubbles depict abundance of the 18 most abundant genera (y-axis) at time point 7 for each center (x-axis). Increasing size of bubbles depict higher abundance (right upper corner legend). Figure excerpted from [213].

Looking at the non-BLG taxa, samples from the center in Klagenfurt were characterized by lower abundance of *Enterococcus* ($p<0.001$) and *Staphylococcus* (p 0.081) signatures, but uniquely carried signatures of *Geobacillus stearothermophilus* and *Streptococcus salivarius subsp. thermophilus.* Samples from the center in Graz revealed overall lower abundance in *Streptococcus* signatures ($p<0.001$).

5 DISCUSSION

Our study revealed that probiotic genera are found in high numbers in stool samples from infants who receive probiotics supplementation. *Bifidobacterium* reads amounted higher numbers in samples from Klagenfurt when compared to *Lactobacillus* reads, even though administered dosage was identical for both probiotic genera. This might be due to bias from extraction method or primer biases, discriminating against *Lactobacillus*. The strong increase of bacterial load in Graz from PIT 1 to PIT 3 (figure 8) appeared simultaneously with increasing *Lactobacillus* reads within the same specimens (figure 10a). No probiotic genera were found in fecal specimens from infants who did not receive probiotics. This supports the previously known findings that *Bifidobacterium* and *Lactobacillus* do not majorly contribute to the gut microbiome of premature infants in early live [225].

Probiotics and antibiotics impacting on bacterial load of fecal specimens needs to be discussed adducing qPCR data. Bacterial load of stool samples from preterm born infants in our study increased significantly at all three centers up to PIT 3, representing approximately the first week of life (5.82 (5; 8)). From that time point on numbers of bacterial load remained more or less constant. One would expect administration of antibiotics resulting in decreased bacterial load, while administration of probiotics would cause bacterial load to increase. However, we found that at the center Leoben administering antibiotics did not lead to substantially lower bacterial loads when compared to the other two centers. Antibiotics, however, may lead to intestinal bacterial death, thus resulting in increased loads of bacterial DNA in stool samples, as discussed below. Throughout the first days of life the increase of bacterial load was delayed in stool samples from Leoben compared to the other centers. From PIT 3 on, following the first week of life, no significant differences in bacterial load could be found in stool samples from Leoben. On the other hand, although expected, supplementation with probiotics in the absence of antibiotics, as facilitated at the center Klagenfurt, did not lead to the highest bacterial increase. Analyzing stool samples from infants who received a combination of antibiotics and probiotics supplementation, as facilitated at the center Graz, revealed the earliest increase in bacterial load. Interestingly, administration of either probiotics or antibiotics did not seem to have any systematic impact on numbers of fecal bacteria of preterm infants throughout the first weeks of life. This finding was confirmed both by numbers of amplicon sequence variants (ASVs) obtained by next generation sequencing and by quantitative PCR of 16S rRNA genes.

ASV numbers seemed to rather support the anticipation of fewer bacteria being present when antibiotics were administered (lower ASV numbers at the center in Leoben). However, ASV numbers are less appropriate to estimate numbers than is qPCR.

Aside from probiotic genera, we observed high numbers of *Geobacillus* signatures in stool samples from the center in Klagenfurt. The origin of these ASVs remains unclear. However, *Geobacillus* represents a thermophilic spore forming bacterium that is known as a “food-spoiler”, especially contaminating milk-powder plants [226]. The feeding regimen at the center in Klagenfurt differs from the other centers by use of formula milk as a main stay, using less breast milk. If the formula milk powder supply had experienced contaminations with *G. stearothermophilus* at the time our samples were collected this might be the origin of the high amounts of *Geobacillus* reads. All we know, *G. stearothermophilus* was not found to be pathogenic or negatively impacting health outcome yet. Thermophilic bacilli are described as non-pathogenic in general [226].

For both probiotic genera, their relative abundances differ a lot. This can be seen not only between centers but also between single infants, displayed by great variances at single time points. Infants supplemented with probiotics including *Lactobacillus*, showing lower *Lactobacillus* read numbers might not respond or respond at a lower level to the probiotic treatment, with restricted colonization of the gut by *Lactobacillus*. However, with our analysis we solemnly measure DNA in stool samples. Therefore, we do not know whether bacteria are dead or alive. Infants showing low abundance may in fact respond well to probiotic supplementation. Low amounts of *Lactobacillus* DNA detectable from stool samples might be caused by deposition of bacteriain certain intestinal habitats without being detectable in stool samples. Infants with high abundance of *Lactobacillus* in stool samples may as well be poor or non-responders to probiotic supplementation, depositing dead *Lactobacilli* in stools. In the literature the phenomenon that some adults do not respond to probiotic supplementation while others do was already described by Reid et al [227]. Our data would also support the findings of Underwood et al [228], who described limited response to probiotic supplementation in premature infants. However, the above mentioned limitations to define whether bacteria from our stool samples were dead or alive pose a clear limitation in this regard.

Over time, relative abundances of probiotic genera ASVs per sample decreased. Since dosage of probiotics supplementation remained constant throughput the study period,

a decline in relative abundances most likely results from growth of different bacteria over time in the intestinal tract. On the other hand, if the administered probiotic genera colonize the intestine and persist, their total numbers should at least stay the same. However, due to multiple factors during processing (PCR over representing high abundant DNA signatures and under representing low abundant DNA; saturation of reactions; normalization steps during analysis) ASV numbers are merely suitable for quantitative estimations.

Probiotic genera are the main drivers of intestinal microbiome development, which is nicely depicted by numbers of ASVs. Aside from *Bifidobacterium* and *Geobacillus*, ASVs were very low at the center Klagenfurt. This might be due to a high bacterial load introduced by probiotics administration, suppressing colonization of the intestinal tract by other bacteria, either through occupying niches or active “defense” mechanisms.

PCoA analysis revealed significant differences between centers in microbiome composition, already present in meconium samples. These findings might reflect the role of the maternal microbiome and environment, contributing to the development of the infant’s microbiome. Excluding BLG led to the loss of group clustering between Graz and Leoben, but not Klagenfurt. The latter, forming a significant cluster, showed that probiotic supplementation does not affect the preterm infants´ fecall microbiome largely throughput the first three days of life. Intestinal passage of orally administered foods, as well as probiotic supplementations, may take up to three days at this early age, thus, most likely, resulting in meconium not being influenced by any orally induced administrations. A possible microbial source contributing to significant differences between centers may be the skin microbiome of infants, as it must be assumed that diverse environmental exposure leads to different skin microbial patterns. Signatures observed in the collected meconium samples could include the early skin microbiome of infants, being transferred to the expelled meconium from diapers. However, we did not find substantial amounts of skin microbes in analyzed samples.

Throughout the first days of life, alpha diversities of infants’ stool samples differ largely between infants from each center, indicating great differences in initial intestinal colonization. However, from PIT 4 on variances diminish showing first similarities. Alpha diversities throughout the first days of life were higher in Klagenfurt when compared to the other centers. This is surprising, since the center in Graz showed the highest bacterial loads. Environmental factors may play a major role in this finding.

However, this can only be hypothesized but remains unproven, as this study was not designed to evaluate environmental influences.

Having mentioned environmental factors influencing the stool microbiome, the center specific clustering of samples shows that the NICUs impact on shaping the neonatal intestinal microbiome significantly. Even with BLG excluded this differentiation persists, leading to the assumption that not only the administration of probiotics but also the environment (nurses, doctors, parents, equipment etc.) and administered medications influence the intestinal microbiome significantly. This hypothesis may be supported by the differentiation over time. The microbiome of meconium samples did not differ significantly between centers but with increasing age and prolonged stay at the NICUs, the fecal microbiome of infants more and more clustered center specific.

The last samples taken at PIT 7 (14.50 (13; 21) days of life) show highest development of microbiomes analyzed, and most appropriately display the impact of hospital regimens on the fecall microbiome. Since the early life's´ microbiome is critical for maturation of a healthy and well-balanced intestinal microbiome [229], differences at this age might impact on the development of the infant´s intestinal microbiome on the long term.

Streptococcus salivarius subsp. thermophilus, which was uniquely found in samples from the center Klagenfurt, is an opportunistic pathogen which can be isolated from human saliva and commonly colonizes the mucosa of preterm infants and newborns within the first days of live [230]. *Lactococcus lactis* was hardly present, which is unexpected as it is a lactic acid bacteria commonly appearing in breast milk [231]. Included infants mostly received formula milk or pasteurized breast milk, both of which might explain the rarity of *Lactococcus lactis* in our samples.

Olm et al identified *Klebsiella* species as a major factor correlating with NEC, as found in fecal samples prior to NEC diagnosis [232]. In our study, however, we isolated signatures of *Klebsiella* in 16 fecal samples taken from nine infants (three infants from Klagenfurt, one from Graz, five from Leoben, table 23), but none of those infants was diagnosed with NEC. All children bearing *Klebsiella* signatures remained healthy.

5.1 LIMITATIONS

Limitations to this study are the relatively low number of included infants and the lack of some samples which should have been analyzed along:
NGS data of the administered probiotics and formula milk would extend our knowledge about what exactly was introduced to the infants' intestinal tract via these components. Especially sequencing of formula milk from the center in Klagenfurt would be of major interest to clarify whether it contained *Geobacillus stearothermophilus* or not. With regard to qPCRs on 16S rRNA gene for total bacterial load, qPCRs specific for *Bifidobacterium* and *Lactobacillus* would aid a more precise numerical estimation of probiotic genera. Furthermore, CFUs cultivated from stool samples would allow evaluating the response to probiotic supplementation by bacterial alive/death differentiation. Additional analyses on metabolomes or metagenomes would enhance conclusions about the impact of the different regimens on the infants´ intestinal microbiomes, enabling identification of clinically relevant data such as the antibiotic resistome or harmful metabolites. Regarding the use of probiotics in VLBW preterm infants, we did not encounter any adverse effects attributable to either probiotics supplementation or hospital regimen. However, numbers of infants included in our study were rather low in regard of elucidating adverse events. Therefore, interpretation of safety must be made conditionally.
Also, we did not question mothers about use of probiotics prior birth. Maternal use of probiotics prior birth may influence the infants´ fecal microbiome.

6 CONCLUSION

Hospital regimens aiming to prevent development of NEC in preterm very low birth weight infants significantly influence the fecal microbiome composition, diversity and absolute numbers of bacteria. Alterations due to these regimens are present right from birth until the end of the second to third week of life. At two to three weeks of life a stable "mature" microbiome is established in preterm infants. A stable "mature" microbiome is established earlier when probiotics are supplemented. Furthermore, probiotics supplementation led to an earlier increase of bacterial load in infants´ stool samples. This in turn may aid preventing neonatal morbidities.
We found five genera dominating intestinal colonization in this study: *Enterococcus, Staphylococcus, Bifidobacterius, Lactobacillus* and *Geobacillus*. Lactobacillus and

Bifidobacterium are no natural residents of the preterm infants´ intestinal microbiome and only appear to be of relevant presence when administered artificially. Meconium was found to contain detectable bacterial DNA. Oral antibiotics were not found to have any negative impact on probiotic strains present in the preterm infants´ intestinal microbiome.

Our findings on hospital regimens shaping the preterm infants stool microbiome should aid and stimulate future research on this topic. Our results should be considered when interpreting microbiome analyses in comparable settings.

7 REFERENCES

1. Blencowe, H., et al., *Born too soon: the global epidemiology of 15 million preterm births.* Reprod Health, 2013. **10 Suppl 1**: p. S2.
2. Blencowe, H., et al., *National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications.* Lancet, 2012. **379**(9832): p. 2162-72.
3. Karnati, S., S. Kollikonda, and J. Abu-Shaweesh, *Late preterm infants - Changing trends and continuing challenges.* Int J Pediatr Adolesc Med, 2020. **7**(1): p. 36-44.
4. Engle, W.A., et al., *"Late-preterm" infants: a population at risk.* Pediatrics, 2007. **120**(6): p. 1390-401.
5. Spong, C.Y., *Defining "term" pregnancy: recommendations from the Defining "Term" Pregnancy Workgroup.* JAMA, 2013. **309**(23): p. 2445-6.
6. Richards, J.L., et al., *Temporal Trends in Late Preterm and Early Term Birth Rates in 6 High-Income Countries in North America and Europe and Association With Clinician-Initiated Obstetric Interventions.* JAMA, 2016. **316**(4): p. 410-9.
7. Davidoff, M.J., et al., *Changes in the gestational age distribution among U.S. singleton births: impact on rates of late preterm birth, 1992 to 2002.* Semin Perinatol, 2006. **30**(1): p. 8-15.
8. Reddy, U.M., et al., *Delivery indications at late-preterm gestations and infant mortality rates in the United States.* Pediatrics, 2009. **124**(1): p. 234-40.
9. Frieden, T.R., C. Centers for Disease, and Prevention, *Public health then and now: celebrating 50 years of MMWR at CDC. Foreword.* MMWR Suppl, 2011. **60**(4): p. 1.
10. Slattery, M.M. and J.J. Morrison, *Preterm delivery.* Lancet (London, England), 2002. **360**(9344): p. 1489-97.
11. Matthews, T.J., M.F. MacDorman, and M.E. Thoma, *Infant Mortality Statistics From the 2013 Period Linked Birth/Infant Death Data Set.* Natl Vital Stat Rep, 2015. **64**(9): p. 1-30.
12. Markestad, T., et al., *Early death, morbidity, and need of treatment among extremely premature infants.* Pediatrics, 2005. **115**(5): p. 1289-98.
13. Wyckoff, M.H., et al., *Outcome of extremely low birth weight infants who received delivery room cardiopulmonary resuscitation.* J Pediatr, 2012. **160**(2): p. 239-244 e2.
14. Bassler, D., et al., *Using a count of neonatal morbidities to predict poor outcome in extremely low birth weight infants: added role of neonatal infection.* Pediatrics, 2009. **123**(1): p. 313-8.
15. Katz, J., et al., *Mortality risk in preterm and small-for-gestational-age infants in low-income and middle-income countries: a pooled country analysis.* Lancet (London, England), 2013. **382**(9890): p. 417-425.
16. Kent, A.L., et al., *Mortality and adverse neurologic outcomes are greater in preterm male infants.* Pediatrics, 2012. **129**(1): p. 124-31.
17. Garfinkle, J., et al., *Trends in sex-specific differences in outcomes in extreme preterms: progress or natural barriers?* Archives of disease in childhood Fetal and neonatal edition, 2020. **105**(2): p. 158-163.
18. Field, D.J., et al., *Survival of extremely premature babies in a geographically defined population: prospective cohort study of 1994-9 compared with 2000-5.* BMJ (Clinical research ed), 2008. **336**(7655): p. 1221-3.
19. Stoll, B.J., et al., *Trends in Care Practices, Morbidity, and Mortality of Extremely Preterm Neonates, 1993-2012.* Jama, 2015. **314**(10): p. 1039-51.
20. Lemons, J.A., et al., *Very low birth weight outcomes of the National Institute of Child health and human development neonatal research network, January 1995 through December 1996. NICHD Neonatal Research Network.* Pediatrics, 2001. **107**(1): p. E1.
21. Fanaroff, A.A., et al., *Trends in neonatal morbidity and mortality for very low birthweight infants.* American journal of obstetrics and gynecology, 2007. **196**(2): p. 147.e1-8.

22. Marlow, N., et al., *Perinatal outcomes for extremely preterm babies in relation to place of birth in England: the EPICure 2 study.* Archives of disease in childhood Fetal and neonatal edition, 2014. **99**(3): p. F181-8.
23. Phibbs, C.S., et al., *Level and volume of neonatal intensive care and mortality in very-low-birth-weight infants.* The New England journal of medicine, 2007. **356**(21): p. 2165-75.
24. Watson, S.I., et al., *The effects of designation and volume of neonatal care on mortality and morbidity outcomes of very preterm infants in England: retrospective population-based cohort study.* BMJ open, 2014. **4**(7): p. e004856.
25. Desplanches, T., et al., *Volume of Neonatal Care and Survival without Disability at 2Years in Very Preterm Infants: Results of a French National Cohort Study.* The Journal of pediatrics, 2019. **213**: p. 22-29.e4.
26. Helenius, K., et al., *Association of early postnatal transfer and birth outside a tertiary hospital with mortality and severe brain injury in extremely preterm infants: observational cohort study with propensity score matching.* BMJ (Clinical research ed), 2019. **367**: p. l5678.
27. Fang, J.L., et al., *Outcomes of outborn extremely preterm neonates admitted to a NICU with respiratory distress.* Archives of disease in childhood Fetal and neonatal edition, 2020. **105**(1): p. 33-40.
28. Ballance, W.A., et al., *Pathology of neonatal necrotizing enterocolitis: a ten-year experience.* J Pediatr, 1990. **117**(1 Pt 2): p. S6-13.
29. Eggesbo, M., et al., *Development of gut microbiota in infants not exposed to medical interventions.* APMIS, 2011. **119**(1): p. 17-35.
30. Jakobsson, H.E., et al., *Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section.* Gut, 2014. **63**(4): p. 559-66.
31. Penders, J., et al., *Factors influencing the composition of the intestinal microbiota in early infancy.* Pediatrics, 2006. **118**(2): p. 511-21.
32. Groer, M.W., et al., *Development of the preterm infant gut microbiome: a research priority.* Microbiome, 2014. **2**: p. 38.
33. Palmer, C., et al., *Development of the human infant intestinal microbiota.* PLoS Biol, 2007. **5**(7): p. e177.
34. Chong, C.Y.L., F.H. Bloomfield, and J.M. O'Sullivan, *Factors Affecting Gastrointestinal Microbiome Development in Neonates.* Nutrients, 2018. **10**(3).
35. Mihatsch, W.A., et al., *Critical systematic review of the level of evidence for routine use of probiotics for reduction of mortality and prevention of necrotizing enterocolitis and sepsis in preterm infants.* Clin Nutr, 2012. **31**(1): p. 6-15.
36. Stewart, C.J., et al., *Development of the preterm gut microbiome in twins at risk of necrotising enterocolitis and sepsis.* PLoS One, 2013. **8**(8): p. e73465.
37. Aagaard, K., et al., *The placenta harbors a unique microbiome.* Sci Transl Med, 2014. **6**(237): p. 237ra65.
38. Torrazza, R.M. and J. Neu, *The altered gut microbiome and necrotizing enterocolitis.* Clin Perinatol, 2013. **40**(1): p. 93-108.
39. Hansen, T.H., et al., *The gut microbiome in cardio-metabolic health.* Genome medicine, 2015. **7**(1): p. 33.
40. Nagpal, R., et al., *Sensitive Quantitative Analysis of the Meconium Bacterial Microbiota in Healthy Term Infants Born Vaginally or by Cesarean Section.* Frontiers in microbiology, 2016. **7**: p. 1997.
41. Mennella, J.A. and G.K. Beauchamp, *Early flavor experiences: research update.* Nutrition reviews, 1998. **56**(7): p. 205-11.
42. Bagci, S., et al., *More than fetal urine: enteral uptake of amniotic fluid as a major predictor for fetal growth during late gestation.* European journal of pediatrics, 2016. **175**(6): p. 825-31.
43. Collado, M.C., et al., *Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid.* Scientific reports, 2016. **6**: p. 23129.

44. Gitlin, D., et al., *The turnover of amniotic fluid protein in the human conceptus.* American journal of obstetrics and gynecology, 1972. **113**(5): p. 632-45.
45. Blakelock, R., et al., *Is a normally functioning gastrointestinal tract necessary for normal growth in late gestation?* Pediatric surgery international, 1998. **13**(1): p. 17-20.
46. Burjonrappa, S.C., E. Crete, and S. Bouchard, *The role of amniotic fluid in influencing neonatal birth weight.* Journal of perinatology : official journal of the California Perinatal Association, 2010. **30**(1): p. 27-9.
47. Mulvihill, S.J., et al., *Trophic effect of amniotic fluid on fetal gastrointestinal development.* The Journal of surgical research, 1986. **40**(4): p. 291-6.
48. Trahair, J.F. and R. Harding, *Restitution of swallowing in the fetal sheep restores intestinal growth after midgestation esophageal obstruction.* Journal of pediatric gastroenterology and nutrition, 1995. **20**(2): p. 156-61.
49. Jimenez, E., et al., *Is meconium from healthy newborns actually sterile?* Research in microbiology, 2008. **159**(3): p. 187-93.
50. Madan, J.C., et al., *Gut microbial colonisation in premature neonates predicts neonatal sepsis.* Arch Dis Child Fetal Neonatal Ed, 2012. **97**(6): p. F456-62.
51. Biasucci, G., et al., *Mode of delivery affects the bacterial community in the newborn gut.* Early Hum Dev, 2010. **86 Suppl 1**: p. 13-5.
52. Di Mauro, A., et al., *Gastrointestinal function development and microbiota.* Ital J Pediatr, 2013. **39**: p. 15.
53. Madan, J.C., et al., *Normal neonatal microbiome variation in relation to environmental factors, infection and allergy.* Curr Opin Pediatr, 2012. **24**(6): p. 753-9.
54. Scholtens, P.A., et al., *The early settlers: intestinal microbiology in early life.* Annu Rev Food Sci Technol, 2012. **3**: p. 425-47.
55. Yatsunenko, T., et al., *Human gut microbiome viewed across age and geography.* Nature, 2012. **486**(7402): p. 222-7.
56. Dai, D. and W.A. Walker, *Protective nutrients and bacterial colonization in the immature human gut.* Adv Pediatr, 1999. **46**: p. 353-82.
57. Oozeer, R., et al., *Intestinal microbiology in early life: specific prebiotics can have similar functionalities as human-milk oligosaccharides.* Am J Clin Nutr, 2013. **98**(2): p. 561S-71S.
58. Putignani, L., et al., *The human gut microbiota: a dynamic interplay with the host from birth to senescence settled during childhood.* Pediatr Res, 2014. **76**(1): p. 2-10.
59. Solis, G., et al., *Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut.* Anaerobe, 2010. **16**(3): p. 307-10.
60. Di Gioia, D., et al., *Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants.* Appl Microbiol Biotechnol, 2014. **98**(2): p. 563-77.
61. Jain, N. and W.A. Walker, *Diet and host-microbial crosstalk in postnatal intestinal immune homeostasis.* Nat Rev Gastroenterol Hepatol, 2015. **12**(1): p. 14-25.
62. Maynard, C.L., et al., *Reciprocal interactions of the intestinal microbiota and immune system.* Nature, 2012. **489**(7415): p. 231-41.
63. Cong, X., et al., *Influence of Feeding Type on Gut Microbiome Development in Hospitalized Preterm Infants.* Nurs Res, 2017. **66**(2): p. 123-133.
64. La Rosa, P.S., et al., *Patterned progression of bacterial populations in the premature infant gut.* Proc Natl Acad Sci U S A, 2014. **111**(34): p. 12522-7.
65. Elgin, T.G., S.L. Kern, and S.J. McElroy, *Development of the Neonatal Intestinal Microbiome and Its Association With Necrotizing Enterocolitis.* Clin Ther, 2016. **38**(4): p. 706-15.
66. Arboleya, S., et al., *Establishment and development of intestinal microbiota in preterm neonates.* FEMS Microbiol Ecol, 2012. **79**(3): p. 763-72.
67. Schwiertz, A., et al., *Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants.* Pediatric research, 2003. **54**(3): p. 393-9.
68. Denning, N.-L. and J.M. Prince, *Neonatal intestinal dysbiosis in necrotizing enterocolitis.* Molecular medicine (Cambridge, Mass), 2018. **24**(1): p. 4.

69. Berrington, J.E., et al., *The neonatal bowel microbiome in health and infection.* Curr Opin Infect Dis, 2014. **27**(3): p. 236-43.
70. Berrington, J.E., et al., *Gut microbiota in preterm infants: assessment and relevance to health and disease.* Arch Dis Child Fetal Neonatal Ed, 2013. **98**(4): p. F286-90.
71. Butel, M.J., et al., *Conditions of bifidobacterial colonization in preterm infants: a prospective analysis.* J Pediatr Gastroenterol Nutr, 2007. **44**(5): p. 577-82.
72. Panigrahi, P., *Probiotics and prebiotics in neonatal necrotizing enterocolitis: New opportunities for translational research.* Pathophysiology, 2014. **21**(1): p. 35-46.
73. Fanaroff, A.A., et al., *Incidence, presenting features, risk factors and significance of late onset septicemia in very low birth weight infants. The National Institute of Child Health and Human Development Neonatal Research Network.* Pediatr Infect Dis J, 1998. **17**(7): p. 593-8.
74. Mai, V., et al., *Distortions in development of intestinal microbiota associated with late onset sepsis in preterm infants.* PLoS One, 2013. **8**(1): p. e52876.
75. Wen, L. and A. Duffy, *Factors Influencing the Gut Microbiota, Inflammation, and Type 2 Diabetes.* The Journal of nutrition, 2017. **147**(7): p. 1468S-1475S.
76. Voreades, N., A. Kozil, and T.L. Weir, *Diet and the development of the human intestinal microbiome.* Frontiers in microbiology, 2014. **5**: p. 494.
77. Spor, A., O. Koren, and R. Ley, *Unravelling the effects of the environment and host genotype on the gut microbiome.* Nature reviews Microbiology, 2011. **9**(4): p. 279-90.
78. Mackie, R.I., A. Sghir, and H.R. Gaskins, *Developmental microbial ecology of the neonatal gastrointestinal tract.* The American journal of clinical nutrition, 1999. **69**(5): p. 1035S-1045S.
79. Odamaki, T., et al., *Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study.* BMC microbiology, 2016. **16**: p. 90.
80. Jandhyala, S.M., et al., *Role of the normal gut microbiota.* World journal of gastroenterology, 2015. **21**(29): p. 8787-803.
81. Nagpal, R., et al., *Ontogenesis of the Gut Microbiota Composition in Healthy, Full-Term, Vaginally Born and Breast-Fed Infants over the First 3 Years of Life: A Quantitative Bird's-Eye View.* Frontiers in microbiology, 2017. **8**: p. 1388.
82. Browne, H.P., et al., *Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation.* Nature, 2016. **533**(7604): p. 543-546.
83. Wampach, L., et al., *Colonization and Succession within the Human Gut Microbiome by Archaea, Bacteria, and Microeukaryotes during the First Year of Life.* Frontiers in microbiology, 2017. **8**: p. 738.
84. Stewart, C.J., et al., *Cesarean or Vaginal Birth Does Not Impact the Longitudinal Development of the Gut Microbiome in a Cohort of Exclusively Preterm Infants.* Frontiers in microbiology, 2017. **8**: p. 1008.
85. Stokholm, J., et al., *Maturation of the gut microbiome and risk of asthma in childhood.* Nature communications, 2018. **9**(1): p. 141.
86. Chu, D.M., et al., *Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery.* Nature medicine, 2017. **23**(3): p. 314-326.
87. Lee, S.A., et al., *Comparison of the gut microbiota profile in breast-fed and formula-fed Korean infants using pyrosequencing.* Nutrition research and practice, 2015. **9**(3): p. 242-8.
88. Martin, R., et al., *Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota.* PloS one, 2016. **11**(6): p. e0158498.
89. Timmerman, H.M., et al., *Intestinal colonisation patterns in breastfed and formula-fed infants during the first 12 weeks of life reveal sequential microbiota signatures.* Scientific reports, 2017. **7**(1): p. 8327.
90. Aloisio, I., et al., *Evaluation of the effects of intrapartum antibiotic prophylaxis on newborn intestinal microbiota using a sequencing approach targeted to multi hypervariable 16S rDNA regions.* Applied microbiology and biotechnology, 2016. **100**(12): p. 5537-46.
91. Backhed, F., et al., *Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life.* Cell host & microbe, 2015. **17**(6): p. 852.

92. Dominguez-Bello, M.G., et al., *Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(26): p. 11971-5.
93. Dominguez-Bello, M.G., et al., *Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer.* Nature medicine, 2016. **22**(3): p. 250-3.
94. Cox, L.M., et al., *Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences.* Cell, 2014. **158**(4): p. 705-721.
95. Negele, K., et al., *Mode of delivery and development of atopic disease during the first 2 years of life.* Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology, 2004. **15**(1): p. 48-54.
96. Fallani, M., et al., *Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics.* Journal of pediatric gastroenterology and nutrition, 2010. **51**(1): p. 77-84.
97. Kim, H.S. and N. Tamiya, *Isolation, properties and amino acid sequence of a long-chain neurotoxin, Acanthophis antarcticus b, from the venom of an Australian snake (the common death adder, Acanthophis antarcticus).* The Biochemical journal, 1981. **193**(3): p. 899-906.
98. Kim, K.H., et al., *Isolation of Clostridium difficile from the environment and contacts of patients with antibiotic-associated colitis.* The Journal of infectious diseases, 1981. **143**(1): p. 42-50.
99. Rousseau, C., et al., *Clostridium difficile carriage in healthy infants in the community: a potential reservoir for pathogenic strains.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 2012. **55**(9): p. 1209-15.
100. Gabriel, I., et al., *The influence of maternal vaginal flora on the intestinal colonization in newborns and 3-month-old infants.* The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians, 2018. **31**(11): p. 1448-1453.
101. Al-Jumaili, I.J., et al., *Incidence and origin of Clostridium difficile in neonates.* Journal of clinical microbiology, 1984. **19**(1): p. 77-8.
102. Guaraldi, F. and G. Salvatori, *Effect of breast and formula feeding on gut microbiota shaping in newborns.* Front Cell Infect Microbiol, 2012. **2**: p. 94.
103. Li, C., et al., *Immunomodulatory constituents of human breast milk and immunity from bronchiolitis.* Ital J Pediatr, 2017. **43**(1): p. 8.
104. Williams, J.E., et al., *Relationships Among Microbial Communities, Maternal Cells, Oligosaccharides, and Macronutrients in Human Milk.* J Hum Lact, 2017. **33**(3): p. 540-551.
105. Perez, P.F., et al., *Bacterial imprinting of the neonatal immune system: lessons from maternal cells?* Pediatrics, 2007. **119**(3): p. e724-32.
106. Pannaraj, P.S., et al., *Association Between Breast Milk Bacterial Communities and Establishment and Development of the Infant Gut Microbiome.* JAMA Pediatr, 2017. **171**(7): p. 647-654.
107. Bezirtzoglou, E., A. Tsiotsias, and G.W. Welling, *Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH).* Anaerobe, 2011. **17**(6): p. 478-82.
108. Benno, Y., K. Sawada, and T. Mitsuoka, *The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants.* Microbiol Immunol, 1984. **28**(9): p. 975-86.
109. Le Huerou-Luron, I., S. Blat, and G. Boudry, *Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects.* Nutr Res Rev, 2010. **23**(1): p. 23-36.
110. Stark, P.L. and A. Lee, *The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life.* J Med Microbiol, 1982. **15**(2): p. 189-203.
111. Cresci, G.A. and E. Bawden, *Gut Microbiome: What We Do and Don't Know.* Nutr Clin Pract, 2015. **30**(6): p. 734-46.

112. Hunt, K.M., et al., *Characterization of the diversity and temporal stability of bacterial communities in human milk.* PLoS One, 2011. **6**(6): p. e21313.
113. Anderson, G.H., S.A. Atkinson, and M.H. Bryan, *Energy and macronutrient content of human milk during early lactation from mothers giving birth prematurely and at term.* Am J Clin Nutr, 1981. **34**(2): p. 258-65.
114. Dritsakou, K., et al., *The impact of maternal- and neonatal-associated factors on human milk's macronutrients and energy.* J Matern Fetal Neonatal Med, 2017. **30**(11): p. 1302-1308.
115. Bauer, J. and J. Gerss, *Longitudinal analysis of macronutrients and minerals in human milk produced by mothers of preterm infants.* Clin Nutr, 2011. **30**(2): p. 215-20.
116. Nommsen, L.A., et al., *Determinants of energy, protein, lipid, and lactose concentrations in human milk during the first 12 mo of lactation: the DARLING Study.* Am J Clin Nutr, 1991. **53**(2): p. 457-65.
117. Makino, H., et al., *Mother-to-infant transmission of intestinal bifidobacterial strains has an impact on the early development of vaginally delivered infant's microbiota.* PLoS One, 2013. **8**(11): p. e78331.
118. Cong, X., et al., *Gut Microbiome Developmental Patterns in Early Life of Preterm Infants: Impacts of Feeding and Gender.* PLoS One, 2016. **11**(4): p. e0152751.
119. Magne, F., et al., *Low species diversity and high interindividual variability in faeces of preterm infants as revealed by sequences of 16S rRNA genes and PCR-temporal temperature gradient gel electrophoresis profiles.* FEMS Microbiol Ecol, 2006. **57**(1): p. 128-38.
120. Underwood, M.A., et al., *Prebiotic oligosaccharides in premature infants.* J Pediatr Gastroenterol Nutr, 2014. **58**(3): p. 352-60.
121. Sela, D.A., et al., *The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome.* Proc Natl Acad Sci U S A, 2008. **105**(48): p. 18964-9.
122. Turroni, F., et al., *Ability of Bifidobacterium breve to grow on different types of milk: exploring the metabolism of milk through genome analysis.* Appl Environ Microbiol, 2011. **77**(20): p. 7408-17.
123. Cabrera-Rubio, R., et al., *The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery.* Am J Clin Nutr, 2012. **96**(3): p. 544-51.
124. Forsgren, M., et al., *Late preterm birth has direct and indirect effects on infant gut microbiota development during the first six months of life.* Acta Paediatr, 2017. **106**(7): p. 1103-1109.
125. Chu, S., et al., *Periconceptional and Gestational Exposure to Antibiotics and Childhood Asthma.* PLoS One, 2015. **10**(10): p. e0140443.
126. Azad, M.B., et al., *Infant antibiotic exposure and the development of childhood overweight and central adiposity.* Int J Obes (Lond), 2014. **38**(10): p. 1290-8.
127. Hviid, A., H. Svanstrom, and M. Frisch, *Antibiotic use and inflammatory bowel diseases in childhood.* Gut, 2011. **60**(1): p. 49-54.
128. Metsala, J., et al., *Mother's and offspring's use of antibiotics and infant allergy to cow's milk.* Epidemiology, 2013. **24**(2): p. 303-9.
129. Bokulich, N.A., et al., *Antibiotics, birth mode, and diet shape microbiome maturation during early life.* Sci Transl Med, 2016. **8**(343): p. 343ra82.
130. Nogacka, A., et al., *Impact of intrapartum antimicrobial prophylaxis upon the intestinal microbiota and the prevalence of antibiotic resistance genes in vaginally delivered full-term neonates.* Microbiome, 2017. **5**(1): p. 93.
131. Hansen, S.M., et al., *Dynamics of Streptococcus agalactiae colonization in women during and after pregnancy and in their infants.* J Clin Microbiol, 2004. **42**(1): p. 83-9.
132. Arboleya, S., et al., *Impact of Prematurity and Perinatal Antibiotics on the Developing Intestinal Microbiota: A Functional Inference Study.* Int J Mol Sci, 2016. **17**(5).
133. Mazzola, G., et al., *Early Gut Microbiota Perturbations Following Intrapartum Antibiotic Prophylaxis to Prevent Group B Streptococcal Disease.* PLoS One, 2016. **11**(6): p. e0157527.

134. Corvaglia, L., et al., *Influence of Intrapartum Antibiotic Prophylaxis for Group B Streptococcus on Gut Microbiota in the First Month of Life.* J Pediatr Gastroenterol Nutr, 2016. **62**(2): p. 304-8.
135. Cotten, C.M., et al., *Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants.* Pediatrics, 2009. **123**(1): p. 58-66.
136. Abdel Ghany, E.A. and A.A. Ali, *Empirical antibiotic treatment and the risk of necrotizing enterocolitis and death in very low birth weight neonates.* Ann Saudi Med, 2012. **32**(5): p. 521-6.
137. Kuppala, V.S., et al., *Prolonged initial empirical antibiotic treatment is associated with adverse outcomes in premature infants.* J Pediatr, 2011. **159**(5): p. 720-5.
138. Alexander, V.N., V. Northrup, and M.J. Bizzarro, *Antibiotic exposure in the newborn intensive care unit and the risk of necrotizing enterocolitis.* J Pediatr, 2011. **159**(3): p. 392-7.
139. Esaiassen, E., et al., *Antibiotic exposure in neonates and early adverse outcomes: a systematic review and meta-analysis.* J Antimicrob Chemother, 2017. **72**(7): p. 1858-1870.
140. Fanaro, S., et al., *Intestinal microflora in early infancy: composition and development.* Acta Paediatr Suppl, 2003. **91**(441): p. 48-55.
141. Brooks, B., et al., *Microbes in the neonatal intensive care unit resemble those found in the gut of premature infants.* Microbiome, 2014. **2**(1): p. 1.
142. Touati, A., et al., *Outbreak of Acinetobacter baumannii in a neonatal intensive care unit: antimicrobial susceptibility and genotyping analysis.* Ann Epidemiol, 2009. **19**(6): p. 372-8.
143. Costeloe, K., et al., *A randomised controlled trial of the probiotic Bifidobacterium breve BBG-001 in preterm babies to prevent sepsis, necrotising enterocolitis and death: the Probiotics in Preterm infantS (PiPS) trial.* Health Technol Assess, 2016. **20**(66): p. 1-194.
144. Wang, Y., et al., *16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis.* ISME J, 2009. **3**(8): p. 944-54.
145. Taft, D.H., et al., *Intestinal microbiota of preterm infants differ over time and between hospitals.* Microbiome, 2014. **2**: p. 36.
146. Gritz, E.C. and V. Bhandari, *The human neonatal gut microbiome: a brief review.* Front Pediatr, 2015. **3**: p. 17.
147. Arrieta, M.C., et al., *The intestinal microbiome in early life: health and disease.* Front Immunol, 2014. **5**: p. 427.
148. Tin, W. and S. Gupta, *Optimum oxygen therapy in preterm babies.* Arch Dis Child Fetal Neonatal Ed, 2007. **92**(2): p. F143-7.
149. Gupta, R.W., et al., *Histamine-2 receptor blockers alter the fecal microbiota in premature infants.* Journal of pediatric gastroenterology and nutrition, 2013. **56**(4): p. 397-400.
150. Romaine, A., et al., *Safety of histamine-2 receptor blockers in hospitalized VLBW infants.* Early Hum Dev, 2016. **99**: p. 27-30.
151. Robinson, J., *Cochrane in context: probiotics for prevention of necrotizing enterocolitis in preterm infants.* Evid Based Child Health, 2014. **9**(3): p. 672-4.
152. Denkel, L.A., et al., *Protective Effect of Dual-Strain Probiotics in Preterm Infants: A Multi-Center Time Series Analysis.* PLoS One, 2016. **11**(6): p. e0158136.
153. Sun, J., et al., *Effects of Probiotics on Necrotizing Enterocolitis, Sepsis, Intraventricular Hemorrhage, Mortality, Length of Hospital Stay, and Weight Gain in Very Preterm Infants: A Meta-Analysis.* Adv Nutr, 2017. **8**(5): p. 749-763.
154. Patel, R.M. and P.W. Denning, *Intestinal microbiota and its relationship with necrotizing enterocolitis.* Pediatr Res, 2015. **78**(3): p. 232-8.
155. Vongbhavit, K. and M.A. Underwood, *Prevention of Necrotizing Enterocolitis Through Manipulation of the Intestinal Microbiota of the Premature Infant.* Clinical therapeutics, 2016. **38**(4): p. 716-32.
156. Costeloe, K., et al., *Bifidobacterium breve BBG-001 in very preterm infants: a randomised controlled phase 3 trial.* Lancet, 2016. **387**(10019): p. 649-60.

157. Underwood, M.A., *Impact of probiotics on necrotizing enterocolitis.* Semin Perinatol, 2017. **41**(1): p. 41-51.
158. Rhee, S.H., *Basic and translational understandings of microbial recognition by toll-like receptors in the intestine.* J Neurogastroenterol Motil, 2011. **17**(1): p. 28-34.
159. Lu, P., et al., *Animal models of gastrointestinal and liver diseases. Animal models of necrotizing enterocolitis: pathophysiology, translational relevance, and challenges.* Am J Physiol Gastrointest Liver Physiol, 2014. **306**(11): p. G917-28.
160. Murgas Torrazza, R. and J. Neu, *The developing intestinal microbiome and its relationship to health and disease in the neonate.* J Perinatol, 2011. **31 Suppl 1**: p. S29-34.
161. Neal, M.D., et al., *Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier.* J Immunol, 2006. **176**(5): p. 3070-9.
162. Sodhi, C.P., et al., *Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis.* Gastroenterology, 2010. **138**(1): p. 185-96.
163. Hackam, D.J., et al., *Innate immune signaling in the pathogenesis of necrotizing enterocolitis.* Clin Dev Immunol, 2013. **2013**: p. 475415.
164. Markel, T.A., et al., *Cytokines in necrotizing enterocolitis.* Shock, 2006. **25**(4): p. 329-37.
165. Sharma, R., et al., *Neonatal gut barrier and multiple organ failure: role of endotoxin and proinflammatory cytokines in sepsis and necrotizing enterocolitis.* J Pediatr Surg, 2007. **42**(3): p. 454-61.
166. Claud, E.C., et al., *Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation.* Proc Natl Acad Sci U S A, 2004. **101**(19): p. 7404-8.
167. Spehlmann, M.E. and L. Eckmann, *Nuclear factor-kappa B in intestinal protection and destruction.* Curr Opin Gastroenterol, 2009. **25**(2): p. 92-9.
168. Neish, A.S., *Molecular aspects of intestinal epithelial cell-bacterial interactions that determine the development of intestinal inflammation.* Inflamm Bowel Dis, 2004. **10**(2): p. 159-68.
169. Neish, A.S., et al., *Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination.* Science, 2000. **289**(5484): p. 1560-3.
170. Mizrahi, A., et al., *Necrotizing Enterocolitis in Premature Infants.* J Pediatr, 1965. **66**: p. 697-705.
171. Fanaroff, A.A., et al., *Trends in neonatal morbidity and mortality for very low birthweight infants.* Am J Obstet Gynecol, 2007. **196**(2): p. 147 e1-8.
172. Stoll, B.J., et al., *Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network.* Pediatrics, 2010. **126**(3): p. 443-56.
173. Kamitsuka, M.D., M.K. Horton, and M.A. Williams, *The incidence of necrotizing enterocolitis after introducing standardized feeding schedules for infants between 1250 and 2500 grams and less than 35 weeks of gestation.* Pediatrics, 2000. **105**(2): p. 379-84.
174. Nowicki, P.T. and C.A. Nankervis, *The role of the circulation in the pathogenesis of necrotizing enterocolitis.* Clin Perinatol, 1994. **21**(2): p. 219-34.
175. Tyson, J.E. and K.A. Kennedy, *Minimal enteral nutrition for promoting feeding tolerance and preventing morbidity in parenterally fed infants.* Cochrane Database Syst Rev, 2000(2): p. CD000504.
176. Sullivan, S., et al., *An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products.* J Pediatr, 2010. **156**(4): p. 562-7 e1.
177. Reisinger, K.W., et al., *Breast-feeding improves gut maturation compared with formula feeding in preterm babies.* J Pediatr Gastroenterol Nutr, 2014. **59**(6): p. 720-4.
178. Ren, H., et al., *Stem Cell Factor/Kit Signal Insufficiency Contributes to Hypoxia-Induced Intestinal Motility Dysfunctions in Neonatal Mice.* Digestive diseases and sciences, 2017. **62**(5): p. 1193-1203.

179. Deplancke, B. and H.R. Gaskins, *Microbial modulation of innate defense: goblet cells and the intestinal mucus layer.* The American journal of clinical nutrition, 2001. **73**(6): p. 1131S-1141S.
180. Anand, R.J., et al., *The role of the intestinal barrier in the pathogenesis of necrotizing enterocolitis.* Shock (Augusta, Ga), 2007. **27**(2): p. 124-33.
181. Watkins, D.J. and G.E. Besner, *The role of the intestinal microcirculation in necrotizing enterocolitis.* Seminars in pediatric surgery, 2013. **22**(2): p. 83-7.
182. Nino, D.F., C.P. Sodhi, and D.J. Hackam, *Necrotizing enterocolitis: new insights into pathogenesis and mechanisms.* Nat Rev Gastroenterol Hepatol, 2016. **13**(10): p. 590-600.
183. Caplan, M.S., D. Simon, and T. Jilling, *The role of PAF, TLR, and the inflammatory response in neonatal necrotizing enterocolitis.* Semin Pediatr Surg, 2005. **14**(3): p. 145-51.
184. Frost, B.L. and M.S. Caplan, *Necrotizing enterocolitis: pathophysiology, platelet-activating factor, and probiotics.* Semin Pediatr Surg, 2013. **22**(2): p. 88-93.
185. Maheshwari, A., et al., *TGF-beta2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine.* Gastroenterology, 2011. **140**(1): p. 242-53.
186. MohanKumar, K., et al., *Smad7 interrupts TGF-beta signaling in intestinal macrophages and promotes inflammatory activation of these cells during necrotizing enterocolitis.* Pediatr Res, 2016. **79**(6): p. 951-61.
187. Kanto, W.P., Jr., J.E. Hunter, and B.J. Stoll, *Recognition and medical management of necrotizing enterocolitis.* Clin Perinatol, 1994. **21**(2): p. 335-46.
188. Heng, Y., et al., *Pneumatosis intestinalis: a review.* Am J Gastroenterol, 1995. **90**(10): p. 1747-58.
189. Rich, B.S. and S.E. Dolgin, *Necrotizing Enterocolitis.* Pediatr Rev, 2017. **38**(12): p. 552-559.
190. Fitzgibbons, S.C., et al., *Mortality of necrotizing enterocolitis expressed by birth weight categories.* J Pediatr Surg, 2009. **44**(6): p. 1072-5; discussion 1075-6.
191. Hintz, S.R., et al., *Neurodevelopmental and growth outcomes of extremely low birth weight infants after necrotizing enterocolitis.* Pediatrics, 2005. **115**(3): p. 696-703.
192. Salhab, W.A., et al., *Necrotizing enterocolitis and neurodevelopmental outcome in extremely low birth weight infants <1000 g.* J Perinatol, 2004. **24**(9): p. 534-40.
193. Bisquera, J.A., T.R. Cooper, and C.L. Berseth, *Impact of necrotizing enterocolitis on length of stay and hospital charges in very low birth weight infants.* Pediatrics, 2002. **109**(3): p. 423-8.
194. Kuschel, C.A. and J.E. Harding, *Multicomponent fortified human milk for promoting growth in preterm infants.* Cochrane Database Syst Rev, 2004(1): p. CD000343.
195. Bury, R.G. and D. Tudehope, *Enteral antibiotics for preventing necrotizing enterocolitis in low birthweight or preterm infants.* Cochrane Database Syst Rev, 2001(1): p. CD000405.
196. AlFaleh, K. and J. Anabrees, *Probiotics for prevention of necrotizing enterocolitis in preterm infants.* Cochrane Database Syst Rev, 2014. **4**: p. CD005496.
197. Bin-Nun, A., et al., *Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates.* J Pediatr, 2005. **147**(2): p. 192-6.
198. Dani, C., et al., *Probiotics feeding in prevention of urinary tract infection, bacterial sepsis and necrotizing enterocolitis in preterm infants. A prospective double-blind study.* Biol Neonate, 2002. **82**(2): p. 103-8.
199. Hoyos, A.B., *Reduced incidence of necrotizing enterocolitis associated with enteral administration of Lactobacillus acidophilus and Bifidobacterium infantis to neonates in an intensive care unit.* Int J Infect Dis, 1999. **3**(4): p. 197-202.
200. Lin, H.C., et al., *Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial.* Pediatrics, 2008. **122**(4): p. 693-700.
201. Lin, H.C., et al., *Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants.* Pediatrics, 2005. **115**(1): p. 1-4.
202. Samanta, M., et al., *Prophylactic probiotics for prevention of necrotizing enterocolitis in very low birth weight newborns.* J Trop Pediatr, 2009. **55**(2): p. 128-31.

203. Sari, F.N., et al., *Oral probiotics: Lactobacillus sporogenes for prevention of necrotizing enterocolitis in very low-birth weight infants: a randomized, controlled trial.* Eur J Clin Nutr, 2011. **65**(4): p. 434-9.
204. Schmolzer, G., et al., *Multi-modal approach to prophylaxis of necrotizing enterocolitis: clinical report and review of literature.* Pediatr Surg Int, 2006. **22**(7): p. 573-80.
205. Adlerberth, I. and A.E. Wold, *Establishment of the gut microbiota in Western infants.* Acta Paediatr, 2009. **98**(2): p. 229-38.
206. Eckburg, P.B., et al., *Diversity of the human intestinal microbial flora.* Science, 2005. **308**(5728): p. 1635-8.
207. Ardissone, A.N., et al., *Meconium microbiome analysis identifies bacteria correlated with premature birth.* PLoS One, 2014. **9**(3): p. e90784.
208. Chassard, C., T. de Wouters, and C. Lacroix, *Probiotics tailored to the infant: a window of opportunity.* Curr Opin Biotechnol, 2014. **26**: p. 141-7.
209. Mai, V., et al., *Fecal microbiota in premature infants prior to necrotizing enterocolitis.* PLoS One, 2011. **6**(6): p. e20647.
210. Morrow, A.L., et al., *Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants.* Microbiome, 2013. **1**(1): p. 13.
211. Lu, C.Y. and Y.H. Ni, *Gut microbiota and the development of pediatric diseases.* Journal of Gastroenterology, 2015. **50**(7): p. 720-726.
212. Pammi, M., et al., *Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis.* Microbiome, 2017. **5**(1): p. 31.
213. Kurath-Koller, S., et al., *Hospital Regimens Including Probiotics Guide the Individual Development of the Gut Microbiome of Very Low Birth Weight Infants in the First Two Weeks of Life.* Nutrients, 2020. **12**(5).
214. Kurath-Koller, S., et al., *Changes of intestinal microbiota composition and diversity in very low birth weight infants related to strategies of NEC prophylaxis: protocol for an observational multicentre pilot study.* Pilot Feasibility Stud, 2017. **3**: p. 52.
215. Klindworth, A., et al., *Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies.* Nucleic Acids Res, 2013. **41**(1): p. e1.
216. Klymiuk, I., et al., *The Human Gastric Microbiome Is Predicated upon Infection with Helicobacter pylori.* Front Microbiol, 2017. **8**: p. 2508.
217. Nadkarni, M.A., et al., *Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set.* Microbiology, 2002. **148**(Pt 1): p. 257-266.
218. Pausan, M.K.-K.V.R., GL; Madl, T; Giselbrecht, E; Weiss, EC; Jantscher-Krenn, E; Moissl-Eichinger, C., *Human Milk Oligosaccharides modulate the risk for preterm birth in a microbiome dependent and independent manner*. 2019, biorxiv: biorxiv.org.
219. Bolyen, E., et al., *Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.* Nat Biotechnol, 2019. **37**(8): p. 852-857.
220. Mahnert, A., et al., *Leaves of Indoor Ornamentals Are Biodiversity and Functional Hotspots for Fungi.* Front Microbiol, 2018. **9**: p. 2343.
221. Davis, N.M., et al., *Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data.* Microbiome, 2018. **6**(1): p. 226.
222. Zakrzewski, M., et al., *Calypso: a user-friendly web-server for mining and visualizing microbiome-environment interactions.* Bioinformatics, 2017. **33**(5): p. 782-783.
223. Wickham, H. and G. Grolemund, *R for data science : import, tidy, transform, visualize, and model data*. First edition. ed. 2016, Sebastopol, CA: O'Reilly. xxv, 492 pages.
224. Wickham, H., *Ggplot2 : elegant graphics for data analysis*. Use R! 2009, New York: Springer. viii, 212 p.
225. Chernikova, D.A., et al., *The premature infant gut microbiome during the first 6 weeks of life differs based on gestational maturity at birth.* Pediatric Research, 2018. **84**(1): p. 71-79.
226. Scott, S.A., et al., *The formation of thermophilic spores during the manufacture of whole milk powder.* International Journal of Dairy Technology, 2007. **60**(2): p. 109-117.

227. Reid, G., et al., *Responders and non-responders to probiotic interventions: how can we improve the odds?* Gut Microbes, 2010. **1**(3): p. 200-4.
228. Underwood, M.A., et al., *A Comparison of Two Probiotic Strains of Bifidobacteria in Premature Infants.* Journal of Pediatrics, 2013. **163**(6): p. 1585-U114.
229. Bisgaard, H., et al., *Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age.* Journal of Allergy and Clinical Immunology, 2011. **128**(3): p. 646-U318.
230. Rotimi, V.O., S.A. Olowe, and I. Ahmed, *The development of bacterial flora of premature neonates.* J Hyg (Lond), 1985. **94**(3): p. 309-18.
231. Martin, R., et al., *Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women.* Res Microbiol, 2007. **158**(1): p. 31-7.
232. Olm, M.R., et al., *Necrotizing enterocolitis is preceded by increased gut bacterial replication, Klebsiella, and fimbriae-encoding bacteria.* Sci Adv, 2019. **5**(12): p. eaax5727.

8 APPENDIX

8.1 Parental information Sheet

Einwilligungsblatt samt Information
Mikrobiomanalyse Frühgeborener <1500g

Liebe Eltern!

Neunzig Prozent der Zellen im Körper sind Mikroorganismen. Sie leben auf unserer Haut, in Mund und Nase und v.a. in unserem Verdauungssystem, wo sie etwa 1-2 kg Biomasse ausmachen. Die Gesamtheit der menschlichen Mikroorganismen hat außerordentlichen Einfluss auf unseren Körper – und entscheidet unter Umständen über unsere Gesundheit und Wohlbefinden.

Bei Frühgeborenen Kindern spielen Darmkeime (Mikroorganismen des Darms) eine wichtige Rolle. Zur Verhinderung schwerer Erkrankungen wie der nekrotisierenden Enterokolitis (einer Entzündung des Darms des Frühgeborenen) werden Antibiotika und sogenannte Probiotika verwendet. Probiotika sind lebende mikrobielle Nahrungszusätze die den Menschen positiv beeinflussen (Milchsäurebakterien, Bifidobakterien usw.).

Mit Hilfe unserer Studie (Stuhlprobenuntersuchung bei Frühgeborenen mit <1500g Geburtsgewicht) möchten wir untersuchen wie sich die Zusammensetzung der Mikrobakterien im Darm frühgeborener Kinder im Verlauf der ersten Lebenswochen verändert. Die Erkenntnisse dieser Studie sollen dazu beitragen in Zukunft die Entwicklung der Darmflora frühgeborener Kinder durch angepasste Therapiestrategien positiv beeinflussen zu können.

Im Rahmen der Studienteilnahme werden aus dem Stuhl den Ihr Kind von selbst absetzt mittels eines sterilen Spatels Proben aus der Windel entnommen und tiefgefroren. In weiterer Folge werden diese Proben auf die Zusammensetzung und Verteilung der darin enthaltenen Darmkeime untersucht. Die Sammlung der Proben erfolgt alle 2 Tage einmalig während der ersten Lebenswochen. Insgesamt werden 7 Stuhlproben entnommen. Jede Probe wird in 2 Probengefäßen gesammelt. Von diesen wird eines zu Untersuchungszwecken verwendet, das zweite für eventuelle spätere Untersuchungen gelagert. Ihrem Kind wird durch die Probensammlung weder Schaden zugefügt, noch wird der Stuhlgang zu Zwecken der Probengewinnung stimuliert. Die Versorgung, Therapie und Pflege Ihres Kindes werden durch die Teilnahme an der Studie nicht verändert, sondern finden in gleicher Weise statt wie sie auch ohne Studienteilnahme erfolgen.

Wir laden Sie ein, Ihr Kind an der oben genannten Studie teilnehmen zu lassen. Die Teilnahme an der Studie erfolgt ausschließlich freiwillig, unentgeltlich und kann jederzeit ohne Angabe von Gründen beendet werden.

Bitte unterschreiben Sie die Einwilligungserklärung nur wenn:

- Sie Art und Ablauf der Studie vollständig verstanden haben und dieser zustimmen
- Sie einverstanden sind, Ihr Kind an unserer Studie teilnehmen zu lassen
- Sie einverstanden sind, dass die entnommenen Proben gelagert und für eventuelle spätere Untersuchungen verwendet werden dürfen und
- Sie sich über Ihre Rechte als Elternteil/Eltern des an dieser Studie teilnehmenden Kindes im Klaren sind.

Studienleiter: Univ. Ass. Dr. Stefan Kurath-Koller
(Tel: 0316 385 82906)

Ich habe die Informationen gelesen und verstanden. Alle meine Fragen wurden zu meiner Zufriedenheit beantwortet und ich habe zurzeit keine weiteren Fragen mehr.

Ich willige hiermit freiwillig ein, dass der Stuhl meines Kindes zu Studienzwecken, wie oben angeführt, gesammelt und verwendet werden darf.

Name des erziehungsberechtigten Elternteils (Blockschrift):

_____

Name und Geburtsdatum des Kindes (Blockschrift):

_____

Ort/Datum: _____

Unterschrift: _____

Aufklärender Arzt:

Name (Blockschrift): _____

Ort/Datum: _____

Unterschrift: _____

8.2 Standardized operating procedures

Standard Operating Procedure (SOP) stool sample collection – University Hospital Graz

Vor Sammlung der Stuhlproben muss sichergestellt werden dass das „Einwilligungsblattes samt Information“ vollständig ausgefüllt und sowohl vom aufklärenden Arzt/der aufklärenden Ärztin sowie dem Elternteil/den Eltern des teilnehmenden Kindes unterschrieben vorliegt.

Jedem Inkubatorbett eines an der Studie teilnehmenden Kindes liegt eine Klarsichthülle bei. In dieser finden sich das „Einwilligungsblatt samt Information“, das Studienprotokollblatt des jeweiligen Kindes, sowie mittels Büroklammer daran angeheftet ein Bogen mit zur Probenbeschriftung zu verwendenden abziehbaren und fortlaufend nummerierten Aufklebern. Das Studienprotokollblatt ist mit Name, Geburtsdatum und Geburtsgewicht des teilnehmenden Kindes zu versehen.

Während des gesamten Vorgangs der Stuhlprobenentnahme müssen aus hygienischen Gründen Einmalhandschuhe getragen werden. An jedem Entnahmetag (siehe Studienprotokollblatt des jeweiligen Kindes) werden mittels sterilem Mikrospatel (Steri Ware Microspatula, Sampling Systems Ltd., UK) aus derselben spontan in die Windel abgesetzten Stuhlmasse 2 (zwei) Proben á 2ml (zwei Milliliter) entnommen und in jeweils einen Probencontainer (2ml CryoTube™ Vials; Thermo Scientific, Denmark) verbracht. Die Probencontainer werden anschließend gut verschlossen und mittels Kipptest (einmaliges Umdrehen des verschlossenen Gefäßes) visuell auf Dichtigkeit überprüft.

Die abziehbaren Aufkleber des Bogens zur Probenbeschriftung sind fortlaufend nummeriert und müssen nach Probenentnahme auf die Probencontainer aufgebracht werden. Für jeden Entnahmetag stehen zwei Aufkleber, entsprechend den zwei zu befüllenden Probencontainern, zur Verfügung. Datum der Entnahme, sowie die Probennummern müssen am Studienprotokollblatt in den dafür vorgesehenen Feldern eingetragen werden.

Der gefüllte, verschlossene und beklebte Probencontainer wird umgehend in Trockeneis gebettet und mit diesem zusammen in einer Styroporbox gelagert. Die Styroporbox wird schnellst möglich vom hausinternen Botendienst an das Labor im Hause (2. Untergeschoß) verbracht, und die Probencontainer dort nach Einbringen in

eine Cryo Box (Thermo Scientific™ Lagerbox, Thermo Fisher Scientific Inc., Waltham, MA USA) im Kühlschrank (FRYKA Kühl-und Gefrierbox B 35-85, Fryka Kältetechnik GmbH, Esslingen, Germany) bei -80°C gelagert.

Beim Einbringen der Cryo Box in den Kühlschrank ist folgendes Vorgehen zu beachten:

- *Entsperren des Türschlosses sowie Öffnen des zusätzlichen Sicherungshebels, wie in der Bedienungsanleitung vorgeschrieben, vor Betätigen des Türgriffs und Öffnens des Schrankes.*
- *Die Öffnungsdauer möglichst kurz halten um den Temperaturanstieg zu minimieren.*
- *Nach Einbringen der Cryo Box in die dafür vorgesehene Halterung innerhalb des Kühlschrankes erfolgt das sachgerechte Verschließen des Schrankes, wie in der Bedienungsanleitung vorgesehen, in rückwärtiger Reihenfolge der oben genannten Schritte (Schließen der Tür mittels des Türgriffes – Verriegeln der Tür mittels Sicherungshebel – Versperren des Türschlosses)*

<u>Vorgehen wenn am Entnahmetag kein Stuhlgang vorliegt:</u>

- *Den nächsten verfügbaren Stuhlgang verwenden und ab diesem Tag im Protokoll weiter gehen. Beispiel: Stuhlentnahme für Dienstag geplant (weitere Entnahmen Donnerstag, Samstag,…), zu diesem Zeitpunkt jedoch kein Stuhl. Nächster Stuhl des Kindes am darauffolgenden Tag, Mittwoch. Der Stuhl am Mittwoch wird verwendet und die weiteren Entnahmen erfolgen weiterhin jeden 2. Tag (im Beispielsfall Freitag, Sonntag,…).*
- *Liegt auch an den folgenden 2 Tagen kein Stuhlgang vor hat der Ausschluss des Kindes aus der Studie zu erfolgen!*

<u>Verwendbares Material:</u>

- *Jeder Stuhl des Kindes, beginnend mit dem Mekonium wird verwendet. Auch Stuhl nach vorherig erfolgter Darmspülung kann verwendet werden. Es gibt keine Mengenuntergrenze! Sollte nur ein kleines Stuhlkügelchen vorhanden sein dieses in Probenröhrchen A füllen und in diesem Fall Röhrchen B leer belassen.*

Standard Operating Procedure (SOP) stool sample collection – General Hospital Klagenfurt

Vor Sammlung der Stuhlproben muss sichergestellt werden dass das „Einwilligungsblattes samt Information“ vollständig ausgefüllt und sowohl vom aufklärenden Arzt/der aufklärenden Ärztin sowie dem Elternteil/den Eltern des teilnehmenden Kindes unterschrieben vorliegt.

Jedem Inkubatorbett eines an der Studie teilnehmenden Kindes liegt eine Klarsichthülle bei. In dieser finden sich das „Einwilligungsblatt samt Information“, das Studienprotokollblatt des jeweiligen Kindes, sowie mittels Büroklammer daran angeheftet ein Bogen mit zur Probenbeschriftung zu verwendenden abziehbaren und fortlaufend nummerierten Aufklebern. Das Studienprotokollblatt ist mit Name, Geburtsdatum und Geburtsgewicht des teilnehmenden Kindes zu versehen.

Während des gesamten Vorgangs der Stuhlprobenentnahme müssen aus hygienischen Gründen Einmalhandschuhe getragen werden. An jedem Entnahmetag (siehe Studienprotokollblatt des jeweiligen Kindes) werden mittels sterilem Mikrospatel (Steri Ware Microspatula, Sampling Systems Ltd., UK) aus derselben spontan in die Windel abgesetzten Stuhlmasse 2 (zwei) Proben á 2ml (zwei Milliliter) entnommen und in jeweils einen Probencontainer (2ml CryoTube™ Vials; Thermo Scientific, Denmark) verbracht. Die Probencontainer werden anschließend gut verschlossen und mittels Kipptest (einmaliges Umdrehen des verschlossenen Gefäßes) visuell auf Dichtigkeit überprüft.

Die abziehbaren Aufkleber des Bogens zur Probenbeschriftung sind fortlaufend nummeriert und müssen nach Probenentnahme auf die Probencontainer aufgebracht werden. Für jeden Entnahmetag stehen zwei Aufkleber, entsprechend den zwei zu befüllenden Probencontainern, zur Verfügung. Datum der Entnahme, sowie die Probennummern müssen am Studienprotokollblatt in den dafür vorgesehenen Feldern eingetragen werden.

Das gefüllte und verschlossene Stuhlgefäß wird umgehend in Trockeneis gebettet und mit diesem zusammen in einer Styroporbox gelagert. Die Styroporbox wird umgehend von hausinternen Botendienst an das Zentrallabor, Institut für Labordiagnostik und Mikrobiologie, Ver- und Entsorgungszentrum (VEZ) verbracht und die Probencontainer dort nach Einbringen in eine Cryo Box (Thermo Scientific™ Lagerbox, Thermo Fisher

Scientific Inc., Waltham, MA USA) im Kühlschrank (FRYKA Kühl-und Gefrierbox B 35-85, Fryka Kältetechnik GmbH, Esslingen, Germany) bei -80°C gelagert.

Beim Einbringen der Cryo Box in den Kühlschrank ist folgendes Vorgehen zu beachten:

- *Entsperren des Türschlosses sowie Öffnen des zusätzlichen Sicherungshebels, wie in der Bedienungsanleitung vorgeschrieben, vor Betätigen des Türgriffs und Öffnens des Schrankes.*
- *Die Öffnungsdauer möglichst kurz halten um den Temperaturanstieg zu minimieren.*
- *Nach Einbringen der Cryo Box in die dafür vorgesehene Halterung innerhalb des Kühlschrankes erfolgt das sachgerechte Verschließen des Schrankes wie in der Bedienungsanleitung vorgesehen in rückwärtiger Reihenfolge der oben genannten Schritte (Schließen der Tür mittels des Türgriffes – Verriegeln der Tür mittels Sicherungshebel – Versperren des Türschlosses)*

<u>Vorgehen wenn am Entnahmetag kein Stuhlgang vorliegt:</u>

- *Den nächsten verfügbaren Stuhlgang verwenden und ab diesem Tag im Protokoll weiter gehen. Beispiel: Stuhlentnahme für Dienstag geplant (weitere Entnahmen Donnerstag, Samstag,…), zu diesem Zeitpunkt jedoch kein Stuhl. Nächster Stuhl des Kindes am darauffolgenden Tag, Mittwoch. Der Stuhl am Mittwoch wird verwendet und die weiteren Entnahmen erfolgen weiterhin jeden 2. Tag (im Beispielsfall Freitag, Sonntag,…).*
- *Liegt auch an den folgenden 2 Tagen kein Stuhlgang vor hat der Ausschluss des Kindes aus der Studie zu erfolgen!*

<u>Verwendbares Material:</u>

- *Jeder Stuhl des Kindes, beginnend mit dem Mekonium wird verwendet. Auch Stuhl nach vorherig erfolgter Darmspülung kann verwendet werden. Es gibt keine Mengenuntergrenze! Sollte nur ein kleines Stuhlkügelchen vorhanden sein dieses in Probenröhrchen A füllen und in diesem Fall Röhrchen B leer belassen.*

Standard Operating Procedure (SOP) stool sample collection – General Hospital Leoben

Vor Sammlung der Stuhlproben muss sichergestellt werden, dass das „Einwilligungsblattes samt Information“ vollständig ausgefüllt und sowohl vom aufklärenden Arzt/der aufklärenden Ärztin sowie dem Elternteil/den Eltern des teilnehmenden Kindes unterschrieben vorliegt.
Jedem Inkubatorbett eines an der Studie teilnehmenden Kindes liegt eine Klarsichthülle bei. In dieser finden sich das „Einwilligungsblatt samt Information“, das Studienprotokollblatt des jeweiligen Kindes, sowie mittels Büroklammer daran angeheftet ein Bogen mit zur Probenbeschriftung zu verwendenden abziehbaren und fortlaufend nummerierten Aufklebern. Das Studienprotokollblatt ist mit Name, Geburtsdatum und Geburtsgewicht des teilnehmenden Kindes zu versehen.
Während des gesamten Vorgangs der Stuhlprobenentnahme müssen aus hygienischen Gründen Einmalhandschuhe getragen werden. An jedem Entnahmetag (siehe Studienprotokollblatt des jeweiligen Kindes) werden mittels Mikrospatel aus derselben spontan in die Windel abgesetzten Stuhlmasse 2 (zwei) Proben á 2ml (zwei Milliliter) entnommen und in jeweils einen Probencontainer (2ml CryoTube™ Vials; Thermo Scientific, Denmark) verbracht. Die Probencontainer werden anschließend gut verschlossen und mittels Kipptest (einmaliges Umdrehen des verschlossenen Gefäßes) visuell auf Dichtigkeit überprüft.
Die abziehbaren Aufkleber des Bogens zur Probenbeschriftung sind fortlaufend nummeriert und müssen nach Probenentnahme auf die Probencontainer aufgebracht werden. Für jeden Entnahmetag stehen zwei Aufkleber, entsprechend den zwei zu befüllenden Probencontainern, zur Verfügung. Datum der Entnahme, sowie die Probennummern müssen am Studienprotokollblatt in den dafür vorgesehenen Feldern eingetragen werden.
Der gefüllte, verschlossene und beklebte Probencontainer wird umgehend im Tiefkühlfach der Station (-20°C) gelagert. Die Prben müssen schnellstmöglich, spätestens jedoch nach 72h, bei -80°C gelagert werden.

Vorgehen wenn am Entnahmetag kein Stuhlgang vorliegt:

- *Den nächsten verfügbaren Stuhlgang verwenden und ab diesem Tag im Protokoll weiter gehen. Beispiel: Stuhlentnahme für Dienstag geplant (weitere Entnahmen Donnerstag, Samstag,…), zu diesem Zeitpunkt jedoch kein Stuhl.*

Nächster Stuhl des Kindes am darauffolgenden Tag, Mittwoch. Der Stuhl am Mittwoch wird verwendet und die weiteren Entnahmen erfolgen weiterhin jeden 2. Tag (im Beispielsfall Freitag, Sonntag,…).

- *Liegt auch an den folgenden 2 Tagen kein Stuhlgang vor hat der Ausschluss des Kindes aus der Studie zu erfolgen!*

<u>Verwendbares Material:</u>

- *Jeder Stuhl des Kindes, beginnend mit dem Mekonium wird verwendet. Auch Stuhl nach vorherig erfolgter Darmspülung kann verwendet werden. Es gibt keine Mengenuntergrenze! Sollte nur ein kleines Stuhlkügelchen vorhanden sein dieses in Probenröhrchen A füllen und in diesem Fall Röhrchen B leer belassen.*