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Antibiotics and probiotics to neonates

Adverse effects, impact on gut microbiota and antibiotic resistome, and Bifidobacterium pathogenicity

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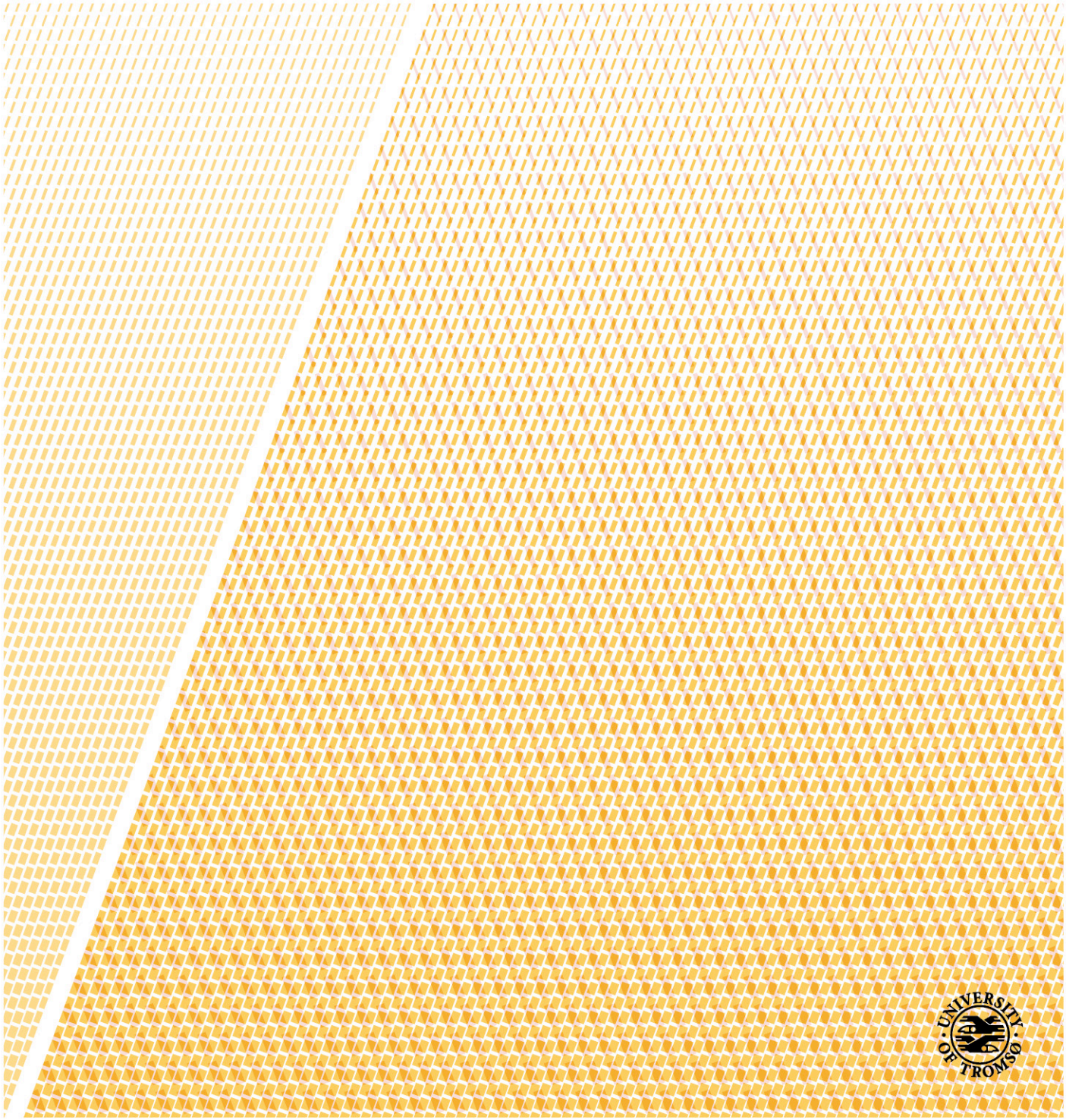
A dissertation for the degree of Philosophiae Doctor – XXXX



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Esaiassen E, Fjalstad JW, Juvet LK, van den Anker JN, Klingenberg C. Antibiotic exposure in neonates and early adverse outcomes: a systematic review and meta-analysis. J Antimicrob Chemother. 2017; 72: 1858-70

Paper II

Fjalstad JW, Esaiassen E, Juvet LK, van den Anker JN, Klingenberg C. Antibiotic Therapy in Neonates and Impact on Gut Microbiota and Antibiotic Resistance Development: A Systematic Review. Accepted for publication in J Antimicrob Chemother. 17th of October 2017

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Esaiassen E, Hjerde E, Cavanagh P, Pedersen T, Andresen J, Rettedal S, Støen R, Nakstad B, Willasen NP, Klingenberg C. Probiotic Supplementation and Development of Preterm Infant Gut Microbiota and Antibiotic Resistome- An Observational Multi-Center Study**.** Submitted 20th of October 2017.

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Esaiassen E, Hjerde E, Cavanagh JP, Simonsen GS, Klingenberg C; Norwegian Study Group on Invasive Bifidobacterial Infections. *Bifidobacterium* bacteremia: Clinical Characteristics and a Genomic Approach to Assess Pathogenicity. J Clin Microbiol. 2017; 55: 2234-48

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Abbreviations

ARG	Antibiotic resistance gene
CFU	Colony forming unit
EBM	Evidence-based medicine
ELBW	Extremely low birth weight infants
EOS	Early onset sepsis
ESBL	Extended-spectrum beta-lactamases
GA	Gestational age
HGT	Horizontal gene transfer
HMO	Human milk oligosaccharides
IFI	Invasive fungal infections
LOS	Late onset sepsis
LPS	Lipopolysaccharide
MDR	Multidrug resistant
NEC	Necrotizing enterocolitis
NRS	Non-randomized studies
NORM	Norwegian Surveillance System for Antimicrobial Drug Resistance
OTU	Operational taxonomic unit
PMA	Postmenstrual age
TLR	Toll-like receptor
VLBW	Very low birth weight infant
WGS	Whole genome sequencing

Abstract

Background and Aims: Antibiotics are the most commonly prescribed drugs in neonatal intensive care units (NICUs). Gut dysbiosis, often induced by antibiotics, and a sudden shift in the microbiota composition is associated with development of necrotizing enterocolitis (NEC) in preterm infants. Probiotics may reduce the incidence of NEC. Still, little is known about the impact of probiotics on early development of gut microbiota composition and resistome in preterm infants supplemented with probiotics. Despite the many health benefits proposed by probiotic bacteria, an increasing number of *Bifidobacterium* bacteraemia episodes have been reported lately, but the pathogenicity of *Bifidobacterium* remains to be elucidated.

The main objective of this thesis was to systematically review potential side effects of antibiotic therapy in neonates and to study in-depth the gut microbiota composition of preterm infants receiving probiotic prophylaxis. My first aim was to perform a systematic review on studies reporting on different categories of antibiotic exposure in neonates and subsequent risks of developing early adverse outcomes (Paper I-II). My second aim was to assess influence of probiotics and antibiotics on gut microbiota composition and resistome in preterm infants (Paper III). Finally, I aimed to study the pathogenic potential of *Bifidobacterium* (Paper IV).

Material and Methods: In the systematic review (Paper I-II), searches were conducted in PubMed, Embase, Medline and the Cochrane Database. Randomised controlled trials (RCTs) and non-randomised studies (NRSs) were eligible for inclusion if they reported on patient groups with different levels of antibiotic exposure in the neonatal period and the outcomes NEC, invasive fungal infections (IFI), death, changes in gut microbiota and/or antibiotic resistance development. When appropriate, meta-analyses using the random effect model or semi-quantitative vote counting were conducted.

In a prospective, longitudinal observational multi-centre clinical trial (Paper III) we enrolled 76 infants from six Norwegian NICUs; 31 probiotic supplemented extremely preterm (PEP) infants < 28 weeks gestation, 35 non-probiotic supplemented very preterm (NPVP) infants 28-31 weeks gestation and 10 full term control (FTC) infants. Faecal samples were collected at 7 days, 28 days and 4 months of age, and analysed with random shotgun metagenome sequencing and subsequent advanced bioinformatic statistics.

In a retrospective cohort study (Paper IV) we investigated *Bifidobacterium* isolates from 15 patients with bacteraemia. We collected detailed clinical characteristics and performed whole genome shotgun sequencing on all blood culture isolates. We also performed a pan-genomic comparison

of invasive and non-invasive *B. longum* isolates based on 65 sequences available from GenBank and the sequences of 11 blood culture isolates from this study.

Results: In the systematic review there was a lack of RCTs and high quality NRSs. Moreover, there was substantial heterogeneity regarding methodology and outcomes among the included studies, limiting our meta-analysis. However, we found prolonged antibiotic exposure to be associated with increased risk of NEC and/or death. Broad-spectrum antibiotic exposure was associated with increased risk of IFI and reduced colonisation of obligate anaerobe commensals such as *Bifidobacterium* and *Lactobacillus* and higher abundance of *Escherichia*. Furthermore, antibiotic exposure, in general, was associated with colonisation with multidrug resistant Gram-negative bacteria. In the multi-centre trial, we found significantly higher relative abundance of *Bifidobacterium* in PEP-infants compared to infants in the two other groups. *Lactobacillus* was only detected in small amounts in all groups, but relative abundance increased up to four months of age in all three groups. There were no differences in distribution of ARG-classes or abundance of ARGs at 28 days and 4 months of age between PEP-infants and the two other groups, despite a much higher antibiotic exposure in the PEP-group. In the retrospective cohort study, *Bifidobacterium* blood culture isolates were predominantly found in immunocompromised patients. Functional annotation identified unique genes in both invasive and non-invasive isolates, but no differences in putative virulence genes.

Main conclusions: Antibiotic exposure appears to induce disease-promoting alterations in the gut microbiota and antibiotic given for longer periods in babies with negative cultures is associated with increased risk of NEC and/or death in preterm infants. The results of our systematic reviews strongly suggest that the use of broad-spectrum antibiotics, particularly third generation cephalosporins or carbapenems, is associated with increased risk of IFI and higher abundance of antibiotic resistance development, the latter also seen in the clinical trial. The high abundance of *Bifidobacterium* in PEP-infants at one week of age suggests that a more gradual increase in probiotic supplementation may replicate the physiological gut microbiota development. In PEP-infants, we found no difference in the abundance of ARGs compared between the three groups of infants, despite the massive antibiotic exposure in the probiotic group compared to the two other groups of infants. Our findings support the potential of probiotics to provide colonisation resistance to reduce spread of antibacterial resistance and thereby infections caused by antibiotic resistant pathogens. *Bifidobacterium* has an invasive potential in the immunocompromised host and may cause a sepsis-like picture, but we could not delineate specific pathogenic traits characterising invasive isolates.

1 Introduction

1.1 Preface

Neonates, and in particular those born prematurely, are frequently exposed to empiric systemic antibiotic therapy for suspected sepsis. Early treatment of a bacterial infection is important and antibiotic therapy has certainly saved many lives. However, clinicians tend to overuse antibiotics in neonates despite the existence of several guidelines on the appropriate use of antibiotics [1]. Unnecessary antibiotic treatment in the neonatal period disturbs the microbial flora leading to gut dysbiosis, and possible colonisation with multi-drug resistant bacteria.

Gut dysbiosis in preterm infants is recognised as a risk factor for developing necrotizing enterocolitis (NEC), a devastating condition with high morbidity and mortality [2]. Over the last 10-15 years, many clinical trials have evaluated whether probiotic supplementation to preterm infants may reduce the risk of NEC. In 2014 a Cochrane report stated that enteral supplementation of probiotics prevents severe NEC and all-cause mortality in preterm infants [3]. There were no serious side effects and no cases of probiotic bacteraemia in more than 2500 infants given probiotic prophylaxis. The authors strongly supported a change in practice by implementing probiotics in routine care. Based on available evidence, a group of Norwegian neonatologist wrote a protocol suggesting that preterm infants with the highest risk of NEC should be offered prophylaxis with a probiotic product containing lactobacilli and bifidobacteria [4].

The overall aim of this thesis was to systematically review potential side effects of antibiotic therapy in neonates and to study in-depth the gut microbiota composition of preterm infants receiving probiotic prophylaxis. First, we developed a prospective protocol and did a systematic review and meta-analysis on side effects of antibiotic therapy in order to inform clinicians about potential detrimental effect of non-optimal antibiotic treatment in the neonatal period. Second, we designed and performed an explorative clinical multi-centre trial investigating the gut microbiota composition using shotgun metagenome sequencing in order to obtain a deeper insight in the mechanisms of probiotic therapy. During the work with this PhD thesis we experienced a small outbreak of *Bifidobacterium* bacteraemia in preterm infants receiving probiotic therapy. We therefore performed, and included in this thesis, a separate study investigating the pathogenic potential of bifidobacteria, as these bacteria are widely used in probiotic products, both in neonates and adults.

1.2 Neonatal sepsis

Neonatal sepsis is a systemic bloodstream infection occurring in infants at ≤ 28 days of life. It is often further categorized as early-onset sepsis (EOS) occurring in the first 72 h of life or late-onset sepsis (LOS) occurring after 72 h of life. This classification is mainly based on the different routes of transmission, between EOS and LOS, and thus the somewhat different pattern of pathogens causing sepsis. Neonatal sepsis is the single most important cause of neonatal deaths worldwide, estimated to cause 12% of the 2.7 million neonatal deaths in 2015 [5]. In a cohort of 400,000 live births in the USA, 389 (0.97/1000 live births) were diagnosed with early-onset sepsis (EOS) with a mortality of 16% and mortality was inversely proportional with gestational age [6]. Incidence rates of neonatal sepsis in very low birth weight (VLBW) infants ranges from 1-5/1000 live births to 49-170/1000 live births [7]. Symptoms and signs are often nonspecific. Neonates are relatively immunocompromised, and the impaired innate immune function, in particular among preterm infants, make them predisposed to invasive infections. In addition, invasive devices, prolonged hospitalization, use of broad-spectrum antibiotics that alters the gut microbiota and potential colonisation of pathogens, increases the risk to already vulnerable infants.

1.2.1 Early-onset sepsis

In Norway, the incidence of culture-proven early EOS is 0.54 per 1000 live born term infants [1]. This is similar to rates reported from other developed countries [6, 8]. Incidence rates of EOS in term infants have been declining over the past 20 years, but EOS is still a leading cause of morbidity and mortality in this population. EOS is most often caused by pathogens acquired through vertical transmission from mother to infant before or during delivery. The organisms most frequently causing EOS in term and preterm infants together are Group B streptococci (GBS) and *Escherichia coli* [9]. However, in a cohort of 238 infants with EOS from Scandinavia, *Staphylococcus aureus* was identified as the most frequently detected pathogen, followed by GBS and *E. coli* [10]. Risk factors for EOS include maternal GBS colonisation, prematurity, early and prolonged rupture of membranes and maternal intra-amniotic infection/chorioamnionitis [11-13]. Clinical signs and the symptoms vary by gestational age and severity of infection. Symptoms are often non-specific and include hypothermia, lethargy, poor feeding and nonspecific signs like acidosis and anuria. Respiratory symptoms are also common [14].

1.2.2 Late-onset sepsis

LOS is predominantly caused by Gram-positive organisms in particular coagulase-negative staphylococci (CoNS), but also *S. aureus* and other Gram-positive bacteria [15]. Gram-negative organisms and *Candida spp* occur less common, but associated with higher mortality [16]. LOS is a frequent complication of extreme prematurity and the risk of LOS increases with decreasing birth weight and gestational age, possibly also due to prolonged hospitalisation [16]. In a cohort of 9575 extremely low birth weight (ELBW) infants, rates of LOS were 58% and 20% in infants of 22 and 28 weeks of gestational age, respectively [17]. Alteration in the gut microbiota development of preterm infants is likely to increase the risk of infections and inflammatory processes, and sepsis is one major threat for preterm infants. Different bacterial species like *Enterobacteriaceae*, staphylococci, enterococci, lactobacilli and bifidobacteria may translocate from the intestinal lumen into the blood stream. However, strictly anaerobe bacteria exposed to oxygen in living tissues seems to be less able to translocate [18]. Immature gut barrier and immune dysfunction of the preterm infant may contribute to translocation. Routes of transmission may also be through contamination of intravenous lines. In addition, preterm infants have a high rate of *Candida* colonisation compared with term infants, due to an immature immune system and impaired skin and mucosal integrity [19, 20]. Candida colonisation is a risk factor for invasive candida infections with high mortality [21-23].

1.3 Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is a gastrointestinal syndrome characterized by transmural inflammation and necrosis of the large or small bowel and subsequent intramural gas-forming organisms into the intestinal wall [24]. Although significant progress has been made in our understanding of NEC, many questions remain regarding optimal preventive strategies, diagnostic considerations, and medical and surgical management. The incidence of NEC is inversely related to birth weight, with the majority of affected being VLBW infants [25, 26]. NEC is one of the four main causes of mortality and morbidity in the neonatal intensive case units (NICUs) and long-term complications include neurodevelopmental impairment, short bowel syndrome, strictures and growth restriction [27]. The signs and symptoms of NEC are often classified by Bells criteria [28], later modified by Neu [29].

NEC is a multifactorial disease and its pathogenesis remains largely unknown. However, emerging evidence suggests a combination of abnormal microbial colonisation ("gut dysbiosis"), gut immaturity and an exaggerated immune response in the intestinal mucosa leading to NEC [2,

30]. Other risk factors include feeding practice, patent ductus arteriosus and packed red blood cell transfusion [31-33]. In addition, host development is found to be an important aspect of the disease, as NEC is most often found in preterm infants. However, its onset does not relate to postnatal age as much as postmenstrual age (PMA) and NEC has a peak incidence around 31 weeks PMA [34]. Inflammation and cell death, including apoptosis of enterocytes are important pathologies in NEC [35]. Several studies have found Toll-like receptor 4 (TLR4) to play an important role in the development of NEC. TLRs are pattern recognition receptors present on various cells that recognise structurally conserved molecules found on microbes. The lipopolysaccharide (LPS) found in Gram-negative bacteria is an important example of ligands for TLR4. Expression of downstream regulators activating TLR4 leads to release of pro-inflammatory cytokines and increased enterocyte apoptosis. Preterm infants exhibit excessive TLR4 signalling in response to LPS compared to term infants [36]. The most abundant LPS-carrying bacteria in preterm infants are of the phylum *Proteobacteria*, of which *E. coli* and *Klebsiella pneumonia* are important members. Recent years of research have focused on inappropriate colonisation and bacterial overgrowth or microbial community dysbiosis as major predisposing factors of NEC [37-41]. A causative bacterial agent for NEC is yet to be discovered, as studies have shown that infants with and without the disease harbour similar species in their gut. However, recent studies have demonstrated an increase of *Proteobacteria* at the time of NEC onset [37, 42], whilst other have reported a bloom of *Proteobacteria* a week prior to the onset of NEC [39, 41]. The increase of *Proteobacteria* coupled with excessive TLR4 signalling triggers the hyper-inflammatory response that may lead to NEC. A recent study, using a metagenome approach with strain-level resolution, identified uropathogenic subtypes of *E. coli* as a significant contributor to the risk of NEC [43]. Furthermore, Wang and colleagues studied differences in gut microbiota composition in preterm infants with and without NEC. They found a less diverse gut flora dominated by *Gammaproteobacteria* (>90%) in patients with NEC [40]. A similar finding with high levels of *Gammaproteobacteria* in patients with NEC was found in a recent study from Sweden [44]. However, many preterm infants are highly colonised with *Proteobacteria* without developing NEC [38].

Overall, progression in the prevention and treatment of NEC has been slow. However, the microbial dysbiosis and bacterial overload in the gut makes probiotics a potential prophylactic approach in order to reduce rates of NEC. Indeed, numerous systematic reviews and meta-analysis have shown a significant reduction in the risk of NEC after probiotic supplementation [3, 45, 46], see further details in chapter 1.9.1 of this thesis. Other preventive strategies include human milk. There is strong evidence favouring the use of human milk to reduce the risk of

NEC in preterm infants. Studies have shown that compared to donor human milk, formula feeding increased the risk of NEC by risk ratio 2.77 [47]. Early enteral feeds with human milk followed by slow advancement of feeding volumes are recommended to reduce the risk of NEC. In established NEC, no specific treatment has proven to alter the outcome, and surgical management approaches are controversial. However, treatment involves antibiotics and discontinuation of enteral feeds. Surgical indications for NEC are the presence of intestinal perforation or clinical deterioration in the face of maximal medical management.

1.4 Antibiotic therapy of neonatal sepsis

Severe infections are among the most common causes of morbidity and mortality among neonates worldwide [48]. Sign and symptoms of sepsis are often non-specific and the fear of potential dramatic consequence leads to empirical use of antibiotics in many uninfected infants. Virtually all ELBW infants receive antibiotics during their first postnatal days even though the incidence of culture proven sepsis is very low in this population [49, 50]. Nevertheless, antibiotics are one of the most valuable resources in managing sick newborns.

Antibiotics are the most commonly prescribed medication in the NICU, and ampicillin and gentamicin are prescribed twice as frequently as the second most common medications [51, 52]. In a population-based study from Norway, 2.3% of all term live born infants were given intravenous antibiotic treatment [1]. The relatively rare cases of culture-proven sepsis are treated with full course of appropriate antibiotics, but the appropriate management and treatment duration of the much more common "suspected (clinical) sepsis" is much more difficult to establish. Therefore, rule-out sepsis courses accounts for the highest antibiotic use in the NICUs [53]. Penicillin or semisynthetic penicillin combined with an aminoglycoside is the drug of choice against microorganisms causing EOS. The National Institute for Health and Clinical Excellence (NICE) guidelines recommend benzylpenicillin and gentamicin whilst The American Academy of Pediatrics (AAP) recommends ampicillin and gentamicin as the first line treatment for EOS, respectively [54, 55]. For treatment of suspected LOS an anti-staphylococcal/beta-lactamase stable penicillin (oxacillin, flucloxacillin) or a first generation cephalosporin (e.g cephalotin) together with an aminoglycoside is often recommended [56]. Vancomycin should be restricted to cases of methicillin-resistant *S. aureus* (MRSA) or MR-CoNS [57, 58]. There are no randomised controlled trials suggesting that one antibiotic regimen is better than the other. However, empiric use of antibiotic regimens does matter in the control of antimicrobial resistance in an intensive-care setting. The empiric use of broad-spectrum antibiotics like third generation cephalosporins

for Gram-negative coverage is usually not recommended due to more rapid development of multi-drug resistant (MDR) bacteria than a regimen containing an aminoglycoside [59-61]. Moreover, virulent late-onset pathogens like non-*E. coli*, *Enterobacteriaceae* and *Pseudomonas* are often not susceptible to third-generation cephalosporins. In addition to selecting the most appropriate antibiotics for use, clinicians must also choose the duration of the empirical treatment. Overuse of antibiotics and prolonged antibiotic treatment has been associated with invasive candidiasis, NEC, LOS, and death [62-64]. Antimicrobial stewardship is being promoted as the general principle to improve antibiotic use and thereby improve the quality of care and limit antibiotic resistance development. Examples include use of biomarkers such as C-reactive protein (CRP) to guide initiation of antibiotic therapy, obtain sufficient blood culture volumes and to discontinue treatment after 36-48 hours unless strong suspicion of bacterial infection. A recent European study using a procalcitonin-guided decision making for duration of antibiotic therapy in neonates found that procalcitonin-guided management was superior to standard care in reducing duration of antibiotic therapy in neonates with suspected EOS [65].

Different interventions to improve antibiotic stewardship have been evaluated in the neonatal population [53, 65]. Cantey reported an overall reduction of 27% in antibiotic usage in a NICU after selecting different targets for an antibiotic stewardship program such as discontinuation after 48 h in the electronic medical record and limiting duration of antibiotic therapy of pneumonia and culture-negative sepsis to five days [53].

1.5 Antibiotic resistance

Increasing antibiotic resistance in human pathogens pose a threat to surviving serious infections, including neonatal sepsis. The number of infections caused by MDR bacteria is increasing, and globally an estimated 200 000 neonatal deaths are attributed to resistant organisms each year [66]. Bacteria possess a wide variety of mechanisms leading to antibiotic resistance (Figure 1). Some bacterial species are innate resistant to different classes of antimicrobial agents. This resistance results from inherent structural or functional characteristics. Of greater concern are cases of acquired resistance, where initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that agent. Bacteria may acquire resistance by mutation in the chromosome (*de novo*), with no risk of transferability, or by horizontal gene transfer where the acquired gene is located on or near transferable elements like conjugative plasmids, prophage/phage elements and transposases.

Antibiotic resistance can be caused by several different mechanisms [67]:

- Enzymatic inactivation of the antibiotic by modification or hydrolysis
- Minimizing the intracellular concentration of the antibiotic as a result of poor penetration into the bacterium or due to efflux pumps
- Modification of the antibiotic target by mutations or post-translational modification of the target
- Using an alternative pathway (i.e. cell wall synthesis)
- Carrying several copies of the target in the chromosome

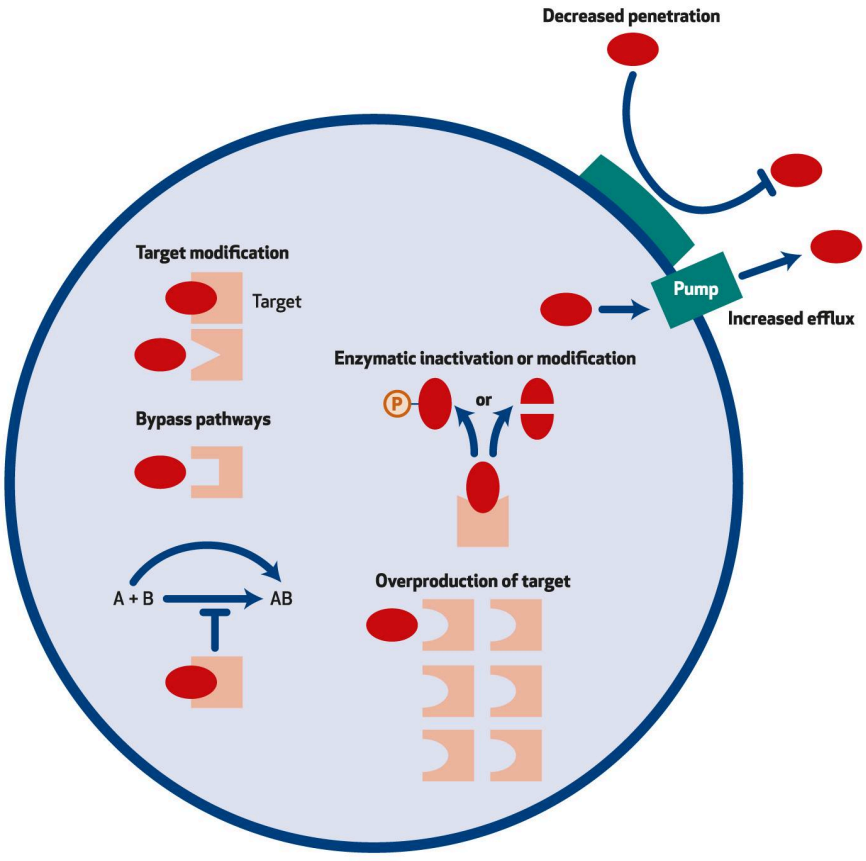

Figure 1. Different molecular mechanisms for antibiotic resistance (figure by Kenneth Kristensen).

1.6 The human gut microbiota and microbiome

"Microbiota" refers to a population of microscopic organisms that inhabits our body; the gut microbiota is the community of organisms found within the gut. The human "microbiome" refers to the collective genome of the microbiota that live inside and on us, and the gut microbiome therefore refers to the total genetic material of the microbial cells residing in the gut. The indigenous gut microbiota is responsible for three main functions; competitive exclusion of pathogens, nutrition and immunomodulation. The diversity of microbes within the gut microbiota can be defined as the number and distribution of distinct types of organisms, also referred as the alpha diversity. The beta diversity represents the differences in species composition among sites/communities.

The developing gut microbiota of infants is characterized by high inter-individual diversity (beta-diversity), but by the end of the first year of life, the microbial ecosystems are converging towards a profile characteristic of the adult gastrointestinal tract [68]. The adult-like structure of the gut microbiota is finally established at around 3 years of age [69, 70].

The prokaryotic diversity found in the human microbiota is classified in 12 bacterial phyla where each phylum represents species that have also been isolated in the human gut. The majority of species isolated in the gut belong to four phyla (*Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*), and are dominated by species from the families *Bacillaceae*, *Enterobacteriaceae*, *Corynebacteriaceae* and *Bacteroidaceae,* respectively (Figure 2) [71]. *Actinobacteria*, followed by *Proteobacteria* and *Firmicutes* are the major phyla during childhood while the phyla of an adult gut microbiota is made up of 80-90% *Bacteroidetes* and *Firmicutes*. The gut microbiota of adults is characterized by high alpha- and beta diversity. A recent large study estimated that the human gut microbiota is composed of 1500-35 000 bacterial species [72], resulting in a bacterial gene content which is 150-fold more than the gene content found in our own human genome [73]. The human microbiome is highly variable with substantial intra-individual variations at different body-sites, inter-individual variation at the same body-site and intra-individual variation at different time points [74]. I addition to large longitudinal differences along the gastrointestinal tract, there is also a spatial difference in the distribution of bacterial species in the gut. *Bacteroides, Bifidobacterium, Streptococcus, Enterobacteriaceae, Clostridium, Lactobacillus* and *Ruminococcus* are dominant in the lumen, while *Clostridium, Lactobacillus* and *Enterococcus* are predominant in the mucosa and mucus [75].

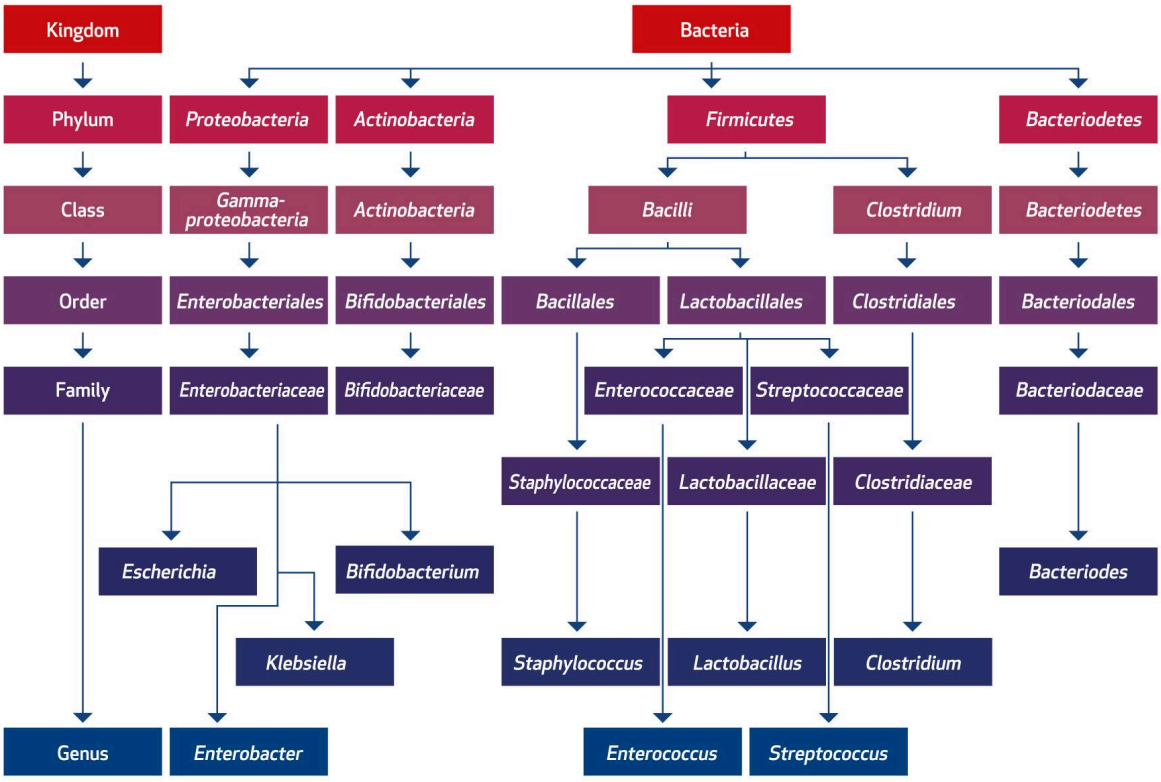

Figure 2. Dominant bacterial kingdom in the gut microbiota (figure by Jon Fjalstad, based on data from www.bacterio.net).

1.6.1 Gut microbiota development in neonates

The establishment of the gut microbiota commences at birth and represents an essential step in the development of the intestine and immune system. However, studies have suggested that the gastrointestinal tract might be colonised even before birth [76-78]. Collado et al recently reported that the placenta, amniotic fluid and meconium all harbour a unique low abundant microbiota with low richness and low diversity [77]. This suggests a foeto-maternal microbial transfer that is initiating the colonisation of the foetal intestine, creating the primary inoculum.

In term infants gut colonisation starts with *Firmicutes*, including aerobes and facultative anaerobes such as *Enterobacteriaceae,* including *E. coli*, *Klebsiella* and *Enterobacter* and *Streptococcus* continuing with obligate anaerobes [79]. The previous dogma was that the pioneer bacteria entering the gut exerted a positive oxidation/reduction potential at birth preventing expansion of obligate anaerobes and gradually, as the consumption of oxygen changes, growth of more anaerobic bacteria such as *Bifidobacterium* and *Bacteroides* were permitted [80]. However, recent years findings have suggested that the reason for the obligate anaerobe preponderance is much more complex.

The source inoculum of the infant gastrointestinal tract is hypothesized to be derived from the maternal gut microbiota, diet and the environment. *Bifidobacterium* is found in human milk, maternal faeces and infant faeces suggesting direct inoculation through mother-infant contact and breastfeeding [81]. Furthermore, human milk exerts a selective pressure promoting growth of certain strains of *Bifidobacterium* that are able to digest human milk oligosaccharides (HMOs) and grow in the presence of HMOs. Mother-infant transmission has been the focus of many recent studies. Makino recently demonstrated several *Bifidobacterium* species transmitted from the mother to vaginally delivered infants, suggesting the mothers intestine as an important source for the infant gut microbiota [82]. Shotgun metagenomic analysis of mother-infant pair samples has revealed vertical transmission of *Bifidobacterium breve* and *Bifidobacterium longum* subsp. *longum* from mother to infant [83].

The profile of the gut microbiota of a full-term, vaginally delivered, breast-fed infant is considered as "ideally healthy" [84]. Several factors influence the assembly of the gut microbiota during infancy. Gestational age, birth mode, antibiotic administration, feeding type and environment of care all have an important influence on the acquisition and shaping of the gut microbiota (Figure 3).

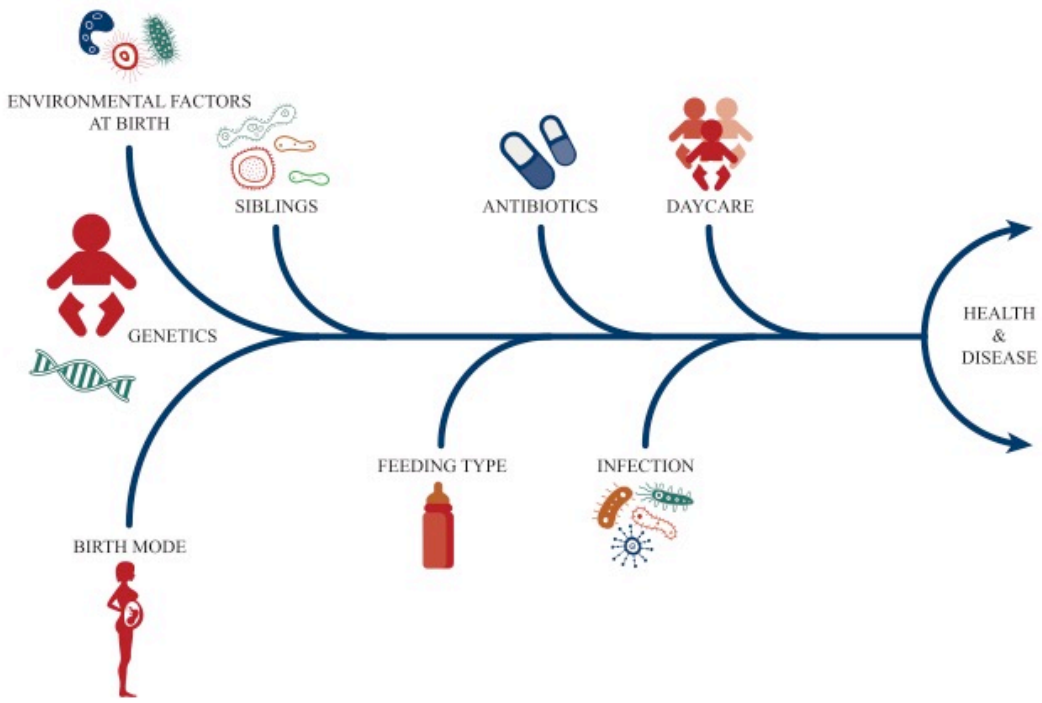

Figure 3. Factors influencing the gut microbiota development (figure by Kenneth Kristensen).

1.6.2 Gut microbiota in preterm infants

By the end of 28 weeks of gestation, the development of the intestinal tract is completed with all cell types found in the adult intestinal lining. However, many of these cells do not possess adult functional patterns. A number of specific biochemical patterns of differentiation occur after birth, often in response to diet. In addition, the gut epithelial barrier function starts maturation from 26 weeks gestation. These maturation mechanisms are altered when the baby is born prematurely, leaving the intestine immature with an incomplete barrier function. In addition, the immature host defence of the preterm infant responds differently to the initial colonisation compared to the full-term infant. Studies have shown that premature enterocytes respond to an inflammatory stimulus with excessive inflammation and can react to commensals with higher levels of inflammation than mature enterocytes [36, 85]. Furthermore, the process of bacterial gut colonisation in preterm infants is more challenging because of several environmental factors influencing, including use of antibiotics, parenteral nutrition and often long-term hospitalization. Studies in human and animal models have found both qualitative and quantitative differences in the gut microbiota between preterm and term infants [86-88]. Term infants usually display a diverse flora with predominance of *Bifidobacterium* and *Lactobacillus,* thought to be protective against colonisations of pathogens. In contrast, preterm infants have only low numbers of *Bifidobacterium* and *Lactobacillus*, coupled with increased colonisation of pathogenic organisms such as *Escherichia* and *Klebsiella* [89-92]. The most notable difference in the succession of bacterial colonisation between preterm and term infants includes enrichment of *Proteobacteria* in preterm infants the first 2 weeks of life whereas *Firmicutes* dominates the initial flora of term infants. The level of *Proteobacteria* in preterm infants is maintained at high level the first month of life. In term infants there is a dramatic increase in the level of *Bifidobacterium* and *Bacteroides* in the first six months of life [93, 94]. Some studies report that preterm infants reach term infants levels of *Bifidobacterium* at approximately six months of life and at this time point significant differences in gut microbiota composition due to gestational age have disappeared [86, 87]

1.6.3 Gut microbiota and mode of delivery

After delivery, the newborn infant is exposed to a variety of microbes, preferentially from the mother. Many studies have found that caesarean section (CS) delivery causes abnormal colonisation of the intestine, and infants are colonised with skin flora from the mother and caregivers rather than vaginal or faecal flora found in infants born by vaginal delivery [95-97]. CS delivery is associated with lower levels of *Bifidobacterium* and *Bacteroides* and more frequent colonisation of *Clostridium* and *Lactobacillus* during the first three months of life [98].

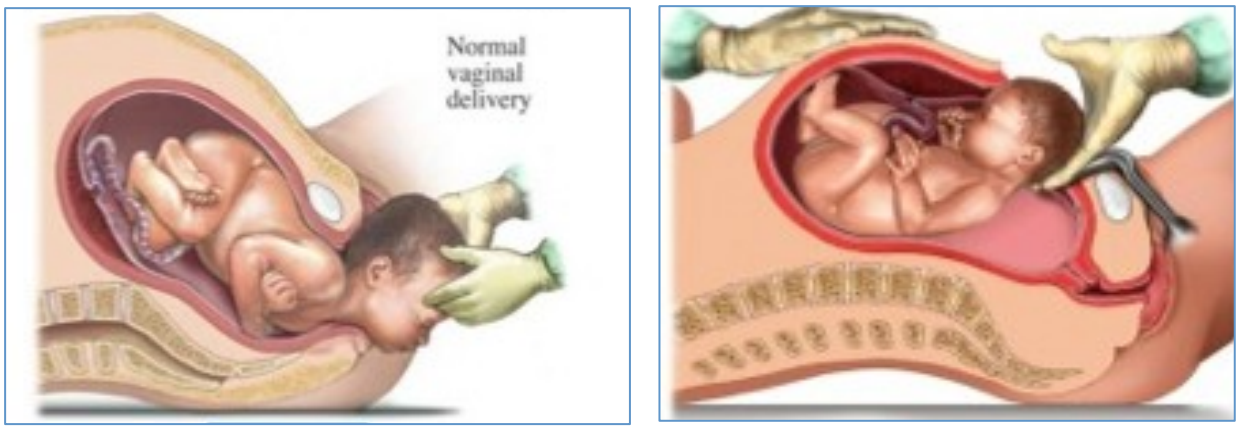

Figure 4. Vaginal delivery versus caesarean delivery (copyright Nucleus Medical Media, Inc).

In the Nordic countries rates of CS deliveries has increased since the mid-1990s and constituted 17% of all deliveries in 2014 [99, 100]. In the US, 32% of all live births were CS deliveries in 2014 [101]. Given the high rate of CS deliveries, recent years of research have focused on how mode of delivery affects the gut microbiota development. It has been thought that the initial microbial exposure is important in defining the successional trajectories leading to a complex and more stable adult ecosystem. However, the clinical context surrounding the decision to deliver via caesarean surgery is often complex with significant potential confounders including underlying maternal or foetal medical conditions or comorbidities, varying use of medications like antibiotics and analgesics. Recently, Azad et al found differences in the infant gut microbiota born by caesarean delivery based on whether or not the mother was in active labour before caesarean surgery, indicating that these differences depended on whether the foetus had descended into the vaginal canal and then had been exposed to vaginal microbes [102]. Moreover, body site specificity served as the major determinant of the bacterial composition and functional capacity and not mode of delivery for maternal-infant pairs during the infants first six months of life [103]. For preterm infants (GA < 33 weeks) mode of delivery does not appear to significantly affect the development of the gut microbiota, but is hypothesized to be highly influenced by the environment, including the profound effects of hospitalization and more use of broad-spectrum antibiotics [104-106]. A recent pilot study investigated vaginal seeding, where vaginal microbes were transferred from the mother to CS delivered infants in attempt to mimic exposures during vaginal delivery to restore an otherwise disrupted gut microbial colonisation [107]. However, further studies are needed to assess the balance of potential risks and benefit for this procedure and its use in clinical practice [108, 109].

1.6.4 Gut microbiota and enteral feeding

Feeding practice also affects the composition of the infant gut microbiota [110-112]. Breast-milk contains a mixture of nutrients and immunological components. Carbohydrates, fatty acids, and lactoferrin along with secretory IgA have a major effect on the milieu within the gut microbiota [113, 114]. Human milk also contains live bacteria, including *Bifidobacterium spp*, *Lactobacillus spp*, *Streptococcus spp* and *Staphylococcus spp*. The exact origin of these bacteria remains to be firmly established, but it has been hypothesized that bacteria translocate from the maternal gut and enters the mammary glands through the blood stream or lymphatic circulation – the so called "entero-mammary pathway" [115, 116]. However, definite proof of this pathway remains to be established. Another explanation is contamination by skin bacteria or transfer from neonatal oral microbiota, which might explain the predominance of *Streptococcus spp*. and *Staphylococcus spp*. in human milk [117, 118].

Breast-milk acts bifidogenic by specialized molecules designated as "bifidus factors". These factors facilitates the colonisation process and in particular enrichment of bifidobacteria. In addition, several components in breast milk, including non-digestible oligosaccharides (incl. HMOs) and lipid-bound glucoconjugates prevent pathogenic bacteria from adhering to the enterocytes through direct binding [119]. Oligosaccharides are the third most abundant component of human milk. The gut microbiota of breast-fed infants is characterised by reduced species diversity and richness, enrichment of bifidobacteria, and lower abundance of *Bacteroidetes* and *Clostridiales* compared to non-breastfed infants [110, 120-122]. Compared to formula, breast-milk is more complex and provides a more optimal nutrient for the infant. Short term benefit of expressed breast milk includes reduced risk of NEC and LOS in preterm infants [123, 124]. In addition, formula feeding induces higher intestinal permeability, increasing the probability of translocation of bacteria to the blood stream [125]. Recently, Gregory and colleagues investigated how different nutritional regimens acted protective against gut immaturity in the preterm infant. They found that the gut microbiota of formula fed infants was most influenced by gestational age while the gut microbiota of infants fed with mothers breast milk was more resilient to the influence of gestational age, suggesting a protective effect against gut immaturity offered by breast milk [126]. Breast-feeding can also modify antibiotic-induced microbiota changes. Fewer antibiotic induced changes in the gut microbiota were detected at 1 year of age in infants who were exclusively breastfed at 3 months of age [102, 127]. However, Penders and colleagues showed that maternal diet did not influence the infants gut microbiota composition [84].

1.6.5 Gut microbiota and effects of early antibiotic treatment

Overuse of antibiotics, particularly broad-spectrum antibiotics, applies a selection pressure that favours antibiotic resistant bacteria and decreases colonisation resistance [59, 128]. However, the relative impact of different types of antibiotic exposure on the actively developing gut microbiota composition and antibiotic resistance development is not fully understood. Several studies have investigated the impact of early antibiotic exposure on gut microbiota composition and antibiotic resistance development [38, 59, 129], but prior to this thesis it had not been systematically reviewed. Some main previous findings describe how antibiotic perturbation of the actively developing gut microbiota can have profound impact on health and disease throughout life, both indirectly due to disruption of the metabolic and immune development but also due to potential enrichment of antibiotic resistance genes (ARGs) available for transfer to pathogens [130]. Antibiotic use can have a detrimental effect on the gut microbiota homeostasis. These changes may further predispose the infant to future episodes of NEC and LOS.

1.6.6 Gut microbiota and antibiotic resistance genes

The human commensal gut microbiota harbours numerous functional ARGs comprising what is coined the "human gut-associated antibiotic resistome". The human gut microbiota has the most accessible reservoir of ARGs due to its likelihood of contact and exchange with human pathogens [131]. Alterations in the gut microbiota during the critical period in neonates and infants are not only related to altered physiologic composition, but also in its associated antibiotic resistome. The developmental trajectory of these community-encoded ARGs is largely unknown and studies investigating the infant gut microbiota and its associated resistance genes are lacking [132]. However, the widespread use of broad-spectrum antibiotics has most likely had a substantial contribution to the changes observed. Exposure to third-generation cephalosporins is a strong predictor of emergence of resistant *Enterobacteriaceae* [59]. Furthermore, infection caused by extended-spectrum beta-lactamase (ESBL) producing Gram-negative bacteria is an emerging clinical problem in NICUs in many countries [133]. However, as the epidemiology of these resistant organisms mature in a NICU setting, they may be acquired under a variety of different settings. ARGs in term infant gut microbiota are established in the first week of life, even in the absence of antibiotic treatment [134, 135]. The preterm infant resistome is also established very early and reflects both antibiotic selections of the colonizing bacteria from other habitats, as well as by direct influence of antibiotic selection in infants. This is illustrated by findings of genes encoding resistance to other antibiotics than those used in the NICUs [106]. ARGs that are

enriched after a specific antibiotic therapy are generally unique to the particular antibiotic given and also largely contributed by a particular bacterial species [106]. Moreover, collateral enrichment of resistance to other antibiotics can also be observed. Although there are suggestions of vertical transmission of resistance genes, recent work has shown that environmental variables and host genetics has greater impact than the maternal influence on the gut-associated resistome in infants [104]. Duration of colonisation varies, but studies have suggested that once the infant is colonised, colonisation is usually very short-lived suggesting that the infant most frequently serves as a transient reservoir or a dead-end host and the duration of colonisation is related to time of hospitalization [136].

With the evolution of multiple antibiotic resistances, the large repertoire of ARGs in the human gut microbiota of healthy individuals could contribute to further emergence of antibiotic resistance in human pathogens. The majority of the human gut-associated resistome is contained within chromosomal DNA, but it may be represented on extrachromosomal replicons like plasmids and phages with the potential of transmission to other pathogens. Earlier, both costs and limitations in the advancement of molecular technology hampered the assessment of resistance genes in the gut microbiota, but new diagnostic tools of functional, or sequence-based metagenomics can now provide novel insight into the diversity of the human gut associated resistome [131].

1.6.7 Current methods to study the human gut microbiota

To study the human gut microbiota, two major technological periods can be distinguished; microscopic observation and traditional culture-based methods were the first to characterise bacterial ecosystems and dominating before 1995 followed by the advent of culture-independent methods. Culture-based methods, despite improvements, are less sensitive, laborious and time consuming [137]. Furthermore, as most of the gut microbes are anaerobes that are difficult to grow outside the body, these methods detect only 10-25% of the microbial diversity blinding us to see the real global picture of the gut microbiota [138]. With the development of next generation sequencing, the gut microbiota can now be studied by direct DNA sequencing called metagenomics. This enables the identification of both cultivable and yet non-cultivable bacteria as well as the functionality of the gut microbiota in an elaborate manner in both health and disease. However, culture-based techniques are still important in order to assess antibiotic susceptibility. Despite a rapidly expanding area and advancement in technology, each of the steps in the pipeline of gut microbiota analysis has the potential of introducing biases in the apparent microbiota composition and offers a major challenge in analysing the gut microbiota [139].

1.6.8 Genomic approach to study the gut microbiota and resistome

Metagenomics, stemming from microbiology, ecology and genomics, has over the last two decades revolutionised microbial research [140, 141]. As it is multidisciplinary, it has been prone to varying definitions. Briefly, metagenomics refers to the study of metagenomes, genetic material recovered directly from environmental samples. It is the analysis of all DNA in an organism isolated from a microbial ecosystem without previous culturing. There are two main approaches for analysing the microbiome, 16S ribosomal RNA (rRNA) gene amplicon sequencing and random shotgun metagenomics. In 16S rRNA gene targeted amplicon sequencing, the 16S rRNA part of the bacterial genome is sequenced. This method is normally used in taxonomic classification and for determining species diversity and has been the standard analysis of prokaryote diversity due to the inherent conservation of 16S rRNA between species. Shotgun metagenomic sequencing involves randomly sequencing all DNA in the sample, without the need to target or amplify a specific gene, also referred to as metagenomic sequencing. This results in DNA sequences (sequence reads) that represent small regions of the genomes present in the sample. Some of these reads will be sampled from taxonomically informative genomic loci (e.g., 16S rRNA), and others will be sampled from coding sequences that provide insight into the biological functions encoded in the genome. Databases applying different algorithms are used to annotate genes enabling us to study the functional potential of the metagenome through identification of metabolic pathways, to identify potential resistance genes and putative virulence genes. Furthermore, functional genome annotation is an important tool in assessing unique features of a particular bacterial niche and the functional diversity between different bacterial species [142]. In short, 16S rRNA sequencing attempts to reveal "who is there" in a microbial community, while shotgun metagenome sequencing can answer the complementary question of "what can they do".

Metagenome sequencing can also provide a more comprehensive understanding of the human gut associated resistome [143]. Three different metagenomic approaches exist to examine the human gut associated resistome: (1) Targeted (PCR-based) metagenomics, (2) sequence-based metagenomics and (3) functional metagenomics. The main drawback of PCR-based metagenomics is that known resistance genes and mechanisms are targeted. However, limited cost makes it a valuable tool in studying the resistome. In sequence-based metagenomics, DNA from an environmental sample is extracted, fragmented and size-separated and randomly sequenced without the need of culturing. However, this approach is also, like PCR based metagenomics, limited to identifying genes that are already known. In functional metagenomics a DNA fragment is cloned into a vector and the subsequent expression is studied in a host (e.g. *E.*

coli). Resistance genes are subsequently screened for by growing the transformant on different antibiotic containing media. With this approach, both known and unique resistance genes can be discovered. However, the method does rely on the genes ability to be expressed in the new host.

1.6.9 Limitations of metagenome sequencing of faecal samples

Despite the powerful technology of next generation sequencing, there are still limitations. In addition to higher costs and a more time-consuming approach, metagenome sequencing requires a higher amount and quality of DNA than 16S rRNA amplicon sequencing. Differences in sequencing platforms, DNA isolations kits and the differences in the complexity of the samples can possibly lead to different or biased findings. One of the major biases in metagenome studies is the sequencing depth. In a complex ecosystem like the human gut microbiota, consisting of 10^{12} bacteria per gram stool, metagenome studies are unable to detect bacteria of $<10^5$ bacteria per gram [144]. Technical preparation of stool samples is a particular important issue. Many studies have investigated the effect of different storage conditions and the overall consensus is that freshly collected samples remain the gold standard where possible [145, 146]. However, a newly developed commercial available tool for stool storage was recently tested and found to be satisfactory and even increased the quality of extracted DNA compared to more traditional sampling with freezing after faecal collection [147, 148]. Ideally, metagenome sequencing strives to embrace all DNA in one sample, but this is not possible due to the extreme microbial diversity and low abundance of certain organisms. Furthermore, different DNA extraction kits will generate different results in terms of amount and quality of extracted DNA and influences on bacterial community composition [149]. Moreover, different organisms, in particular Gram-positive bacteria, are difficult to lyse in the extraction process and metagenome-samples may be contaminated by host DNA.

A recent study found that 16S rRNA sequencing can capture broad shifts in the community over time, but with limited resolution and lower sensitivity compared with metagenome sequencing [150]. A reason for the difference in taxonomic findings between the two methods could be the known primer biases towards certain taxa in 16S rRNA analysis. Another explanation could be the difference in the reference databases used for the two methods. While databases used for 16S rRNA analysis are composed of 16S rRNA sequences from a high diversity of taxa, the databases used for metagenome sequences are based on whole- or draft genomes from fewer or less diverse taxa.

1.6.10 Statistical approaches to study the gut microbiota

After sequencing and production of processing reads, the next phase involves generation of data sets based on the shotgun reads [151]. These datasets are then compared to large databases such as Genbank [152], Kyoto Encyclopedia of Genes and Genomes (KEGG)[153] or Clusters of Orthologous Groups of proteins (COG)[154], using e.g. the Basic Local Alignment Search Tool (BLAST)[155] listing genes and the number of matched reads (Figure 5). However, not all reads will map to sequence databases because not all organisms have previously been sequenced. In addition, the reads may map to genes with unknown function. The next step involves finding the right numerical tool for exploring these large datasets in order to present the data as trees, similarity curves, abundance, diversity, and other ecological and statistical descriptors of community structure.

The alpha-diversity is the microbiota diversity within the same sample. The alpha-diversity is calculated e.g. using the Chao1 index (which estimates the numbers of different operational taxonomic units (OTUs) present within that sample) or the Shannon diversity index (which evaluates both the number of OTUs and the evenness of their distribution) [156]. The beta-diversity is the difference in microbiota community composition across different samples or environments. There are two main approaches for measuring beta-diversity; those that take into account the phylogenetic differences and those who do not (non-phylogenetic beta-diversity) [157]. One example of phylogenetic beta-diversity is UniFrac (unique fraction) metrics that are based on the fraction of branch length shared or the "unique evolution" between two communities within a phylogenetic tree constructed from all the communities being compared. A small UniFrac distance implies that the two communities are compositionally similar. A non-phylogenetic approach to calculate the beta-diversity is the Bray-Curtis dissimilarity. To visualize distances/dissimilarities between samples/groups different ordination techniques are often used, the most commonly being non-metric multidimensional scalings (NMDS) and principal coordinate analysis (PCoA).

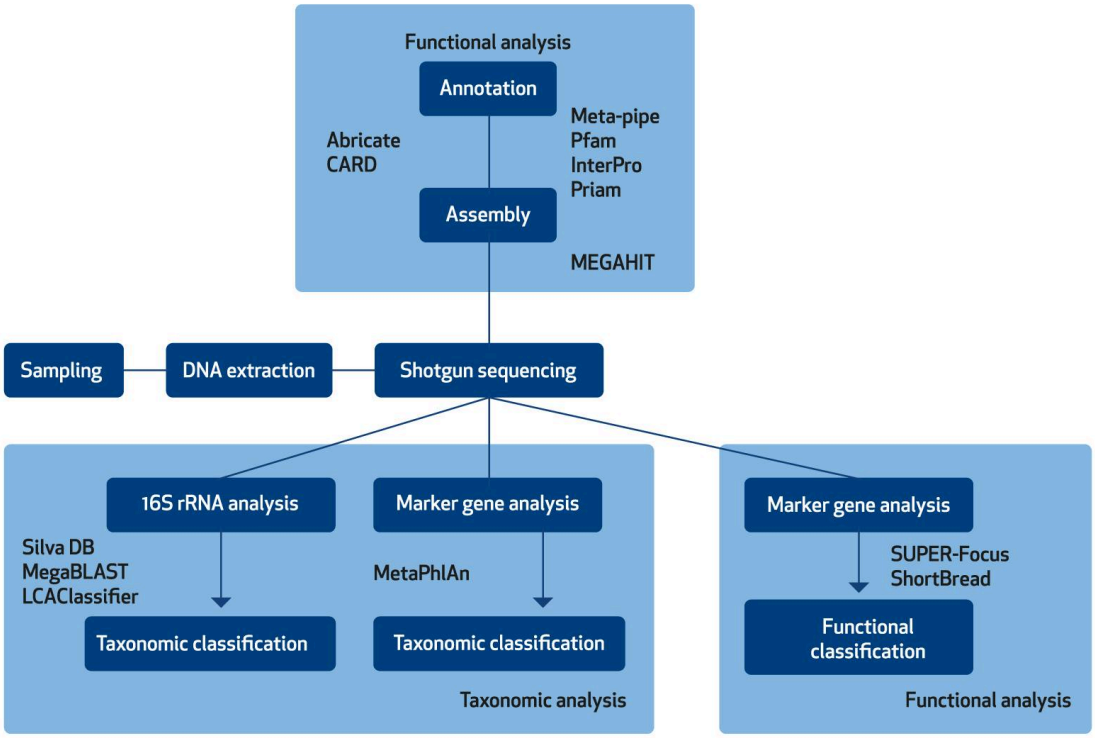

Figure 5. Metagenomic workflow: processing a sample from raw data to a complete taxonomical and functional analysis.

1.7 Probiotics

An intervention that has caused overwhelming interest in clinical medicine over the past two decades is the use of probiotics. Probiotics are defined as "live micro-organisms which when administered in adequate amounts, confer a health benefit to the host (WHO 2001)". The word probiotic means "for life" and it is used in reference to bacteria associated with beneficial effects on humans and animals for disease management, infectious control and health improvement. Probiotics have been used in a wide range of diseases including diarrhoea prevention and control after antibiotic treatment, irritable bowel disease, *Helicobacter pylori* infection, colon cancer and prevention of atopy, food allergies and eczema and prevention of necrotizing enterocolitis in preterm infants [158-162]. Required validation of bacteria used as probiotic agents includes resistance to gastric acidity, bile acid resistance, adherence to mucus and/or human epithelial lining, antimicrobial activity against potentially pathogen bacteria, ability to reduce pathogen adhesion to surfaces, bile salt hydrolase activity and resistance to spermicides (vaginal use) [163]. There are a number of different organisms that can be classified as probiotics including *Lactobacillus, Bifidobacterium, Streptococcus, Propionibacterium, Bacillus* and *Leuconostoc mesenteroides* but the most commonly used strains belong to the genera *Lactobacillus* and *Bifidobacterium*.

1.7.1 Why use probiotics in preterm infants?

Given the evolution of the intestinal flora in preterm neonates, *Bifidobacterium* and *Lactobacillus* are often the species of choice in probiotics administered to preterm infants. The competitive advantage of *Bifidobacterium* results in decreased diversity and fewer luminal pathogens. Studies also reveal that *B. infantis* grown on HMOs are better able to bind intestinal epithelial cells, including Caco-2 cells and HT-29 cells than when grown on other commercial prebiotic products like oligofructose [164, 165]. In addition to the advantage in colonisation in the presence of human milk, bifidobacteria also confer other beneficial properties. The hallmark of NEC is an excessive inflammatory response due to immaturity of specific innate immune response genes. *B. infantis* reduce the inflammatory response of IL-6 and IL-8 to stimulus of lipopolysaccharides (LPS) [166]. Furthermore, *B. infantis* induces lower expression of inflammatory response genes and stimulate genes promoting the integrity of the mucosa barrier, e.g. tight junctions [164]. Moreover, genes involved in chemokine expression, playing an active role in the development of NEC [167], have been suppressed in response to *B. infantis* in both human and mouse models [168, 169].

Lactobacillus is not a major component in the infant gut microbiota, but in combination with *Bifidobacterium*, it offers an environment to promote growth of autochthonous lactic-acid bacteria by formation of short-chain fatty acids and facilitates uptake of butyrate by host colonocytes [170]. *L. acidophilus* produces a variety of bacteriocins and suppresses pro-inflammatory cytokines, including IL-8 [171].

1.7.2 When probiotic organisms become invasive

Probiotic bacteria are traditionally considered non-pathogenic commensals that rarely cause human infections. In a systematic review of more than 20 probiotic trials in neonates, there was no evidence that probiotic organisms could cause invasive infections [3]. In a large cohort study focusing on blood stream infections caused by probiotic bacteria in 3500 hematopoietic transplant recipients the authors did not find any cases of *Bifidobacterium* bacteraemia [172]. However, the pathogenic potential of probiotic bacteria remains unclear and although the true incidence of probiotic-associated bloodstream infections is unknown, *Bifidobacterium* species are estimated to represent 0.5-3% of anaerobic blood culture isolates [172, 173]. Among adults only 15 cases of *Bifidobacterium* bacteraemia had been reported in the literature until 2015 [174], predominantly among patients with underlying gastrointestinal disease and/or impaired

immunity. *B. longum* and *B. dentium* are the most frequently reported species to cause bifidobacterial infections [174, 175]. There are now twelve published cases of bacteraemia in infants supplemented with probiotics; of these eight were caused by *Bifidobacterium spp.* [176-180] and four by *Lactobacillus spp* [181-183]. These case reports include all from mild to serious systemic infections after ingestion of probiotic bacteria. Over the last years an increasing number of *Bifidobacterium* blood culture isolates have also been reported to the Norwegian Organization for Surveillance of Antimicrobial Drug Resistance (NORM), but reasons for this change in epidemiological pattern is unknown [184].

1.7.3 Bifidobacteria

In this thesis, I have focused in particular on the *Bifidobacterium* species, and I will therefor present a more detailed description of this bacterial species.

Bifidobacteria are Gram-positive obligate anaerobic, non-motile, non-spore forming rods and members of the family *Bifidobacteriacea* belonging to the *Actinobacteria* phylum. The bifidobacteria display different morphologies, but the bifurcated or "bifido" shape is the most common. Its discovery was attributed to Henry Tissier who first isolated the bacteria from faeces of breast-fed infants in 1899 [185]. The average size of the bifidobacterial genome is 2.2 Mb, although considerable variation exists among the different species. The G+C content varies between 59.2% (*B. adolecentis*) to 64.6% (*B. scardovii*) and the average number of genes is 1825 [186]. The pan-genome represents the total number of different genes encoded by a certain species, consisting of a core genome shared by all isolates. Recent investigation has revealed that the core genome of the *Bifidobacterium* consists of around 400-450 genes [187, 188].

Bifidobacteria are among the most abundant constituents of the human gut microbiota [189], but are also habitants of the vaginal tract and oral cavity in humans. It is mostly found in humans and social animals, whose offspring are dependent of parental care, which implies a special route of transmission. Currently, there are 58 recognised (sub)species of *Bifidobacterium*, including nine subspecies. (http://www.bacterio.net/bifidobacterium.html). *B. longum* is represented by three subspecies (*longum, infantis* and *suis*), but recently a fourth subspecies was suggested [190]. Species distribution is different in infants and adults; *B. adolecentis* and *B. longum* subsp. *longum* are the major bifidobacterial species in the adult intestinal flora and *B. longum* subsp. *infantis*, *B. bifidum* and *B. breve* are the predominant species in the intestinal tract of human infants [191]. In breastfed infants, bifidobacteria constitute more than 80% of the intestinal microbiota whereas bifidobacteria comprise only 3-6% of the adult faecal flora (Figure 6) [84, 192, 193].

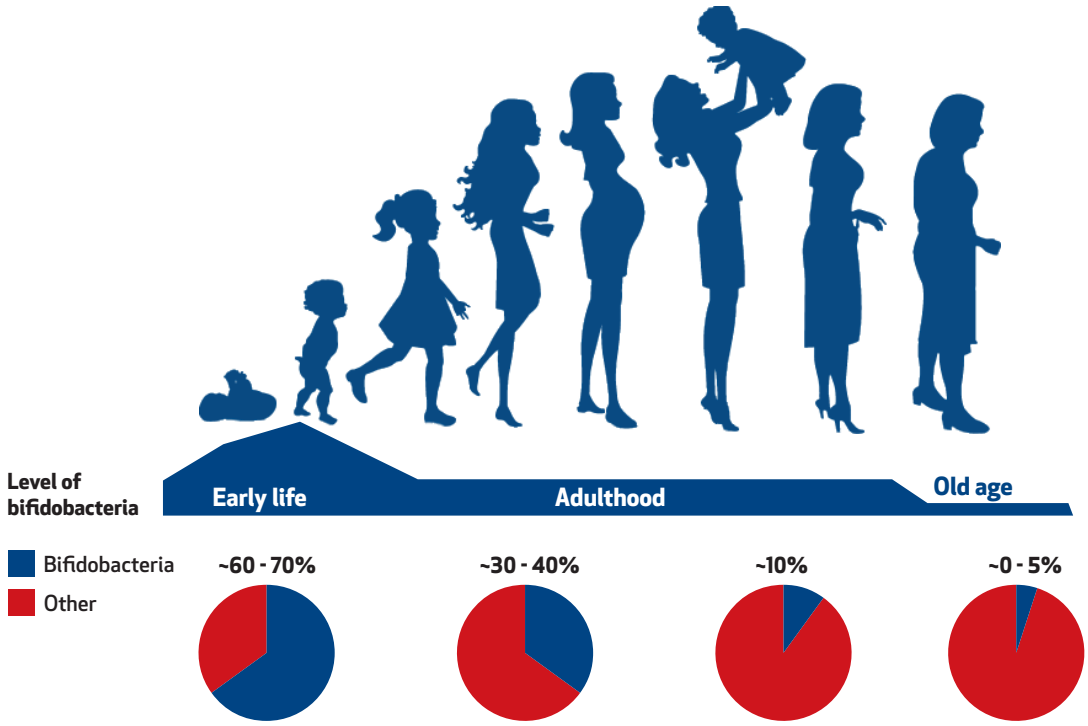

Figure 6. Levels of *Bifidobacterium* during different stages in life (figure by Kenneth Kristensen).

The ability of bifidobacteria to compete with other members of the intestinal microbiota and their ubiquitous colonisation of the gut is largely attributed to their unique saccharolytic features. One of the major forces that drive *Bifidobacterium* predominance in the infant gut is its unique ability to consume HMOs, a feature it shares only with *Bacteroides*. Pan-genome analysis has suggested that up to 14 % of the identified genes in the bifidobacterial genome are related to carbohydrate metabolism [194]. In bifidobacteria, glycosyl-hydrolases (GH) are the most prevalent carbohydrate modifying enzymes, GH13 being the most representative [195]. In contrast, the human genome encodes only eight GHs that are directly involved in carbohydrate metabolism. It is therefore reasonable that this paucity is compensated by members of the gut microbiota, including the *Bifidobacterium*, thus allowing the human host to digest otherwise non-digestible complex carbohydrates.

Bifidobacterium metabolize HMOs present in human milk. Amongst the bifidobacteria, *B. longum* subsp. *infantis* and *B. bifidum* utilize HMOs most efficiently, explaining the dominance of especially *B. longum* subsp. *infantis* in the gut microbiota of breast-fed infants. After weaning, the population of bifidobacterial species changes towards species more capable of metabolizing plant-derived sugars.

1.7.4 *Bifidobacterium* and antibiotic resistance

Despite the proposed health-promoting effects of *Bifidobacterium* [196], antibiotic resistance determinants in commensals are of great concern as they can serve as a reservoir of resistance genes to intestinal pathogens [131]. However, the possible threat of transfer of antibiotic resistance genes (ARGs) is related to the genetic basis of the resistance mechanism. *Bifidobacterium* often displays resistance against many of the antimicrobials in use today, the most common trait being resistance to tetracycline, metronidazole, penicillin and ciprofloxacin. Nonetheless, despite many reports on the susceptibility pattern of *Bifidobacterium*, there is lack of information regarding their resistome. Most data on antibiotic resistance determinants have been limited to macrolides and tetracycline [197]. The *tet* genes, encoding proteins that protect the ribosomes from the action of tetracycline, are the most abundant genetic resistance determinants among bifidobacteria and the *tet(W)* gene has been the one most commonly found [198-200].

Only a very small fraction (<1%) of the bifidobacterial resistome is predicted to reside on mobile genetic elements [201]. Furthermore, conjugative plasmids in bifidobacteria have not yet been reported. The repertoire of ARGs in the bifidobacteria may therefore represent microbe-host coevolution to selective pressure imposed by extensive use of antibiotics. Moreover, a study comparing the repertoire of bifidobacterial ARGs between infants and adults showed that adults possess a much larger arsenal of bifidobacterial ARGs compared to infants. This reinforces the concept that the infant gut microbiota are more prone to dysbiosis induced by antibiotics than the gut microbiota of adults [201]. A recent pan-genome analysis of *B. adolecentis*, a species mainly represented among adults, indicates that this species has a greater genetic diversity compared to other human bifidobacterial species, including those found in infants [202].

1.8 Evidence-based medicine

Evidence-based medicine (EBM) can be defined as ”the conscious, explicit and judicious use of current best evidence in making decisions about the care of individual patients” [203]. In a clinical setting, to fulfil the means of EBM, the practitioner should combine his clinical expertise and looking at all evidence and judging it fairly while considering the patients best interest. This is called practicing EBM. However, healthcare providers, researchers, consumers and policy makers are provided with unmanageable amounts of information, including evidence from healthcare research. To alleviate the process, systematic reviews attempts to collate all empirical evidence that fits pre-specified criteria in order to answer pre-specified research questions [204]. Systematic reviews often use a statistical technique, the meta-analysis, to combine eligible results from different studies. The aim of the meta-analysis is to increase the statistical power of the measure that is being investigated. Systematic reviews and meta-analysis are recognised as the highest standard of EBM. Furthermore, conducting a systematic review can offer the opportunity to acquire high level of methodological expertise, but also the capacity to learn and solve problems by using critical and analytical thinking. This capacity is considered one of the key generic and transferable skills for future researchers. Moreover, literature searches may provide a thorough understanding of the electronic databases [205].

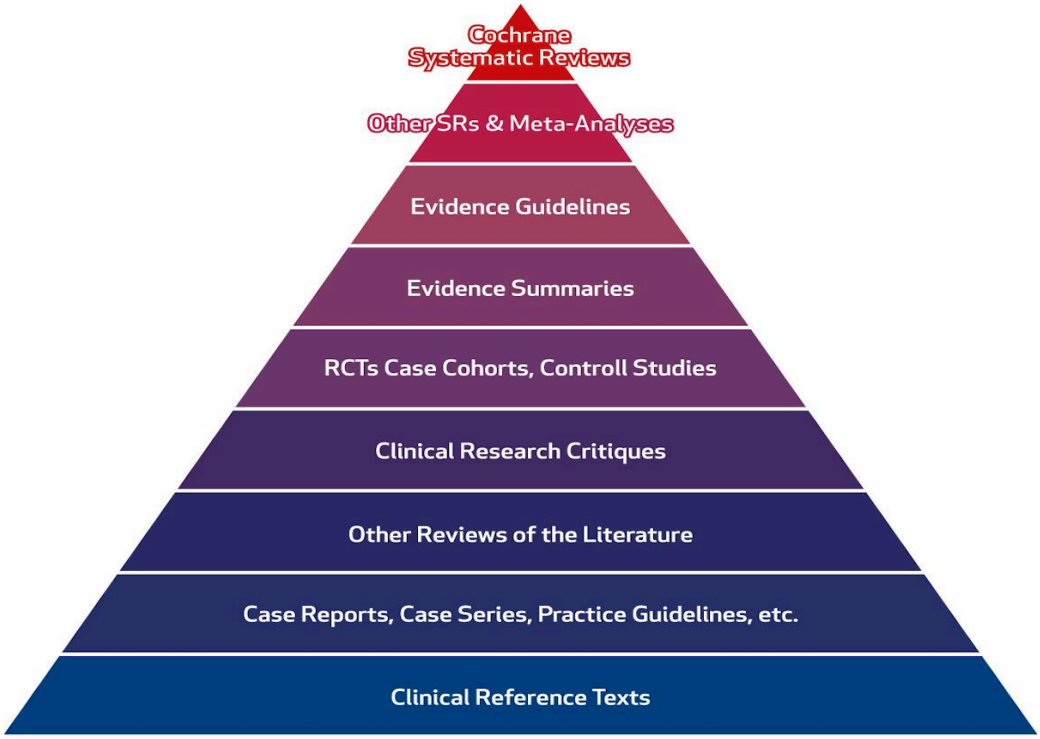

Figure 7. The evolution of evidence-based medicine showing the levels of evidence pyramid (adopted and modified from University of Washington Health Links).

In order to trust and convey EBM, one must maintain a transparent, safe and efficient way in medical science. One way of doing this is to register trials on easy accessible databases and use systematic schemes for reviews.

1.8.1 Systematic reviews; risk of bias assessment and GRADE

To what extent a systematic review is to be trusted depends mostly on the validity of data and results included from different studies. The validity has two dimensions. The first is the external validity, which is whether the study asks the appropriate research question. The second dimension is to what extent the study minimizes systematic error or biases, called internal validity. A bias can be defined as a deviation from the truth, leading to an overestimation or underestimation of the truth, in results or inferences. Bias is a systematic error and should not be confused with imprecision or random error [204]. To what extent biases have affected the results in one particular study are almost impossible to determine and it is therefore more appropriate to determine risks of bias. To assess the risk of bias, systematic reviews applies different tools, like scales or checklists to assure the quality of the studies included. The Cochrane Collaboration classifies different types of biases into selection, performance, detection, reporting and confounding [204]. In general, randomized clinical trials (RCTs) and non-randomized studies (NRS) differ in several ways in respect to their risk of bias, and NRS may have higher risks of bias. However, biases found in a NRS may be present in much the same way as in poorly designed or conducted RCTs. A common adjunct to risk of bias assessment is quality assessment. However, "quality" relates more to the extent that the study design, conduct, analysis, and presentation is appropriate to answer its research question [206]. 'Quality of evidence' reflects to what extent one can be confident that the estimate of an effect is near the true value. Several guidelines have been developed to rate the quality of evidence, but users are often faced with challenges in understanding the message that grading systems try to communicate [207]. The Grading of Recommendations, Assessment, Development and Evaluations (GRADE) approach is a systematic, transparent and explicit approach about quality of evidence and strength of recommendation which is increasingly being adopted worldwide [208]. This approach specifies four levels of quality from high to very low, which define the degree to which its estimates of effects or associations can be trusted. In the context of a systematic review, quality reflects our confidence that the estimates of effects are true. By using GRADE, quality means more than risk of bias and can be compromised by many other factors including imprecision, indirectness and inconsistency of study results.

1.8.2 Summary of evidence including meta-analyses

Research synthesis can be performed either qualitatively, in the form of a narrative review, or quantitatively, by employing various statistical methods for the integration of results from individual studies. The most commonly quantitative approach is the meta-analysis, defined as a statistical combination of results from two or more separate studies. Important considerations when applying the meta-analysis are the heterogeneity across studies and the type of data that is presented in the individual studies (dichotomous or continuous). The result of the individual studies and the overall estimate from the meta-analysis is usually presented in a Forest plot and provide a visualization of the effect estimate and the heterogeneity between studies. The effect measure (e.g. odds ratio) is often presented as a square with horizontal lines representing the confidence interval. The area of each square represents the studies weight in the meta-analysis and a diamond-shape represents the overall meta-analysis effect [204]. Systematic reviews frequently need to synthesize evidence where quantitative synthesis technique, like the meta-analysis, is not possible. Therefore, other ways of expressing and synthesizing the results of studies collected together for review are needed. We often describe these methods as 'narrative' analysis or synthesis. Research reviews in ecology and evolutionary biology have traditionally been carried out either in the form of narrative reviews, or by "vote counting," where the number of statistically significant results for and against a hypothesis are counted and weighed against each other.

1.8.3 Example 1: Use of probiotics to prevent development of NEC

Several randomized-controlled trials and cohort studies have demonstrated a decrease in the incidence of NEC in preterm infants following administration of probiotics, and the latest English language meta-analysis all have similar conclusions [3, 46, 209-211]. Current evidence-based guidelines justify routine use of this intervention. Routine administration of probiotics has therefore been strongly suggested [3, 212]. Although probiotics have been described as safe and well tolerated, data addressing the safety of probiotics is still sparse and the administration of live bacteria to immune-incompetent patients such as very preterm infants cannot be taken lightly. Cross-contamination and sample size limits the value of traditional RCTs. Moreover, due to lack of direct comparisons between different probiotic products, there is still question of duration of treatment, which probiotic product to chose and optimum dose to provide. Most current probiotic products were developed years ago and based on stability and ease of industrial production rather than specific mechanistic criteria [213].

Lack of evidence, specifically in extremely preterm infants, is often referred as a problem in adopting routine probiotic supplementation in this population. In the latest systematic review there were only 5 out of 23 RCTs reporting outcome on ELBW infants [211]. At the same time, this particular population are those who have the highest incidence of NEC and therefor may benefit most from probiotic supplementation. Right now there are RCTs including 800 extremely preterm infants assuring the safety of probiotics to this population [3, 45, 46], but in order to properly answer this question a placebo-controlled trial of probiotic supplementation including at least a few thousand extreme preterm infants would be needed. Considering all the evidence in favour of probiotics many would argue that it is beyond equipoise to enrol more patients in placebo-controlled RCTs. Therefore, starting a new RCT may be considered both ethically and practically challenging when the current available evidence is shared with parents prior to consent.

1.8.4 Example 2: Use of antibiotics in neonates

Systematic reviews have compared the effect of different antibiotic regimens on EOS [214] and LOS [215]. These reviews found lack of evidence or high quality research in favour of any antibiotic regimen in the treatment of both EOS and LOS and highlight the need of studies addressing the impact of different antibiotic regimens. Still, there are many guidelines based on both expert opinion and other evidence than from RCTs on how to administer antibiotics to neonates [54, 55, 216].

However, systematic evidence for adverse effects associated with antibiotic exposure in this vulnerable population is lacking. Therefore, we performed the first systematic review examining the relationship between antibiotic exposure in early life and five different adverse outcomes of interest.

2 Aims of the thesis

Overall aim:

The overall aim of this thesis was to study the clinical and microbiological effects of antibiotics and probiotics in neonates, with a particular focus on the developing gut microbiota.

Specific aims:

- To perform a systematic review and synthesize evidence from studies reporting different categories of antibiotic exposure in neonates and the subsequent risk of developing the following five adverse outcomes; death, NEC, invasive fungal infections (IFI), antibiotic resistance and alterations in gut microbiota composition.

- To assess influence of probiotics and antibiotics on gut microbiota composition and antibiotic resistome in extremely preterm infants supplemented with probiotics and compare data with very preterm infants not supplemented with probiotics.

- To assess the pathogenic potential of invasive *Bifidobacterium* blood culture isolates by analysing clinical characteristics of patients with *Bifidobacterium* bacteraemia and by using a genomic approach to assess pathogenicity.

3 Materials and methods

3.1 Materials

3.1.1 Study groups

In the systematic review leading to **Paper I-II** we included studies reporting data on neonates, preterm and/or term born up to 44 weeks (w) PMA, with different categories of intravenous antibiotic exposure and if the study reported adverse clinical outcomes including NEC, fungemia, death, changes in gut microbiota composition and/or antibiotic resistance development.

In the Preterm Infant Gut (PINGU) study, a multi-centre observational clinical trial leading to **Paper III**, we recruited eligible neonates from six different Norwegian NICUs (Table 1). We aimed to include three convenient groups of infants:

- 30 probiotic supplemented extremely preterm (PEP) infants; GA 25-27 w/BW < 1 kg
- 30 not probiotic supplemented very preterm (NPVP) infants; GA 28-31 w/BW 1.0-1.5 kg
- 10 healthy full-term control (FTC) vaginally delivered infants

Exclusion criteria were: GA < 25 w or GA 25-31 w with severe lethal complication/poor prognosis around one week of age and all infants with severe congenital malformations.

All the 10 FTC-infants were recruited from the maternity ward at University Hospital of Northern Norway, Tromsø, Norway. The hospital/NICU location of PEP- and NPVP-infants is presented in Table 1.

Table 1. Number of infants enrolled from each participating hospital/NICU

City/Location	Probiotic Extremely Preterm (PEP) infant group (n = 31)	Non Probiotic Very Preterm (NPVP) infants groups (n= 35)
Lørenskog/Oslo	2	5
Tromsø	6	1
Bergen	12	12
Trondheim	3	6
Oslo	5	5
Stavanger	3	6

Lørenskog/Oslo; Akershus University Hospital, Tromsø; University Hospital of Northern Norway, Bergen; Haukeland University Hospital, Trondheim; St. Olav's University Hospital, Oslo; Oslo University Hospital-Ullevål, Stavanger; Stavanger University Hospital.

Information about the infants was collected using data from the Norwegian neonatal network. Further data was collected using detailed questionnaires given to the mothers when the infants were 4 months old.

In the retrospective cohort study leading to **Paper IV** ("The Norwegian Bifidobacterium study") we included all 15 patients with *Bifidobacterium* bacteraemia reported to the Norwegian Organization for Surveillance of Antimicrobial Drug Resistance (NORM) during 2013-2015; [184]. Clinical characteristics from medical records were collected including age, sex, underlying medical conditions, symptoms and signs prompting blood culture, use of antibiotics and outcome.

3.1.2 Biological samples

In the PINGU study (**Paper III**) we collected faecal samples around seven, 28 days and 4 months of age. Samples collected at seven and 28 days of age were collected by a nurse at the local hospital, and samples at 4 months were collected by the parents after careful instructions.

In "The Norwegian Bifidobacterium study" (**Paper IV**) a collection of 15 *Bifidobacterium* blood culture isolates were identified for further analyses. The isolates were recovered from nine different hospital laboratories. Subsequently, all *Bifidobacterium* isolates were analysed at the laboratory of Department of Microbiology and Infection Control at the University Hospital of Northern Norway. Isolates were eligible for inclusion in this study if there was one blood culture set with presence of *Bifidobacterium*.

3.2 Methods

3.2.1 Study designs

The systematic review leading to **Paper I-II** is based on a published research protocol. When appropriate, meta-analysis using the random-effect model or a semi-quantitative vote-counting analysis were conducted.

The PINGU study leading to **Paper III** is an explorative multi-centre study using clinical data and predominantly data generated from metagenome sequencing of faecal samples. The

background for the PINGU study was the decision in 2014 to implement a national Norwegian consensus-based protocol recommending prophylactic probiotic supplementation to preterm infants at highest risk for NEC (gestational age < 28 weeks/birth weight < 1000 g). After considering the safety profile, a widely used probiotic (Infloran®) was selected which contains *Lactobacillus acidophilu*s (ATCC4356) and *Bifidobacterium longum* subsp. *infantis* (ATCC15697).

"The Norwegian Bifidobacterium study" leading to **Paper IV** is a retrospective cohort study using clinical data and genomic data generated from sequencing of *Bifidobacterium* blood culture isolates.

3.2.2 Approvals and protocols

The protocol for **Paper I-II** was prospectively registered in a trial register for systematic reviews (PROSPERO; study protocol registration number: PROSPERO CRD42015026743 [217].

The Norwegian Regional Ethical Committee approved the PINGU study (Approval number 2014/930) leading to **Paper III**. An informed written consent was obtained from the parents (Consent scheme attached as Appendix 2). The PINGU-study was registered at www.clinicaltrails.gov (ClinicalTrials.gov Identifier: NCT02197468).

The Norwegian Regional Ethical Committee approved the collection of blood culture isolates and clinical characteristics (Approval number 2016/1001) for **Paper IV**. Patients received written information about this retrospective study. Participation was voluntary with an opt-out option provided (Consent scheme attached as Appendix 3).

3.2.3 Methods used for faecal sampling and storage

The technical preparation of faecal samples is a critical step in the pipeline of gut microbiota analysis. The most important consideration involves how the faecal samples is to be stored and this may involve logistical challenges due to geographical distances from sample point to processing laboratory. To assure the quality of the collection kit used in the PINGU study (**Paper III**), we performed a validation-pilot study where we compared species distribution after isolating DNA from faeces stored in a commercial available kit (DNA Genotek OMNIgen GUT kit, Ottawa, Canada) to a standard procedure freezing faeces at -70°C immediately after faecal collection using sterile Eppendorf tubes. The DNA Genotek OMNIgen GUT kit consists of a tube with a metal ball and a stabilization buffer. Once the faecal sample is placed in the tube and

homogenized, it remains stable at ambient temperature, eliminating the freezing step or the need for urgent same-day DNA extraction. The quality was measured by the quality of DNA extracted and the taxonomic composition after sequencing. To further assess the preservative ability of the stabilization buffer we made a "cocktail" of different known bacterial species and evaluated the microbial composition due to different times of storage. The bacterial composition in the cocktail was made based on a representative selection of Gram-positive and Gram-negative bacteria commonly found in the human gut microbiota of infants. Samples were analysed by metagenome sequencing using the Illumina sequencer. Pilot data results showed that both sampling procedures displayed good concordance. Furthermore, there was no difference in microbial composition between different times of storage. Ease of use and the possibility of storage at ambient temperature for 7-14 days offered an apparent solution to logistical issues in our trial and was therefore chosen (**Paper III**).

3.2.4 DNA isolation

Faecal samples were processed within 14 days after sampling, preferentially within the first week after storage in ambient temperature, as per manufacturers instructions (**Paper III**). Faecal microbial DNA was extracted using the semi-automated NorDiag Arrow Stool DNA Extraction kit (NorDiag®), according to instructions from the manufacturer. In this protocol, we modified the DNA isolation and added an extra bead for bead beating step facilitating cell lysis as studies have shown that this can increase extraction of DNA from Gram positive bacteria [218].

DNA was extracted from pure cultures of *Bifidobacterium* spp. blood culture isolates using Gentra Puregene yeast/bacterial kit (Qiagen®), according to manufacturer's instructions (**Paper IV**).

3.2.5 Quantification of DNA

The extracted DNA was quantified using a second-generation Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY, USA) using a protocol based on the manufacturer´s recommendations for the Qubit dsDNA HS kit (High Sensitivity, Invitrogen). DNA quality was assessed by spectrophotometry using Nanodrop instrument (Thermo Scientific, Wilmington, MA, USA) (**Paper III and IV**). This was done according to the recommendations for DNA preparation suggesting that one should include a combination of Nanodrop and Qubit to assess the purity and quantity of dsDNA, respectively [219]. The Qubit fluorometer is based on dyes that emit fluorescence when binding to DNA [220]. The Nanodrop uses ultraviolet light at 260 nm and measures the amount of light absorbed by single stranded or double stranded DNA [220].

3.2.6 Whole genome sequencing, assembly and annotation

Bacterial DNA was prepared for whole genome sequencing (WGS) using the Nextera XT Kit (Illumina, San Diego. California, USA), according to the manufacturer's instructions. The fragment size distribution (500-1000 bp) was analysed using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany). In **Paper III**, we pooled samples at concentration of 4nM per sample. The samples were sequenced by the Illumina Miseq platform using v3 reagents with 2 × 300 cycles according to the manufacturer's instructions. In **Paper IV**, this yielded an average of 3.09 mill reads per bacterial isolate. In **Paper IV**, 184 samples were sequenced to a depth of 4.84 million reads in average per sample (ranging from 1.82-12.6 million reads) for microbiota and functional analysis. All samples in **Paper III** were screened for human decontamination. Assembly was performed de novo on trimmed reads using MEGAHIT [221] in **Paper III** and using SPAdes 3.5.0 in **Paper IV** [222]. Structural and functional annotation was performed using an in-house genome annotation pipeline, the META-pipe (Department of Chemistry, University of Tromsø [https://arxiv.org/abs/1604.04103]).

3.2.7 Species identification and taxonomy

In **Paper III**, identification was based on DNA from multiple organisms from the complex gut microbiota community. For metagenome faecal samples, we used the phylogenetic analysis tool, MetaPhlAn, to identify the taxonomic profile by using clade-specific marker genes. To calculate longitudinal changes, sequences were reconstructed using LCAclassifier [223] using the Lowest Common Ancestor (LCA).

In **Paper IV**, species identification was first performed at the microbiology departments at participating centres with traditional phenotyping techniques and supplemented with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Samples were then shipped to Tromsø for retesting. We re-analysed all 15 strains and *Bifidobacterium* species was confirmed using MALDI-TOF MS using a Microflex LT instrument (Bruker Daltonics, Bremen, Germany), Flex Control software and the MALDI Biotyper 3.1 software (Bruker Daltonics, Bremen, Germany). WGS was used for further subtyping of *B. longum*.

3.2.8 Antibiotic resistance (phenotypic and genotypic methods)

In **Paper II**, different studies included in the systematic review used different methods to analyse and define antibiotic resistance. We defined multi-drug resistant (MDR) bacteria as bacteria resistant to ≥ 2 different, unrelated classes of antibiotics or resistant to broad-spectrum antibiotics. Included in this category were ESBL-producing Gram-negative bacteria, carbapenem-resistant *Acinetobacter baumannii* (CRAB), and Gram-negative bacteria resistant to third-generation cephalosporins.

Given the metagenomic nature of genes in the PINGU study (**Paper III)** we used the resistance gene identifier in the comprehensive antibiotic resistance database (CARD)(version 1.1.1; Department of Biochemistry and Biomedical Science; McMaster University [https://card.mcmaster.ca/home)] [224] to predict genes presumed to confer antibiotic resistance in all faecal samples; the gut antibiotic resistome. Resistome prediction in CARD was performed on assembled genes using Abricate [https://github.com/tseemann/abricate]. To extend and obtain quantitative measures of functional ARG analysis to all metagenome sequenced preterm infant microbiomes, we used Short, Better Representative Extract Dataset (ShortBRED) against the formatted CARD database.

In the "The Norwegian Bifidobacterium study" (**Paper IV)**, the antibiotic susceptibility and resistome (ARGs) of bifidobacteria were obtained in order to clarify the relationship between phenotypic and genotypic susceptibility, as this is not always a simple one-to-one correspondence. The phenotypic susceptibility to nine antibiotics (penicillin G, metronidazole, clindamycin, tetracycline, meropenem, cefotaxime, ciprofloxacin, piperacillin-tazobactam and vancomycin) was determined using minimum inhibitory concentration (MIC) gradient strips, according to manufacturer's instructions (Liofilchem® Roseto degli Abbruzzi, Italy). Based on WGS data, we determined the antibiotic resistome from the 15 blood culture strains using the same CARD database as for **Paper III**.

3.2.9 Comparative genomics/ Pan-genome analysis

In **Paper IV** we performed a pan-genome analysis of the genomes from the 76 available *B. longum* isolates. This included all 65 available *B. longum* genomes of both human and animal origin from Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and the 11 *B. longum* genomes sequenced in the framework of this study. A gene cluster incorporating at least one representative from each isolate was defined as being part of the core genome, while gene clusters defying this definition

were part of the accessory genome and could be further sub-divided. Gene clusters represented in ≥ 72 isolates were regarded as soft core, ≤ 2 regarded as shell, and the rest of the accessory genome as cloud. To compare invasive and non-invasive isolates, we performed a pan-genome analysis at subspecies level for *B. longum* subsp. *longum* (n=34) and *B. longum* subsp. *infantis* (n=13) and compared invasive isolates of subsp. *longum* (n=7) and subsp. *infantis* (n=6) versus non-invasive isolates of subsp. *longum* (n=27) and subsp. *infantis* (n=7).

3.2.10 Virulence and other functional genes

To further elucidate the pathogenic potential of *Bifidobacterium*, putative virulence genes were determined using the virulence factor database (VFDB)(2016, Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College [http://www.mgc.ac.cn/VFs/]) [225] and numbers were compared between invasive and non-invasive isolates of *Bifidobacterium* recovered from Genbank (**Paper IV**).

3.2.11 Statistical analysis

In **Paper I,** most NRSs were not pooled for meta-analysis because of marked clinical and methodological diversity regarding e.g. interventions, antibiotics used, study design and the outcomes reported. We meta-analysed adverse outcomes of interest from studies considered sufficiently homogeneous to provide a meaningful summary, and calculated combined effect estimates. In the meta-analyses we pooled RCTs and NRSs; the latter only if clinical base line characteristics of patient groups that experienced different antibiotic exposures were similar, and the studies reported dichotomous outcomes. Subgroup analysis was performed for RCTs and NRSs. We quantified inconsistency between the results of the studies by using the I^2 test. Interpretation of thresholds for statistical heterogeneity was: I^2 values between 0-40 % might not be important, whereas higher I^2 values may represent moderate (30-60 %), substantial (50-90 %) or considerable heterogeneity (75-100 %)[204]. Data entry and meta-analysis were performed using RevMan version 5.3 (The Nordic Cochrane Centre, Copenhagen, Denmark). We calculated odds ratios (ORs) with 95% CI for the outcomes of interest. We present the effect-estimates by using the random-effect model due to assumption of clinical and methodological diversity among the studies, subsequently often leading to statistical heterogeneity.

In **Paper II**, the two outcomes of interest did not provide dichotomous results like in Paper I. The diversity of included studies and outcomes made traditional meta-analysis difficult. We therefore applied a simple vote-counting meta-analysis to investigate whether the different categories of antibiotic exposures had any effect on the outcomes of interest. Studies were classified based on whether they showed a reduction in the outcome measure, no effect, or an increase in the outcome measure following antibiotic exposure.

In **Paper III** we used both bioinformatic and statistical tools to analyse the data. The Mann-Whitney U test for two independent groups or a Kruskal-Wallis test for multiple independent groups were used to compare differences in metagenomic results between groups of infants. Corrections based on multiple testing were performed by the Benjamini-Hochberg false discovery rate (FDR) procedure. FDR provides an important tool when performing multiple comparisons for minimalizing rates of false rejections of null hypothesis (type 1 error) [226]. We used generalised linear model with the Poisson family to calculate trends in bacterial relative abundance and ARG abundance. Alpha diversity was assessed by calculating Shannon Index with the diversity function from MEGAN (v5.10.6). Linear mixed model was used to assess difference in alpha diversity and influence of antibiotic treatment over time and between the three groups.

In **Paper IV**, only descriptive statistical methods were used. Percentage distribution of unique genes were calculated and presented in bar charts.

3.2.12 Systematic review structure

The systematic reviews **(Paper I-II)** were reported according to the preferred reporting items for systematic reviews and meta-analysis (PRISMA)[227]. The PRISMA checklist is a guide on how to develop a systematic protocol and what to include when writing up a systematic review. The reviews were also performed according to the recommendations given by the Cochrane Handbook for Systematic Reviews and Interventions [204]

3.2.13 Literature search

Our search strategy **Paper I-II** for was developed in consultation with an epidemiologist, a librarian, a paediatric pharmacologist and a neonatologist. We searched PubMed, Embase, Medline and the Cochrane Database using MeSH-terms and text words from the inception of each database up to December 2016. The target was human studies written in English. There was no publication period restriction. We did not contact authors for supplemental information, and we did not perform searches in the grey literature. The first search was conducted using MeSH-terms. The search strategy in PubMed, Medline, and the Cochrane Database was as follows: ”Infant, Newborn” and ”Anti-Bacterial Agents” with one of the following outcome terms: "Enterocolitis, Necrotizing", "Fungemia", "Candidiasis, Invasive", "Meningitis, Fungal", "Mortality", "Drug Resistance, Bacterial" or "Microbiota". The Embase database uses its own key words, and ”Newborn” and ”Antibiotic Agent” were combined with one of the following outcome terms: "Necrotising Enterocolitis", "Fungemia", "Invasive Candidiasis", "Fungal Meningitis", "Mortality", "Drug Resistance, Bacterial" or "Microbiota". The second search was conducted using free text in PubMed, Medline and Embase combining the following keywords: ”Infant, Low Birth Weight” or ”Infant, Postmature” or ”Infant, Premature” or "Infant, Newborn” with: ”Anti-Bacterial Agents” or ”Antibiotics”, and one of the following combinations: "Necrotizing Enterocolitis" or "Fungaemia" or "Fungemias" or "Candidemia" or "Invasive Candidiasis" or "Fungal Meningitis" or "Mortality" or "Antibiotic Resistance" or "Antibacterial Drug Resistance" or "Microbiota" or "Microbiome" or "Microbiomes" or "Gut Flora". Finally, we looked at reference lists and citations of included studies and relevant previous reviews to identify any additional eligible studies. All citations were then combined and duplicates/triplicates were excluded.

3.2.14 Study selection and eligibility criteria

We only included studies **(Paper I-II)** if it reported on groups of neonates, preterm and/or term infants, with different categories of intravenous antibiotic exposure and the adverse clinical outcomes NEC, IFI, death, changes in gut microbiota composition and or antibiotic resistance occurring in the neonatal period or up to discharge from the neonatal unit. We considered both NRSs (cohort, case-control, cross-sectional) and RCTs comparing three different antibiotic exposures as mentioned below.

i) yes versus no antibiotics

ii) long versus short duration of antibiotics

iii) broad-spectrum versus narrow spectrum antibiotics

For category (ii), we suggested in advance that "prolonged" antibiotic exposure was always ≥ 3 days or the longest regimen amongst two antibiotic regimens compared. For category (iii), we always defined regimens including third-generation cephalosporins or carbapenems as a broad-spectrum regimen when compared to regimens containing aminoglycosides for coverage against Gram-negative bacteria. This definition was also based on the fact that empiric treatment using a third-generation cephalosporin for Gram-negative coverage induces significantly more antibiotic resistance than a regimen containing an aminoglycoside [59]. NEC was defined as Bell's stage 2-3[228]. IFI was defined as fungaemia or detection of fungi in otherwise sterile body sites. Death, as an adverse outcome, was defined as any cause of death including death attributed to infection during antibiotic therapy in the neonatal period or up to discharge from the neonatal unit. Microbiota analyses were based on faecal samples using both standard culture-based methods and culture-independent methods relying on DNA amplification and sequencing [229]. We defined microbial load as the total number of bacteria in a sample, microbial diversity as the number of bacterial genus or species in a sample, and microbial composition as the taxonomical composition in a sample. Antibiotic resistance development was based on detection of antibiotic susceptibility patterns in bacteria isolated from blood, urine, cerebrospinal fluid, faces, tracheal aspirates, and/or the skin surface. We included case-control studies reporting on pre-specified adverse outcomes, if data on antibiotic exposure prior to the outcomes were presented as extractable data in cases and controls, respectively. We excluded studies investigating antenatal antibiotics, oral antibiotics, low-dose intravenous vancomycin prophylaxis in preterm neonates and studies with a non-neonatal population.

3.2.15 Data extraction

The following information was extracted from the articles; author, year and country; study design; study population, including gestational age (GA) and birth weight (BW), comparison of outcomes between groups with different categories of antibiotic exposure, and if available risk estimates with 95% confidence intervals (CIs) for the specific outcome.

3.2.16 Quality assessment

We assessed methodological quality by using the Cochrane Handbook of Systematic Reviews of Interventions [204] which we adapted and clarified to also assess observational studies [230]. Disagreements in the categorization process were resolved after discussion.

Five domains related to risk of bias were assessed for each study included: (1) Selection, (2) Performance, (3) Detection, (4) Reporting and (4) Confounding. Risks of bias were low, high or unclear and judged based on the following:

Selection bias

- High or low, if patients had been or not been enrolled as consecutively observed based on a pre-existent study protocol and if numbers and reasons for possible exclusions were not reported specifically. Inappropriate selections of controls in a case-control study.

Performance bias

- High risk if systematic difference in the care provided to participants, including if there were different hospitals/centres included and trials with a before and after study design.
- Low risk if only one hospital/centre was included.

Detection bias

- High risk if retrospective study design or if systematic differences in outcome assessment among the groups being compared or erroneous use of statistical analysis.
- Low risk if prospective study design

Reporting bias

- **Paper I**: High risk if not reporting on all of the three adverse outcomes; NEC, IFI and or death.
- **Paper II**: High risk if use of culture-based techniques. Unclear risk if studies applied 16SrRNA sequencing techniques, and low risk if studies applied metagenome sequencing techniques.

In addition to assessing the risk of bias, we applied the GRADE approach to rate the quality of evidence (QoE) for each relevant outcome category in **Paper II**. RCTs started as high QoE while observational studies started as low QoE, and several factors could either downgrade or upgrade the quality rating. Factors lowering the quality of evidence included risk of bias, inconsistency, indirectness, imprecision and publication bias. Factors increasing the quality of evidence included high large effect size, evidence of dose response curve and if all plausible residual confounding would reduce a demonstrated effect. We used guidelines from Balshem et al to rate the quality of evidence [208, 231].

Table 2. A summary of GRADE`s approach to rate the quality of evidence.

A summary of GRADE's approach to rating quality of evidence

Study design	Initial quality of a body of evidence	Lower if	Higher if	Quality of a body of evidence
Randomized trials	High ⇒	Risk of Bias −1 Serious −2 Very serious Inconsistency −1 Serious −2 Very serious Indirectness −1 Serious −2 Very serious Imprecision −1 Serious −2 Very serious Publication bias −1 Likely −2 Very likely	Large effect +1 Large +2 Very large Dose response +1 Evidence of a gradient All plausible residual confounding +1 Would reduce a demonstrated effect +1 Would suggest a spurious effect if no effect was observed	High (four plus: ⊕⊕⊕⊕) Moderate (three plus: ⊕⊕⊕◯) Low (two plus: ⊕⊕◯◯) Very low (one plus: ⊕◯◯◯)
Observational studies	Low ⇒			

4 Summary of main results

4.1 Paper I

Antibiotic exposure in neonates and early adverse outcomes: a systematic review and meta-analysis. J Antimicrob Chemother. 2017; 72: 1858-70

Of the 47 studies meeting our inclusion criteria, there were 9 RCTs and 38 observational non-randomised studies. Of the included studies, 20, 24 and 21 reported on the risk of NEC, IFI and death, respectively. Many studies had high to moderate risk of bias. Meta-analysis was limited by substantial heterogeneity between studies and small number of RCTs.

Necrotizing enterocolitis (NEC): Of the 20 studies reporting different categories of previous antibiotic exposure and subsequent risk of NEC, five studies reported composite outcomes of NEC or death or NEC, LOS or death. In 13 of the included studies, NEC was clearly defined using Bell`s criteria. Six studies reported risk of NEC after antibiotic exposure but the results were divergent. Ten observational studies reported on duration of antibiotic exposure and risk of NEC. There was a significant association between prolonged antibiotic exposure and an increased risk of NEC in five observational studies (5003 participants). Seven studies investigated risk of NEC after exposure to broad versus narrow spectrum antibiotics including one large retrospective cohort comparing ampicillin and cefotaxime to ampicillin and gentamicin and found higher rates of NEC in the group receiving ampicillin and gentamicin. However, six studies found no difference in the risk of NEC when comparing broad versus narrow antibiotic regimens.

Invasive fungal infections (IFI): Eighteen out of the 24 studies focused on preterm infants. Most studies assessed either antibiotic therapy duration or compared different antibiotic regimens, and only two studies assessed antibiotic yes/no. Of the studies reporting on use of broad versus narrow spectrum antibiotics and the risk of IFI, the majority of studies used third generation cephalosporins or carbapenems as broad-spectrum antibiotics. Ten observational studies reported a significant increased risk of IFI after exposure to third-generation cephalosporins.

Death: Five studies including 13 534 infants reported increased risk of death after prolonged duration of antibiotic therapy, compared to shorter duration.

Data from meta-analyses: Meta-analysis was limited by substantial heterogeneity between studies and small number of RCTs. None of the meta-analyses reported significant differences in outcomes after different types of antibiotic exposure, exemplified by Figure 8a-b.

Conclusions: Prolonged antibiotic exposure in uninfected preterm infants is associated with an increased risk of NEC and/or death, and broad-spectrum antibiotic exposure is associated with an increased risk of IFI.

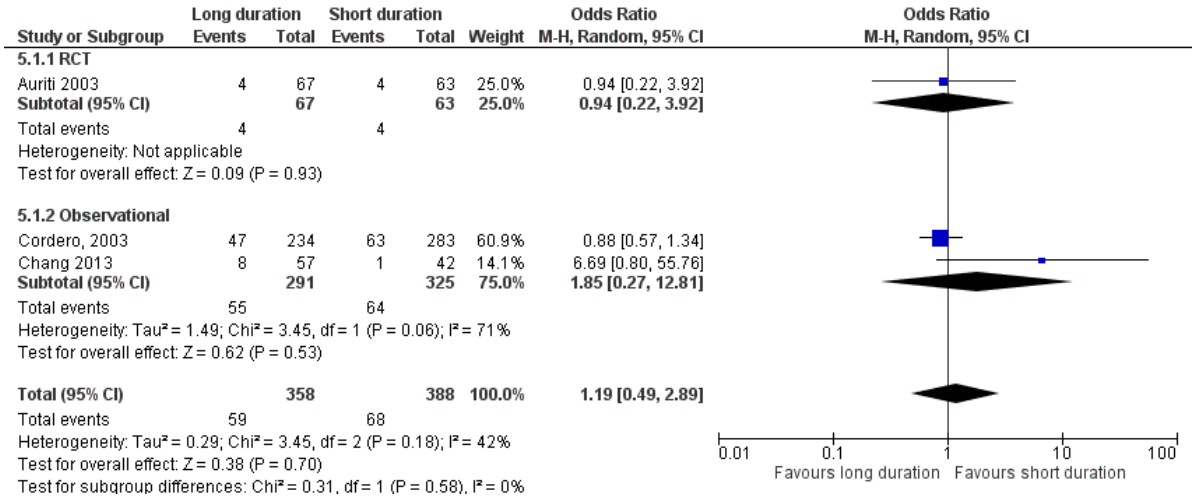

Figure 8a. Forest plot, pooled results of three studies comparing risk of death between children who received prolonged or shorter duration of antibiotic therapy. Subgroup analysis of RCTs and observational studies. The sizes of the squares are proportional to study weights. Diamond markers indicate pooled effect sizes.

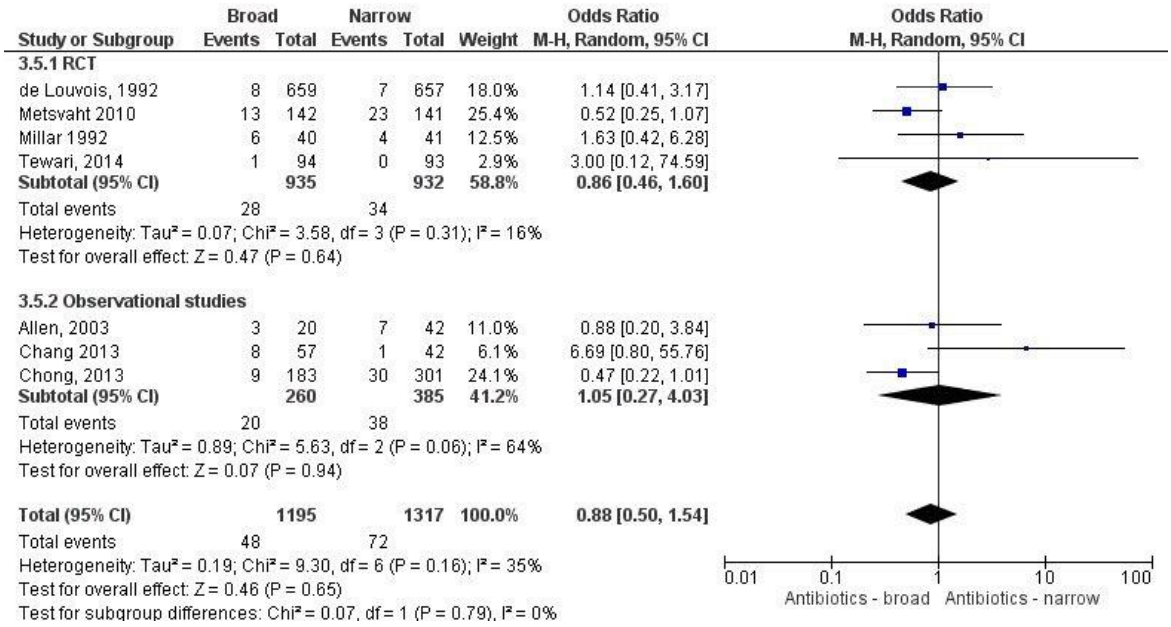

Figure 8b. Pooled results of eight studies comparing risk of death between children who received broader- versus narrower-spectrum antibiotic regimens. Subgroup analysis of RCTs and observational studies. The sizes of the squares are proportional to study weights. Diamond markers indicate pooled effect sizes.

4.2 Paper II

Antibiotic therapy in Neonates and Impact on Gut Microbiota and Antibiotic Resistance Development: A Systematic Review. Accepted for publication in J Antimicrob Chemother. 17th October 2017.

Of the 48 studies meeting our inclusion criteria, there were three RCTs and 45 observational NRSs. Lack of RCTs and diverse outcomes made meta-analysis impossible to perform. We graded quality of evidence (QoE) as very low for all outcomes presented in the gut microbiota category. In contrast, we considered the QoE as moderate in the antibiotic resistance category due to large size effects and a dose-response effect.

Gut microbiota composition: Nineteen studies evaluated the influence on gut microbiota composition after antibiotic exposure and the majority of studies used either culture-based techniques or 16S rRNA gene sequencing analysis. Three studies found reduced bacterial diversity following prolonged antibiotic exposure. Four out of five studies reported reduced colonisation of protective commensal anaerobic bacteria after antibiotic exposure (Figure 9a).

Antibiotic resistance: Thirty-one studies investigated risk of antibiotic resistance development after antibiotic exposure. MDR resistant bacteria were varyingly defined among studies. Studies focused mainly on resistance in Gram-negative bacteria. Thirteen studies reported data after exposure to broad spectrum versus narrow-spectrum antibiotics, and the overwhelming majority reported higher rates of MDR Gram-negative bacteria after exposure to broad-spectrum antibiotics (Figure 9b). In addition, the majority of studies assessing duration of antibiotic therapy and antibiotic resistance development found significantly more MDR Gram-negative bacteria after longer exposure.

Conclusions: We are moderately confident that antibiotic treatment leads to antibiotic resistance development in neonates, and it may also induce potentially disease-promoting gut microbiota alterations. Our findings emphasize the need to reduce unnecessary antibiotic treatment in neonates.

Study	Abundance and/or colonization rates Lower Unchanged Higher	Specific outcome	Abundance or colonization rates
Bennet, 1986-87		*Bifidobacterium* spp.	Colonization rates
		Lactobacillus spp.	
		Bacteriodes spp.	
Blakey, 1982 *		*Lactobacillus* spp.	Colonization rates
		Bacteriodes spp.	
Butel, 2007		*Bifidobacterium* spp.	Colonization rates
Fouhy, 2012		*Bifidobacterium* spp.	Colonization rates
		Lactobacillus spp.	
Hall, 1990		*Bifidobacterium* spp.	Colonization rates
		Lactobacillus spp.	

We graded quality of evidence as very low due to inclusion of observational studies with very serious risks of bias.

Figure 9a. Vote count on gut microbial composition after antibiotic exposure – compared to no antibiotic exposure; Commensal obligate anaerobes (5 studies; 304 neonates)

Study	Infection and/or colonization rates Lower Unchanged Higher	Risk estimates	Specific outcomes	Coloniz or infec
MM		OR 4.9, 95% CI 1.1-21.5†	ESBL-producing *K. pneumoniae*	Infection
Acolet, 1994		NDA	Cefotaxime-resistant *E. cloacae*	Coloniza
Calil, 2001		NDA	MDR *E. cloacae*	Coloniza
De Araujo, 2007		NDA	MDR Gram-negatives	Coloniza
De Champs, 1994		NDA	MDR *E. cloacae*	Coloniza
De Man, 2000		RR 3.14, 95% CI 1.76-5.56	Cefotaxime-resistant Gram-negatives	Coloniza
Le, 2008		OR 33.73, 95% CI 1.02-1136.20†	ESBL producing *Enterobacteriaceae*	Infection
Linkin, 2004		NDA	ESBL producing *Enterobacteriaceae*	Infection
Mammina, 2007		NDA	MDR Gram-negatives	Coloniza
Millar, 2008		NDA	MDR *Enterobacteriaceae*	Coloniza
Pessoa-Silva, 2003		OR 4.60, 95% 1.48-14.31	ESBL-producing *K. pneumoniae*	Coloniza
Thatrimontrichai, 2013		NDA	Carbapenem-resistant *A. baumannii*	Infection
Thatrimontrichai, 2016		OR 4.4; 95% CI 1.2-15.6†	Carbapenem-resistant *A. baumannii*	Infection

We graded quality of evidence as moderate due to inclusion of observational studies with large effect estimates.

Figure 9b. Vote count on infection and/or colonisation with MDR Gram-negative bacteria following antibiotic exposure; Broad spectrum– compared to narrow spectrum (13 studies; 4016 neonates)

4.3 Paper III

Probiotic Supplementation and Development of Preterm Infant Gut Microbiota and Antibiotic Resistome-An Observational Multi-Center Study. Submitted October 2017

The PINGU study cohort comprise 76 infants born in 2015, including 31 extremely preterm infants supplemented with probiotics (probiotic extremely preterm-PEP), 35 very preterm infants not supplemented with probiotics (non-probiotic very preterm –NPVP) and 10 full-term vaginally delivered infants as control (FTC). The PEP infants received more antibiotic therapy than the NPVP infants. Clinical characteristics are reported in Table 3.

Table 3. Study population and clinical characteristics in the PINGU study cohort

	Probiotic Extremely Preterm (PEP) Infants (n= 31)	Non-Probiotic Very Preterm (NPVP) Infants (n=35)	Full Term Control (FTC) Infants (n=10)
Birth weight, g, mean (SD)	825 (178)	1290 (220)	3651 (463)
Gestational age at birth, weeks, mean (SD)	26 (1)	29 (1)	40(1)
Gender; male/female	13/18	20/15	(3/7)
Route of delivery; Caesarean/vaginal	21/10	20/15	0/10
CRIB score, mean (SD)	11 (2)	5 (2)	-
Antenatal antibiotic exposure, *n*	8/31	12/35	0
Antibiotic exposure* first week of life, days, median (IQR), *n*	6 (4-7), 30	4 (3-5), 27	-
Antibiotic exposure after first week of life, days, median (IQR), *n*	6.5 (2.75-13), 22	10 (5.5-14), 5	
Ampicillin or Penicillin + Gentamicin after first week, median (IQR), *n*	6 (3-12), 16	9.5 (6-10), 4	-
Third-generation cephalosporin, median (IQR) ,*n*	7 (6-7), 7	6,1	-
Vancomycin, median (IQR) ,*n*	7 (7-14), 7	4 (4.5-4.5), 2	-
Meropenem, median (IQR), *n*	1	13, 1	-
Total days of antibiotic exposure, median (IQR), *n*	9.5 (6-18), 30	4 (3-6), 27	-
Probiotic supplementation, days, median (IQR)	46 (40-57)	-	-
Parenteral nutrition, days, median (IQR), *n*	9 (6-13), 31	5 (3.25-8), 16	-
Exclusive human milk nutrition until discharge	17/31	16/35	

* Only ampicillin or penicillin + gentamicin in first week of life

Gut microbiota composition: At all three time points for faecal sampling there were large intra-individual differences between infants, in particular at 7 days and 28 days of age. On day 7, we found that PEP-infants had significantly higher levels of *Bifidobacterium* compared to infants in the two other groups and higher abundance of *Lactobacillus* compared to NPVP infants (Fig 10a). On day 28, NPVP-infants displayed a similar abundance of bifidobacteria compared to PEP-infants. PEP-infants had lower abundance of *Bifidobacterium* and higher levels of *Escherichia* compared to microbial composition at seven days of age. FTC-infants had significantly higher levels of

Lactobacillus compared to NPVP-infants (Fig 10b). At four months of age, there were no statistical differences in relative abundance between the three groups of infants (Fig 10c).

Exposure to broad-spectrum antibiotics after first week of life had significant impact on gut microbiota composition in PEP-infants at four months of age showing reduced abundance of *Lactobacillus* and *Veilonella*. Furthermore, a trend towards reduced abundance of *Bifidobacterium* was found in both preterm groups (PEP- and NPVP-infants) at 28 days and four months of age after exposure to broad-spectrum antibiotics compared to more narrow-spectrum regimens.

Alpha diversity (Shannon index) increased significantly with age in both preterm infant groups. Significant differences in beta diversity using PerMANOVA were detected at 7 days and 28 days of age. Different categories of antibiotic exposure after first week of life did not influence diversity.

Antibiotic resistance and gut resistome: We identified a non-redundant set of 99 different ARGs conferring resistance to nine different classes of antibiotics. Among these, 28/99 (28%) represented ARGs located on mobile genetic elements. A substantial number of ARGs identified encoded genes to other antibiotics than those used in the NICU. Only 24 % of ARGs identified changed significantly during the period of observation ($p<0.05$). Genes encoding ESBLs were represented at all three time points, but were not detected in PEP-infants. The methicillin resistance gene (*MecA*) was identified at 7 days and 28 days in both preterm groups. Nine different vancomycin ARGs were detected at four months of age in all three groups of infants. On day 28, we detected higher abundance of four classes of ARGs in infants exposed to broad-spectrum antibiotics compared to infants treated with more narrow-spectrum regimens.

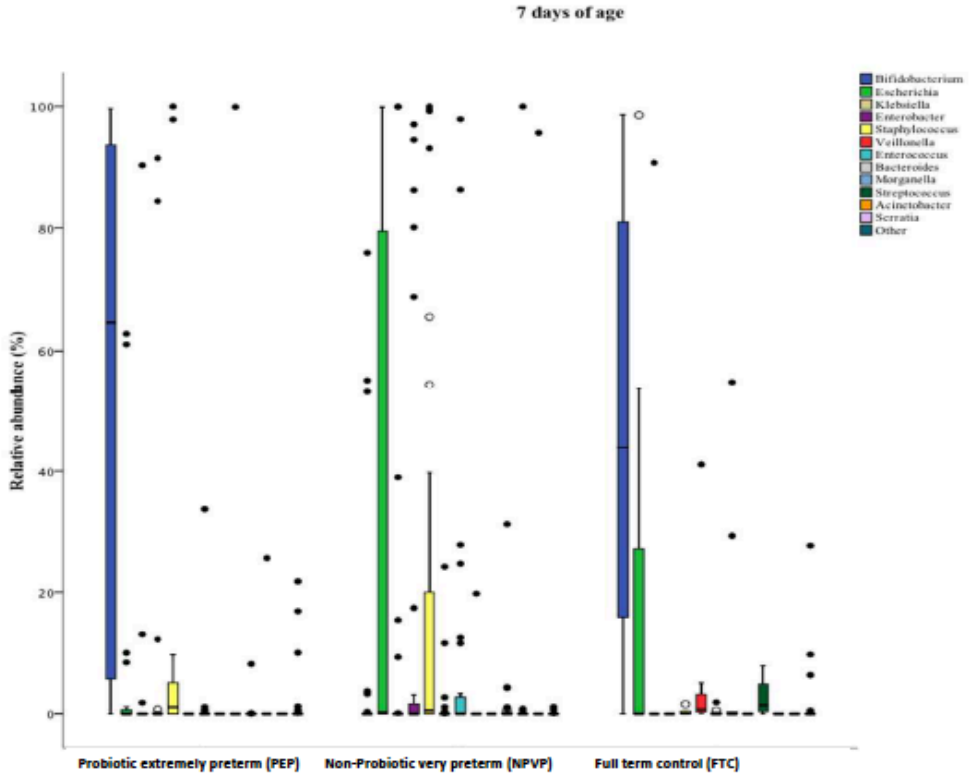

Figure 10a. Relative abundance of dominant taxa (>0.5%) at genus level at 7 days of age.

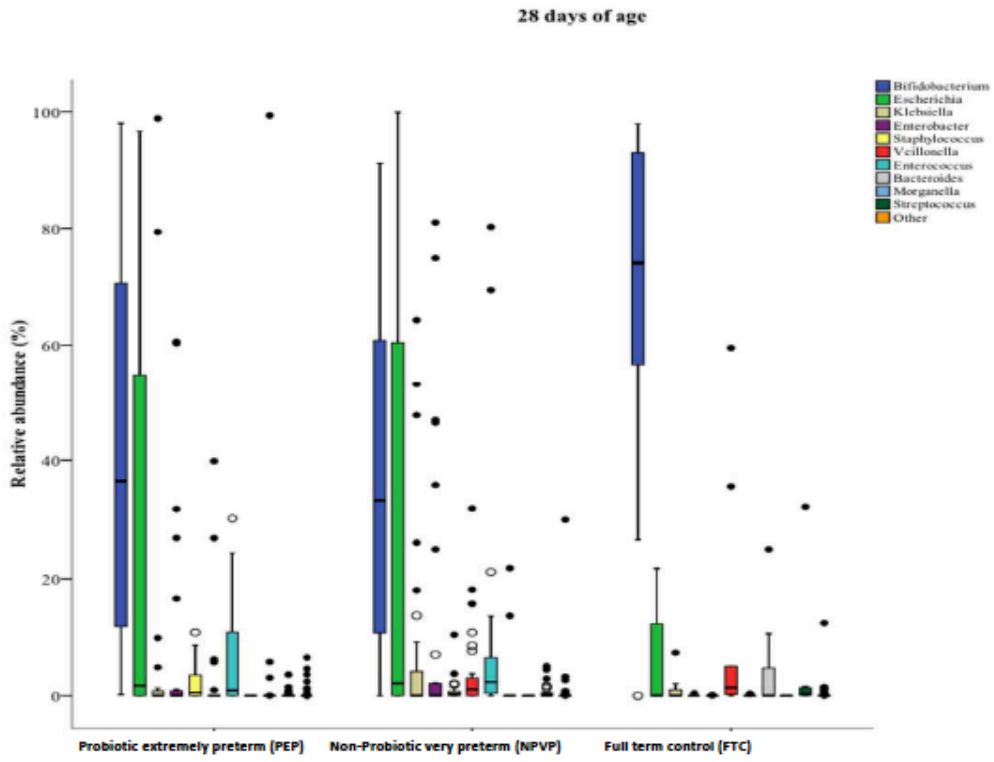

Figure 10b. Relative abundance of dominant taxa (>0.5%) at genus level at 28 days of age.

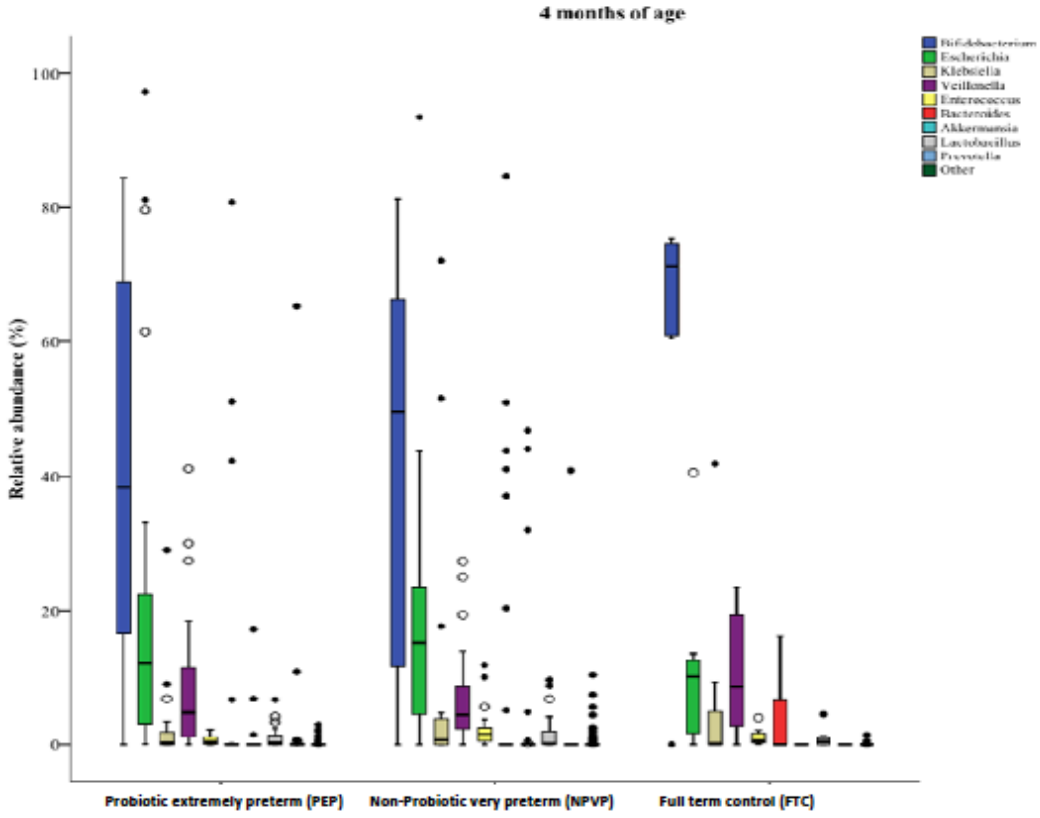

Figure 10c. Relative abundance of dominant taxa (>0.5%) at genus level at four months of age.

4.4 Paper IV

***Bifidobacterium* bacteremia: Clinical Characteristics and a Genomic Approach to Assess Pathogenicity. J Clin Microbiol. 2017; 55: 2234-48**

We used clinical characteristics of patients with *Bifidobacterium* bacteraemia and WGS-data of 15 blood culture isolates to assess the pathogenic potential of *Bifidobacterium.* The majority of patients were in the lower or upper age spectrum, and most were severely immunocompromised or had signs of serious underlying medical conditions. Ten patients had gastrointestinal-tract related conditions. *Bifidobacterium* bacteraemia was considered cause of death in 2/4 patients who died.

Taxonomic composition: Using MALDI-TOF we identified 11 *B. longum*, 2 *B. breve* and 2 *B. animalis* that were subjected to WGS. The 11. *B. longum* were further subdivided into subspecies level; *B. longum* subsp. *infantis* (n=4) and *B. longum* subsp. *longum* (n=7) (Figure 11).

Antibiotic resistance and resistome: All isolates displayed low MIC to vancomycin, meropenem and piperacillin-tazobactam. Nine and six isolates displayed high MIC for ciprofloxacin and metronidazole, respectively. Genes encoding efflux pumps were found in all isolates. All isolates harbored the *mfd* gene and mutations in *gyrA*. Mutations in these genes are associated with resistance to fluoroquinolones, and 12 of 15 bifidobacterial isolates had MIC ≥ 4 mg/L to ciprofloxacin. There were some discrepancies between phenotypic and genotypic findings.

Comparative genomics and virulence genes: We performed pan-genome analysis of all invasive and non-invasive isolates of *B. longum* including 65 available sequences from Genbank and eleven blood culture isolates from this study. Most of the functional classes identified represented genes involved in housekeeping functions. When looking for specific traits characterizing invasive isolates, we found clusters of unique functional genes in both invasive and non-invasive isolates. Moreover, phylogenetic tree based on the accessory genome of *B. longum subsp. longum* visualized clustering of 5/6 invasive isolates (Figure 12). However, number of putative virulence genes was not different between invasive and non-invasive isolates.

Conclusions: *Bifidobacterium* has an invasive potential in the immunocompromised host and may cause a sepsis-like picture. Using comparative genomics we could not delineate specific pathogenicity traits characterizing invasive isolates.

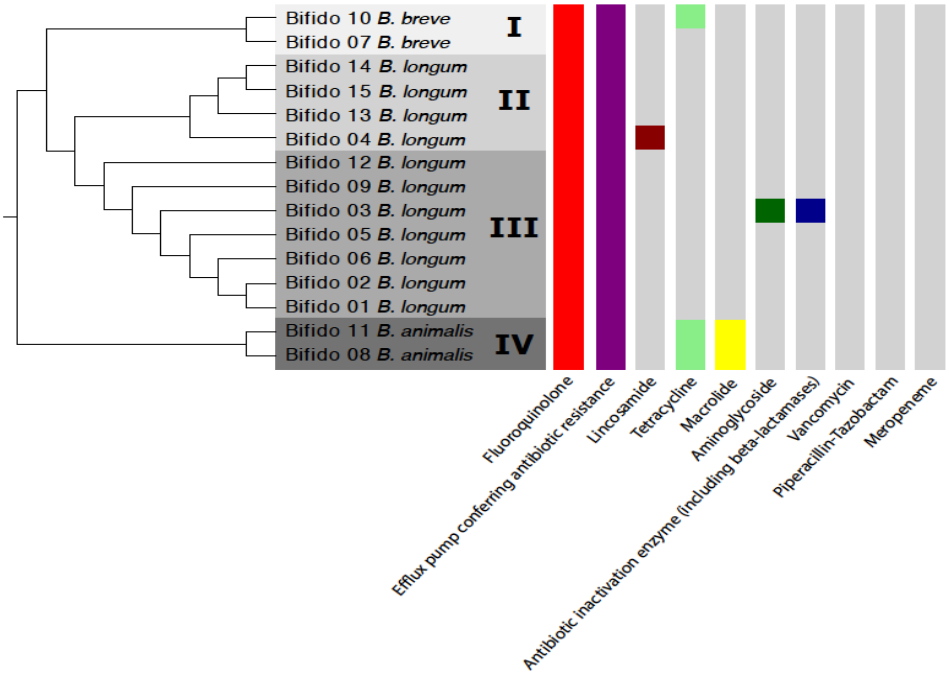

Figure 11. Dendrogram representing the arrangements of clusters between the 15 strains of *Bifidobacterium* and prevalence of genes encoding groups of antibiotic resistance.

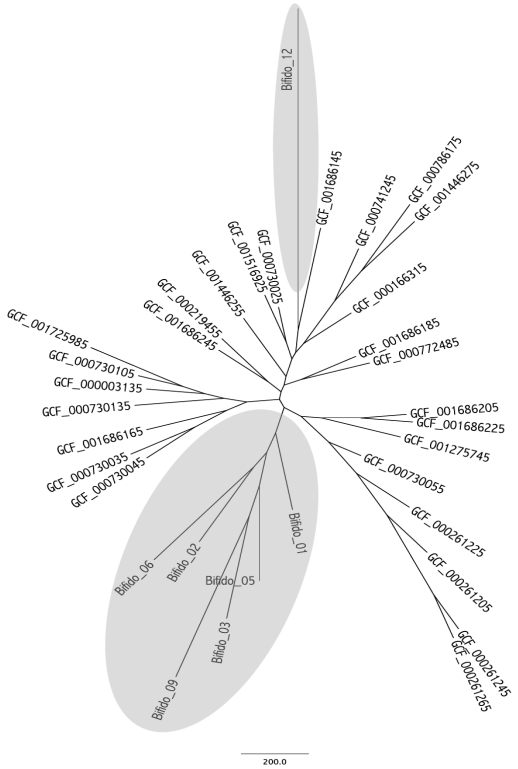

Figure 12. Phylogenetic tree based on the accessory genome of *B. longum* subsp. *longum*.

5 General discussion

5.1 Discussion of results

The studies included in this thesis focus on adverse effects of antibiotic treatment in neonates (**Paper I and II**), the influence of probiotics (**Paper III**) and antibiotics (**Paper II and III**) on gut microbiota composition in preterm and term infants, and finally the pathogenic potential of *Bifidobacterium*, a commonly used probiotic bacteria (**Paper IV**).

We found prolonged antibiotic exposure to be associated with increased risk of NEC and/or death (**Paper I**). Broad-spectrum antibiotics were associated with increased risk of IFI (**Paper I**) and reduced colonisation of obligate anaerobe commensals such as *Bifidobacterium*, *Lactobacillus* and *Veilonella* and higher relative abundance of *Escherichia* (**Paper II and III**). Furthermore, antibiotic exposure was associated with increased colonisation rates with MRD Gram-negative bacteria (**Paper II**).

During the course of our multi-centre clinical trial, assessing the influence of probiotics on the gut microbiota, *Bifidobacterium* was detected in blood cultures of three preterm infants supplemented with probiotics (**Appendix 3**). However, using whole-genome sequencing and comparative genomics, we could not find any specific pathogenic traits characterising invasive strains of *Bifidobacterium* (**Paper IV**).

In probiotic supplemented preterm infants, we found that bifidobacteria strongly dominated the gut microbiota composition only few days after commencing supplementation, and probiotic supplemented preterm infants had even higher relative abundance of bifidobacteria than the full-term control infants at seven days of age (**Paper III**). Despite heavy antibiotic exposure in probiotic-supplemented infants, there were no significant differences in relative abundance of ARGs at 28 days and four months of age compared with more mature preterm infants and the full-term control group (**Paper III**).

5.1.1 Early adverse effects of antibiotics

Many studies have assessed adverse effects of antibiotic exposure early in life, but to our knowledge **Paper I** and **Paper II** are the first to systematically review early adverse outcomes (NEC, IFI, death, gut microbiota composition and/or antibiotic resistance development) of antibiotic exposure in the neonatal period. One of the primary findings was the lack of RCT's and

high quality observational studies, and the heterogeneity regarding methodology and outcomes among the included studies.

Antibiotic therapy may alter several basic equilibriums in the human gut microbiota, including diversity and taxonomic composition. In our systematic review (**Paper II**) we found that antibiotic exposure was associated with reduced gut microbiota diversity, in line with findings from many other studies [43, 91, 232-234]. Reduced diversity following antibiotic treatment creates a window for opportunistic pathogens and inflammation as the microbiota repopulates. Furthermore, microbial diversity is characteristically reduced in infants at risk of developing NEC [40]. Two studies [129, 232] did not detect any significant changes in diversity after antibiotic exposure, in line with our findings in **Paper III**. However, in **Paper III**, the infants most heavily exposed to antibiotic treatment were also supplemented with probiotics. Indeed, probiotics have shown to alleviate the potential loss of microbial diversity created by antibiotic treatment. Concurrent treatment with probiotics and antibiotics in mice lead to an increase in gut microbial diversity, albeit not statistically significant [235].

Antibiotic therapy perturbs the resilient early-life microbiota through their effect on the trajectory of microbial colonisation with delayed commensal colonisation and predominance of more pathogenic bacteria, especially in preterm infants (**Paper II**). Here, we found that four out of nine studies reported increased abundance and/or colonisation of *Enterobacteriaceae* following antibiotic treatment in neonates [38, 129, 233, 236]. In the majority of these studies, the empiric regimens consisted of ampicillin and gentamicin. We speculate that intravenous ampicillin also has an impact on Gram-positive gut bacteria despite being mainly secreted in the kidneys, while intravenous gentamicin mainly covering Gram-negative bacteria in the blood stream has low penetration to the gut, favouring Gram-negative bacteria like *Enterobacteriaceae*. Our findings from **Paper II** also suggested antibiotic exposure in the neonatal period to be strongly associated with reduced abundance of protective commensal bacteria such as bifidobacteria, lactobacilli and/or bacteroides [129, 236, 237]. These bacteria provide colonisation resistance against potentially pathogenic bacteria such as *Enterobacteriaceae*. In **Paper III**, we found a clear trend towards reduced colonisation of *Bifidobacterium*, *Lactobacillus and Veilonella* and increased colonisation of *Escherichia* in infants exposed to broad-spectrum antibiotics compared to infants exposed to more narrow-spectrum regimens. Although the pathogenesis of NEC is currently not well understood, bacterial colonisation is thought to be a critical element in the development of the disease, supported by the fact that NEC cannot be produced in germ free animals [40, 238]. The first and recently published systematic review investigating intestinal dysbiosis preceding NEC found increased relative abundance of *Proteobacteria* and reduced relative abundance of *Firmicutes* and

Bacteroidetes before NEC onset. Furthermore, antibiotic usage was associated with increased abundance of *Proteobacteria* [30]. Taken together, these data may partly explain the increased risk of NEC in infants receiving prolonged antibiotic therapy (**Paper I**).

We found that exposure to third-generation cephalosporins or carbapenems were associated with increased risk of IFI, mainly Candida infections (**Paper I**). Preterm infants are more prone to early colonisation by *Candida* than term infants, due to an immature immune system and impaired skin and mucosal integrity [19]. Furthermore, prolonged use of antibiotics may foster invasive *Candida* infections by suppressing normal flora and allowing *Candida* to occupy muco-epithelial niches that facilitate invasion and dissemination. Cephalosporin use has been associated with intestinal colonisation by *Candida* among neonates [239] and colonisation is a risk factor for invasive *Candida* infections [21-23].

We found an increase in all-cause mortality after prolonged antibiotic therapy in preterm infants (**Paper I**). This may be due to several reasons, including higher risk of NEC and increased risk of other later infections and/or immune-related diseases secondary to a certain degree of immune suppression [240].

5.1.2 Antibiotics and antibiotic resistance

Of studies reporting rates of colonisation and/or infection with MDR Gram-negative bacteria after antibiotic exposure, we found an overwhelming majority reporting higher rates of MDR Gram-negative bacteria, especially ESBL-producing bacteria (**Paper II**). Overuse of antibiotics, in particularly broad-spectrum antibiotics in the most preterm infants, applies a selection pressure favouring antibiotic resistant bacteria and decreases colonisation resistance [128]. Antibiotic treatment appears to reduce colonisation resistance through collateral destruction of obligate anaerobic bacteria. A critical point is that the antibiotic that exerts selection pressure and expansion of an antibiotic-resistant bacterial species may not be the one it is resistant to, but rather an antibiotic that kills bacteria that provides colonisation resistance [241].

In line with others (**Paper II**), we detected significant higher abundance of ARGs in infants receiving broad-spectrum antibiotics compared to narrow-spectrum regimens (**Paper III**). Gibson and co-workers also showed that broad-spectrum antibiotic therapy administered to preterm infants, was associated with enrichment of specific ARGs [106]. In our study, overall there were no differences in distribution of ARG-classes or abundance of ARGs at 28 days and 4 months of age between PEP-infants exposed to massive antibiotic therapy after first week of life

and the two other groups with limited or no antibiotic exposure. Although not a new idea, the potential use of probiotics to re-establish microbiota-mediated colonisation resistance after antibiotic treatment in order to reduce colonisation of antibiotic resistant pathogens and thereby reduce infection rates, has gained interest. It is not clear how probiotic supplementation influences gut colonisation by antibiotic resistant pathogens in preterm infants, but maintaining a balanced gut microbiota under antibiotic treatment may provide opportunities for reducing spread of antibiotic resistance [128]. Furthermore, indirectly, production of bacteriocins and improving mucosal integrity can also be effective means for probiotic bacteria to reduce pathogenic bacterial population and thereby antibiotic resistance [128]. Lactobacilli can also increase the susceptibility of Gram-negative bacteria to antimicrobial agents [242]. Obligate anaerobes are the major contributors to colonisation resistance among the commensal bacteria of the gut [243-245].

5.1.3 Probiotics - a recommended supplement to preterm `high risk` infants?

The hypothesis supporting the use of probiotic bacteria to prevent NEC and sepsis is that their administration to the preterm infant will encourage gut microbiota resembling that of the term infant, strengthen intestinal barrier function, and, thereby, protect the infant. We have found that bifidobacteria strongly dominates the gut microbiota composition in extremely preterm infants only a few days after commencing probiotic supplementation. This finding was already evident at 7 days of age, despite the fact that supplemented infants were more immature and heavily exposed to antibiotic treatment (**Paper III**). Indeed, relative abundance of *Bifidobacterium* was higher at seven days of age in PEP-infants compared to FTC-infants. High levels of probiotic bacteria during supplementation are not necessarily indicative of colonisation, but may simply represent the passage of DNA from the administered species through the host [246].

Among very preterm infants not receiving probiotics, bifidobacteria were rarely present at one week of age. The gut microbiota of preterm infants has consistently been shown to have higher proportions of *Proteobacteria* and a bloom of *Bifidobacterium* occurring in later stages compared to those of full term infants [40, 247]. This finding was evident in the very preterm infants not receiving probiotics (**Paper III**). Furthermore, there may be a gestational age threshold for colonisation with certain microbes: 33 weeks appears to be the milestone for the colonisation of *Bifidobacterium* species [90], explaining the ”catch-up” colonisation in the non-supplemented moderate preterm infants at one month of age (**Paper III**). No bifidobacteria were

detected in a study of ELBW infants not supplemented with probiotics in which GA was less than 32 weeks, further supporting this hypothesis [248]. However, some of the bifidobacterial species represented at 28 days and four months of age in NPVP infants could simply represent cross-colonisation where probiotic bacteria is transferred from supplemented to non-supplemented infants in a neonatal unit where probiotics is being administered. Indeed, rates of cross-colonisation have been varyingly reported from 44% [249] to 7% [250], but data addressing this issue is limited.

Lactobacillus was scarcely detected in all three groups of infant, however, PEP-infants had significantly higher relative abundance of *Lactobacillus* at seven days of age compared to NPVP-infants. Relative abundance of lactobacilli increased up to four months of age in all three groups. High levels of *Bifidobacterium* and barely detectable levels of *Lactobacillus* have been reported earlier [251]. A possible explanation for this somewhat surprising observation is the spatial organization of intestinal bacteria, where lactobacilli are found in intestinal crypts and therefore less accessible to luminal contents [252].

There is no clear dosage guidance for probiotics nor evidence of lethal or toxic doses of probiotics, but evidence indicates that to be functional, probiotics have to be viable and in sufficient dosage levels, typically 10^7 to 10^9 colony-forming units (CFU) [30, 253]. One study from India compared standard and high-dose probiotic regimens and found no difference in proportion of infants colonised or quantitative colonisation rates with probiotic species between groups [254]. The two largest randomized trials (the PiPs trial and the ProPrems Study) used doses of 1 x 10^8 and 1 x 10^9 CFU, respectively [255, 256]. Infloran® capsules administered in our clinical trial contained 10^9 CFU of both *B. longum* subsp. *infantis* and *Lactobacillus acidophilus*, respectively. We speculate that the high abundance of *Bifidobacterium* in the probiotic supplemented group, observed in our study, could be non-physiologic or non-beneficial for the gut ecosystem and immature immune system of the extreme preterm infant in the first days of life. We therefore suggest that a rather gradual increase in probiotic supplementation concomitantly with increased enteral nutrition may replicate the physiological gut microbiota development. Moreover, live probiotics have the potential to replicate in the gut and lead to bacteraemia. Previously there were occasional reports on bacteraemia with lactobacilli, and until 2015, only two *Bifidobacterium* bacteraemia cases in premature newborns had been reported [176, 180]. Although most case reports on neonatal *Bifidobacterium* bacteraemia have reported clinically mildly affected infants, our case series presented three cases of *Bifidobacterium* bacteraemia from the strain in the probiotic Infloran®, of which two cases had a severe clinical course (**Appendix 1**). Furthermore, over the last few years, an increasing number of blood cultures with growth of

Bifidobacterium have been reported to the Norwegian Organization for Surveillance of Antimicrobial Resistance [257]. However, the apparent increase in *Bifidobacterium* bacteraemia observed may have other reasons. In the recent past, the exact diagnosis of *Bifidobacterium* from a blood culture isolate relied on biochemical tests for species identification with known limitations. Thus, blood cultures with growth of *Bifidobacterium* may have been identified only as Gram-positive rods with no further specification of the species. This may have led to an underestimation of the incidence of *Bifidobacterium* bacteraemia. However, new diagnostic tools such as the MALDI-TOF MS, improve species detection and its introduction in routine use may be one reason for the apparent increase observed (**Paper IV**).

Although probiotic products are generally regarded as safe, vigilance regarding their potential virulence, antibiotic resistance, and adverse metabolic activity should be maintained [258]. Careful consideration is therefore important when supplementing probiotics to this high-risk population with associated poor nutrition, impaired immune status and frequent exposure to infectious agents. However, there are no reported cases of sepsis with probiotic organisms in any of the RCTs, among these the PIPs trial comprising 650 VLBW infants (GA < 30 weeks) supplemented with probiotic containing *Bifidobacterium breve* [255]. Furthermore, no probiotic sepsis cases were reported among the 2761 probiotic supplemented infants in the updated Cochrane review [3].

Our clinical findings from patients with *Bifidobacterium* bacteraemia, both neonates and adults, were much in line with previous reports on patients with invasive *Bifidobacterium* infections indicating that they seem to be opportunistic infections in immunocompromised patients, probably secondary to bacterial translocation from the gut (**Paper IV**). *B. longum* and *B. dentium* are the species most frequently reported to cause bifidobacterial infections [174, 175]. In our study, we recovered three different species: *B. breve*, *B. longum* and *B. animalis*.

We identified several *Bifidobacterium* genes playing an important role in bacterial virulence, including genes encoding proteins involved in adhesion, anti-phagocytosis, immune evasion, iron uptake and bile resistance (**Paper IV**). However, our findings must be interpreted with caution as these virulence factors actually are essential features for most commensals and important features for colonisation resistance against pathogens [128]. In fact, most of the mechanisms involved in adhesion of commensal bifidobacteria to host tissue are similar or even identical to those employed by pathogens to cause disease [259]. In order to further explore the virulence potential of *Bifidobacterium* we performed an in-silico comparative analysis of all available invasive blood culture isolates and commensal isolates of *B. longum*. Here we detected unique clusters among

invasive and non-invasive isolates. However, in the virulence prediction, we found limited variation in the putative virulence content, as most genes were present in both invasive and non-invasive isolates.

5.1.4 *Bifidobacterium* and antibiotic resistance

Our antibiotic susceptibility findings were similar across all three *Bifidobacterium* species, much in line with previous studies [199, 260, 261] (**Paper IV**). However, there were discrepancies between phenotypic and genotypic findings. We identified ARGs conferring resistance to fluoroquinolones, tetracycline, lincosamides in addition to efflux pumps and antibiotic inactivation enzyme (**Paper IV**). It has been suggested that ARGs frequently may be transferred between bacteria within the gut microbiota, a process accelerated by the selective pressure of antibiotics, but also recently demonstrated in the gut microbiota of infants in absence of antibiotic treatment [262]. Furthermore, *in vitro* experiments have demonstrated transfer of antibiotic resistance determinants from one *Lactobacillus* to pathogenic bacteria [263] and opposite from enterococci to lactobacilli [264]. In *Bifidobacterium*, only a very small fraction (<1%) of the bifidobacterial resistome is predicted to reside on mobile genetic elements [201]. Furthermore, conjugative plasmids in bifidobacteria have not yet been reported. Although *Bifidobacterium,* together with other probiotic bacteria, harbour ARGs that potentially may be transferred to other gut bacteria, the occurrence of ARGs in probiotic bacteria may also give them selective advantages and improve their colonisation and persistence in the gut. However, accumulation of probiotics "filled with" ARGs may have long-term evolutionary consequences with risk of increased trans-conjugation employed by heavy antibiotic pressure [265]. Moreover, a study comparing the repertoire of bifidobacterial resistance genes between infants and adults showed that adults possess a much larger arsenal of bifidobacterial ARGs compared to infants [201]. However, none of the ARGs or susceptibility results from our study indicate that *Bifidobacterium* confer resistance to the commonly used antibiotic regimens in NICUs, such as ampicillin, penicillin or other beta-lactams. Nonetheless, these bacteria among other microbial populations in the gut microbiota, are commensal bacterial species that have co-evolved as part of the human super-organism over millions of years and thus their safety might be considered well established.

5.2 Strengths and limitations

Systematic reviews represent an important tool in appraising and synthesizing research-based evidence and if possible provide recommendations informed by the empirical evidence. To what extent conclusions can be drawn depends on the validity of included studies. The strengths in our systematic reviews include our rigorous search strategies following an a priori registered protocol. However, while selecting appropriate studies meeting our eligibility criteria, we discovered the lack of RCTs and a substantial diversity in the non-randomized studies in terms of methodology, quality, type of antibiotic exposure and sample size. This made meta-analysis possible in only a subset of studies included (**Paper I**) or we had to apply a semi-quantitative vote counting approach (**Paper II**). However, vote counting fails to take into account the methodological quality of pooled studies. Most studies included were non-randomized. However, even though their quality of evidence is considered low with high risk of bias and confounding, many studies attempted to adjust these limitations by performing multivariable logistic regression analyses. Our intention was to collect as much evidence as possible related to the targeted outcomes. We therefore included both randomized and non-randomized studies, this in line with suggestions from the Cochrane group stating that systematic reviews of rare adverse effects usually need to include non-randomized studies in addition to RCTs, as the latter primary focus on effectiveness and not adverse effects [204, 266].

Our definition of broad-spectrum and narrow-spectrum antibiotics is somewhat arbitrary and clearly has limitations, as most of the narrow-spectrum antibiotics covered both Gram-positive and Gram-negative bacteria (**Paper I-III**). However, our study confirms previous findings that antibiotic regimens containing third generation cephalosporins or carbapenems are more frequently associated with antibiotic resistance development than regimens with aminoglycosides for Gram-negative coverage [59].

It is well known that each of the steps in the pipeline of WGS and gut microbiota analysis has the potential to introduce biases and results need to be interpreted with certain scepticism [139]. In addition to great intra-individual variations between infants gut microbiota, separate analyses from the same person can also vary considerably, even from the same stool sample (**Paper III**). Furthermore, using databases in search for homologous functional genes can be speculative as closely related genes might cloak important differences and sequence homology between different bacteria do not always predict function [267], especially for less characterized bacteria such as the *Bifidobacterium* (**Paper III and IV)**.

At the time of this study, probiotic supplementation to extremely preterm infants was considered standard of care in Norway. We were therefore beyond equipoise perform a randomized study comparing probiotic to no probiotic supplementation in extremely preterm infants. The NPVP-infant group has limitations as a control group particularly due to maturational differences and the difference in antibiotic exposure compared to the PEP-infant group. However, more antibiotic exposure in the PEP-group would most likely have led to less diversity and higher abundance of ARGs. Still, we found few differences between the two preterm groups at 28 days and 4 months of age, suggesting a protective effect of probiotics in the PEP-infant group.

5.3 Methodological considerations

5.3.1 Faecal sampling

We used a standardized sampling technique allowing us to place the samples immediately in tubes that could be stored at room temperature for up to a week. This was essential to maintain high quality results in a multi-centre design, and avoiding the problems with immediate freezing of samples. However, infants harbours a much lower microbial diversity in the gut compared to adults and this means that any variation in the gut microbiota composition caused by storage in the stabilising buffer would have a proportionally greater effect on the infant gut microbiota composition [147]. Studies have shown that the specimen itself and the DNA isolation method can affect the DNA quality and quantity and inferred microbial composition as well as the microbial richness and diversity [218]. Inherent specimen properties may also influence the DNA isolation efficacy leading to a biased microbial community composition.

5.3.2 DNA isolation

Many studies have demonstrated how different DNA extraction kits will generate different results in terms of amount and quality of extracted DNA depending on the samples bacterial composition [268, 269]. We did not perform PCR and therefore possible inhibitors from DNA isolation on PCR reactions will not be further discussed. In contrast to pure cultures where DNA is isolated from one bacterial species, the complex faecal matter with different bacterial species, endogenous human DNA and dietary components, makes DNA isolation from faeces

particularly difficult [270]. The essence is obtaining a sufficient DNA quantity representing as many species as possible with optimal DNA purity for the sequencing approach extracted from this heterogeneous material. Moreover, when performing studies on microbial communities with multiple samples, processing time and cost must also be taken into consideration [271].

5.3.3 Species identification

Earlier, species identification relied mostly on Gram staining and biochemical tests with known limitations. Various molecular techniques including PCR, DNA sequence analysis, microarray analysis and fluorescence in-situ hybridization provide a more accurate identification, but often requires 12-24 hours for a final identification. Therefore, many clinical laboratories have not routinely implemented these methods, also due to higher costs. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has emerged as a robust, rapid and specific low-cost identification tool for bacterial species [272, 273]. There are two different protocols for bacterial treatment prior to MALDI identification; an on-plate method that is more simplified compared to the second method involving an extra in-tube extraction. The on-plate method is a direct smear technique with addition of 70% formic acid for cell wall denaturation before the spectra is acquired by the mass-spectrometer and compared with the database. The tube-based extraction includes an extra protein-step before the media is placed on the plate for MALDI identification. Studies have shown that these two methods often provide consistent results, but the in-tube extraction can sometimes provide greater utility, especially for Gram-negative bacteria [274, 275]. The simple extraction method is the most common routine in clinical microbiology laboratories.

5.3.4 Whole genome sequencing

Whole genome sequencing differs in its complexity depending on whether it is a genomic sample (containing DNA from one organism) or metagenomic sample (DNA from several different organisms). Metagenomic samples represent different bacteria with different abundance, meaning that high abundant organisms will represent sufficient data coverage while low abundant organisms are more difficult to assemble [271].

5.3.5 Comparative genomics

Bacterial genome data have been used in order to investigate bacterial evolution through identification of genes, constituting the core named cluster of orthologous genes (COGs), which appear to be conserved among bacteria.

In assessing the potential pathogenicity of *Bifidobacterium*, a classical risk approach similar to that used for true pathogens must be interpreted with caution as most of the factors detected as putative virulence determinants are actually essential features for commensal bacteria [276]. Therefore, a more appropriate approach is to compare clinical isolates with gastrointestinal commensals as controls, as we did in **Paper IV**.

The pan-genome represents the total number of genes encoded by a certain species in the gene repertoire of currently sequenced representatives of a species [277]. Therefore, investigation of the bacterial pan-genome represents a sophisticated approach to detect unique features of a species and differences between particular isolates, e.g. invasive versus non-invasive.

5.3.6 Risks of bias and quality of evidence in systematic reviews

Heterogeneity and their susceptibility to different biases is a well recognised problem in systematic reviews of observational studies. This was indeed a substantial challenge in both **Paper I** and **Paper II**. For both of these papers we used the Cochrane Handbook of Systematic Reviews and Interventions to assess risk of bias with each of the included studies. In **Paper II** we also included studies without clear dichotomous outcomes. We therefore decided to include the GRADE approach for quality of evidence assessment. This was not specified in our prospective research protocol, and the fact that we did not use the GRADE approach in **Paper** I is a limitation with this paper.

6 Concluding remarks

Our systematic reviews highlight the potential detrimental effect of antibiotic treatment in neonates, especially preterm infants and emphasize the need to reduce unnecessary antibiotic exposure in neonates. We found that antibiotic exposure appears to induce disease-promoting alterations in the gut microbiota and prolonged antibiotic therapy is associated with increased risk of NEC and/or death in preterm infants. Exposure to broad-spectrum antibiotics, particularly third generation cephalosporins or carbapenems, are associated with increased risk of IFI, increased risk of colonisation with antibiotic resistant bacteria and higher abundance of ARGs in the gut.

The high abundance of *Bifidobacterium* in probiotic-supplemented infants at one week of age suggests that a more gradual increase in probiotic supplementation may replicate the physiological gut microbiota development. Use of broad-spectrum antibiotics was associated with higher abundance of ARGs. However, in the probiotic-supplemented group, we found no difference in the abundance of ARGs compared to other groups of infants, despite the massively antibiotic exposure in the probiotic group. Our findings support the potential of probiotics to provide colonisation resistance, to reduce spread of antibiotic resistance and thereby infections caused by antibiotic resistant pathogens.

Bifidobacterium has an invasive potential in the immunocompromised patients or in patients with a compromised intestinal barrier, including preterm infants. Using comparative genomics, we could not delineate specific pathogenic traits characterizing invasive isolates. However, a possible phylogenetic separation was detected between invasive and non-invasive isolates of *B. longum* subsp. *longum* isolates.

7 Future aspects

In our multicentre observational trial (**Paper III**), we are planning a follow-up study from faecal samples collected at 1 year of age. Here we aim to investigate colonisation persistence of probiotic bacteria and the antibiotic resistome. Furthermore, exploring functional analysis like how metabolic pathways in the preterm infant gut microbiota are influenced by probiotic therapy is still in its infancy [188]. This may provide important information of the mechanisms of action of probiotics and need to be further assessed.

Animal models have shown that probiotics reduce fungal colonisation and IFI. In a recent systematic review, only five studies were found to report on fungal colonisation after probiotic supplementation [278]. Current evidence is therefore limited to draw any firm conclusions on the effect of probiotics on this particular outcome and this may be an important a subject for further investigation.

Do resistance genes present in commensal bacteria threaten human health or are they destined to stay sequestered in host bacteria unlikely to cause disease? New sequencing techniques might help us answer this question. Binning ARGs to its neighbouring sequence could help us determine the type of bacteria hosting it. This could answer the complementary question in functional analysis of "who is doing what?"

Future advancement in cultivation methods provide an important tool in augmenting metagenomic studies by providing in vivo fitness models providing a richer and detailed view of the dynamic infant gut microbiota and its resistome.

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Intentional introduction of disease has been rare (*3*). Consequently, the incident identified by Thalassinou and her colleagues arouses readers' interest and inspires speculation.

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Bifidobacterium longum Subspecies *infantis* Bacteremia in 3 Extremely Preterm Infants Receiving Probiotics

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To the Editor: Metaanalysis of randomized trials that tested different probiotics showed a reduction of ≈50% in necrotizing enterocolitis and all-cause deaths in preterm infants (*1*). Use of probiotics is increasing worldwide (*2*,*3*), and cases of probiotic sepsis were not reported among >5,000 infants in an updated review (*1*).

In Norway, a consensus-based protocol recommending prophylactic probiotic supplementation for preterm infants at highest risk for necrotizing enterocolitis (gestational age <28 weeks, birthweight <1,000 g) was introduced in 2014. After considering the safety profile, we investigated use in preterm infants of a widely used combination of oral probiotics (Infloran; Laboratorio Farmacéutico Specialità Igienico Terapeutiche, Mede, Italy) that contained 10^9 *Lactobacillus acidophilus* (ATCC 4356) and 10^9 *Bifidobacterium longum* subspecies *infantis* (ATCC 15697).

B. longum is a microaerotolerant, anaerobic bacterium susceptible to many antimicrobial drugs (Table). This bacterium is a rare cause of neonatal infections; until 2015, only 2 *Bifidobacterium* bacteremia cases in premature newborns had been reported (*4*,*5*).

A total of 290 extremely preterm infants received oral probiotics during April 2014–August 2015 in Norway. Three patients were given a diagnosis of *B. longum* bacteremia: 2 patients in a neonatal unit in which 17 patients were given oral probiotics and 1 patient in a neonatal unit in which 31 patients were given oral probiotics (Table).

All 3 infants had respiratory distress syndrome and received mechanical ventilation after birth. Enteral feeding with human milk was begun on day 1. Oral probiotics (½ capsule, 1×/d) were given during the first week of life and increased to 1 capsule/day after 4–7 days.

We identified *B. longum* in blood cultures by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics, Billerica, MA, USA). Whole-genome sequencing (MiSeq, Illumina, San Diego, CA, USA) and comparative analysis of nucleotide-level variation by using variant cell format in SAMtools (http://samtools.sourceforge.net) showed that all 3 blood culture isolates and a *B. longum* strain cultured from an oral probiotic capsule were identical.

Patient 1 had sepsis and severe hypotension 8 days after birth. A blood culture was prepared, and the patient was given antimicrobial drugs and vasoactive support. Abdominal distention, gastric residuals, and feed intolerance developed the next day, but the patient was cardiorespiratory stable. On day 12, abdominal radiographs showed pneumoperitoneum. Surgery showed multiple ileal perforations and bowel necrosis. Histologic analysis showed classical features of necrotizing enterocolitis. The patient received an ileostoma and improved after treatment with antimicrobial drugs. Blood culture was positive for gram-positive rods, which were identified as *B. longum*. Subsequent clinical course was uneventful.

Patient 2 had apnea, bradycardia, and temperature instability 12 days after birth. A blood culture was prepared, and the patient was given antimicrobial drugs. Blood culture was positive for gram-positive rods, which were identified as *B. longum*. Use of oral probiotics was discontinued. The patient recovered rapidly, and subsequent clinical course was uneventful.

Patient 3 had sepsis and necrotizing enterocolitis 9 days after birth. Ultrasound showed free abdominal fluid. A blood culture was prepared, and the patient was given antimicrobial drugs. Surgery showed 2 separate bowel perforations, and

Table. Characteristics of 3 extremely preterm infants with *Bifidobacterium longum* subspecies *infantis* bacteremia, 2015*

Characteristic	Patient 1	Patient 2	Patient 3
NICU	A	B	A
Sex	M	M	F
Date of onset	Apr	Jul	Sep
Gestational age, wk	24	23	24
Birth weight, g	730	500	697
Mode of delivery	Vaginal	Vaginal	Caesarean section
Apgar score at 1, 5, and 10 min after birth	4, 5, 5	Unknown, 0, 4	2, 2, 3
Reason for prematurity	Preterm rupture of membranes, maternal infection	Sudden preterm rupture of membranes, delivery not attended by healthcare personnel	Placental abruption
Age at onset of sepsis, d	8	12	46
Maximum CRP level, mg/L, ≤48 h of symptom onset	147	25	242
Age at discharge, wk	40	41	43
Weight at discharge, kg	3.3	3.4	3.3
Bacterial culture medium and conditions	BacT/ALERT,† aerobic, 36°C	BACTEC Plus,† aerobic, 35°C	BacT/ALERT,† aerobic, 36°C
Bacterial growth in blood culture, d	2	3	2

*Patients were given ½ to 1 capsule/day of oral probiotics (Infloran; Laboratorio Farmacéutico **Specialità Igienico Terapeutiche,** Mede, Italy) that contained 10^9 *Lactobacillus acidophilus* (ATCC 4356) and 10^9 *B. longum* subspecies *infantis* (ATCC 15697). MICs (mg/L) for antimicrobial drugs tested were 0.016 for meropenem, 0.032 for ampicillin, 0.064 for penicillin, 0.064 for piperacillin/tazobactam, 0.250 for cefotaxime, 0.250 for clindamycin, 0.250 for vancomycin, and 4.000 for ciprofloxacin. All bacterial strains were inherently resistant to aminoglycosides. ATCC, American Type Culture Collection (Manassas, VA, USA); CRP, C-reactive protein; NICU, neonatal intensive care unit.
†bioMérieux (Marcy l'Étoile, France).

the patient received an ileostoma and colostoma. Histologic analysis did not show necrosis or inflammation. *Enterococcus faecalis* grew in the blood culture obtained on day 9. The patient had a complicated clinical course and received prolonged mechanical ventilation. However, the patient gradually tolerated full feeds. Use of oral probiotics was continued.

On day 46, the condition of patient 3 suddenly deteriorated; hypotension and metabolic acidosis developed, and the patient was again given antimicrobial drugs. A blood culture was positive for *B. longum*. Supplementation with oral probiotics was discontinued. The patient recovered from the infection, but secondary ileus developed. The patient had a complicated clinical course until discharge.

Recently, 5 other *B. longum* bacteremia cases among 5 preterm infants at 26–31 weeks gestation were reported (*6*,*7*). All 5 infants had received oral probiotics; 3 had severe gastrointestinal complications, similar to patient 1 in our report, and 2 patients were moderately compromised, similar to patient 2 (*6*,*7*).

We do not know whether *Bifidobacterium* organisms in blood culture for patient 1 were a consequence of intestinal necrosis and bacterial translocation or the cause of necrotizing enterocolitis. Patient 3 probably had a leaky gut that predisposed this patient to bacterial translocation. All 3 patients were extremely premature (23–24 weeks gestation) and had impaired immune systems, which predisposed them to infections with bacteria with low virulence. A recently published case of *Bifidobacterium* bacteremia in a 2-year old boy with leukemia highlights impaired immunity as a risk factor (*8*).

Only aerobic blood cultures are prepared for neonates. We detected *Bifidobacterium* bacteremia by using 2 automated blood culture systems and aerobic bottles. However, the sensitivity of these systems for detecting *Bifidobacterium* bacteremia is unknown. Thus, the incidence of *Bifidobacterium* bacteremia is theoretically underestimated. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry improves species detection and its use might be 1 reason for the apparently recent increase in probiotic-associated bacteremia.

We report that systemic infection with probiotic bacteremia might have a severe clinical course in extremely preterm infants. Clinical suspicion and appropriate blood culture conditions are essential for proper diagnosis and management.

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Multidrug-Resistant *Staphylococcus aureus*, India, 2013–2015

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To the Editor: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a versatile pathogen capable of causing a wide variety of human diseases. Increased frequency of *S. aureus* infections imposes a high and increasing burden on healthcare resources. In many countries, MRSA infections in hospitals are common. Data from the National Nosocomial Infections Surveillance system suggest that, in the United States, incidence of nosocomial MRSA infections is steadily increasing and that these infections account for >60% of intensive care unit admissions (*1*,*2*). *S. aureus* has developed resistance to several antimicrobial drugs, including second- and third-line drugs. Only a few drugs, such as vancomycin (a glycopeptide), daptomycin (a lipopeptide), and linezolid (an oxazolidinone), have been approved for the treatment of serious infections caused by MRSA. Another drug, tigecycline (a glycylcycline), has shown good activity against MRSA strains in vitro (*3*). The epidemiology of MRSA is constantly changing, which results in variation in its drug-resistance patterns throughout regions and countries (*4*). Therefore, to support clinicians in preventing and treating infection, epidemiologic surveillance is essential. We report resistance patterns of *S. aureus* collected over 2 years (December 2013–November 2015) from blood samples of patients admitted to 1 hospital in Odisha, eastern India.

A total of 47 *S. aureus* isolates were collected; only 1 isolate per patient was included in the study. Susceptibility of the isolates was tested against antimicrobial agents according to the Clinical and Laboratory Standards Institute broth microdilution procedure and interpretation criteria (http://clsi.org/). MICs for the isolates were confirmed by using a Vitek 2 Compact automated system (bioMérieux, Marcy l'Étoile, France). *S. aureus* ATCC 25923 was used as a control strain. *S. aureus* identification was confirmed by using a Vitek 2 system, by hemolytic activity on blood agar, and by positive catalase activity test results. Clinical MRSA isolates were analyzed by using PCR with specific primers: *mecA* (*5*), *cfr* (*6*), and *VanA* (*7*).

Among the 47 *S. aureus* isolates, 28 (60%) were resistant to oxacillin (MICs 4–64 mg/L) and cefoxitin (MICs 8–64 mg/L). All MRSA isolates were able to grow in selective medium containing either aztreonam (75 mg/L) or colistin (10 mg/L). Screening of MRSA isolates showed that 2 isolates were highly resistant to vancomycin (MIC ≥100 mg/L) (Figure). Further screening showed that both vancomycin-resistant isolates were also resistant to linezolid (MIC ≥100 mg/L) (Figure). PCR amplification of both isolates indicated presence of all 3 genetic determinants: *mecA* (methicillin resistance), *cfr* (linzolid resistance), and *VanA* (vancomycin resistance). Among the 3 isolates that showed resistance to tigecycline (MIC ≥50 mg/L), 1 isolate was susceptible to vanocmycin and linezolid (Figure). Unlike previously reported isolates, these 2 MRSA isolates showed resistant phenotypes to linezolid, tigecycline, and vancomycin.

MICs observed in this study were higher than those previously reported. Vancomycin-resistant *S. aureus* has been identified in many other countries. Most linezolid-resistant *S. aureus* has been isolated from patients in North America and Europe (*8*). The tigecycline-resistant *S. aureus* isolate (MIC >0.5 mg/L) reported from Brazil was also susceptible to linezolid, teicoplanin, and vancomycin (*9*).

This study indicates the emergence of multidrug-resistant *S. aureus* with co-resistance to methicillin, vancomycin,

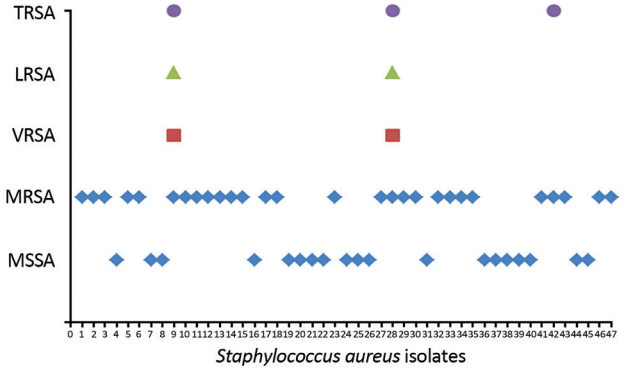

Figure. Distribution of various resistance types of *Staphylococcus aureus* isolates collected in eastern India, 2013–2015. LRSA, linezolid-resistant *S. aureus*; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S .aureus*; TRSA, tigecycline-resistant *S. aureus*; VRSA, vancomycin-resistant *S. aureus*.

Forespørsel om deltakelse i forskningsstudie

Undersøkelse av tarmflora til for tidlig fødte barn innlagt på nyfødtavdelinger i Norge og friske barn på barselavdelinger.

Bakgrunn og hensikt

Dette er et spørsmål til dere om å delta i en forskningsstudie hvor vi ønsker å undersøke tarmfloraen til for tidlig fødte barn og friske fullbårne barn. For tidlig fødte barn har ofte en umoden tarmflora sammenlignet med fullbårne friske barn. Dette kan skyldes at de har fått antibiotika, at det har tatt lang tid før de tolererer mat i magen og at de er innlagt på sykehus der man ofte har en egen "sykehusflora". De mest for tidlig fødte barna, født før svangerskapsuke 28 eller med fødselsvekt under 1000 gram, vil få tilførsel av probiotika. Probiotika er levende «snille» bakterier som man mener kan ha en helsefremmende effekt. I Norge gis morsmelk, enten mors egen melk eller såkalt bankmelk (melk fra en morsmelkbank) til nesten alle for tidlig fødte barn. Nytten av probiotika til barn som får full ernæring morsmelk er ikke så godt undersøkt. Det er også lite undersøkelser på om probiotika-bakterier som gis til noen barn i en sykehusavdeling kan endre sykehusfloraen for andre barn i avdelingen.

Hva innebærer studien?

Barn som er aktuelle for å være med i denne studien skal være født før svangerskapsuke 32 og ha en fødselsvekt som er lavere enn 1500 g. I tillegg ønsker vi å ha med en gruppe friske barn som er født til termin og som ammes fullt; disse skal være en kontrollgruppe.

Fra barna som blir med i studien vil vi samle inn en avføringsprøve på to tidspunkt i nyfødtperioden mens barnet er innlagt på sykehus; ved slutten av første leveuke og ved slutten av fjerde leveuke. Vi ønsker også å samle inn avføringsprøver fra barnet ditt ved 6 og 12 måneders alder. Avføringsprøvene vil bli frosset ned og deretter i første omgang lagret i en biobank. Når studien er ferdig vil vi analysere tarmbakteriefloraen fra alle avføringsprøvene med moderne laboratorieteknikker.

Det vil ikke bli tatt noen ekstra blodprøver og studien innebærer ingen ekstra smertefulle undersøkelser eller inngrep. Ved 6 og 12 måneders alder vil vi gjerne få lov til å ringe dere som foreldre og be om svar på noen korte spørsmål rundt barnets ernæring, vekst og eventuelle allergier/eksem. Vi vil videre sende dere små prøveglass for å få tatt avføringsprøve når barnet er rundt 6 og 12 måneder gammelt.

Mulige fordeler og ulemper

Barnet deres vil få helt lik medisinsk behandling som alle andre barn i avdelingen. Utover avføringsprøvene vil det ikke være noen andre spesielle undersøkelser eller medisinske tiltak. Svar på avføringsprøvene vil først foreligge lenge etter at deres barn er utskrevet fra sykehus og vil ikke få noen konsekvens for behandlingen eller videre oppfølging.

Hva skjer med informasjonen om ditt barn?

Informasjonen som blir samlet om deres barn er bakgrunnsdata (fødselsvekt, alder, sykelighet under opphold på nyfødtavdelingen) samt data fra tarmbakterieanalysene.
Alle opplysninger og prøvesvar vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode vil bli knyttet til alle opplysninger og prøvesvar. Koden vil bli knyttet til en navneliste. Det er kun autorisert personell knyttet til prosjektet som har adgang til navnelisten og som kan finne tilbake til deres barn. Det vil ikke være mulig å identifisere deres barn i resultatene av studien når disse publiseres.

Frivillig deltakelse

Det er frivillig å delta i studien. Dette vil ikke få konsekvenser for videre behandling av deres barn om dere velger å delta eller ikke. Dersom dere som foreldre ønsker at barnet deres deltar, undertegner dere samtykkeerklæringen på siste side og leverer denne til sykepleier på Nyfødtavdelingen.

Om dere nå sier ja til å delta, kan dere senere trekke tilbake samtykke uten at det påvirker barnets øvrige behandling.

Hvis dere ønsker å delta i studien, eller få nærmere informasjon, så vennligst ta direkte kontakt med en av følgende ansatte på Nyfødtavdelingen (se navneliste) eller hør med en sykepleier om vedkommende kan formidle kontakt. Dersom dere senere har spørsmål til studien, kan dere også kontakte:

- Claus Klingenberg, overlege, Nyfødt Intensiv, UNN. Tel 77 66 98 45, e-post: claus.klingenberg@unn.no
- Eirin Esaiassen, assistentlege/stipendiat, Nyfødt Intensiv, UNN. Tel 77 66 98 45, e-post:

Samtykke til deltakelse i studien

Jeg er villig til at mitt barn deltar i studien

(Signert av mor, dato)

Jeg er villig til at mitt barn deltar i studien

(Signert av far, dato)

Jeg bekrefter å ha gitt informasjon om studien

(Signert, rolle i studien, dato)

Innhenting av medisinske opplysninger i forbindelse med forskningsprosjekt

Bifidobakterier fra blodkulturer i Norge

Bakgrunn:

Mitt navn er Eirin Esaiassen og jeg jobber som stipendiat ved Universitetssykehuset Nord-Norge. I mitt doktorgradsprosjekt undersøker jeg bifidobakterier og deres evne til å forårsake sykdom hos mennesker. I den forbindelse har jeg fått opplysning om at du tidligere har gjennomgått en infeksjon forårsaket av bifidobakterier. Derfor ønsker jeg å kontakte deg.

Bifidobakterier finnes normalt i tarmen hvor de ansees som ”gode bakterier” som blant annet hjelper til med fordøyelsen og hindrer etablering av mer farlige bakterier. På grunn av av sin gunstige virkning er bifidobakterier også en vanlig bestanddel i probiotika. Probiotika er levende ”snille” bakterier som man mener kan ha en helsefremmende effekt og finnes i gjærede meieriprodukter, men også håndkjøpsmedisin.

Man har i de siste årene observert en økende forekomst av infeksjoner forårsaket av bifidobakterier, både hos barn og voksne. Årsaken er foreløpig ukjent, men økt bruk av probiotika kan være en forklaring. Det kan imidlertid også skyldes at bifidobakterier over tid har endret sine egenskaper slik at de lettere gir sykdom hos mennesker.

Hva innebærer studien?

Vi ønsker å undersøke bifidobakterier funnet i blodkulturer i forbindelse med infeksjon hos pasienter ved ulike sykehus i Norge. Vi har derfor samlet inn alle blodkulturer med oppvekst av bifidobakterier i tidsrommet 2014-2015. Disse har vi analysert med moderne laboratorieteknikker. For å kunne tolke våre funn på en best mulig måte er der derfor viktig å knytte disse opp mot kliniske data fra den enkelte pasient de tilhører. Dette er informasjon som: alder, kjønn, grunnlidelse, alvorlighet av infeksjon forårsaket av bifidobakterie og evt hvilken type antibiotikabehandling

som ble gitt og varighet av denne. Alle opplysninger vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger.

Hva innebærer dette for deg?

Data vil innhentes ved de respektive sykehusenes hvor du som pasient har vært innlagt. **Du trenger derfor ikke å foreta deg noe.**
Studien inkluderer ikke noe undersøkelser eller behandling, men vil være en viktig del av kvalitetsarbeidet rundt diagnostikk av infeksjonspasienter. Informasjon vedrørende den enkelte pasient vil derfor være avgjørende.

Studien er godkjent av Regional komite for medisinsk og helsefaglig forskningsetikk (REK). Dersom du ikke ønsker at opplysninger innhentes kan du ta kontakt med undertegnede (se kontaktinformasjon nedenfor). Dersom du har andre spørsmål kan du også ta kontakt.

På forhånd takk!

Med vennlig hilsen

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Universitetssykehuset Nord-Norge

Claus Klingenberg (Prosjektansvarlig)
Prof. Overlege
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