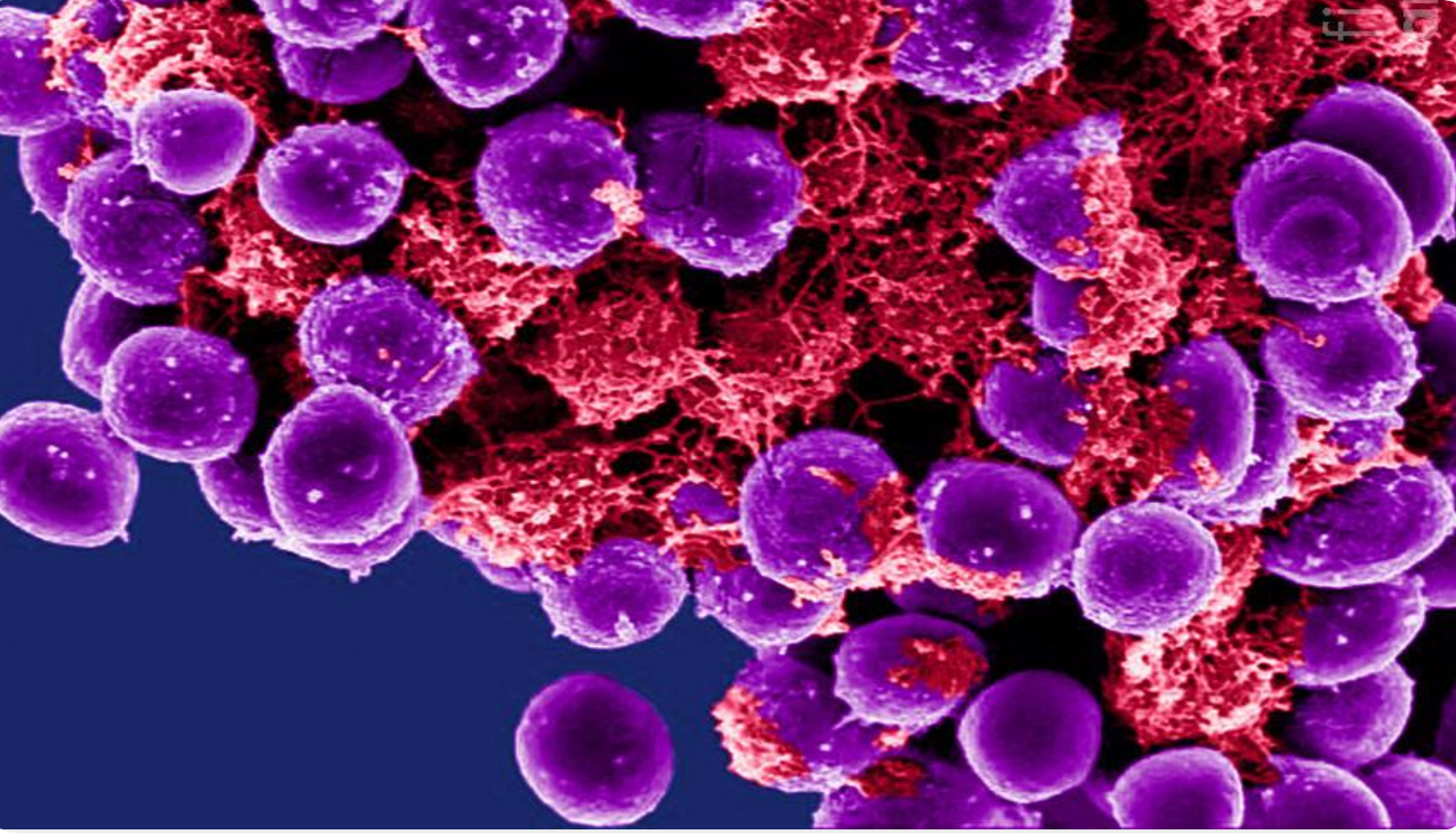

The premature microbiome and late-onset neonatal sepsis

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ABSTRACT

Neonatal sepsis is a systemic infection and is one of the leading causes of mortality and morbidity in prematurely born infants worldwide. Neonates which contract sepsis within three days post-partum are diagnosed with early-onset neonatal sepsis, and after diagnosed with late-onset neonatal sepsis.

The prevalence of late-onset sepsis is increasing, and survivors are at risk of developing multiple life-threatening conditions. One factor that can protect the baby is the gut microbiome, which is a critical arbiter in maintaining host health. Imbalanced gut microbiota in neonates can play a factor in the pathogenesis of late-onset sepsis. Because of this imbalanced state, often called dysbiosis, supplementation of probiotics to the neonatal gut microbiota has been thoroughly inspected.

Causality of neonatal dysbiosis in relation to late-onset sepsis has been established in a mouse model by translocation of *Klebsiella pneumoniae* from the neonatal dysbiotic microbiome to the liver. Second, the bacteria most commonly responsible for sepsis can be found in the neonatal gut mucous layer, as opposed to only the skin which was thought previously. Moreover, preterm neonates have a far lower viscosity of the mucous in the epithelial layer of the gut, resulting in an increased permeability of the epithelial layer. Neonatal dysbiosis is directly linked to that of maternal dysbiosis and preterm birth. Probiotics such as lactobacilli and bifidobacteria do not show abundant growth in dysbiotic microbial circumstances of premature neonates when treated with faecal transplants, while *Escherichia coli* does. This indicates that preceding probiotic treatment of Lactobacilli and Bifids, treatment with *E. coli* might create an environment where Lactobacilli, Bifids and other probiotic bacteria can grow in the gut more abundantly. In contrast, prophylactic antibiotics lowers incidence of early-onset sepsis but raises risk of late-onset sepsis due the created microbial dysbiosis.

The most prevalent bacterium found in blood cultures from neonates with sepsis is *Staphylococcus epidermidis,* which may be resistant to specific antibiotics that are used in the treatment of early-onset sepsis. Antibiotics must be chosen with care, to reduce the rise in antibiotic resistant bacteria and prevent late-onset sepsis from neonatal dysbiosis of the gut.

Dysbiosis has a direct causation in late-onset sepsis. This includes translocation of a pathobiont from the gastrointestinal tract to the liver and the amount of intestinal oxygen present. Studies shows that coagulase-negative staphylococci that are responsible for sepsis can be found in the neonatal gut and not only on the skin as thought previously. Lastly probiotics can be used to prevent neonatal sepsis through regulation of the dysbiosis of the microbiome, as the statistically significant meta-analysis shows the positive effect of probiotics on blocking the development of sepsis. This body of evidence suggests that neonatal gut microbiota dysbiosis might be responsible for late-onset sepsis.

LIST OF ABBREVIATIONS

BPD	Bronchopulmonary dysplasia
CI	Confidence interval
CoNS	Coagulase-negative staphylococci
EOS	Early-onset sepsis
GBS	Group B streptococcus
GFP	Green fluorescent protein
HCAI	Healthcare associated infections
LOS	Late-onset sepsis
MRSE	Methicillin resistant *Staphylococcus epidermidis*
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
PMDZ	Pimonidazole
RR	Relative risk
SCFA	Short chain fatty acid
Spp.	Several different unknown species under known genus
VLBW	Very-low birthweight

LIST OF BACTERIA AND THEIR SPECIFICATIONS

Bifidobacterium spp.	Gram-positive bacteria from maternal milk, common probiotic.
Escherichia coli	Gram-negative bacteria from lower intestine, possible probiotic.
Enterobacteriaceae	Family of Gram-negative bacteria common in neonatal sepsis.
Enterococcaceae	Family of Gram-positive bacteria common in neonatal sepsis.
Staphylococcaceae	Family of Gram-positive bacteria common in neonatal sepsis.
Lactobacillus spp.	Gram-positive bacteria from maternal milk, common probiotic.
Staphylococcus epidermidis	Gram-positive skin & mucous bacteria, common in neonatal sepsis.

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1 INTRODUCTION

Currently, 15 million preterm babies are born across the globe annually, and this number is rising (1). Care for these vulnerable babies causes new problems, for instance neonatal sepsis. This is a systemic infection which transpires in infants and is one of the leading causes of their mortality and morbidity worldwide, especially in the prematurely born.

Neonatal sepsis can be divided into two groups based on the time of presentation after birth. Neonates which contract sepsis within three days post-partum are diagnosed with early-onset neonatal sepsis (EOS), whereas late-onset neonatal sepsis (LOS) has been defined to be after 72 hours (2). EOS is caused by bacterial pathogens which are transmitted vertically from mother to infant, while the cause of LOS is speculated to be from the environment. And while the incidence of EOS has decreased over the years, the incidence of LOS has increased. This is in stark contrast to the improved survival rates of premature infants. On the cause of LOS is currently not much known, but two hypotheses have been proposed on the pathogenesis (3).

The first hypothesis suggests that LOS is associated with the postnatal community or nosocomial environment, because of the intensive care the neonates need and horizontal transfer of pathobionts from doctors to the neonates (4, 5).

The second hypothesis on the pathogenesis of LOS proposes that the commensal microbiota of the baby itself causes LOS. The different state of the premature microbiome, as compared to that of the normal term infant's gut microbiota, has been speculated to enable the systemic spread of commensal bacteria from the intestine of premature infants, resulting in LOS (6).

The ambition of this report is to determine whether the old hypothesis of LOS has been correct in that the pathogenic origin lies in horizontal transfer from the environment, or whether the new hypothesis of systemic spread from the intestine of the baby is responsible for LOS. Is the pre-term neonatal microbiome an element determining the pathogenesis of late-onset sepsis?

2 NEONATAL SEPSIS

Neonatal sepsis, also known as Sepsis Neonatorum, is an invasive infection, which is most commonly of bacterial origin, during the neonatal period (first 28 days after birth). The groups which are at highest risk of developing neonatal sepsis are neonates which are preterm, are of very-low-birthweight (VLBW), have depressed function at birth and neonates with maternal perinatal risk factors. Males are at more risk of LOS than females (7). The neonatal period represents a time in which the most drastic physiologic changes occur of human life (8). This chapter will discuss LOS aetiology in context of neonatal healthcare.

2.1 LATE-ONSET SEPSIS

LOS is a leading cause of morbidity and mortality in the neonatal intensive care unit, with incidence rates in preterm infants varying between 20 to 38% and mortality rates in these infants ranging from 13 to 19% (9). Survivors of LOS are at risk for prolonged hospitalisation, development of multiple life threatening conditions and neurodevelopment impairment (10). The diagnosis of LOS in clinical practice is challenging, as symptoms have limited specificity and sensitivity. Moreover, the lack of an accepted consensus definition for LOS slows down the effort to improve diagnostics (2).

The presence of a positive blood culture represents the best way to determine the presence of sepsis, but this can only be determined after severe clinical signs (2, 11). The use of common laboratory tests including white blood cell indices and heart rate characteristics have proven insufficient diagnostic accuracy for neonatal sepsis (12, 13). Some clinical manifestations in preterm neonates aren't a dependable indicator of ailment. Often the early signs of infection in neonates are not specific and can also be common in premature neonates and the development to extrauterine life. Contrarily, a study from Johnson et al. (1997) showed that asymptomatic presentation doesn't rule out infection completely in a high-risk setting. The Johnson et al. study investigated over 5.000 cases of EOS, where positive blood cultures were identified in 0.5% in asymptomatic infants and in symptomatic infants 3.2%, respectively. This makes diagnosis hard, especially in the early stages where it is most vital (14, 15), and withholding antimicrobial therapy can lead to dismal outcomes (16).

However, this liberal use of antibiotics has detrimental side effects that must be considered. Epidemiological data on VLBW infants shows that coagulase-negative staphylococci (CoNS) are the predominant pathogens in LOS, followed by Gram-negative bacilli and fungi. LOS is therefore most commonly caused by commensals of the gut or the skin. Group B streptococcus (GBS) and *Escherichia coli* are the most common cause for EOS, and pre-term mothers in labour who are GBS-positive receive antibiotics to reduce risk of EOS (4, 6, 17).

Paradoxically, the use of antibiotics decreases the incidence of EOS, but increases the risk of LOS, which could be explained by the alteration of the neonatal gut microbiome (18, 19).

2.2 NEONATAL DYSBIOSIS

Healthy gut microbiota is a critical arbiter in maintaining host health, and an imbalance of gut microbiota (henceforth called "dysbiosis") can play a factor in the pathogenesis of a number of diseases (20). The premature neonates often have an odd ratio of microbiota which could be in a state called dysbiosis regardless of LOS. Even maternal dysbiosis has an influence on the gut of the baby, as it could act as a trigger for premature birth. Subsequently, premature birth can cause infants to experience overgrowth of a distinct facultative anaerobe bacteria instead of a healthy multispecies distribution. These facultative anaerobes include *Enterobacteriaceae*, *Enterococcaceae* and *Staphylococcaceae* and an overgrowth of these may result in other probiotic bacteria not being able to settle in the gut microbiota.

The inability of the gut microbiome to adapt after the initial facultative anaerobes have settled is therefore called dysbiosis (21).

The early appearance of obligate anaerobes in the microbiome correlates with longer gestation (22). In term infants show normal succession of facultative anaerobes by consecutive colonisation of the gut by obligate anaerobes such as *Lactobacillus* spp., *F. prausnitzii* and *Akkermansia muciniphila* (23-25). Preterm and VLBW infants however, seem to have merely a fraction of obligate anaerobes, even weeks after birth (21). *Enterobacteriaceae* and *Enterococcaceae* are far more abundant in preterm colonisation (26). The wide use of antibiotics in neonatal intensive care units (NICU) contributes to the creation of a limited diversity in the microbial population (27).

Due to the associations between LOS and dysbiosis, the addition of probiotics has been under thorough examination, due to their potency and the current gaps in knowledge. Current research is mostly focussed on determining their beneficial effect towards a healthy preterm gut microbiome. The probiotics used were most commonly *Lactobacillus* spp. alone or combined with *Bifidobacterium* spp. or other commensals which were thought to have beneficial effects (28).

Neonatal dysbiosis plays a role in the pathophysiology of LOS and is associated with other serious illnesses which are related to premature birth. Preterm infants are at risk of developing bronchopulmonary dysplasia (BPD) or necrotizing enterocolitis (NEC), the latter being the most lethal gastrointestinal disease in neonates (29).

2.3 THE HEALTH COMPLICATIONS OF PREMATURE BIRTH

Premature neonates with low birthweight are prone to multiple health problems, and premature birth raises the risk of serious illnesses. There are both short- and long-term complications linked to preterm birth. Short-term complications which premature babies can show include problems of the immune system, gastrointestinal problems, low body temperature, heart problems, brain problems, laboured breathing or respiratory problems and lack of reflexes for sucking and swallowing, which lead to feeding difficulties (30).

NEC is one of these short-term complications, with the highest mortality rate of 50% or more based on the severity. NEC typically occurs in the second to third week of life in prematurely born babies. NEC is characterised by damage to the intestinal tract, which can range from mucosal injury to perforation and full-thickness necrosis (31). The cause of NEC is still unknown.

Long-term complications of preterm birth include cerebral palsy, impaired learning, vision problems, hearing problems, dental problems, behavioural and psychological problems and chronic health issues such as sudden infant death syndrome and BPD. The latter is characterised by impaired angiogenesis and dysregulated alveolarization (formation of alveoli) and is the most common complication in preterm births (30). BPD incidence is 50% in extremely preterm neonates and is associated with persistent lung impairment later in life (32).

LOS has been directly associated with NEC and BPD (31), making LOS a huge problem in preterm neonates. Studies show that many of the complications from preterm neonates have a relation to the microbiome, including NEC which is plays a huge role in the mortality of low-birthweight preterm infants. Lastly, neonatal dysbiosis has been established in neonates with NEC (33).

3 DYSBIOSIS MICROBIOME AND INFECTION

Our gut contains many different species that makeup our microbiota which aids us in different essential functions to help us retain health. These functions include short chain fatty acid (SCFA) production, regulation of host gene expression and vitamin production. This chapter will discuss the impact of dysbiosis of the microbiome and the commensal bacteria which are responsible for neonatal sepsis.

3.1 COMMENSAL ORIGIN IN NEONATAL SEPSIS

LOS is most often caused by Gram-positive bacteria, which are commensals typically from either the skin or the gut. CoNS were the most commonly found pathogens with 48% being the cause of the infection. Of the CoNS group, *Staphylococcus epidermidis* was the most commonly found pathogen. The colonisation of *S. epidermidis* start immediately after birth. It is the most common isolated species of the skin microbiome. More recent data indicates that *S. epidermidis* may be part of the abundant bacterial genera in the mucous of the airway and gut microbiome, especially in preterm neonates (34, 35). As compared to other pathogens such as *S. aureus* and *Escherichia coli*, *S. epidermidis* is underestimated based on its role in neonatal morbidity, as it is the predominant pathogen of sepsis in preterm infants (36). Furthermore, recent studies show evidence implicating an association between *S. epidermidis* sepsis and sepsis-related neonatal diseases such as NEC and BPD. The pathogenesis of each entity is hypothesised to be a multi-hit process with inflammation as the primary downstream mechanism (37-39). Antibiotic resistance appears to be widespread for *S. epidermidis,* methicillin in particular. Methicillin resistant *S. epidermidis* (MRSE) are strains that encode the mecA gene, which is located on mobile genetic elements, making the resistance transferable to other bacterial strains. MRSE is now treated with vancomycin.

3.2 MATERNAL DYSBIOSIS INCREASES RISK OF NEONATAL DYSBIOSIS

Many aspects of early postnatal life have an influence on the neonatal microbiome, but the pathogenesis of neonatal dysbiosis can commence during pregnancy. For instance, the occupation of pathobionts in the amniotic fluid (protective liquid in the amniotic sac), can trigger the innate immune response. This response causes a rise in the production of prostaglandins which increases contractility of the uterus, directly promoting premature birth. Rising incidence of infections of the uterus are responsible for the vast majority of spontaneous preterm births. (40).

During pregnancy, one or only a few *Lactobacillus* species dominate the healthy vagina. These protect against colonisation by pathobionts by excretion of antimicrobial compounds and by producing lactic acid. Vaginal microbiota which are abnormal, or an active bacterial infection can promote the acquisition of neonatal flora which promote preterm delivery (41). Factors which are associated with premature birth such as formula feeding, antibiotic exposure and caesarean section have strong evidence showing a negative impact on the neonatal microbiome diversity (20, 42).

Neonatal microbial colonisation in a healthy full-term vaginally delivered infant plays an important role in mucosal immunity, energy regulations and nutrient absorption and digestion (20). In a healthy full-term infant, gut microbiota is acquired by maternal vaginal and faecal microbiota during birth, and thereafter from breastmilk. Furthermore, all infants get gut microbiota from their environment, including family. A balanced gut microbiota is characterised by a diverse species colonisation including Bifidobacteria, which are capable to digest human milk oligosaccharides (43). Epithelial barrier function and integrity are influenced by host-microbiome interactions, dysbiosis may lead to structural disruptions which increase intestinal permeability, resulting in translocation of bacteria to the bloodstream (44).

3.3 STUDY SHOWS PREVENTION OF DYSBIOSIS PROTECTS AGAINST LOS

A study of Singer et al. has used neonatal mice models which showed a similar delay in the appearance of obligate anaerobes as is common in premature neonates. Many features of intestinal development in humans take place *in utero*, while these changes happen postnatally in rodents. The succession pattern of gut microbiota reflects intestinal developmental maturity, reflecting the gestational age. The study has shown that the dysbiosis in neonates indeed led to LOS in the absence of adequate host clearance after translocation (Singer et al.).

The Singer study describes that full term-mice were used to model LOS as their intestines resemble those of preterm infants. Pups of 5-day old were intragastrically infected with virulent *Klebsiella pneumoniae*, with two strains that were engineered to express green fluorescent protein (GFP) for bioluminescence. The primary sites of infection were determined to be localised to the colon, the cecum and in minor extend to the small intestine. The strain was engineered so it could be tracked through live-animal imaging, histological imaging, real-time colonisation and dissemination. A strong relationship between the isolated *K. pneumoniae* colony forming units and *ex vivo* tissue luminescence corroborated this approach. During the monitoring of the luminescent bacteria, it was ensured that the dosing was limited to the stomach. The hypothesis of Singer et al. was that if neonatal dysbiosis precedes sepsis, translocation would occur where the pathobiont colonisation was the densest. This hypothesis has been proven to be correct, as after 24 hours co-colonisation of the strains showed that translocation correlated with bioluminescence imaging and the GFP strain was found in the liver of the septic pups, but not in non-septic pups, as seen in Figure 2. Therefore, *K. pneumoniae* dysbiosis did indeed lead to intestinal translocation and subsequently to LOS . Not all pups with dysbiosis ended up with sepsis, but neither do all premature neonates with dysbiosis. (6)

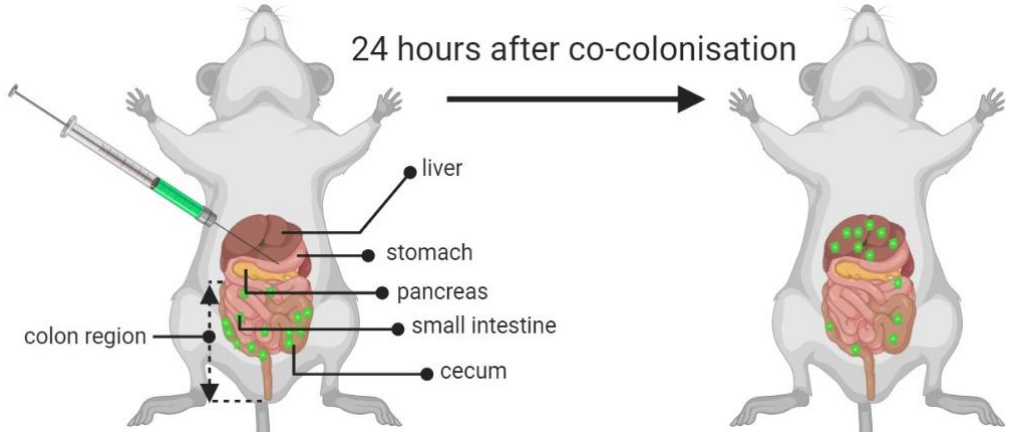

Figure 1: The translocation in the 5-day old pups which were carried full-term from the study of Singer et al. 2019. The hypothesis of neonatal dysbiosis preceding sepsis was proven to be correct, as the co-colonisation strands of the gut-injected K. pneumoniae did translocate after 24 hours (6).

A major drive of the colonisation dynamics was intestinal oxygen, and luminal oxygen is known to drive dysbiosis in the context of infection, inflammation and antibiotic use (45). The Singer et al. study determined whether intestinal oxygen levels decreased in mice as they aged, and if that would have an effect on the susceptibility to *K. pneumoniae* dysbiosis.

To measure epithelial hypoxia, the redox chemistry of pimonidazole (PMDZ) was used (46). This staining demonstrated that control mice showed greater epithelial hypoxia the older they were, signifying that lowering luminal oxygen levels are the start of the transition to dominance of obligate anaerobes in the microbiome, creating a diverse gut microbiota. This is the transition from susceptibility for dysbiosis to protection in mice. Furthermore, the study also showed that not Lactobacillus, but *E. coli* was present in high abundance after faecal transplantation. *E. coli* was then used as a probiotic, and it showed that it could prevent LOS. These results show that different bacterial strains may confer colonisation resistance against sepsis-causing pathobionts (6).

4 PREVENTION OF LATE-ONSET SEPSIS

The current prevention of LOS is based on the theory that LOS is caused by horizontal transfer of commensals from the parents or medical staff (Ref). Unlike for EOS, where an antibiotic treatment did indeed decrease the overall incidence, LOS incidence only rose more in recent years. This is partly because VLBW infants now have higher survival chances and because of their need for continuous and invasive monitoring and support. LOS is not only increasing in incidence, it is also a significant cause of mortality and morbidity, which in turn leads to high pressure at NICU units for the staff and the families involved (16). Many strategies to prevent LOS are the same for all healthcare associated infections (HCAI), which include measures such as good hand hygiene, aseptic precautions and specific techniques to reduce contaminations from the catheter (47). While it cannot be ruled out that the neonates can get an HCAI, more recent studies have suggested that the primary origin of the infection is the gut of the baby itself and that the mother's microbiome plays an important role on the evolution of the baby's microbiome and health during the neonatal period (6, 48, 49). This chapter will discuss the role of the microbiome in preventing LOS.

4.1 PRENATAL AND POSTPARTUM ASPECTS OF LATE-ONSET SEPSIS

During the previous chapters, it was established that LOS is a result of a multitude of problems which can all attribute to the pathogenesis. The first of these problems being the preterm birth itself. As discussed in chapter 3.2, maternal dysbiosis has been shown to result in premature birth, which can result in dysbiosis of the neonate's microbiome. It has been discussed that a way to treat the maternal dysbiosis and hopefully prevent preterm birth could be the use of probiotics during the pregnancy. Probiotics are living micro-organisms which when administered in the correct amount could confer a health benefit to the host (50). The most common used probiotics are bifidobacteria, lactobacilli and non-pathogenic fungi. Such probiotics have been thought to protect the host against vaginal infections, which may cause lower preterm births (51).

The birth itself can bring complications that have an influence on LOS. Preterm birth means the mother may not be able to nurse the baby herself, while maternal milk contains many peptides, proteins and probiotics that sustain immune maturation and a healthy gut microbiota composition (52). Some studies have argued the beneficial effects of donor milk, but several classes of protective elements can be destroyed during the pasteurisation process needed to preserve the milk (53). One of these protective elements that gets destroyed during pasteurisation is lactoferrin. Lactoferrin is a glycoprotein with an avidity in iron binding, lactoferrin works as part of a host defence mechanism. Experimental studies and *in vivo* experiments have shown inhibitory effect of lactoferrin on bacterial invasion and attachment, which showed a reduction in sepsis severity (54, 55).

Postpartum aspects that play a role in LOS pathogenesis are antibiotic use (which is also a prenatal factor when taking into account the mothers dysbiosis) and environmental factors such as transmission of pathogens from nursing staff of the NICU (4).

Another parameter specifically important to the NICU unit is that these bacteria must be contained due to the neonates not having a developed immune system, making good hygiene detrimental to the survival of these neonates. This not only prevents the bacteria from the environment to get to the neonate, but also prevent NICU staff from spreading these pathogenic bacteria from one of the infants to the next (56). The combination of all of these circumstances create an overview of what needs to be addressed to reduce the frequency of LOS in neonates. An overview of all the aspects that play a role in the pathogenesis of LOS can be found in Figure 2 below.

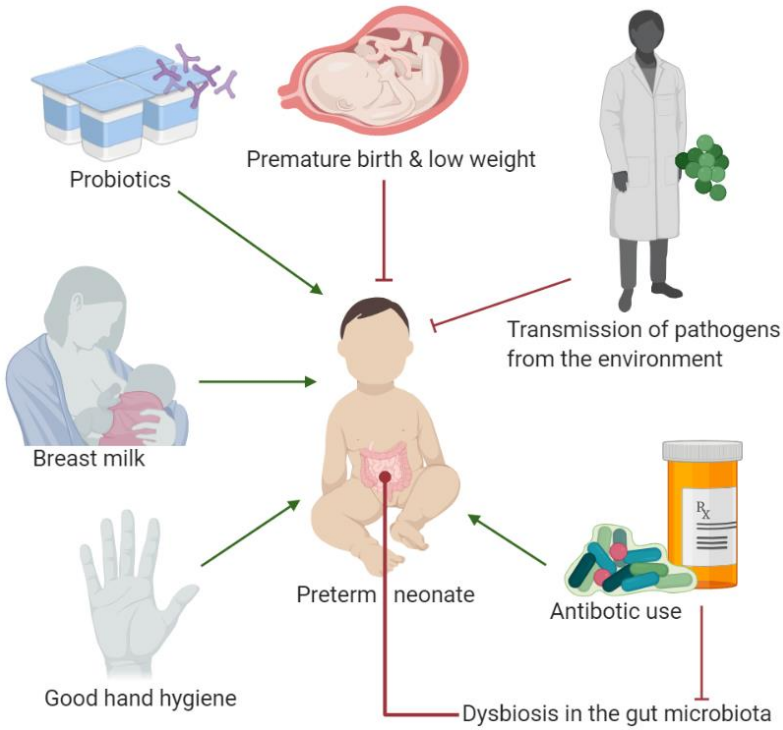

Figure 2: Multifactorial origins of LOS. Premature birth, low weight, transmission of pathogens from the environment all have a direct negative effect on the pathogenesis of LOS and are represented by a red line. Probiotics, maternal breast milk and good hygiene all are represented by a green arrow, showcasing promising results when battling LOS incidence. Antibiotic use is paradoxically both good and bad, as the specific antibiotics may kill the pathogen causing neonatal sepsis, but also cause dysbiosis in the gut microbiota, which can lead to LOS (74, 75).

4.2 THE DEFINITION OF A HEALTHY GUT IN NEONATES

In adults, around 80% of the gut microbiota consists of three dominant phyla: *Bacteroidetes*, *Firmicutes,* and *Actinobacteria* (57). At one year of age, the microbiota of a baby is similar to that of an adult. Before this, the neonatal microbiota is vastly different as compared to the adult one. Neonatal microbiota is characterised by rapid changes, as the new-born is exposed to multiple different bacteria including *enterobacteria, enterococci* and *staphylococci* that colonise the GI tract (58).

Under normal circumstances of the first days of life, the population of the GI tract should consist of *Bifidobacteriales*, *Lactobacillales,* and *Clostridiales*. Moreover, *bifidobacteria* are the most common bacteria in the GI tract of healthy infants. The use of antibiotics during pregnancy or postnatally seems to reduce the amount of *bifidobacteria* (58). Bifidobacteria and lactobacilli are maternally obtained and often used as probiotics.

The glycoprotein lactoferrin, previously mentioned in 4.1, is considered as a growth promoter of *bifidobacteria*, which is the predominant beneficial microorganism in the GI tract. Bacteria specifically obtained from breast milk affect the gut microbiota composition in infants, protecting against infections and promoting maturation of the immune system (52). Other nutrients from breast milk includes oligosaccharides and sIgA have an effect on the proliferation of healthy microbiota.

SIgA production, activation of T regulatory cells and the anti-inflammatory response are all stimulated by intestinal bacteria. The faecal concentration in the first month of lactoferrin increases in the first month after birth, which promotes the growth as well as differentiation of the immature intestine. As a result, this protein is implied to promote the closure of the enteric gap junctions regulating the postnatal intestinal development (50). From this can be determined that appropriate gut colonisation acquired from breastfeeding is involved in the proper development of the immune system and needed for the interception of pathogens.

Another important protective barrier in the GI immune barrier is intestinal mucous. Large glycoproteins called mucins are produced and secreted by goblet cells to create a physical barrier, remove bacteria and concentrate enzymes close to the surface of the epithelial to facilitate in host nutrient digestion. The mucus produced in preterm infants is different than that of adults however, especially in viscosity. The proposed mechanism of the pathobiont using the lowered viscosity and enteric gap junctions of the GI tract present in premature neonates can be seen Figure 3 (59) .

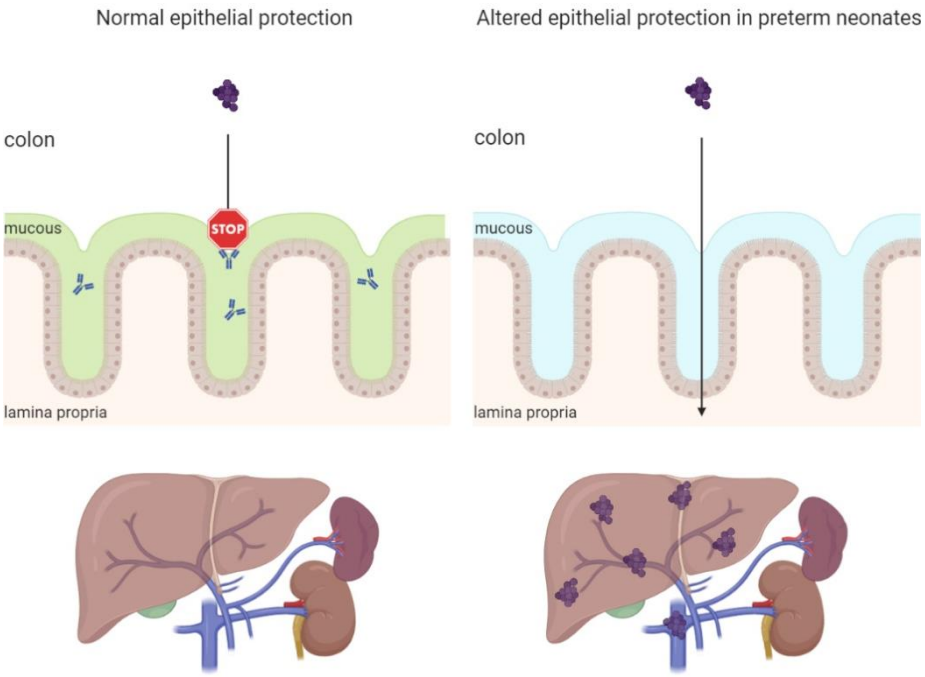

Figure 3: A simplified overview of the normal epithelial immune barrier containing intestinal mucous on the left and the preterm neonate's epithelial layer being of a different viscosity and level of protection on the right. The normal epithelial protection can prevent pathobionts from migrating by the thick, viscous layer of healthy mucous, which contains defensins and sIgA (as seen in the picture, the blue antibodies on the left). The altered epithelial layer on the right, however, does not have the same viscosity and antibodies and defensins, making it possible for pathobionts to translocate through the mucous layer to the liver, and subsequently to the bloodstream (6, 59).

In the pAnda study, a mixture of probiotics (*Bifidobacterium bifidum*, *Bifidobacterium lactis*, and *Lactococcus lactis*) was administered to pregnant women and their infants during the first year of life. This reduced the incidence of eczema in the first 3 months of life as compared to the placebo group (60). The prophylactic use of probiotic supplementation in neonates with infantile colic and other GI problems has been under examination, especially *Lactobacillus reuteri*. This probiotic supplement has shown significant reduction of crying episodes, and when orally supplemented in the first three months of life, reducing the overall incidence of colic episodes and other GI disorders (61, 62).

The Singer et al. study from 3.3 showed that a selection of lactobacilli is capable of preventing LOS in mice models (6). *Lactobacillus murinus* was prophylactically supplemented to susceptible pups. These pups showed drastically reduced overgrowth of the *K. pneumoniae* strain compared to control mice, therefore establishing a potent probiotic effect. Pups that received *Lactobacillus johnsonii* were also protected, but not to the same degree (6). The probiotic activity shown by different *Lactobacillus* spp. were therefore highly variable and need to be researched more.

4.3 META-ANALYSES ON PROBIOTIC SUPPLEMENTS IN LATE-ONSET SEPSIS

Earlier, a Crochan Review from 2014 with 19 studies (*N* = 5338) while they did find significant preventative effect on NEC, the review did not find benefits of probiotic supplementation in lowering the risk of LOS in premature infants (relative risk (RR) 0.91; 95% confidence interval (CI), 0.80–1.03; 19 studies, *N* = 5338) (63), which was also a similar result to another meta-analysis of that time RR, 0.919; 95% CI, 0.823–1.027; *P* = 0.137, 17 randomised controlled trials, *N* = 5215) (64).

In contrast, a more recent meta-analyses suggest the opposite is true. The meta-analysis done by Shripada et al. in 2016 used a combination of 37 randomised controlled trials (*N* = 9416) which were focussed on probiotic supplementation that were reported on LOS. LOS was only the primary outcome of 9 studies, whereas it was a secondary outcome in the remaining 28. Single-strand probiotics were supplemented in 23 studies, the remaining 14 used multiple strains. *Bifidobacterium* was used in 22 studies as probiotic, *Lactobacillus* was supplemented in 21. The pooled meta-analysis of Shripada et al. that compared "probiotics" with "placebo" and "no probiotics" showed probiotic supplementation to result in statistically significant reduction in the incidence of LOS (675/4852 [13.9%] vs 744/4564 [16.3%]; RR 0.86; 95% CI, 0.78–0.94; *P* = .0007). The results remained significant even after excluding studies with a high risk of bias (65).

4.4 SAFETY OF PROBIOTICS IN INFANTS

Probiotics supplemented in both the perinatal and postnatal periods are suggested to give a positive effect on the future health of neonates. The administration of probiotics is considered safe, but despite many studies using probiotic supplementation in these periods, few of them tested for safety. A randomised, double-blind, placebo-controlled trial from Allen et al. tested for possible negative effects of a probiotics mixture containing lactobacilli and bifidobacteria in pregnant women and their infants postnatally. None of these negative effects were attributed to the use of the probiotics (66). Furthermore, Baldassarre et al. confirmed in their double-blind, randomised, controlled trial that early administration of probiotics during the pregnancy had no side effects in either mother or child (50). A study which involved 256 pregnant women and their infants, showed no adverse side effects when administered of a probiotic mixture of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis Bb12* (67).

Contrary to the previous studies, a study from Kuitunen et al. suggests that early supplementation of probiotics do have an adverse effect on the haematologic values in infants. During this trial, infants supplemented with probiotics during pregnancy and after birth showed significantly lower haemoglobin levels at 6 months of age as compared to their placebo counterparts (68).

5 CONCLUSION & DISCUSSION

The aim of this report was to determine whether the neonatal microbiome could play a factor in the pathogenesis of late-onset sepsis. To determine this, the old hypothesis of transmission of environmental pathogens being the cause LOS has been under critical evaluation. The entire developmental window, from the maternal gut influence during pregnancy to morbidity due to LOS related illnesses has been considered in this report, to perhaps enlighten the path to stop the increasing prevalence of LOS.

The first step in lowering the rising incidence of LOS means that a general consensus on the diagnosis is needed (4, 12, 17). Most studies consider LOS to be after 72 hours, which should be kept as timeframe to determine the incidence of LOS as opposed to EOS. Neonatologists and researchers must communicate the exact diagnosis in a way that all studies can determine LOS from a clinical report. The NICU has to start monitoring premature neonates for these studies before they have shown symptoms, in a biobanking way. The origin of the disease still has not been acknowledged or agreed on, since the pathophysiology of the disease is too diverse. Earlier, research had focussed on the bacteria causing LOS which were believed to be only commensal to the skin. Researchers determined horizontal transfer from the environment to be the explanation as to why LOS suddenly had appeared in premature infants (4, 5, 9). In more recent years, the gut microbiota has been speculated to play a role in neonatal dysbiosis, which could be a possible explanation to LOS as well. While studies have both reasoned environmental factors as well as the infant's own gut to be the source of the invasive infection, the first hypothesis raises a few more questions. If environmental factors had been at play, then improved hygiene should also have resulted in a lowered incidence of LOS, which has not been the case. Moreover, the "bad hygiene" theory does not seem to answer as to why EOS did decrease as did overall infant death.

CoNS such as *S. epidermidis* have been the most common cause by far in LOS and have recently been shown to be not only a skin bacterium, but also present in mucous and in the GI tract. Therefore, *S. epidermidis* could be part of the abundant bacterial genera of the gut microbiome and in the airway, especially in preterm neonates. *S. epidermidis* is regarded as a harmless commensal, but the unique circumstance of neonatal innate immunity can contribute to the development of *S. epidermidis* as a significant nosocomial pathogen (36). This evidence cannot be ignored in finding the aetiological agent responsible for LOS. Do these pathobionts come from the skin, or are they part of the neonatal gut microbiota and can they get to the stomach where they are redistributed through the permeable gut to the liver as seen in the Singer et al. study (6)?

The permeation of a pathobiont in the gut is the molecular mechanism which the second hypothesis relies on but is not yet understood. From the gathered evidence in this review, the premature gut is already more permeable due to the lack of an innate immune system and the lack of sIgA and other nutrients which come from mother milk. sIgA plays an important role in the immune function of mucous (69), the layer in the GI tract where recent research has placed *S. epidermidis* to be present in neonates (36). This would explain both the route the pathobiont takes to permeate the gut, as well as why *S. epidermidis* is so common in LOS.

The Singer study mentioned is a reliable study that has shown a causality of neonatal dysbiosis in relation to LOS by translocation of the *K. pneumoniae* from the neonatal dysbiotic gut to the liver (6). However, while mice are often used to study the impact of diet and other environmental factors on the microbial diversity, there are great differences between size, metabolic rate and dietary habits, so the translation of the translocalisation and the subsequent connection to sepsis does need to be confirmed in humans. Only a low percentage of bacterial genes are shared between mice and man, but an important one is *Lactobacillus* spp. Mice models have other multiple benefits, for their many anatomical, histological and physiological similarities to man (70).

Studies show that organoids representing the GI tract might be a suitable replacement for mice studies to study the microbiome in the future, but sepsis is also related to the cardiovascular system, and making such a complex tissue organoid will take a long time to develop (71).

The concept of using probiotics to correct dysbiosis in the intestinal microbiota is a promising field and multiple randomised placebo-controlled clinical trials have been performed. Probiotics in preterm infants are hypothesised to enhance the gut barrier, modulate the immune response and offer competitive inhibition of colonisation by pathogens, therefore habituating a balance in the gut (72). The most common probiotics such Lactobacillus spp. and Bifidobacterium spp. showed upon ingestion that they protect infants from developing sepsis and NEC by maintaining the intestinal barrier. These probiotics showed suppression of the growth of various pathogenic bacteria. More than 25 systemic reviews and meta analyses with over 12.000 participants almost unanimously showed that probiotics were a safe and effective treatment to reduce the risk for LOS, NEC and all-cause mortality (50, 63, 73). In addition, the newest and largest meta-analysis (N = 9416) showed that probiotic supplementation in neonates resulted in statistically significant (P = .0007) reduction in the incidence of LOS (65).

Interestingly, original maternal probiotics such as *Lactobacilli* and *Bifido* bacteria have not shown abundant growth in dysbiotic microbial circumstances when treated with faecal transplants (transplantation of faecal matter from a healthy person to a patient), while *E. coli* did. This might insinuate that a cocktail of probiotics could prove a better way to treat microbial dysbiosis, first treatment with *E. coli* and subsequently with *Lactobacilli* and *Bifido* bacteria. *E. coli* might create an environment where other probiotic bacteria might settle better as compared to direct insertion into a dysbiotic environment in the gut (6).

Lowering risk of neonatal sepsis with prophylactic antibiotics in the NICU has signified a lowered incidence of EOS, but also paradoxically raises the risk of the development of LOS. *S. epidermidis* is resistant to specific antibiotics which are used in the prophylactic treatment of EOS. Antibiotics use should be reduced and chosen with care to stop the rise in antibiotic resistant bacteria and lower the prevalence of LOS. As LOS has been successfully treated with vancomycin, this may be the preferable antibiotic to use as preventative strategy for EOS as well (6, 16, 27). When prevention has failed, LOS patients that survive are at risk of developing BPD and NEC. NEC is a GI disease in which part of the GI tract dies, which can also be traced back to a dysbiosis in the gut (17, 33).

The main question was whether the neonatal microbiome should be taken into consideration in the pathogenesis of LOS. Recent discoveries indicate that the second hypothesis could explain the possible pathophysiology. To sum up: The Singer et al. study shows that translocation of the pathobiont from the GI tract to the liver is possible, and therefore the pathobiont can get to the bloodstream, resulting in sepsis (6). New intelligence succeeding the Singer study indicates that the CoNS most commonly responsible for sepsis (*S. epidermidis)* can be found in the neonatal gut mucous (36). Moreover, preterm neonates have a far lower viscosity of the mucous in the epithelial layer of the gut, have an increased permeability of the gut and are incapable of obtaining nutrients from mother milk (54, 55). These nutrients help probiotic intestinal bacteria, which in term produce sIgA and activate T-regulatory cells and the anti-inflammatory response. sIgA is in healthy infants present in the mucous of the gut, which explains the extra permeability of the gut in preterm infants, as well as *S. epidermidis* being the most common pathobiont found in LOS (20, 36, 69). Finally, the statistically significant meta-analysis shows the positive effect of probiotics on blocking the development of sepsis, which suggests a direct causation.

This summary indicates the growing body of evidence in favour of the second hypothesis, that neonatal gut microbiota dysbiosis is not only a factor in LOS but could be the aetiological agent responsible.

6 RECOMMENDATIONS

While the evidence most certainly points that new and exciting methods are on the horizon to combat the LOS incidence, this report has a few recommendations which will hopefully build a foundation towards a LOS-free future.

First off, the priority should lie on the elimination of maternal factors that could lead to premature birth. Premature birth plays the most important aspect in the development of all neonatal sepsis variants and their related illnesses such as NEC and BPD, which have a very high mortality rate. A method should be used to check the mother for dysbiosis of the gut and proper action should be taken, such as a personalised probiotics treatment based on the bacterial diversity from a faecal sample of the mother.

Another recommendation is concerning the pathogenesis of the disease. Recent research found that the commensal bacteria causing sepsis is not only present on the skin as was thought earlier, but also in the mucous membrane of the respiratory system and the gut. While faecal samples tell us a lot about the abundance of the bacteria in the microbiome, a method to analyse whether or not this bacterium is from this mucous layer, the skin or from the respiratory system is still not available. The origin of this might answer the question as to where this pathobiont is from. This does not make the translocation of the pathobiont in the Singer study any less relevant, as this has proven the direct causal role of the dysbiosis in the gut microbiota to LOS. Moreover, the role of intestinal oxygen should be tested in premature neonates, as was described in the Singer study as well. More research concerning the difference in mucous of the gut between healthy infants and infants with LOS might also provide answers on the translocation of the pathobiont, especially sIgA, the innate immune system and the mucous viscosity.

Prevention with probiotics should be vigorously tested based on which cocktail should reduce the risk of neonatal sepsis in premature neonates the most. Lactobacillus and Bifids are great as a second or third addition, but bacteria which can compete with the imbalanced gut environment creating *Enterobacteriaceae*, *Enterococcaceae* and *Staphylococcaceae*. One of these could be *E.coli*.

As mentioned in the previous chapter, more care needs to be taken when using antibiotics in the mother and infant, due to the dysbiosis leading to risk of LOS.

With all these aspects considered, this report hypothesises that LOS incidence should decrease over the years, when the research concerning which probiotics can reduce dysbiosis of the microbiome in both the mother and the infant is well established and practiced in hospitals.

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Picture front page from Fine Art America

Figure 1-3 all made in Biorender.

To look into the literature used for this study:

PubMed advanced search was used for specific terms. These terms for the introduction and chapter 2 were “Neonatal sepsis” in combination with “Late onset”.

For chapter 3 these included “Neonatal sepsis” in combination with Microbiome” and “Dysbiosis” with “Premature birth”.

Finally, for chapter 4 the search terms used were “Infant” and “Probiotics”, “Preterm/Premature” in combination with “Probiotics”, and a lone search for “Maternal dysbiosis”, “Neonatal dysbiosis”,

“Neonatal sepsis” and “Late onset sepsis” searches were performed for the use of all chapters.