The Safety of Probiotic Prophylaxis in Preterm Infants for the Prevention of Necrotising Enterocolitis

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Abstract

Background: Necrotising enterocolitis (NEC) is a common and potentially devastating disease in very preterm infants. The use of probiotics halves the incidence of NEC, and they are routinely used in preterm infants in Australasia. However, there remain concerns about their safety, including the reliability of the contents of probiotic products, the risk of the probiotic organisms infecting the preterm infant, and the potential for the probiotic organisms to colonise the neonatal unit.

Aims: To determine: Aim 1) the concentration of probiotic organisms present in a probiotic product commonly used in preterm babies and if there was any contamination over a decade of surveillance; Aim 2) the incidence of probiotic bacteraemia in very preterm or very low birth weight infants, who received prophylactic probiotics. And Aim 3) if the neonatal unit environment is contaminated with organisms from the prophylactic probiotic routinely administered to very low birth weight infants for prevention of necrotising enterocolitis.

Methods: Aim 1) Reviewed a decade of surveillance on the concentration of probiotic organisms and presence of contamination in probiotic products commonly used in preterm infants in Australasia. Aim 2) A retrospective observational cohort study of the incidence of bacteraemia with probiotic organisms in preterm infants eligible for probiotic prophylaxis for seven years before and after the introduction of routine probiotic use. Aim 3) A multi-centre study testing multiple surfaces in the neonatal unit for the presence of the probiotic organisms.

Results: Probiotic samples did not consistently match label claims, with the concentration of organisms reducing over time. Routine probiotic use was associated with probiotic bacteraemia, incidence 0.2%, all cases had a full recovery. The study investigating the third aim was paused due to COVID-19 related restrictions, the protocol is presented in this thesis.

Conclusion: In this thesis we have shown that the contents of probiotic products do not consistently correlate with label claims and that the use of prophylactic probiotics in this vulnerable population is associated with a rare risk of probiotic bacteraemia. These are both concerning and warrant further consideration. However, they do not outweigh the significant benefits of probiotic use in this population.

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Abbreviations

rRNA	Ribosomal ribonucleic acid
ANZNN	Australian and New Zealand Neonatal Network
APHA	American Public Health Association
BPD	Bronchopulmonary Dysplasia
BSID	Bayley scales of infant and toddler development
BW	Birth weight
CFU	Colony forming unit
CMMEF	Compendium of methods for the microbiological examination of foods
COVID-19	Coronavirus disease 2019
CPAP	Continuous positive airway pressure
DNA	Deoxyribonucleic acid
EBM	Expressed breast milk
ELBW	Extremely low birth weight
GA	Gestational age
ISO	International organisation for standardisation
IUGR	Intrauterine growth retardation
IVH	Intraventricular haemorrhage
LOS	Late onset sepsis
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
NEC	Necrotising enterocolitis
NHI	National health index number
NICU	Neonatal intensive care unit
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
RCT	Randomised controlled trial
RDS	Respiratory distress syndrome
TLR4	Toll-like receptor 4
VLBW	Very low birth weight

1 Literature review

1.1 Preterm birth

Preterm birth is a significant national and global health issue, with rates increasing globally (Blencowe et al., 2012). Preterm birth, is defined as less than 37 weeks gestational age (GA) (World Health Organization, 1970). It is further subdivided into extremely preterm (less than 28 weeks GA), very preterm (28 to 31+6 weeks GA), and moderate to late preterm (32 to 36+6 weeks GA) (Blencowe et al., 2012). Moderate and late preterm infants comprise over 80% of all preterm births (Davidoff et al., 2006). Preterm birth is the most common cause of neonatal death, and is among the leading causes of child death (Black et al., 2010). Preterm birth rates vary from 5 to 18% worldwide and are higher in lower income countries (World Health Organization, 2018). The prognosis varies widely, with a 90% mortality rate of extremely preterm infants born in low-income countries, compared to less than 10% born in higher income countries (World Health Organization, 2012). As in other countries, preterm birth remains a significant issue in New Zealand. Of the 60,026 live born infants born in 2017, 6.2% were moderately preterm, and 1.3% were very or extremely preterm (Ministry of Health, 2019). In New Zealand, rates of preterm birth varied across different demographic groups, with the highest rate occurring in women over 40 years old (Ministry of Health, 2019) . Multiple factors can contribute to causing a baby to be born preterm, 70% are considered spontaneous and 30% are induced due to maternal or fetal medical indications (Goldenberg et al., 2008). Common medical causes of preterm birth include infection, intrauterine growth retardation (IUGR) or pre-eclampsia (R. L. Goldenberg et al., 2008).

1.1.1 Short term complications of preterm birth

Even in developed countries, preterm infants have significantly increased rates of morbidity and mortality compared to term infants. Acute morbidity includes, although is not limited to; respiratory distress syndrome (Sweet et al., 2019), necrotising enterocolitis (NEC)(Battersby, Longford, Mandalia, Costeloe, & Modi, 2016) (section 1.3), late-onset sepsis (Stoll et al., 2002), patent ductus arteriosus (PDA) (Hamrick & Hansmann, 2010), intraventricular haemorrhage (McCrea & Ment, 2008) and anaemia (Strauss, 2010). The infant mortality rate increases with reducing gestation, with mortality of only 2.1 per 1000 live births at term, compared to 187 per 1000 live births for infants born less than 32 weeks gestation (Ely &

Driscoll, 2019). Survivors continue to have high morbidity rates, the severity of which also correlates with increasing prematurity. Respiratory distress affects 93% of VLBW infants (Stoll et al., 2010). Thirty percent of these infants go on to develop bronchopulmonary dysplasia (BPD) (Hilgendorff et al., 2014). While the definition of BPD is under frequent revision, a currently accepted version for infants born less than 32 weeks gestation is defined as requiring oxygen for at least 28 days, with a further severity classification depending on the presence and type of oxygen requirement at 36 weeks (Davidson & Berkelhamer, 2017). BPD is caused by multiple factors which act to inhibit the normal maturation of preterm lungs (Davidson & Berkelhamer, 2017). Multiple factors also contribute to feeding difficulties in preterm infants, in particular, an immature suck swallow breathe coordination (Lau et al., 2007). This contributes to lower rates of breastfeeding in infants born preterm (Callen & Pinelli, 2005) and hence higher rates of non-human milk feeds, which increases the risk of developing NEC (Maffei & Schanler, 2017). VLBW infants have high rates of late-onset sepsis (LOS) at 21%, incidence of which increase with reducing gestational age and birth weight (Stoll et al., 2002). LOS is associated with increased length of hospital stay and increased mortality (Stoll et al., 2002). Anaemia is very common in preterm infants (Strauss, 2010). There is growing interest in the relationship between anaemia, red blood cell transfusion, and development of NEC (Maheshwari, Patel, & Christensen, 2018) which is currently being investigated (Gale et al., 2019; NICHD Neonatal Research Network, 2012). PDA is another significant health issue in preterm infants, occurring in one quarter (Lokku et al., 2017). PDA is associated with an increased risk of developing NEC, and while treatment of the PDA does not increase risk of NEC it does not reduce the risk either (Jones, Craven, Attia, Thakkinstian, & Wright, 2011; Mitra et al., 2018).

1.1.2 Chronic health complications of preterm birth

Chronic health complications of preterm birth include BPD (Hilgendorff et al., 2014), neurodevelopmental impairment (Larroque et al., 2008), behavioural disorders (Yates et al., 2020) and an increased risk of cardiometabolic disease (Markopoulou, Papanikolaou, Analytis, Zoumakis, & Siahanidou, 2019). Intraventricular haemorrhage occurs in 20-25% of VLBW infants (McCrea & Ment, 2008), the severity of which correlates with the degree of later neurodevelopmental impairment (Nongena et al., 2010). 6.8% of very preterm infants develop cerebral palsy (Pascal et al., 2018), compared to only 0.1% of infants born at term (Himpens, van den Broeck, Oostra, Calders, & Vanhaesebrouck, 2008; Wu, Croen, Shah, Newman, &

Najjar, 2006). Very preterm infants also have an increased risk of developing attention deficit hyperactivity disorder and other mental health issues including anxiety (Yates et al., 2020). There is an increased risk for cardiovascular disease and metabolic syndrome in adults who were born very preterm (Markopoulou et al., 2019).

1.1.3 Interventions to improve outcomes of preterm birth

Several antenatal and postnatal interventions that improve outcomes for infants born very preterm warrant particular mention. Antenatal corticosteroids reduce multiple adverse preterm birth complications including RDS, neonatal death, NEC, and likely IVH (McGoldrick, Stewart, Parker, & Dalziel, 2020). Antenatal magnesium sulphate reduces cerebral palsy (Chollat, Sentilhes, & Marret, 2019). Surfactant has been a well-established therapy since the '90s for its prevention of mortality, BPD, pulmonary interstitial emphysema, and pneumothorax (Soll & Özek, 1997) with recent evidence continuing to support its use despite the changes in the management of RDS (Course & Chakraborty, 2016). Kangaroo mother care reduces neonatal mortality, sepsis, hypothermia, hypoglycaemia, hospital readmission, and increases breastfeeding rates (Boundy et al., 2016). Continuous positive airway pressure (CPAP) has become an established method of respiratory support. Compared to intubation, resuscitation with CPAP reduces the combined outcome of mortality or bronchopulmonary dysplasia or both (Schmölzer et al., 2013). In addition, the use of CPAP in low- and middle-income countries reduces the need for mechanical ventilation (Martin, Duke, & Davis, 2014). Management of the umbilical cord has also been shown to impact neonatal outcomes. Delayed cord clamping reduces mortality, without influencing the risk of NEC (Fogarty et al., 2018; Rabe, Gyte, Díaz-Rossello, & Duley, 2019) and, hygienic cord care reduces neonatal infection related morbidity and mortality (Stewart et al., 2016). The importance of thermoregulation for reducing neonatal mortality was established in the '50s (Silverman, Fertig, & Berger, 1958), however despite efforts continues to be a struggle (Laptook et al., 2018; Laptook, Salhab, & Bhaskar, 2007; Lyu et al., 2015).

1.2 Microbiome

1.2.1 Definition and history

Understanding of the human microbiome and its importance on health has undergone huge evolution over recent decades. The microbiome is defined as "The entire habitat, including the

microorganisms (bacteria, archaea, lower and higher eurkaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions" whereas, microbiota refers to solely the organisms in this environment (Marchesi & Ravel, 2015). Understanding of the significance of microbes to human health developed from Kochs postulates (Koch, 1890). While this resulted in huge advances in medicine, the initial understanding of microbes equalling disease did not encompass the symbiotic relationship we know about today. Around a similar time to Kochs postulates, scientists were recognising the importance of microbes in the environment and how these acted together rather than individually, which later influenced our understanding of the microbiome (Gibbons & Gilbert, 2015).

1.2.2 Contents of the microbiota and metabolic roles

The human microbiome project resulted in significant advances in the understanding of the composition of the microbiota (Proctor, 2016). Sequencing 16S rRNA, a culture independent technique, has been key in the identification of microbes (Schloss & Handelsman, 2004). We now know the main phyla in the human gastrointestinal microbiome are the Bacteroidetes (gram-negative) and the Firmicutes (gram-positive) (Cockburn & Koropatkin, 2016).

An important role of microbiota is the metabolism of carboydrates including dietary polysaccharides and endogenous glycans (Cockburn & Koropatkin, 2016). Polysaccharides are the main carbohydrate source in our diet. The most abundant animal polysaccharides are glycosaminoglycans (Imberty, Lortat-Jacob, & Pérez, 2007), whereas cellulose is the most abundant plant polysaccharide. Humans rely on the microbiota to digest these polysaccharides (Holscher, 2017). Bacterial polysaccharides also from a large component of the human diet (Cockburn & Koropatkin, 2016), while mechanisms exist to resist their metabolism, some microbiota can break down this bacterial cell wall allowing its digestion (van Bueren, Saraf, Martens, & Dijkhuizena, 2015). When the microbiota break down these carbohydrates, they produce lactate as well as the short chain fatty acids acetate (the most abundant), butyrate, and propionate (Ríos-Covián et al., 2016).

The short chain fatty acids produced by the microbiota have many vital roles. They are the main energy source of colonic enterocytes (van der Beek et al., 2015). They can regulate pH with resultant improment in electrolyte absorption (Markowiak-Kopeć & Śliżewska, 2020). Short chain fatty acids can increase the production of mucin (Willemsen, Koetsier, van Deventer, & van Tol, 2003) which is an essentiall component of the immune defense barrier.

Short chain fatty acids also have anti-inflammatory properties through reduction of inflammatory mediators, which reduces the activity of NFκB macrophages (Markowiak-Kopeć & Śliżewska, 2020), and are being investigated for their role in reducing colonic cancer risk (Wong, de Souza, Kendall, Emam, & Jenkins, 2006).

1.2.3 Development of the microbiome

The traditional concept of a 'sterile womb' has been increasingly challenged over recent years, though remains a highly debated topic (Bushman, 2019; Perez-Muñoz, Arrieta, Ramer-Tait, & Walter, 2017; Stinson, Boyce, Payne, & Keelan, 2019). The largest initial exposure to microbes is during birth with the maternal vaginal flora shown to influence the infant's gastrointestinal microbiome (Gabriel, Olejek, Stencel-Gabriel, & Wielgoś, 2018).

After delivery, skin to skin is another modulator of the infant microbiome, allowing further transfer of microbes from the parent to infant (Widström, Brimdyr, Svensson, Cadwell, & Nissen, 2019). Of great impact to the microbiome is the effect of breast feeding, both from areolar contact and from the components of the breast milk. A recent longitudinal study found that in healthy infant mother pairs, in the first month of life, 10.4% of infant gastrointestinal bacteria was from areolar skin and 27.7% from breast milk (Pannaraj et al., 2017). Breast milk contains human-milk oligosaccharides which promote growth of Bifidobacterium species (Coppa, Bruni, Morelli, Soldi, & Gabrielli, 2004). The CHILD study reviewed components and influencers of human breast milk (Moossavi et al., 2019). They found the microbiome of breast milk is influenced by various factors including maternal breast-feeding practices, BMI, atopic disease, smoking. The most abundant microbes in breast milk, in order of decreasing prevalence, were Proteobacteria, Firmicutes, Actinobacteria and Bacteroides.

Various factors can influence this development of the infant microbiota, including mode of delivery, antibiotic exposure and type of feeding. Debate exists around the effect of caesarean delivery on the infant microbiome, though most research indicates that infants born through caesarean section do have an altered microbiome (Mueller, Bakacs, Combellick, Grigoryan, & Dominguez-Bello, 2015; Shao et al., 2019; Stinson, Payne, & Keelan, 2018). Antibiotic exposure, either antenatally or prenatally can also influence the microbiome (Fouhy et al., 2012; Tanaka et al., 2009). Compared to formula fed infants, breast fed infants had more bifidobacteria and less Bacteroides, *Clostridium coccoides*, and Lactobacillus species (Fallani et al., 2010).

All the factors explained above, including higher rates of caesarean delivery, more antibiotic exposure, and less breast feeding adversely affect the gastrointestinal microbiome of preterm infants, addition to several others. Gestational age is one of the strongest influencers of the infant microbiome (Korpela et al., 2018). The preterm infant microbiome is shown to have more Enterbacter, Enterococcus, and less Bifidobacterium than term infants (Arboleya et al., 2012; Schwiertz et al., 2003). The hospital environment has a significant impact on the preterm infant microbiome, with shared microbes being found in both infant stool microbiome and the neonatal environment (Brooks et al., 2014). The composition of breast milk for preterm infants has also been shown to have differences, including with varying levels of human milk oligosaccharides (Granger et al., 2021) which as discussed in 1.4.11 act as a prebiotic. Furthermore, respiratory support can also affect gastrointestinal microbiome (Henderickx, Zwittink, van Lingen, Knol, & Belzer, 2019a).

While infants are breast fed their microbiome is predominated by bifidobacteria (Moore & Townsend, 2019). This evolves as their diet transitions to more solid foods, gradually changing to microbes that are better able to digest solid foods, with major alterations being due to differences in health or diet (Koenig et al., 2011).

1.2.4 Role of the microbiome in diseases other than NEC

The importance of the microbiome has been shown in various diseases, including inflammatory bowel disease, obesity, metabolic disease, and asthma. Patients with inflammatory bowel disease can have a dysregulated microbiome (Glassner, Abraham, & Quigley, 2020). Several disease characteristics suggest the importance of the microbiome in inflammatory bowel disease including disease being most severe in areas with higher bacterial activity, effectiveness of antibiotic therapy in treating inflammatory bowel disease, and the use of fecal diversion in treating chrons disease (Glassner et al., 2020). There has been a drastic growth in the understanding of the importance of the microbiome in obesity and metabolic disease over the past decade (Sanz, Olivares, Moya-Pérez, & Agostoni, 2014; Zhao, 2013). The role of the microbiome in obesity is likely multifactorial. This includes the metabolism of bile acids affecting lipid and glucose homeostasis, short chain fatty acid production affecting gut hormones such as leptin which influence satiety, and the presence of endotoxins which influence levels of lipopolysaccharide and hence inflammation (Tseng & Wu, 2019). Similarly, there has been increasing interest over the past decade in the role of the lung microbiome in respiratory illnesses. Previously the lungs were thought to sterile, however over the past decade

it has been accepted that this is not the case (Dickson et al., 2017) and there has been an established consensus on what constitutes a healthy lung microbiota (Moffatt & Cookson, 2017). Asthmatic patients can have a dysbiosis with predominance of potentially pathogenic organisms such as Haemophilus and less commensals (Hilty et al., 2010).

1.3 Necrotising enterocolitis

1.3.1 Timing

Necrotising enterocolitis (NEC) is the most frequent serious acquired gastrointestinal disease of the newborn (Lee & Polin, 2003). The classic triad of symptoms is abdominal distension, bloody stool, and bile-stained aspirates (Brook, 2008), however symptoms are often non-specific and can be difficult to distinguish from other neonatal illnesses. Disease severity ranges from mild feed intolerance to profound shock (Lin & Stoll, 2006). The timing of onset is also very variable with a trend to occurring later in infants with a lower birthweight, at a mean of 32 days for more premature infants with birth weight less than 1000g and 7 days for infants with birth weight over 1000g (Yee et al., 2012).

1.3.2 Symptoms, diagnostic criteria, and incidence

Symptoms, diagnostic criteria, and incidence of NEC can all vary. A surgical or post-mortem specimen showing the classic features of necrotic, inflamed, infarcted intestine allows a definitive diagnosis. However, many infants will only require medical management, so this is not always available. The Modified Bell Staging is the most widely used diagnostic criteria in clinical practice, though this varies with other definitions used in high income countries including the International Classification for Diseases code, or the Centers for Disease Control and Prevention definition (Battersby, Santhalingam, Costeloe, & Modi, 2018). The modified Bell staging relies on systemic, intestinal, and radiologic signs to grade NEC as stage 1 (suspected), 2 (proven), or 3 (advanced) (Walsh & Kliegman, 1986). Despite its widespread use, some now suggest the Modified Bell staging is outdated. Due to the non-specificity of symptoms of NEC, and variations in diagnostic criteria used, it can be difficult to assess trends over time and compare incidence between different centres. Incidence varies considerably between countries and neonatal units, with between 2 to 7% of very preterm infants in high-income countries developing NEC (Battersby et al., 2018).

1.3.3 Aetiology

Despite extensive research a definitive cause of NEC has not yet been identified, and studies suggest it is likely multifactorial. The leading influential factor is preterm birth, which predisposes to an undeveloped intestinal epithelial barrier, an abnormal gastrointestinal microbiome, and an immature immune system (Lin & Stoll, 2006).

The importance of the gastrointestinal microbiome is becoming increasingly understood. As discussed further in Chapter 1.2.3 preterm infants have been shown to develop dysbiosis, with a less diverse microbiome with more potentially harmful microbes than in term infants (Schwiertz et al., 2003). Multiple factors contribute to this difference, including the NICU environment, antibiotic use, respiratory support, and type of feeding (Henderickx, Zwittink, van Lingen, Knol, & Belzer, 2019b). Debate exists around the effect of mode of delivery on this dysbiosis and potential causes for this, though most research indicates that infants born through caesarean section do have an altered microbiome (Mueller et al., 2015; Shao et al., 2019; Stinson et al., 2018). Through various methods such as altered intestinal permeability and inflammatory signaling, this dysbiosis contributes to a reduced intestinal barrier function and immune effectivity (Bergmann et al., 2013). Gene sequencing of stool from preterm infants shows a change in bacterial composition over time in preterm infants, with a tendency towards a predominance of gram-negative bacteria before developing NEC (Warner & Tarr, 2016) suggesting that dysbiosis contributes to the development of NEC.

In addition to preterm birth, there are multiple other risk factors which each play a role in the development of NEC. The list is extensive and includes low birth weight, non-human milk feeds, H2 blockers, chorioamnionitis, sepsis, prolonged antibiotic use, patent ductus arteriosus, indomethacin use, mechanical ventilation, red blood cell transfusions, perinatal asphyxia, cyanotic congenital heart disease, IUGR, low Apgar score (Gephart, McGrath, Effken, & Halpern, 2012). Factors that are shown to be protective against developing NEC including feeding with human-derived milk, following standardised feeding guidelines (Gephart et al., 2012), and of most impact, probiotics (Sharif, Meader, Oddie, Rojas-Reyes, & McGuire, 2020), which are discussed in further detail below (chapter 1.4).

There is increasing interest in the role of the Toll-like receptor 4 (TLR4) in the pathogenesis of NEC. TLR4 is a lipopolysaccharide receptor which is involved in regulation of gastrointestinal development by communicating with stem cells, and therefore is expressed in a higher concentration in the preterm intestine (Egan et al., 2016; Neal et al., 2012). Multiple

animal studies are showing increasing evidence for the role of TLR4 in the development of NEC (Jilling et al., 2006; Leaphart et al., 2007).

TLR4 is a proinflammatory. Activation of TLR4 upregulates inflammatory pathways including IL-17, IL-22, and TH17 cells promoting the inflammatory cascade that results in NEC (Egan et al., 2016). Triggering of TLR4 results in intestinal cell death (Neal et al., 2013) and reduces their repair and regeneration with healthy cells (Neal et al., 2012) therefore causing irreparable damage to the intestinal wall. Cell death causes a loss of enteric glia, resulting in reduction of glial peptide brain-derived neurotrophic factor, and is thought to occur early in the development of NEC. The lower levels of this glial peptide brain-derived neurotrophic factor result in inflammation and abnormal motility of the gut (Kovler et al., 2021), this could potentially explain why abdominal distension and ileus are often seen early in NEC (Hackam & Sodhi, 2022).

The importance of TLR4 in the pathogenesis of NEC aligns with our understanding of the importance of the microbiome, the changes seen in gut perfusion, the propensity of NEC to occur in preterm infants, and the protective nature of feeding with expressed breast milk. Analysis of microbiome changes in the lead up to NEC has shown that infants who go on to develop NEC are more likely to have a predominance of microbes that express lipopolysaccharide, which binds to and stimulates TLR4 and likely to have less CpG DNA, which inhibits TLR4 (Shaw et al., 2021). TLR4 expression can also modify gut vascular perfusion, as activation of TLR4 reduces the expression of endothelial nitric oxide synthase (eNOS) which has vasodilation properties (Yazji et al., 2013), contributing to the loss of gastrointestinal perfusion that can occur in NEC. The preterm intestine has higher concentrations of TLR4 due to its role in gut development. When infants are born premature, their intestine becomes colonised with microbes. The presence of microbes in a preterm intestine that would otherwise have very minimal microbe interaction in utero, results in increased activation of TLR4, and the resultant cascade of intestinal cell apoptosis, reduced proliferation, and resultant disruption of the intestinal barrier with increased bacterial translocation (Hackam, Good, & Sodhi, 2013). Breast milk has been shown to inhibit glycogen synthase kinase-3β which results in inhibition of TLR4 signalling (Good et al., 2015), potentially explaining a causal factor in the protective nature of expressed breast milk in preventing NEC.

1.3.4 Treatment

The foundation of treatment for NEC is medical stabilisation (Nolan, Goree, & Good, 2021). This is achieved with wide spectrum antibiotic use (generally combining several agents), gut rest with a nasogastric tube on free drainage, nil by mouth, and parental nutrition. In addition, correction of any metabolic, hematologic, or circulatory compromise. Intestinal perforation is an absolute indication for surgery, otherwise, surgery is generally reserved for infants who deteriorate despite medical management. Surgical treatment options are laparotomy with excision of necrotic intestine, or primary peritoneal drainage (Lee & Polin, 2003; Rao, Basani, Simmer, Samnakay, & Deshpande, 2011).

1.3.5 Long term consequences

The consequences of NEC are significant, with an overall mortality of 28% . Half of the infants who develop NEC will require surgery (Hull et al., 2014). Among these infants, complications include recurrence in 8%, intestinal strictures in one quarter, and intestinal failure in 13% (Hau et al., 2019). One-third of VLBW infants with surgically treated NEC will die,(Hull et al., 2014) increasing to 50% in ELBW infants (Blakely et al., 2006). The mortality rate is lower for infants with medically treated NEC at 21%. NEC survivors are 2.6 times as likely to suffer cognitive impairment (Shah et al., 2011), with risk being greatest in infants with earlier onset of the NEC, requirement for more red cell transfusions, and more hemorrhagic lesions on bowel specimens (Garg et al., 2021). NEC is also associated with lung injury, with increased rates of chronic lung disease in survivors (Jia et al., 2019; Laughon et al., 2009).

1.4 Probiotics

Probiotic bacteria are defined as live microbial supplements that colonize the gastrointestinal tract, potentially conferring advantage to the host (Millar & Wilks, 2003). Over the past decade there has been extensive research into probiotic use for very preterm infants, which has shown a reduction in necrotising enterocolitis and potentially also late onset sepsis and mortality (Sharif et al., 2020). Despite the significant benefits of their use, concerns persist around safety elements and unknowns of probiotic use, resulting in hesitancy of their uptake in many neonatal units (Barbian, Buckle, Denning, & Patel, 2019).

1.4.1 History

The first cohort study on probiotic use in preterm infants was in Bogota, Colombia in 1999. Following the observation of changes in the gastrointestinal microflora in relation to developing NEC, all 1237 infants admitted to the neonatal unit over a one-year period were administered *Lactobacillus acidophilus* and *Bifidobacterium infantis*. The incidence of NEC was compared to infants the year prior. They found significantly reduced NEC and NEC related mortality in the intervention group (Hoyos, 1999). In the same year the first animal model study showed that the administration of *Bifidobacterium infantis*, compared to *Escherichia coli* or saline controls reduced the incidence of NEC in newborn rats (NEC, 29.2% *Bifidobacterium infantis*, vs 69.5% *Escherichia coli*, vs 70% controls, P <0.01) (Caplan et al., 1999). These pioneering researchers paved the way to a subject that has become one of the most studied areas in neonatology.

1.4.2 Probiotics effect on necrotising enterocolitis

The ProPrems trial was a multicentre RCT in New Zealand and Australia, from 2007 – 2011 (Jacobs et al., 2013). This trial investigated probiotic supplementation with *Bifidobacterium infantis, Streptococcus thermophilus*, and *Bifidobacterium lactis* (ABC Dophilus Probiotic Powder) in 1100 VLBW infants and demonstrated a significant reduction in NEC with relative risk (RR) of 0.46, 95% confidence interval (CI) 0.23 to 0.93, with no difference in risk of late onset sepsis, or mortality. The PiPS trial, another RCT, ran from 2010 to 2013 (Costeloe, Hardy, Juszczak, Wilks, & Millar, 2016). This investigated supplementation of the probiotic *Bifidobacterium breve* for infants 23 – 30 week's gestation, including 1315 infants from 24 hospitals in England. The PiPS trial found no significant difference in risk of NEC, late onset sepsis, or mortality with the use of probiotics, contributing to debate around probiotic use. There have since been numerous RCTs (Härtel et al., 2014; Janvier, Malo, & Barrington, 2014; Singh et al., 2019) and systematic reviews of multiple trials (Alfaleh & Anabrees, 2014; Liu, Wang, Lu, & Pei, 2022; Morgan et al., 2020; Rao, Athalye-Jape, Deshpande, Simmer, & Patole, 2016; Sharif et al., 2020) showing the benefit of probiotics in reducing NEC. The most recent Cochrane review shows the use of probiotics in very preterm or very low birth weight infants reduces NEC with RR 0.54, 95% CI 0.45 to 0.65. While earlier Cochrane reviews found that probiotics also reduce the risk of mortality (Alfaleh & Anabrees, 2014), and late onset sepsis (Rao et al., 2016), the latest review concluded that these effects no longer remained significant when excluding trials at high risk of bias (Sharif et al., 2020). Following these meta-

analyses, most institutions throughout Australasia commenced routine use of prophylactic probiotics for VLBW very preterm infants, though other parts of the world such as North America have displayed more hesitance over introducing probiotics (Janvier et al., 2014).

Systematic reviews of non-randomised clinical trials are important for assessing the impact of probiotics when used under clinical conditions. A 2021 review, which included 77,018 infants, showed that probiotics reduce both at least stage 2 NEC (6.8% vs 4.2%), late-onset sepsis (11% vs 9.5%), mortality (5.9% vs 4.6%) and time to full feeds (-1.23 days) (Deshmukh & Patole, 2021b). They were powered for subgroup analysis of extremely low birth weight infants which showed a reduction in NEC (4.5% versus 7.9%), but no effect on mortality or late-onset sepsis, though quality of evidence grade for this extremely low birth weight cohort was either low or very low.

1.4.3 Probiotic organism used

Organisms used as probiotics to prevent NEC in preterm infants include the bacteria Bifidobacterium, Lactobacillus, *Streptococcus thermophiles*, and less commonly *Escherichia coli, Enterococcus faecalis*, and the yeast *Saccharomyses boulardii* (Wassenaar, Gu¨, & Klein, 2008). Probiotics with combinations of strains are likely more beneficial than single strain products (Chang et al., 2017; Jiang, Zhang, Xu, Li, & Yang, 2020). However, despite multiple studies reviewing this there is not a clear consensus on the most effective strain or combination as outlined below.

Whilst certain immunologic effects of probiotics are strain specific, other benefits occur through mechanisms that are shared between higher taxonomic levels (Sanders, Benson, Lebeer, Merenstein, & Klaenhammer, 2018). One example of this is the Bifidobacterium genus all producing short chain fatty acids, which have an anti-inflammatory effect (Sanders et al., 2018). There is a paucity of data on optimal dosing regimens, with the data that is available being derived from achieving adequate faecal concentrations of the probiotic, rather than achieving the desired clinical outcomes (Boyle, Robins-Browne, & Tang, 2006). Current experts recommend using similar dosing regimens to that used in clinical trials, most of which are in the range of 10^8 to 10^9 CFU (van den Akker et al., 2020).

Despite multiple trials investigating this, there is not yet a consensus on the optimal probiotic organism for preventing NEC. A 2018 strain specific network meta-analysis concluded that only certain probiotic strains are effective in reducing NEC, mortality and late-onset sepsis

(van den Akker et al., 2018). Seven probiotic regimes were found to reduce the incidence of NEC (*Bifidobacterium lactis* Bb-12 or B94, *L reuteri* ATCC 55730 or DSM 17938, *Lactobacillus rhamnosus GG*, combination of *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, and *Lactobacillus acidophilus*, combination of *Bifidobacterium infantis* Bb-02, *Bifidobacterium lactis* Bb-12, and *Streptococcus thermophilus* TH-4, combination of *Bifidobacterium longum* 35624 and *Lactobacillus rhamnosus* GG), two for reducing LOS and three for mortality. Despite including 51 RCTs, most strains were only assessed in a small number of trials, many of which had small numbers of infants, resulting in the conclusion that they were unable to determine the optimal strain based on current evidence. Another 2020 network meta-analysis compared single versus multi-strain probiotics for reducing NEC (Morgan et al., 2020). They found that combinations of at least one Lactobacillus species with at least one of Bifidobacterium species, Bifidobacterium animalis lactis, Lactobacillus reuteri, or Lactobacillus rhamnosus significantly reduced severe NEC. When including all outcomes they concluded that the combination of a lactobacillus species with a Bifidobacterium species to be superior to single or other multiple strain combinations. While helpful, multiple reasons including differences in study aims and design mean caution is required in interpreting network meta-analysis for determining the optimal probiotic strain (Deshmukh & Patole, 2021a). The 2020 position statement from the European Society for Paediatric Gastroenterology Hepatology and Nutrition Committee on Nutrition, and the European Society for Paediatric Gastroenterology Hepatology and Nutrition Working Group for Probiotics and Prebiotics, recommend the use of Lactobacillus rhamnosus GGATCC5310 with dose of one to six billion CFU, or, the combination of Bifidobacterium infantis Bb-02, Bifidobacterium lactis Bb-12 and Streptococcus thermophilus TH-4 with dose of 3 to 3.5 x10^8 CFU per strain. Interestingly they concluded that due to conflicting evidence on the commonly used combination of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* they were unable to make a recommendation regarding its use.

While there is minimal evidence on long term outcomes of very preterm infants administered probiotics, the available data shows they are safe. A 2 to 5 year follow up of children who were born very preterm and involved in a RCT of probiotic use as part of the ProPrems trial found no difference in survival free of neurodevelopmental impairment, and interestingly, lower rates of deafness in the routine probiotic group (Jacobs et al., 2017a). This finding of comparable neurodevelopmental outcomes is comparable with other studies (Akar et al., 2016).

1.4.4 Mechanism of action

Probiotics have a multifactorial mechanism of action, involving both the innate and humoral immune systems (Halloran & Underwood, 2019). In the innate immune system, probiotics have effects on dendritic cells and epithelial cells (Frei, Akdis, & O'Mahony, 2015). Dendritic cells are key in the primary immune response, with roles as surveyors of the microbial surroundings and production of many immunoreactive mediators including the metabolism of vitamin A to retinoic acid which has important roles in immune homeostasis. Probiotic microorganisms can stimulate the production of retinoic acid (Konieczna et al., 2013). Probiotic organisms have also been shown to increase dendritic cell signalling of TREG cells through activation of the dendritic cell enzyme heme oxygenase (Karimi, Kandiah, Chau, Bienenstock, & Forsythe, 2012). Dendritic cell MHC-II presentation of certain microorganisms can cause production of anti-inflammatory cytokines (Dasgupta, Erturk-Hasdemir, Ochoa-Reparaz, Reinecker, & Kasper, 2014). Fermentation of fibers such as prebiotics in the colon results in the production of short chain fatty acids including butyrate, acetate, and propionate (Frei et al., 2015). These short chain fatty acids have multiple immunomodulatory effects. Butyrate can alter the production of histone deacetylase (HDAC) resulting in the reduction of proinflammatory cytokine release from dendritic cells and increased TREG stimulation (Frei et al., 2015).

Probiotics have important effects on intestinal epithelial barrier. Intestinal epithelial cells are a key barrier for physical defence and have crucial roles in nutrient absorption. Essential to the immune barrier is mucus, epithelial junction complexes, and antimicrobial peptides (Ohland & MacNaughton, 2010). Probiotics can influence the secretion of antimicrobial peptides such as defensin and cathelicidins (Habil, Abate, Beal, & Foey, 2014). Probiotics can also increase goblet cell production of mucin (Wang et al., 2014) and strengthen the epithelial junctions (Hummel, Veltman, Cichon, Sonnenborn, & Schmidt, 2012). Pathogens can reduce nutrient absorption through the intestines, this effect has been shown to be reduced by *Bifidobacterium infantis* (Symonds et al., 2012). Probiotic organisms can down regulate the production of proinflammatory cytokines from epithelial cells (Boonma, Spinler, Venable, Versalovic, & Tumwasorn, 2014).

The adaptive immune system can be affected by probiotics through their effects on T lymphocytes, natural killer cells, and B cells (Frei et al., 2015). Certain probiotic organisms can increase Treg cells (Frei et al., 2015) and, reduce the proinflammatory Th17 cells and IL-

17 production (Tanabe, 2013). Natural killer cells are central in intestinal inflammatory response (Frei et al., 2015) and are influenced by the microbiome (Olszak et al., 2012).

1.4.5 Manufacturing

There remain unknowns in how to faultlessly manufacture probiotic products. The process includes the selection of the desired strain, isolation of this, fermenting the product to cause multiplication, centrifuging out unwanted by-products, then preparing the probiotic for storage with cryoprotection, freezing, blending with other desired strains, and finally packaging (Fenster et al., 2019). Ideally, the probiotic organisms would survive production, storage, and transit through the gastrointestinal tract, and be absent of pathogenic features. Unfortunately, multiple factors throughout this process threaten to affect the product, including thermal and oxidative stress, or cell dehydration during the production phase; acidity, moisture, oxygen, and competition with other organisms in the storage phase; or the effect of gastric acidity, bile salts, and enzymes once the probiotic have reached the gastrointestinal tract (Ganguly et al., 2011; Lacroix & Yildirim, 2007).

1.4.6 Potential harms

1.4.6.1 Case reports of probiotic bacteraemia

The most concerning potential harm from probiotic use in neonates is the risk of sepsis from a probiotic organism. The recent Cochrane reviews on probiotic use in preterm infants for preventing NEC, which combined have included thousands of infants administered probiotics reported no infants were identified as having developed sepsis from the probiotic organism, therefore this is assumed to be rare (Sharif et al., 2020). However, there are now multiple case reports of blood culture-proven sepsis from a probiotic organism in the literature as outlined in Table 1.

Anaerobic blood culture bottles are generally needed for detecting obligate anaerobic organisms such as Bifidobacterium (Sato et al., 2016). Probiotic organisms can be grown in paediatric culture bottles, but will take longer than if anaerobic culture bottles are used. This indicates the requirement for laboratories to have this longer incubation time (Sakurai et al., 2022) and may contribute to underreporting of cases.

The following table represents a summary of a literature search of the features of the current cases of bacteraemia from a probiotic organism.

Table 1 Case Reports of Probiotic Bacteraemia in the Literature

Reference	GA	BW (grams)	Sex	Comorbidities	Probiotic & when started	Day of bacteraemia with probiotic organism	Symptoms on day of blood culture isolate	Organism isolated	Number of positive blood cultures with probiotic organism	If blood culture isolate tested to be same organism as in probiotic
(Ohishi et al., 2010)	37	2060	F	Omphalocoele, repaired day of birth.	*Bifidobacterium breve*, day 2	Day 10	Bilious gastric fluid	Bifidobacterium breve	1	Yes by randomly amplified polymorphic DNA analysis.
(Dani et al., 2016)	39	1060	F	Trisomy 18, triple X syndrome. Respiratory failure requiring mechanical ventilation. Congenital heart disease. Epilepsy. Recurrent infections. Antibiotic associated diarrhoea.	*Lactobacillus rhamnosus* GG, day 9	Day 97	Fever and tachycardia	*Lactobacillus rhamnosus*	1	Identified by MALDI-TOF mass spectrometry. Isolates compared with probiotic strain by pulsed field gel electrophoresis showing identical profile
(Dani et al., 2016)	23	660	M	RDS, PDA closed pharmaceutically, *Staphylococcus haemolytic* sepsis.	*Lactobacillus rhamnosus* GG (Dicoflor), day 2	Day 18	Apnoea with metabolic acidosis	*Lactobacillus rhamnosus*	3 (day 18, day 26 and day 34)	As above
(Bertelli et al., 2014)	26	867	F	RDS, PDA closed with indomethacin	Infloran, day 5	Day 14	Tachycardia, desaturation, and ileus.	*Bifidobacterium infantis*	1	Yes, with genome sequencing
(Bertelli et al., 2014)	28+6	1090	F	Dizygotic twin, RDS	Infloran, day 5	Day 10	Abdominal distension, tenderness, ileus, rapid deterioration, diagnosed with NEC stage III.	*Bifidobacterium infantis*	1	Yes, with genome sequencing
(Esaiassen E, Cavanagh P, Hjerde	24	730	M	RDS	Infloran, started in first week	Day 8	Sepsis with severe hypotension, abdominal distension, gastric	*Bifidobacterium longum*	1	Yes, with whole genome sequencing

E, Simonsen GS, Støen R, 2016)							aspirates and feed intolerance. Diagnosed with NEC on day 12.			
(Esaiassen E, Cavanagh P, Hjerde E, Simonsen GS, Støen R, 2016)	23	500	M	RDS	Infloran, started in first week	Day 12	Apnoea, bradycardia and temperature instability	*Bifidobacterium longum*	1	Yes, with whole genome sequencing
(Esaiassen E, Cavanagh P, Hjerde E, Simonsen GS, Støen R, 2016)	24	697	F	RDS, NEC on day 9 with stoma formation	Infloran, started in first week	Day 46	Hypotension, metabolic acidosis.	*Bifidobacterium longum*	1	Yes, with whole genome sequencing
(Zbinden, Zbinden, Berger, & Arlettaz, 2015)	30	1200	F	RDS, PDA treated, prior episodes of infection	Infloran, day 1	Day 20	Abdominal distension, mottled skin (had treated omphalitis on d13)	*Bifidobacterium longum*	1	Yes, with 16S RNA sequencing
(Zbinden et al., 2015)	28	850	M	RDS	Infloran, day 3	Day 20	-	*Bifidobacterium longum*	1	Yes, with 16S RNA sequencing
(Zbinden et al., 2015)	29	1230	F	RDS	Infloran, day 1	Day 11	Acute deterioration with NEC stage III on day of blood culture isolate	*Bifidobacterium longum*	1	Yes, with 16S RNA sequencing
(Land et al., 2005)	Term, now 6 weeks	3200	M	6 week old male, term, congenital heart disease. Developed antibiotic associated diarrhoea.	*Lactobacillus GG* for antibiotic associated diarrhoea, commenced on day 79	Day 99	Fever, tachypnea and tachycardia	Lactobacillus species	3	Yes, DNA extraction then diversilab kit

(Kunz, Noel, & Fairchok, 2004)	36	-	M	Short gut secondary to congenital ileal atresia and volvulus. TPN dependant.	*Lactobacillus GG*, day 95	Day 118	Fever and diarrhoea.	Lactobacillus species	1	No
(Kunz et al., 2004)	34	-	M	Gastroschisis with infarcted bowel at birth and resultant short gut. TPN dependant. Cholestatic liver disease.	*Lactobacillus GG*, day 17	Day 186	Temperature, tachycardia, and apnoea.	*Lactobacillus rhamnosus* GG	1	Yes, with pulsed field gel electrophoresis
(Guenther, Straube, Pfister, Guenther, & Huebler, 2010)	28	935	-	Rotavirus and adenovirus gastroenteritis on day 13.	Escherichia coli NISSLE 1917, day 15	Day 25	Fever, apnoea, hypotension.	Escherichia coli Nissle 1917	1	Yes, with pulsed field gel electrophoresis
(Jenke, Ruf, Hoppe, Heldmann, & Wirth, 2012)	27	600	F	PDA closed with indomethacin	Infloran, day 9	Day 18	Gastric aspirates, abdominal distension and tenderness, apnoeas.	Bifidobacterium species	1	Identified with mass spectrometry, further typing with PCR showed the presence the Bifidobacterium infantis strain.
(Chiang et al., 2021)	26	749	F	RDS	*Lactobacillus rhamnosus*, day 14	Day 26	Apnoea, bradycardia, desaturation and abdominal distension	*Lactobacillus rhamnosus*	2, day 28 and day 32	Yes, with whole genome sequencing.
(Brecht, Garg, Longstaff, Cooper, & Andersen, 2016)	25+6	970	M	RDS. PDA treated with ibuprofen, ileal perforation on day 6 which was resected, another ileal perforation on day 11 treated with resection	Infloran, day 18	Day 63	Apnoea, lethargy	*Lactobacillus rhamnosus*	1	Yes, with MALDI-TOF and 16S rRNA gene sequencing

				of the previous anastomosis and creation of an ileostomy.						
(Pillai, Tan, Paquette, & Panczuk, 2020)	27+6	1240	F	RDS	Florababy Pro (*Lactobacillus rhamnosus,* Bifidobacterium species) day 1	Day 7	Abdominal distension, diagnosed with NEC requiring surgery	*B. longum*	1	Yes, with molecular typing of probiotic and blood culture isolate
(Sato et al., 2016)	36	2249	M	Cloacal exstrophy, omphalocele, imperforate anus, treated with cystourethroplasty and colostomy	*Bifidobacterium breve* BBG-01, day 1	Day 8	Abdominal distension with eventual intestinal obstruction, surgically removed	*Bifidobacterium* spp	2. Day 8 and day 11	Yes, with random amplified polymorphic DNA (RAPD) analysis
(Roy et al., 2017)	27	825	M	-	*Saccharomyces boulardii*	-	-	*S. cerevisiae* (N CCPF 920006)	1	Yes, with association study by Fluorescent amplified fragment length polymorphism (FAFLP)
(Roy et al., 2017)	31	1500	M	-	*Saccharomyces boulardii*	-	-	*S. cerevisiae* (N CCPF 920007)	1	As above
(Lungarotti, Mezzetti, & Radicioni, 2003)	30	-	M	IUGR, feeding intolerance	*Saccharomyces boulardii* ,third week	In third week, 4 days after starting probiotic	'Symptoms suggesting sepsis'	*Candida albicans* at start of illness, *S. cerevisiae* two weeks later	1	-
(Cavicchiolo et al., 2019)	25+6	770g	F	-	*Lactobacillus rhamnosus* GG, day 3	Day 18	Abdominal distension, raised CRP	*L. rhamnosus*.	2 peripheral, 1 central	Yes, with random amplification of polymorphic DNA (RAPD)
(Cavicchiolo et al., 2019)	-	-	M	'Premature'	Same room as above infant, not on probiotics	-	Abdominal distension, feeding intolerance, raised CRP	*L. rhamnosus*.	1	Yes, with random amplification of polymorphic DNA (RAPD)

(Cavicchiolo et al., 2019)	-	-	M	'Premature'	Same room as above infant, not on probiotics	-	Abdominal distension, feeding intolerance, raised CRP	*L. rhamnosus*.	1	Yes, with random amplification of polymorphic DNA (RAPD)
(Sakurai et al., 2022)	38	2741	F	Esophageal atresia, aspiration pneumonia	Bifidobacterium breve BBG-01, day 3	Day 11	Apnoea, respiratory distress, fever, tachycardia	*Bifidobacterium breve*	1	Yes, with PCR
(Sakurai et al., 2022)	25	380	M	Necrotizing enterocolitis	Bifidobacterium breve BBG-01, day 0	Day 5	Apnoea, respiratory distress, tachycardia, abdominal distention	*Bifidobacterium breve*	1	Yes, with PCR
(Sakurai et al., 2022)	34	1413	M	Necrotizing enterocolitis, congenital heart disease	Bifidobacterium breve BBG-01, day 0	Day 27	Apnoea, respiratory distress, fever, tachycardia, abdominal distention, bloody stool	*Bifidobacterium breve*	1	Yes, with PCR
(Sakurai et al., 2022)	33	2085	M	Food protein-induced enterocolitis syndrome	Bifidobacterium breve BBG-01, day 0	Day 7	Apnoea, respiratory distress, fever, vomit, bloody stool	*Bifidobacterium breve*	1	Yes, with PCR
(Sakurai et al., 2022)	31	1490	F	Ileal volvulus Food protein-induced enterocolitis syndrome	Bifidobacterium breve BBG-01, day 0	Day 8	Vomit, bloody stool	*Bifidobacterium breve*	1	No

GA = Gestational age, F = Female, M = Male, RDS = Respiratory distress syndrome, PDA = patent ductus arteriosus, IUGR = Intrauterine growth retardation, NEC = Necrotising enterocolitis, PCR = polymerase chain reaction

As included in the above table, a recent study reviewed the incidence of bacteraemia with probiotic organisms over the five-year period from 2014 to 2019 in a neonatal unit that routinely supplemented with BBG-01 (Sakurai et al., 2022). They identified 6 cases, corresponding to 2% of the infants who were receiving the probiotic. Interestingly, the time taken for blood cultures isolates to become positive varied between 4 and 7 days, highlighting the importance of a longer duration of culture isolation to detect these slow growing anaerobic isolates. This is a significantly different finding to the PiPS trial (Costeloe et al., 2016) which administered the same probiotic but found no cases of bacteraemia, one contributor to this difference may be the exclusion of infants with lethal or gastrointestinal malformations in the PiPS trial, whereas they were included in the trial by Sakurai et al.

A recent systematic review has identified 32 infants with probiotic bacteraemia (Kulkarni et al., 2022). Most cases occurred in very preterm infants. Bifidobacterium was the most common causative organism, isolated in 19 cases. With lactobacillus being identified in 10 cases and non-pathogenic fungi including Saccharomyces in 3 cases. One death was attributed to sepsis with the probiotic organism *Lactobacillus reuteri*. 12 of the 32 infants had gastrointestinal co-morbidities. Of note in this systematic review, was a paper that reported three cases of sepsis with *Lactobacillus rhamnosus* in a room where only one of the three infants was intentionally being administered a liquid probiotic containing *Lactobacillus rhamnosus* GG (Cavicchiolo et al., 2019). This highlights the potential for nosocomial infection of probiotic organisms in infants not intentionally being administered probiotics and the need to investigate potential vectors of their spread.

Therefore, it is apparent that sepsis from a probiotic organism is a potential risk from probiotic administration in very low birth weight infants. Whilst this is assumed to be rare, the incidence is unknown which contributes to caution regarding probiotic use. Further research is needed to determine the risk of this adverse outcome and potential predisposing factors.

1.4.6.2 Pathogenic features of probiotic organisms

There are multiple potential virulent features that ideally probiotic organisms would not have, including the ability to: translocate; produce toxins; survive and multiply in the blood; incorporate virulence genes from the environment into their DNA; and conversely pass their virulence genes onto other organisms (Wassenaar et al., 2008). While the presence of these features can be confirmed, it is difficult to exclude them (Wassenaar et al., 2008). Studies have

found that probiotic organisms can be resistant to commonly used antibiotics (Temmerman, Pot, Huys, & Swings, 2003), and outbreaks of resistant organisms have occurred in neonatal units during probiotic use (Topcuoglu, Gursoy, Ovali, Serce, & Karatekin, 2015). Despite these concerns, most scientists agree that probiotics have a transient presence in the gastrointestinal tract (Boyle et al., 2006; Brigidi, Swennen, Vitali, Rossi, & Matteuzzi, 2003), therefore even if they were able to uptake resistance genes, the modified bacteria would be replaced with unmodified bacteria (Wassenaar et al., 2008).

Several characteristics of the preterm infant intestine make is vulnerable for bacterial invasion and hence potential transmural spread of the probiotic organism (Fleming, Berrington, & Jacobs, 2019). They have increased intestinal wall permeability (Stratiki et al., 2007; van den Berg et al., 2006), making it more susceptible to bacterial translocation. In addition, preterm infants have a reduced mucin layer (Halpern & Denning, 2015), meaning there is less physical separation between bacteria in the intestine and epithelial cell wall. These factors coupled with the immature immune response in preterm infants makes them more vulnerable to infection from the organisms in the intestine. Meaning that normally non-pathogenic organisms such as bifidobacteria or lactobacilli can potentially pose an infection risk. This correlates with the observation of over a third of the probiotic bacteraemia cases in a recent review having gastrointestinal co-morbidities (Kulkarni et al., 2022) and the finding of a friable intestinal mucosa in two infants who had recently had a episode of bacteraemia with a probiotic organism (Kunz et al., 2004).

1.4.6.3 Environmental contamination

Environmental contamination could be through staff members or, aerosolised spread of the probiotic organism when opening the probiotic capsule (Gengaimuthu, 2018). Opening packets of the fungal probiotic *Saccharomyces boulardii* led to environmental contamination (assessed by culture of swabs) of surrounding air, the arm of the simulated patient, and the table surface (Hennequin et al., 2000). The organism persisted on the technician's hands even after vigorous hand washing (Hennequin et al., 2000). Saccharomyces septicaemia can cause significant harm (Hennequin et al., 2000). Probiotic organisms are often part of the microflora and hence may have similar characteristics to resident skin flora, reducing the effectiveness of alcohol gel in cleaning them off hands (Gengaimuthu, 2018). The effectiveness of handwashing techniques for bacterial probiotic products has not been researched. There have been reports of both gastrointesintal colonisation (Costeloe et al., 2016) and probiotic sepsis (Cavicchiolo et al.,

2019), in infants who are not intentionally being administered a probiotic. This shows that probiotic organisms do have the potential to spread around the neonatal unit and that this can have adverse outcomes. Further research is needed to determine the vectors of spread of bacterial probiotic products and hence how this can be reduced.

1.4.7 Content of probiotic products

Prior studies on the microbiological composition of probiotic products used in adults and in foods, have found that the product contents do not consistently correlate with the label claims (Barbian et al., 2019). Current studies have reviewed a wide range of probiotic products available on the market (Aureli, Fiore, Scalfaro, Casale, & Franciosa, 2010; Drago, de vecchi, Nicola, Colombo, & Gismondo, 2004; Drago, Rodighiero, Celeste, Rovetto, & de Vecchi, 2010; Fasoli et al., 2003; Lewis et al., 2015; Theunissen, Britz, Torriani, & Witthuhn, 2005), including food produce (Temmerman et al., 2003), there is a paucity of studies that have focused on probiotics used in neonates (Lewis et al., 2015; Vermeulen, Luijendijk, Toledo, Kaam, & Reiss, 2020). All studies concluded that the probiotic products frequently had a different number of probiotic organisms than advertised, sometimes with complete absence of listed organisms, and not infrequently were contaminated with undeclared species or strains. This contamination of probiotic products can be with pathogenic organisms, with potentially lethal consequences (Vallabhaneni et al., 2015).

The variation in the number of probiotic organisms contained is also concerning. While the optimal dosing regime is yet to be determine, the current recommendation is to use similar dosing regimens to that used in clinical trials, most of which are in the range of 10^8 to 10^9 CFU (van den Akker et al., 2020). Doses below this may not achieve a clinical benefit, leaving these infants not protected from necrotising enterocolitis, late-onset sepsis, or death. Conversely, doses higher than the recommended have the potential to cause harm. One study that focused on a neonatal probiotic was conducted in Rotterdam and evaluated the microbiological composition of Infloran^{TM} (Vermeulen et al., 2020) . Infloran^{TM} capsules from three different lots at one point in time were cultured. Their findings of the absence of labelled strains and contamination with pathogenic bacteria were considered so concerning, that their unit has ceased administration of probiotics until a safer reliable product is available.

1.4.8 Testing Methods for Identification and Enumeration of Probiotic Organisms

Various testing methods, each with their own merits and limitations, exist for identification and enumeration of probiotic organisms. Traditionally culture-based techniques have been the most commonly used. There are several limitations with this approach including that; only cells in states of active replication will replicate and therefore be counted however, cells that are not in states of active replication can still have metabolic activity (Davis, 2014). An addition, different probiotic organisms require different culture media (van de Casteele et al., 2006) and quantification is achieved by counting colony-forming units (CFU) per gram or ml of the original sample, however a colony can be formed from an individual cell or from clusters of cells (Davis, 2014). Non-culture-based methods can be divided into imaging (flurescence in-situ hydridisation, live–dead staining and microscopic counting), molecular biology (PCR, 16S ribosomal ribosomal nucleac acids (16S rRNA), MALDI-TOF mass spectrometry), and cell sorting (Flow cytometry) (Davis, 2014). In general, non-culture-based methods are more expensive, although quicker and more accurate for enumeration (Davis, 2014). Previous studies reviewing the microbiological composition of probiotic products have used a variety of these techniques, both culture-dependent (Aureli et al., 2010; Drago et al., 2004; Drisko et al., 2005; Huys et al., 2006; Temmerman et al., 2003; Theunissen et al., 2005) and independent (Drago et al., 2010; Elliot & Teversham, 2004; Fasoli et al., 2003; Vermeulen et al., 2020).

1.4.9 Regulation

Various studies reviewing probiotic products have concluded that regulations around probiotic use need to change (Aureli et al., 2010; Drago et al., 2004; Drisko et al., 2005; Elliot & Teversham, 2004; Fasoli et al., 2003; Huys et al., 2006; Temmerman et al., 2003; Theunissen et al., 2005; Vermeulen et al., 2020). Globally there are diverse approaches to regulating probiotics however, generally, probiotics are considered dietary supplements, or similar variations thereof, which do not have the stringent premarketing safety checks of medications. In the United States of America, probiotics require approval by the Food and Drug Administration unless they have been regarded by experts as safe for use (Wassenaar et al., 2008). In Australia, probiotics are regarded as complementary medicines and require review by the Therapeutic Goods Administration (Health Products Regulation Group, 2019). In New Zealand probiotics are regulated under the 1985 Dietary Supplements Regulations (Beattie, 2016). This requires the product label to have; the name of the supplement or adequate description including ingredients, weight, manufacturer name and address, batch number,

recommended dose and use-by date (or similar). However, as in other countries, this does not require regular safety testing or the compulsory adverse event reporting of medications. There are proposals to change probiotics to be regulated under a new Therapeutic Products Bill (Barnes, 2018; Ministry of Health, 2022), which aims to increase the safety of products. However, this has not yet come into effect. Arguably, while the law does not require strict safety testing of probiotics, this needs to be done by the institutions that use these products (Vermeulen et al., 2020).

1.4.10 Other Benefits of Probiotic Use

The role of probiotics in preventing and treating allergic conditions has been extensively researched, with promising but conflicting results (Sestito et al., 2020). This includes that probiotics may reduce symptoms of cows milk allergy in children (Tan-Lim & Esteban-Ipac, 2018). The administration of probiotics both prenatally and postnatally can reduce allergic diseases (Zhang et al., 2016). And, that probiotics can improve atopic dermatitis (Kim et al., 2014). Despite some papers finding benefits of probiotics in allergic disease this is not a consistent finding, with others finding no effect and concluding that further research is required (Plummer et al., 2020).

Probiotics are being investigated into their role in cardiovascular health and cancer prevention and treatment, including in colorectal cancer and cervical cancer. The role of probiotics in colorectal cancer is attributed to intestinal dysbiosis and the resultant proinflammatory state which can be altered by probiotic use (Rossi, Mirbagheri, Keshavarzian, & Bishehsari, 2018). Probiotic use in cervical cancer can improve treatment outcomes and reduce side effects (Jahanshahi et al., 2020). Probiotic supplementation has been shown to reduce blood pressure and cholesterol (Dixon et al., 2020).

Probiotics have also been shown to have beneficial effects in several gastrointestinal conditions. Probiotics have been shown to be beneficial in preventing and reducing duration of paediatric antibiotic-associated diarrhoea (Guo, Goldenberg, Humphrey, el Dib, & Johnston, 2019) and effective in preventing Clostridim difficle-associated diarrhoea in both adults and children (J. Z. Goldenberg et al., 2017). They can also improve symptoms in irritable bowel syndrome (Didari, Mozaffari, Nikfar, & Abdollahi, 2015).

1.4.11 Prebiotics

Prebiotics are defined as ‘a substrate that is selectively utilized by host microorganisms conferring a health benefit’ (Gibson et al., 2017). Human milk contains oligosaccharides which are not digested in the upper GI tract and therefore can be selectively digested by the colonic bacteria, in particular the probiotic bacteria Bifidobacterium and lactic acid species (Manning & Gibson, 2004; Mohanty, Misra, Mohapatra, & Sahu, 2018). Oligosaccharides are short chain polysaccharides, they occur naturally in some foods or can be manufactured by hydrolysing polysaccharides (Manning & Gibson, 2004). Examples of manufactured oligosaccharides include galacto-oligosacchardies (a type of human milk oligosaccharide) and fructo-oligosaccharides (from vegetables) (Kona & Matlock, 2018).

The oligosaccharide prebiotics have a multifactorial mechanism of action. Digestion of the oligosaccharides by probiotic organisms results in the production of short chain fatty acids which are used as an energy source by intestinal epithelial cells and improve the intestinal barrier (Kona & Matlock, 2018). Prebiotics also reduce the ability of pathogens to adhere to the intestinal wall. The oligosaccharides structurally resemble the receptor sites on the intestinal cell walls used by pathogens for adherence. Through competitive inhibition, pathogens bind to the prebiotic oligosaccharide rather than the intestinal cell wall. The oligosaccharide and bound pathogen is then flushed out of the GI tract, removing the pathogen rather than it initiating infection via the intestinal cell wall (Shoaf, Mulvey, Armstrong, & Hutkins, 2006).

Prebiotics have been shown to have health benefits in preterm infants. In addition to increasing GI colonisation of the probiotic organisms (Srinivasjois, Rao, & Patole, 2013), the use of prebiotics shows promising beneficial immune properties (Kona & Matlock, 2018) including the reduction of late-onset sepsis (Chi, Buys, Li, Sun, & Yin, 2019; Tarnow-Mordi et al., 2020) though not necrotising enterocolitis when they are used without probiotics (Chi et al., 2019; Pammi & Abrams, 2019; Tarnow-Mordi et al., 2020)

1.4.12 Synbiotics

Synbiotics are “a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host” (Swanson et al., 2020). They are divided into complementary and synergistic synbiotics. Complementary synbiotics contain

a probiotic and a prebiotic, whereas a synergistic symbiotic contains a probiotic plus a substrate specifically utilised by that probiotic organism.

1.5 Summary

Preterm birth is common health issue globally with many longstanding health impacts (World Health Organization, 2018). NEC is a devastating potential complication of preterm birth, and carries significant morbidity and mortality, as well as a substantial economic burden on the health system (Meyer et al., 2020). Probiotics have been shown to reduce the risk of NEC and potentially late-onset sepsis and mortality in very preterm infants (Sharif et al., 2020) resulting in their widespread use. Despite these benefits of probiotics, concerns persist regarding the potential harms and unknowns, which contributes to some neonatal units displaying hesitancy in introducing routine probiotic prophylaxis (Barbian et al., 2019). These include the reliability of probiotic product contents, the risk of sepsis from a probiotic organism, the potential for the probiotic organisms to be colonising the neonatal unit, uncertainty of the optimal organism or dose, and low evidence data in very low birth weight infants. Further research is needed to explore these concerns.

1.6 Thesis objectives

Probiotics reduce the incidence of necrotising enterocolitis in very preterm infants and may also reduce late onset sepsis and mortality. Despite these proven benefits, there are safety concerns about probiotics which limits their use. The most serious potential harm of probiotic use is sepsis from either the probiotic organisms or an organism that has contaminated the probiotic sample. While this is assumed to be rare, the frequency is unknown. Probiotic product contents have been shown to vary from their label claims with variations in the presence and concentration of listed organisms and contamination with potentially pathogenic organisms, this has not been extensively reviewed for a probiotic product commonly used in preterm infants. Potential sources of bacteraemia from a probiotic organism include environmental contamination with the probiotic organisms causing true infection, or from contamination of equipment used in blood cultures and hence a false positive blood culture. Prior studies on probiotics used in research conditions have shown contamination of the neonatal unit environment and of the microbiome of babies not intentionally being administered a probiotic, this has not been reviewed when probiotics have been used under clinical conditions.

The aim of the study discussed in chapter 3 was to determine the concentration of probiotic organisms present in a probiotic product commonly used in preterm babies and if there was any contamination over a decade of surveillance. It is unknown what the risk of sepsis from a probiotic organism is for infants being administered probiotics, and therefore the study described in chapter 4 aimed to determine the incidence of probiotic bacteraemia in very preterm or very low birth weight infants, who received prophylactic probiotics. The aim of the study presented in chapter 5 is to determine if the NICU environment is contaminated with organisms from the prophylactic probiotic routinely administered to very low birth weight infants for prevention of necrotising enterocolitis. Due to the pause in research in the study centre resulting from COVID-19 restrictions, chapter 5 presents the study protocol rather than the results.

These data will aid in decisions regarding safety of probiotic use. If safety concerns are proven then this could result in the implementation of changes to reduce these safety concerns. Conversely, if results show minimal safety concerns this could contribute to the introduction of probiotics in countries not currently using them, and therefore a reduction in morbidity and mortality for those very preterm infants.

2 Methods

2.1 Study centres

The three studies discussed in 3, 4 and 5 were conducted in the Neonatal Intensive Care Unit in Starship Child Health, Auckland District Health Board. The study protocol presented in 5 will also be conducted in the Neonatal Intensive Care Unit in Middlemore Hospital, Counties Manukau District Health Board. Both neonatal units are within the Auckland Region.

Auckland NICU has 46 cots, with 16 level 3, and 30 level 2 spaces. Level three cots are for infants less than 32 weeks gestation, ventilation, or otherwise sick enough to require 1:1 or 1:2 nursing intensive care. There are approximately 900 admissions per year, of which 160 are VLBW infants. Auckland NICU introduced routine probiotic prophylaxis in 2012. This is prescribed as Infloran™. Infloran is advertised as containing no less than one billion colony forming units of *Bifidobacterium bifidum* and *Lactobacillus acidophilus*(Laboratorio Farmaceutico SIT S.r.l., 2019) per 250mg capsule for samples produced in *Laboratorio Farmaceutico,* Italy. Another variety of Infloran available is from Berna Biotech Ltd, Berne, Switzerland, this is less used in Auckland and contains *Bifidobacterium infantis* and *Lactobacillus acidophilus* (Rerksuppaphol & Rerksuppaphol, 2010). Infloran™ is mixed in with feed, administered at a dose of 250mg daily, from birth until 36 weeks or discharge (whichever is first) (Newborn Services Clinical Practice Committee & Alsweiler, 2020). Local practice is for gloves to be worn when preparing and administering EBM however, this is not required for formula. The bed space is cleaned by the infant's nurse once per shift, with cleaning of other surfaces and floors by a cleaner once daily. Cleaning of surfaces is routinely with Clinell wipes (active ingredients quaternary ammonium compounds and a polymeric biguanide, gama healthcare Ltd) (GAMA Healthcare Ltd, 2021) during the COVID-19 pandemic 0.1% sodium hypochlorite was used instead.

Middlemore NICU has 36 cots, half of which are level 3 spaces, and admits approximately 90 VLBW infants per year. Middlemore NICU introduced probiotic prophylaxis in 2009 and added prebiotics in 2011. They administer Dicoflor 60 ™ (AG Pharma S.r.l., 2020), at a daily dose of 6 billion CFU of *Lactobacillus rhamnosus GG.* In addition, the prebiotic Lactoferrin ™ is given. Both are administered daily from birth until discharge from the neonatal unit. Every space in the neonatal unit is cleaned daily.

Middlemore NICU level 3 has an open plan layout, whereas the level 3 in Auckland NICU is comprised of nine discrete rooms each containing two cots. In Auckland NICU the probiotic is stored in a fridge in the patient's room until it is used, whereas in Middlemore NICU the probiotic is stored in a fridge in a separate medication room within the neonatal unit.

In both centres, the criteria for receiving probiotics are birth weight less than 1500g or gestational age less than 32 weeks. The probiotic is typically mixed with expressed breast milk (EBM) or formula (Newborn Services Clinical Practice Committee & Alsweiler, 2020) (or if insufficient milk available then with water), then administered to the patient. The preparation is done in the patient's room by their nurse immediately prior to administration.

2.2 Testing Methods for Detecting Probiotic Organisms

All three studies presented throughout this thesis used laboratory-based testing methods for identifying and/or enumerating the probiotic organisms. The type of method used varied across the three studies, and as described below progressed from a traditional culture-based method in the first study, to a combination of culture with more advanced identification methods in the second study, then even further advanced methods in the third which combine PCR and MassARRAY technologies.

The study presented in chapter 3 reviewed the concentration of probiotic organisms present in a probiotic product commonly used in preterm babies and if there was any contamination over a decade of surveillance. The products were analysed by an external laboratory who used culture-based techniques to detect and enumerate the probiotic organisms and potential contaminants. Methods used were a combination of international standard organisation methods and American public health association methods from the Compendium of Methods for the Microbiological Examination of Foods (Salfinger & Tortorello, 2015). These are detailed further in chapter 2.3.4 but generally consisted of addition of a diluted sample of the probiotic product to a specific agar plate, followed by a period of incubation, with identification confirmation varying between the methods from morphological appearance based for probiotic organisms, to morphological based or biochemical testing or PCR for contaminants. Then finally a calculation based on the initial dilution factor to determine the original concentration in colony forming units per gram, allowing a direct comparison to the concentration reported on the product label which was also in colony forming units per gram.

The study presented in chapter 4 also used culture-based techniques, however with more advanced identification methods. The aim was to determine the incidence of probiotic bacteraemia in very preterm or very low birth weight infants, who received prophylactic probiotics. This was reviewed from 2004 to 2011 and 2013 to 2021. Blood culture isolates initially underwent identification testing with rapid-ID 32 A, in 2013 this was changed to matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), a more sophisticated identification technology. The Rapid-ID 32 A strip contains 32 cupules, 29 of which are test cupules containing enzymes such as urease or alkaline phosphatase. The product initially has anaerobic incubation on an agar plate such as blood agar. Samples of this are then added to an inoculator tray, where they undergo homogenisation and are filled into the cupules. There is then a further four-hour incubation process, this time under aerobic conditions. Reagents are then added to the specific cupules to facilitate enzymatic reactions. The reaction results from each cupule are then recorded with each result being coded a numeric profile. This profile is compared to their database allowing for identification of the microbe (bioMérieux SA, 2006; King & Phillips, 1996). The MALDI-TOF MS process is different. The probiotic sample and a matrix (organic compound of low mass) are both added to a metal plate, with resultant crystallisation occurring. This matrix is required for the later ionisation of the sample because it provides protons and acts as a scaffold. The crystalline compound is then irradiated with a UV laser beam resulting in the compound turning into a gas plume that contains ions from both the probiotic sample and the matrix. These ionised gases are analysed by the mass analyser which determines their mass to charge ratio (the speed at which the ions move through a the time of flight (TOF) tube to the detector). This mass to charge ratio is then compared to a database resulting in identification of the microbe (Clark, Kaleta, Arora, & Wolk, 2013).

The study protocol presented in chapter 5 aims to determine if the NICU environment is contaminated with organisms from the prophylactic probiotic routinely administered to very low birth weight infants for prevention of necrotising enterocolitis. This study has a more advanced method to identify the probiotic organisms, using MassARRAY technology, iPLEX chemistry. MassARRAY technology combines PCR with mass spectrometry to enable multiple simultaneous reactions with high accuracy and speed (Agena Bioscience Inc, 2019). The process for this study is as described below (sourced from personal communication, Copedo, J); A 6-plex PCR assay has been established for identifying the probiotic organisms, as outlined in Table 6, chapter 5.2.2. Five of these amplicons are forward and reverse primer sets to

specifically target the probiotic organisms. The sixth amplicon was designed to target a synthetic DNA sequence which has been incorporated into an IDT plasmid. The first step in the iPLEX chemistry pathway is amplifying the 6 amplicons by a standard PCR reaction. Following step one, step two is the addition of shrimp alkaline phosphatase. This dephosphorylates any remaining unincorporated nucleotides in the solution, making them unable to interfere with further reactions. Step three is an extension reaction. A specific primer has been designed to bind to a certain location on the amplicons. Once bound, extension occurs with a polymerase and termination nucleotide, allowing for only a single nucleotide to bind to the extension primer. The extension process is repeated until all the extension primers have a single nucleotide added to them. Following these initial three steps, the product undergoes a MALDI-TOF like reaction. The product is added to a spectro-chip with a matrix to allow ionisation. A laser ionises the compound which the ions then accelerating through a vacuum to a detector. The smaller fragments are detected first. This creates a spectrum with peaks at the mass locations. If no bacteria were present, the spectrum would show a peak of the un-extended extension primer because it was unable to bind to one of the products from our first PCR, and hence has not been extended by one nucleotide. If the bacteria is present then the result will show a peak shift to the mass of the extended extension primer.

2.3 Statistical analysis

Data were analysed on JMP 13 (SAS Institute Inc, Cary, NC, USA). Categorical data were compared by Chi Square or logistic regression for multivariate analysis. Parametric continuous data were analysed by Student T test or linear regression. Non-parametric data were log transformed if possible or analysed by Wilcoxon test. Data are presented as mean (SD), median (IQR), number (%), or odds ratio or mean difference with 95% confidence interval as appropriate. A p value of <0.05 was considered statistically significant.

2.4 Methods for the Microbiological Surveillance of Prophylactic Probiotics used to Prevent Necrotising Enterocolitis in Preterm Infants

2.4.1 Study design

This was a retrospective observational study conducted in Auckland NICU. Auckland NICU monitored the content of the probiotic capsules by routinely sending samples to an external laboratory, Eurofins Food Analytics (Eurofins Scientific, 2021) for analysis.

Infloran™, from Laboratorio Farmaceutico Italy, was the usual probiotic product used in the study center to prevent necrotising enterocolitis in very preterm infants (Newborn Services Clinical Practice Committee & Alsweiler, 2020). From February 2017 to August 2018 there was a shortage of Infloran™, resulting in the substation of a different probiotic, Labinic™, from Biofloratech Ltd. (*Lactobacillus acidophilus, Bifodobacterium bifidum, Bifodobacterium longum subsp. Infantis)* (Biofloratech Ltd, 2016) and a pause in analysis of Infloran ™ samples.

Samples were examined to determine the concentration of probiotic organisms, and to assess for contamination with Salmonella, Listeria, *Bacillus cereus*, Coagulase Positive Staphylococci, Coliforms, Enterococci, *Escherichia coli*, Yeasts or Moulds. Samples were assessed prior to their expiry date, stored at 3°C couriered from the study centre hospital pharmacy to the laboratory.

2.4.2 Study period

Probiotic product contents were analysed every three months between June 2012 and January 2022.

2.4.3 Primary outcome

Infloran is advertised as containing no less than one billion colony forming units of each of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* (Laboratorio Farmaceutico SIT S.r.l., 2019) per 250 mg capsule. One billion per 250 mg capsule equates to four billion colony forming units per gram. Labinic™ is advertised as containing 0.67 billion colony forming units of each organism per 0.2ml (5 drop) dose. However, as the concentration from the laboratory analysis was reported as colony forming units per gram rather than per ml, we were unable to determine the proportion of Labinic that contained the advertised concentration. Therefore, we

defined our primary outcome as the proportion of Infloran samples where the concentration of colony forming units per gram was at least the 4 billion advertised.

2.4.4 Laboratory methods

This study used pre-existing data obtained by an independent commercial laboratory. The laboratory had analysed the probiotic samples using a combination of methods from both the International Standard Organisation (ISO), and the American Public Health Association (APHA) methods found in the Compendium of Methods for the Microbiological Examination of Foods (CMMEF) (Salfinger & Tortorello, 2015).

2.4.4.1 Presumptive Lactobacillus acidophilus ISO 20128

Enumeration of *Lactobacillus acidophilus* was as per the method in ISO 20128 (International Organization for Standardization, 2006). De Man Rogosa Sharpe agar was prepared with added clindamycin and ciprofloxacin to inhibit growth of other microorganisms that are common in milks. Samples of diluted probiotic product were added to this agar. These were incubated for 72hrs (+/- 3 hrs) at 37 degrees Celsius. Following incubation, colonies that displayed the typical macroscopic appearance of *Lactobacillus acidophilus* were identified, to allow enumeration of presumptive *Lactobacillus acidophilus.* The typical appearance was of flat, irregularly edged, white grey colonies 1 to 3mm in diameter. This does does not distinguish between *Lactobacillus johnsonii, Lactobacillus gasseri* and *Lactobacillus crispatus,* hence they were labelled presumptive *Lactobacillus acidophilus.* The number of colonies with this typical appearance were used in a calculation that factored in the original dilution, to determine the original concentration in CFU/g.

2.4.4.2 Presumptive Bifidobacterium

An internal laboratory method was developed for enumeration of Bifidobacterium. This included initial standard preparation with weighing, homogenising and creation of a suspension. Dilutions of this suspension were inoculated onto reinforced clostridial medium agar. Once the agar was set the plates underwent anaerobic incubation at 37 degrees Celcius for three days. Colony identification occurred through gram stain with gram positive rods being labelled as presumptive bifidobacteria. Enumeration was calculated based on the dilution factor. Bifidobacteria was identified at the genus but not species level. The two different

sources of Infloran ™ had different species of bifidobacteria (*Bifidobacterium bifidum* and *Bifidobacterium infantis),* and the laboratory method did not distinguish between these species.

2.4.4.3 Salmonella

Detection contamination with Salmonella species was as per either the ISO method 6579-1:2017 (International Organization for Standardization, 2017a) or the Food and Drug Administration Bacteriology Analytical Manual method (Andrews et al., 1998). Both methods are very similar and are described below;

Samples undergo pre-enrichment by addition to buffered peptone water then incubation at between 34 – 38 °C for 18 hours. Following pre-enrichment samples were added to either Rappaport-Vassiliadis medium with soya broth or Modified Semi-solid Rappaport-Vassiliad agar, as well as Muller-Kauffmann tetrathionate-novobiocin broth. Samples were then incubated at 41.5 °C for 24 hours for Modified Semi-solid Rappaport-Vassiliadis agar or Rappaport-Vassiliadis medium with soya broth, or 37°C for 24 hours for the Muller-Kauffmann tetrathionate-novobiocin broth. Following this initial incubation, samples were inoculated onto xylose lysine deoxycholate agar and incubated at 37°C for 24 hours, in addition another solid selective medium complementary to xylose lysine deoxycholate agar was inoculated and incubated according to its manufacturing instructions. Finally, colonies of suspected salmonella were sub-cultured, following which they underwent identification confirmation with biochemical (urease test) or serological (polyvalent flagellar H) testing. If this result was negative for Salmonella then four additional colonies from other media were tested. This test was for presence or absence of Salmonella species only rather than enumeration, therefore colony counts were not performed.

2.4.4.4 Listeria

Detection of contamination with Listeria species was as per ISO method 11290-1:2017 (International Organization for Standardization, 2017b). This involved primary enrichment at 30 °C for 24 to 26 hours in half concentration Fraser broth. Then secondary enrichment in full concentrations of Fraser broth at 37 °C for 24 hours. Following secondary enrichment, the sample was added to two agar plates. To agar listeria and then incubated for 48 hours at 37 °C, as well as to another agar of the laboratories choosing that is complementary to Listeria and incubated for its appropriate time. Following incubation, a colony is added to pre-dried tryptone

soy yeast extract agar and incubated at 37°C for 18 to 24hrs. Colonies with the typical appearance of 1 – 2mm diameter size with a convex, colourless appearance that when held to light appear blue-grey and granular, undergo confirmation testing with several options available.

2.4.4.5 Bacillus cereus

Detection of Bacillus cereus was as per the method described in CMMEF Chapter 31.6 (Bennett, Tallent, & Hait, 2015). Diluted sample is added to mannitol-egg yolk-polymixin or Kim-Goepfert agar. Agar plates are then incubated at 30 to 32 °C for 20 to 24 hours. Following incubation colonies with the typical appearance of a pink, violet colour are counted. This number is multiplied by the reciprocal of the dilution to give the presumptive B. cereus count.

2.4.4.6 Coagulase Positive Staphylococci

Enumeration of coagulase positive staphylococci was as per the method described in CMMEF chapter 39 (Bennett, Hait, et al., 2015).

2.4.4.7 Escherichia coli and Coliforms

Detection of *Escherichia coli* and Coliforms was as per the method described in CMMEF chapter 9.935 (Kornacki, Gurtler, & Stawick, 2015a). This technique allows for enumeration of both *Escherichia coli* and coliforms simultaneously. The product sample is added to petriform *Escherichia coli* count agar plate and incubated at 35°C for 24 +/- 2 hours prior to being examined for colonies. *Escherichia coli* are distinguished from other coliforms based on their morphological appearance. Non-Escherichia *coli* coliforms appear as red colonies, associated with one or more gas bubbles within one colony diameter of the colony. However, *Escherichia coli* colonies appear as blue colonies associated with one or more gas bubbles within one colony diameter of the colony. To allow for colonies that may be slow producers of β-D-glucuronidase the plates are incubated for an extra 24 +/- 2 hours and re-counted. This is regarded as a confirmation test with no further testing required.

2.4.4.8 Enterococcus species

Enumeration of Enterococci was as per the method described in CMMEF Chapter 10.51 (Kornacki et al., 2015b).

2.4.4.9 Enterobacter sakazakii

Detection of contamination with Enterobacter Sakazakii was as per the method described in the FDA Bacteriology Analytical Manual, Chapter 29 (Hammack et al., 1998). This included addition of the sample to chromogenic agar, as well as Brilliance Enterobacter sakazakii agar and, Enterobacter sakazakii chromogenic plating agar. The agar plates are then incubated for 24 hours at 36 °C. Following incubation, suspected colonies (green appearance on Brilliance Enterobacter sakazakii agar, or a blue to black/grey appearance on Enterobacter sakazakii chromogenic plating agar) undergo confirmation testing with either Rapid ID 32 A or a PCR assay.

2.4.4.10 Yeasts or Moulds

Detection of contamination with yeasts and moulds was as per the method described in the CMMEF, Chapter 21.51. This included preparing agar plates by adding chloramphenicol to Dicloran Rose Bengal Chloramphenicol agar, then allowing this to dry. The product sample was added to the agar plates which were then incubated for five days at 25°C. Following this colony enumeration occurred. Colonies with typical appearance of mould (cotton-like) are labelled as mould and counted. The remaining colonies are examined morphologically to determine if they are yeast or bacteria, allowing for the enumeration of yeasts.

2.4.5 Statistical analysis

For general statistical analysis see chapter 2.3. Due to variations in the number of samples sent per year the annual mean probiotic concentration was used to analyse change over time. Non-parametric data were log transformed, and then back transformed for data presentation. Log transformed continuous data were analysed by linear regression to determine change over time. Data are presented as mean (SD). A p value of <0.05 was taken as statistically significant.

2.5 Methods for the Neonatal Bacteraemia with Bifidobacteria or Lactobacillus Species After the Introduction of Prophylactic Probiotics: A Retrospective Observational Cohort Study

2.5.1 Study design and periods

This was a retrospective, observational cohort study of preterm babies eligible for probiotics before and after the introduction of routine prophylactic probiotics.

Auckland NICU participated in the ProPrems trial, a randomized control trial on probiotic use in VLBW infants for preventing late-onset sepsis, from February 2011 to November 2011 (Jacobs et al., 2013). Prior to participation in the trial no babies in the NICU received probiotics. In July 2012, after the completion of the ProPrems trial, the NICU introduced routine probiotic prophylaxis for infants born less than or equal to 32 weeks gestation, or VLBW (Newborn Services Clinical Practice Committee & Alsweiler, 2020). Our study excluded this trial period and included very preterm or VLBW infants from the seven years before (January 2004 to January 2011, no probiotic cohort) to the seven years after (January 2013 to January 2020, routine probiotics cohort) routine probiotic use.

Infloran™ (*Bifidobacterium bifidum, Lactobacillus acidophilus)* (Newborn Services Clinical Practice Committee & Alsweiler, 2020) , was the probiotic used in the study center, apart from during February 2017 to August 2018 during which time there was a shortage of Infloran™, when Labinic™ (*Lactobacillus acidophilus, Bifodobacterium bifidum, Bifodobacterium infantis)* (Bartle, Knight, & Cairns, 2017) was substituted. Each 250mg Infloran™ capsule is said to contain no less than one billion *Bifidobacterium bifidum* and *Lactobacillus acidophilus* (Laboratorio Farmaceutico SIT S.r.l., 2019). Infloran™ is commenced with the first feed and continued until 36 weeks gestation, or discharge, whichever is first (Newborn Services Clinical Practice Committee & Alsweiler, 2020).

All infants admitted to Auckland NICU have their medical information stored on a prospectively maintained database. Medical information is accrued on this medical database from their admission until their discharge from the unit. A cohort of eligible infants (probiotic criteria) for each time period were generated from the database.

2.5.2 Outcomes

The primary outcome was defined as bacteraemia with bifidobacteria or lactobacillus species

on blood cultures taken for clinical suspicion of infection from birth until 36 weeks gestational age.

The secondary outcomes were;

- NEC from birth until 36 weeks gestational age, either:

  Clinical NEC (modified Bell stage 2 or 3) (M. C. Walsh & Kliegman, 1986) OR

  Histological NEC confirmed by laparotomy, histology, or autopsy, or, if no tissue evidence was available, the reported primary cause of death on the death certificate (Battersby et al., 2016)

- Late onset sepsis, defined as per the Australia and New Zealand Neonatal Network definition of an episode of infection after 48 hours of age, with positive blood or cerebrospinal fluid culture or PCR resulting in antibiotic treatment, not including skin flora contaminants (Australian and New Zealand Neonatal Network., 2022)

- Death, both before 36 weeks gestational age and before discharge from NICU.

- Fully breastfeeding on discharge.

- Length of stay (discharged to home).

- Survival free of neurosensory impairment at two years' corrected age (defined as survival without cerebral palsy, deafness (defined as requiring hearing air or cochlear implant), or blindness (defined as vision ≤6/60 in better eye), and with BSID II or III cognitive, language and motor composite scores greater than 85 (Balasundaram & Avulakunta, 2020).

2.5.3 Participants

Inclusion criteria were infants born between 01 January 2004 to 31st January 2011 (no probiotic cohort), and 01 January 2013 to 31st January 2020 (routine probiotic cohort), who were born ≤ 32 weeks' gestational age or ≤1500g birthweight.

Exclusion criteria were out-born infants transferred to Auckland NICU more than 24 hours after birth, or infants with gastrointestinal or cardiac malformations requiring surgery within 28 days after birth.

2.5.4 Sample size

The sample size was limited by the number of babies born during the two cohorts. For our 2004 to 2010 cohort, we estimated 1240 participants. There were less admissions from 2013 to 2019, we estimated 965 participants in this cohort. For our primary objective our sample size would give us 84% power with alpha 0.05 to detect an increase from 0.1% to 1% in the incidence of bifidobacteria or lactobacillus bacteraemia. The incidence of NEC in infants <1500g varied from 1 to 5 % in our 2004 -January 2011 cohort (K. Walsh et al., 2011). Meta-analyses have shown probiotics halve the incidence of NEC (Alfaleh & Anabrees, 2014). Our sample size was calculated to provide 80% power to detect a reduction in NEC incidence from 5% to 2.5%.

2.5.5 Method for assessing the primary outcome of bacteraemia

To assess for our primary objective a list of all blood cultures positive for Bifidobacterium or lactobacillus, from infants in our cohorts, were generated from the hospital laboratory. Blood culture isolates in the hospital laboratory were identified using a Rapid ID 32 A (bioMérieux SA, France) from 2004 to 2013, in 2013 this was changed to the MALDI-TOF mass spectrometry (Shimadzu Europa GmbH). Both have databases that contain *Lactobacillus acidophilus* and Bifidobacterium species. MALDI-TOF mass spectroscopy does not differentiate between Bifidobacterium species, the Rapid ID 32A was able to identify *Bifidobacterium bifidum*, with other species of Bifidobacterium being labelled as Bifidobacterium species. Refer to chapter 2.2 for further details on laboratory testing method.

2.5.6 Method for assessing secondary outcomes

To assess for our secondary objectives we accessed data from hospital records. This included baseline demographics such as antenatal corticosteroid use, caesarean delivery, multiple birth, sex, gestation, birthweight z score, 5 minute Apgar score, respiratory support and non-human milk feeds. To evaluate neurodevelopmental outcomes we reviewed their hospital records.

All VLBW infants at Auckland City Hospital are offered routine neurodevelopmental follow up at two years corrected age by a developmental psychologist and neonatal paediatrician, infants in the cohort who were <32 weeks GA but had a BW >1500g were not eligible for neurodevelopmental follow up. The neurodevelopmental assessment includes the Bayley Scales of Infant and Toddler Development (BSID II from 2004 to 2007 (Nancy Bayley, 1993), BSID III 2007-2020(N Bayley, 2006)), and clinical assessment of cerebral palsy, deafness, or

blindness. The Bayley Scales of Infant and Toddler Development were updated from the second (Bayley II) to third (Bayley III) edition in 2006, the study centre changed editions in 2007 (Balasundaram & Avulakunta, 2020). Subsequently, part way through the first cohort infants were changed between Bayley II and Bayley III. Bayley II had a combined language and cognitive score, whereas these were separated in Bayley III.

To evaluate for characteristics of the infants who developed bacteraemia from bifidobacteria or lactobacillus, we reviewed their; gestational age and birth weight (to retain their anonymity these will be recorded as categories), gender, probiotic use and duration, clinic features of infection, blood culture isolate, concurrent infection concerns, antibiotic used for treatment, and outcome of infection.

2.5.7 Data management

Data obtained from the laboratory and infant database included NHI numbers so that individual patient's clinical records could be accessed. After this information was collected from the clinical records, the data was de-identified with only a study number matched to the patient data. A separate document had a list showing which study numbers correspond to which NHI numbers to allow the clinical records to be re-reviewed if required. Each document was be stored in a different folder, with a different name. Both documents were password protected, each with a different password.

2.5.8 Statistical analysis

For general statistical analysis see chapter 2.3. Demographic categorical data were compared by Chi square and continuous data by Student t test. Outcome data were adjusted for gestational age and birth weight z score (based on Fenton (Fenton, 2003)) compared by logistic regression (categorical) or linear regression (continuous). Subsequent analyses were planned for other variables if there was more than a 10% difference between the groups including antenatal corticosteroid use, caesarean section delivery, ethnicity, out born, assisted ventilation and non-human milk feed.

2.5.9 Ethics and funding

We obtained ethics approval from The Auckland Health Regional Ethics Committee prior to commencing the study. Approval number AH1168.

As this was a retrospective observational study, participants were not informed of the study and consent was not obtained.

3 Microbiological Surveillance of Prophylactic Probiotics used to Prevent Necrotising Enterocolitis in Preterm Infants

3.1 Introduction

Probiotic bacteria are live microbial supplements that colonise the gastrointestinal tract, potentially conferring advantage to the host (Millar & Wilks, 2003). Prophylactic probiotics reduce the incidence of necrotising enterocolitis (NEC) in preterm neonates (Meyer et al., 2020; Sharif et al., 2020). Probiotics have also been shown to reduce neonatal sepsis and mortality, but these effects no longer remain statistically significant after excluding trials at high risk of bias (Sharif et al., 2020). A concerning potential harm from probiotic use in neonates is the risk of sepsis from the probiotic organism (Bertelli et al., 2014; Dani et al., 2016; Ohishi et al., 2010).

There remains uncertainty on how to safely manufacture probiotic products for neonates, with multiple factors throughout production threatening to affect the viability of the probiotic organisms (Lacroix & Yildirim, 2007). Prior studies on the microbiological composition of probiotic products used in adults, have found that the contents do not consistently correlate with the label claims, often containing a different number of probiotic organisms than advertised (Aureli et al., 2010; Drago et al., 2004, 2010; Fasoli et al., 2003), sometimes with a complete absence of listed organisms (Aureli et al., 2010; Drago et al., 2004, 2010; Fasoli et al., 2003; Temmerman et al., 2003; Theunissen et al., 2005; Vermeulen et al., 2020), and not infrequently contaminated with undeclared species or strains (Drago et al., 2010; Temmerman et al., 2003; Theunissen et al., 2005). Contamination of the probiotic products can be with pathogenic organisms, with potentially lethal consequences (Centers for Disease Control and Prevention, 2015). A probiotic dose lower than intended may not achieve a clinical benefit, conversely, a dose higher than that recommended may increase the risk of harm.

Previous analysis of the contents of a probiotic used in a neonatal intensive care unit found only one of the three lots analysed contained both the advertised probiotic microorganisms. The other two lots contained missing strains and contamination with potentially pathogenic organisms (*Streptococcus oralis*, *Lactococcus garvieae*, *Enterococcus feacalis*, *Enterococcus faecium* and *Lactococcus lactis*) (Vermeulen et al., 2020) . Previously, there has not been an analysis of neonatal probiotic product contents over an extended time period.

Current guidelines recommend routine microbiological surveillance for probiotic products used in preterm babies (Deshpande, Rao, Keil, & Patole, 2011). We aimed to determine the concentration of probiotic organisms present in a probiotic product commonly used in preterm babies and if there was any contamination over a decade of surveillance.

3.2 Methods and Materials

3.2.1 Probiotic product and analysis

The routine probiotic product used in the study center was Infloran™.(Newborn Services Clinical Practice Committee & Alsweiler, 2020) Each 250 mg capsule was labelled in the product information sheet as containing no less than one billion colony forming units of each of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* (Laboratorio Farmaceutico SIT S.r.l., 2019).

From February 2017 to August 2018 there was a shortage of Infloran™, and a different probiotic, Labinic™, from Biofloratech Ltd., (*Lactobacillus acidophilus*, *Bifodobacterium bifidum,*, *Bifodobacterium longum subsp. Infantis* (Biofloratech Ltd, 2016)) was substituted. Labinic™ is advertised as containing 0.67 billion colony forming units of each organism per 0.2ml (5 drop) dose. However, as the concentration from the laboratory analysis was reported as colony forming units per gram we were unable to determine the proportion of Labinic that contained the advertised concentration.

Probiotic product contents were analysed every three months between June 2012 and January 2022 to determine the concentration of probiotic organisms, and to assess for contamination with Salmonella, Listeria, *Bacillus cereus*, Coagulase Positive Staphylococci, Coliforms, Enterococci, *Escherichia coli*, Yeasts or Moulds. Samples were analysed prior to their expiry date and stored at 3°*C*.

Probiotic samples were analysed by an external laboratory using the International Standard Organisation (ISO), and the American Public Health Association (APHA) methods from the Compendium of Methods for the Microbiological Examination of Foods (CMMEF). Refer to Chapter 2.4.4 for details on analysis of samples.

3.2.2 Primary Outcome

The proportion of Infloran™ samples that contained at least the advertised concentration of organisms (at least four billion CFU/g for each of *Bifidobacterium bifidum* and *Lactobacillus acidophilus*).

3.3 Results

3.3.1 Infloran™ samples

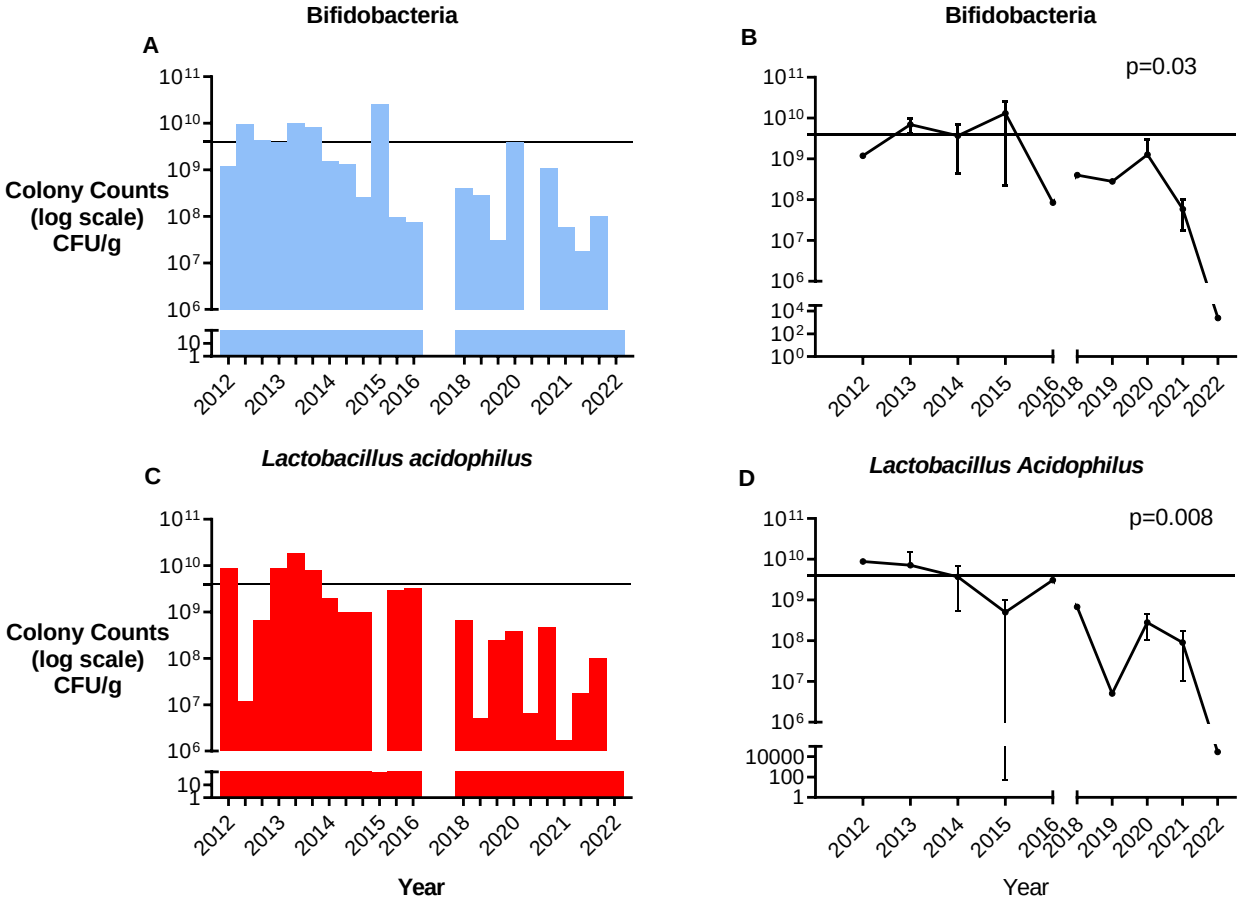
Twenty three samples of Infloran™ were analysed between June 2012 and January 2022. The frequency of analysis varied from every three to eight months. One sample from December 2015 had no detectable *Lactobacillus acidophilus* but contained the advertised concentration of bifidobacteria and was included in the analysis. One sample in July 2019 reported no detectable *Lactobacillus acidophilus* and Bifidobacterium. The batch was re-tested the following fortnight with the subsequent result showing a more typical concentration, and the original sample was excluded from the analysis. Likewise, one sample in January 2022 contained no detectable *Lactobacillus acidophilus* or Bifidobacterium. The batch was re-tested the following week with the subsequent sample showing low numbers of both organisms, this repeat was included in the analysis with the original sample being excluded.

The advertised concentration of bifidobacteria species was present in 7/22 (32%) samples (figure 1A). The advertised number of *Lactobacillus acidophilus* CFU/g was present in 4/22 (28%) samples (figure 1C).

There was a reduction over time in both bifidobacteria (figure IB) and *Lactobacillus acidophilus* (figure ID).

There was no detection of microbiological contamination of probiotic products by Salmonella, Listeria, *Bacillus cereus*, Coagulase Positive Staphylococci, Coliforms, Enterococci or *Escherichia coli*. In February 2013 there was detection of yeast at 30 CFU/g and mould at 10 CFU/g. The specific organism was not determined. No other samples detected either yeast or mould.

Figure 1 Concentration of Probiotic Organisms Present in Samples of Infloran™ Over a Ten Year Period

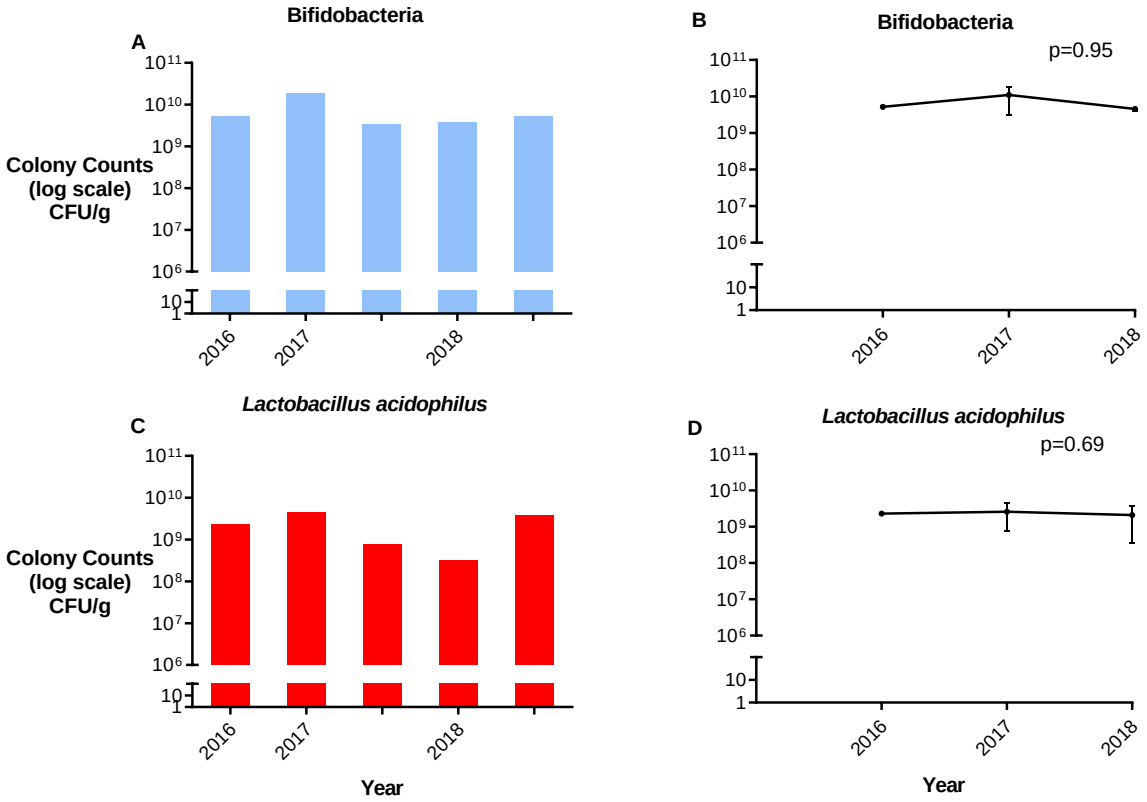


Bifidobacterium concentrations; in each sample (A), annual mean with standard deviation (B). *Lactobacillus acidophilus* concentrations; in each sample (C), annual mean with standard deviation (D). The horizontal line represents the advertised concentration.

3.3.2 Labinic™ samples

Five samples of Labinic™ were assessed between November 2016 to July 2018. Variation was observed in the concentration of organisms present (figures A – D) however, the concentration of organisms present did not change over time (figure 2B and 2D). No contamination was detected in the Labinic™ samples.

Figure 2 Concentration of Probiotic Organisms Present in Samples of Labinic™ Over Time



Bifidobacterium concentrations; in each sample (A), annual mean with standard deviation (B). *Lactobacillus acidophilus* concentrations; in each sample (C), annual mean with standard deviation (D).

3.4 Discussion

This study reports the microbiological surveillance over a decade of prophylactic probiotic use in a neonatal intensive care unit. We found a wide variation in probiotic organism concentration, in two probiotic products. The majority of probiotic concentrations did not meet the advertised colony count and the concentration of both of the organisms within Infloran™ reduced over time. Overall, there was a low rate of contamination with only one Infloran™ sample being possibly contaminated with a small concentration of yeast and mould.

Current recommendations for the probiotic dose in very preterm infants for prevention of NEC is 3×10^9 colony forming units/day (Deshpande et al., 2011). However, this dose is based on the median dose used in clinical trials, with the safest most effective dose unknown (van den Akker et al., 2020). There is also minimal evidence on the most effective probiotic organism or combination of organisms to reduce NEC and mortality. The number of live organisms

present in probiotic products reduces over time (Aureli et al., 2010), and the World Health Organisation recommendation is for the labelled concentration to represent that at the end of the shelf life (Drago et al., 2010). Therefore, we defined contents matching the advertised colony count as having at least the advertised amount present. Other studies reviewing probiotic contents have used various definitions including the percentage that met the national guideline criteria (Aureli et al., 2010) or the presence of the labelled organisms rather than quantifying their concentration (Vermeulen et al., 2020), or a similar definition to our study (Drago et al., 2010). This varying reporting makes comparing the products used in the different studies challenging. However, our findings of less than half of the contents of the products sampled matching the label claims is consistent with other studies (Aureli et al., 2010; Drago et al., 2010; Vermeulen et al., 2020).

The extensive duration of this study allowed assessment of trends over time. There was a reduction in the concentration of one of the probiotic organisms over time. This change observed over time raises concern that the quality of probiotics may be deteriorating. In New Zealand, probiotics are regulated under the 1985 Dietary Supplements Regulations (Beattie, 2016), which do not have the regular safety testing requirements of medications. Internationally, probiotic regulations vary, but generally have similar requirements (de Simone, 2019). The combination of a product with reducing reliability, regulated by a law not requiring regular testing, is concerning when, although not yet known, there will likely be a minimum effective dose. This supports the requirement for regular monitoring of probiotic contents while regulation remains minimal. A recent study including all New Zealand neonatal intensive care units, all except one using Infloran™, showed that the rates of NEC reduced in the immediate years after introducing probiotics (2013-2015) (Meyer et al., 2020). The reduction we have observed in the probiotic concentration over time raises the concern that the reduction in NEC may not be maintained.

Contamination of probiotic products with pathological organisms can have fatal consequences as demonstrated by a neonatal death from a probiotic contaminated with the mould *Rhizopus oryzae* (Centers for Disease Control and Prevention, 2015). Our study only found possible contamination in one sample, which was with yeasts and mould, the specific organism was not determined. This is less contamination than has been reported in other studies (Drago et al., 2010), though still enough to highlight the importance of monitoring for contamination as preterm infants are vulnerable to infection. A recent study identified Infloran™ contamination with *Streptococcus oralis, Lactococcus garvieae, Enterococcus feacalis, Enterococcus*

faecium and *Lactococcus lactis* (Vermeulen et al., 2020). We tested for a set panel of organisms (organisms chosen for being known to cause sepsis in preterm infants) which did not include Streptococcus, Lactococcus, or Enterococcus species. Extending the contamination panel to include these organisms should be considered for future microbiological surveillance.

A limitation of this study was the use of culture-based rather than molecular biology techniques to determine the concentration of probiotic organisms. Culture-based methods have been traditionally used and are cheaper, although they have several limitations. Only cells in states of active replication will replicate and therefore be counted. However, cells that are not in states of active replication can still have metabolic activity (Davis, 2014). Quantification is achieved by counting colony-forming units (CFU) per gram or ml of the original sample. However, a colony can be formed from an individual cell or clusters of cells (Davis, 2014). Compared to culture-based, PCR-based techniques are quicker and more accurate for both identification and enumeration, although more expensive. Prior studies have shown that PCR based methods can be accurately used for reviewing the (Elliot & Teversham, 2004; Fasoli et al., 2003)s(Elliot & Teversham, 2004; Fasoli et al., 2003) For these reasons, it is likely that future studies will trend towards using PCR rather than culture-based methods.

Conclusion

The extensive duration of this study allowed a comprehensive review of probiotic contents and trends over a decade of surveillance. There was a wide variability and a reduction over time in the concentration of organisms present in a commonly used neonatal probiotic. This is concerning when although not yet known, there will likely be a minimum effective probiotic dose for preventing necrotising enterocolitis. Contamination with potential pathogens was less than suggested in other studies although still did occur. These findings support the need for a standardized neonatal probiotic product and the ongoing monitoring of probiotics products until this occurs.

4 Neonatal Bacteraemia With Bifidobacteria or Lactobacillus Species After the Introduction of Prophylactic Probiotics: A Retrospective Observational Cohort Study.

4.1 Introduction

Necrotising enterocolitis (NEC) is the most frequent serious acquired gastrointestinal disease of the newborn (Lee & Polin, 2003). Approximately 3% of very preterm infants develop NEC (Battersby et al., 2016). The biggest risk factor for developing NEC is prematurity, with its associated undeveloped epithelial barrier, abnormal gastrointestinal microbiome and immature immune system (Lin & Stoll, 2006). Another significant risk factor is non-human milk feeds which compared to donor expressed milk increase the risk of NEC (RR 1.87, 95% CI 1.23 to 2.85, number needed to treat for harmful outcome 33) (Quigley, Embleton, & McGuire, 2019). NEC can be devastating with an overall mortality of 28% (Hull et al., 2014), increasing to 50% in extremely low birth weight infants (Blakely et al., 2006). Survivors have increased risk of gastrointestinal complications including strictures and intestinal failure (Hau et al., 2019), as well as neurodevelopmental impairment (Schulzke, Deshpande, & Patole, 2007).

Prophylactic probiotics have been shown to halve the incidence of NEC in preterm babies in multiple meta-analyses of randomised controlled trials, involving thousands of infants (Sharif et al., 2020). Probiotics have also been shown to reduce the incidence of late onset sepsis and mortality (Alfaleh & Anabrees, 2014; Rao et al., 2016). However, when only including studies at low risk of bias the effects on late onset sepsis and mortality were no longer statistically significant (Sharif et al., 2020). Subsequently, the use of prophylactic probiotics for VLBW or very preterm infants has become routine in many countries.

While probiotics have significant beneficial effects, there have been multiple case reports of neonatal sepsis with probiotic organisms, Table 1, Chapter 1.4.6.1. These include case reports from different probiotic products and with various organisms including Bifidobacterium, lactobacillus, fungi, and Eshcerichia Coli (Kulkarni et al., 2022). Most of these cases were either in infants born extremely premature or those with other significant comorbidities, particularly gastrointestinal (GI) such as omphalocele (Ohishi et al., 2010), short gut syndrome from gastroschisis or congenital ileal atresia (Kunz et al., 2004), or with a stoma due to prior NEC or ileal perforation (Brecht et al., 2016; Esaiassen et al., 2016). A large meta-analysis

including 16 randomised controlled trials of infants administered probiotics reported that no infants developed sepsis from the probiotic organism (Alfaleh & Anabrees, 2014). Therefore, the risk of neonatal sepsis due to a probiotic organism is assumed to be rare. However, this is currently unknown. This study aimed to determine the incidence of probiotic bacteraemia in very preterm or very low birth weight infants, who received prophylactic probiotics.

4.2 Methods and Materials

4.2.1 Study centre and time periods

This was a retrospective, observational cohort study conducted in the Auckland City Hospital NICU. Refer to chapter 2.1 for further information on the study centre and to chapter 2.5 for further details on study method. In July 2012, the NICU introduced routine probiotic prophylaxis for infants born less than or equal to 32 weeks gestation, or VLBW (Newborn Services Clinical Practice Committee & Alsweiler, 2020). Our study included very preterm infants from the seven years before (January 2004 to January 2011 = no probiotic cohort) to the seven years after (January 2013 to January 2020 = routine probiotics cohort) routine probiotic use. Infloran (*Bifidobacterium bifidum, Lactobacillus acidophilus)* (Newborn Services Clinical Practice Committee & Alsweiler, 2020) was the probiotic used in the study centre, apart from during February 2017 to August 2018 during which time there was a shortage of Infloran, when Labinic (*Lactobacillus acidophilus, Bifodobacterium bifidum, Bifodobacterium infantis)*(Bartle et al., 2017) was substituted.

Cohorts of infants that met the inclusion criteria were obtained from a prospectively maintained database. To assess for probiotic bacteraemia a list of all blood cultures positive for bifidobacterium or lactobacillus species from infants in the cohorts was generated from the hospital laboratory. Neurodevelopmental outcomes were sourced from their routine neurodevelopmental follow up at two years corrected age.

4.2.2 Participants

Inclusion criteria were infants born between 01 January 2004 to 31st January 2011, and 01 January 2013 to 31st January 2020, who were born ≤ 32 weeks' gestational age or ≤1500g birthweight. Exclusion criteria were out-born infants transferred to Auckland NICU more than 24 hours after birth, or infants with gastrointestinal or cardiac malformations requiring surgery within 28 days after birth.

4.2.3 Outcomes

The primary outcome was bacteraemia with bifidobacteria or lactobacillus species from birth until 36 weeks gestational age. See chapter 2.5.2 for a list of the secondary outcomes and 2.3 for details on the statistical analysis.

4.3 Results

There were 1536 infants in the no probiotic cohort and 1298 infants in the routine probiotic cohort (Figure). Infants in the routine probiotic cohort were less likely to be from a multiple birth, more likely to be Asian and less likely to be Caucasian, likely to have a 5-minute Apgar score <7, less likely to be fed at least 80% expressed breast milk and less likely to require inotropic support (Table 2).

Figure 3 Eligible Infants in No Probiotic Cohort and Routine Probiotic Cohort

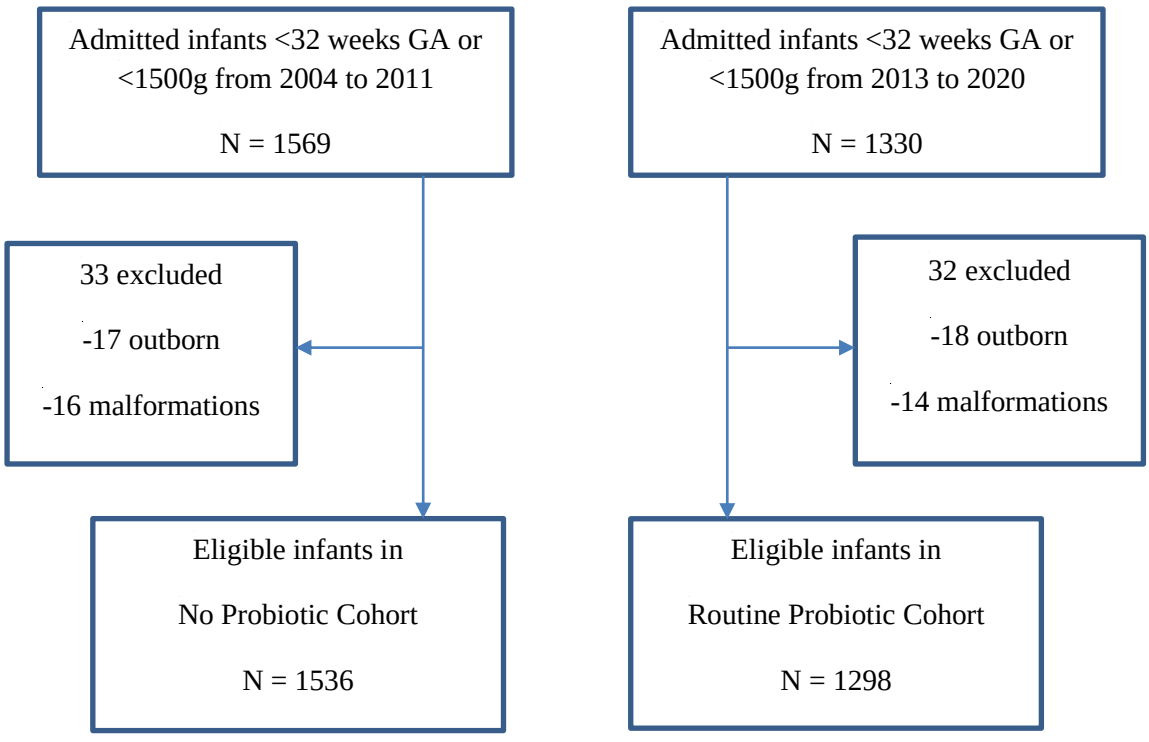


Table 1 Baseline Demographics of Infants in the No Probiotic, and Routine Probiotic Cohorts

	No probiotic N = 1536	Routine probiotics N = 1298	P value
GA (weeks)	30.0 (27.6 - 31.6)	30.1 (27.7 - 31.7)	0.72
Birth weight			
Grams	1310.0 (975.0 – 1600.0)	1282.0 (987.5 – 1580.0)	0.58
Z score	-0.05 (0.96)	-0.09 (1.06)	0.32
Male	836 (54.4)	688 (53.0)	0.45
Multiple birth	471 (30.7)	333 (25.6)	0.003
Antenatal Steroids, any	1386 (90.2)	1197 (92.2)	0.06
Ethnicity			<0.0001
Māori	289 (18.8)	232 (17.9)	
Pacifica	194 (12.6)	166 (12.7)	
Asian	260 (16.9)	339 (26.1)	
Other	18 (1.1)	21 (1.6)	
Caucasian	775 (50.4)	540 (41.6)	
Out born (admitted before 24hrs old)	86 (5.6)	79 (6.1)	0.58
Caesarean Section	972 (63.3)	804 (61.9)	0.46
5 minute Apgar score <7	166 (10.9)	253 (19.6)	<0.0001
Feeds in hospital <80% breast milk	433 (28.9)	463 (36.3)	<0.0001
Required ventilation	639 (41.6)	538 (41.45)	0.93
Required inotropes	183 (11.9)	104 (8.0)	0.0005

Data are mean (SD), median (IQR) or n (%) as appropriate

No infants in the pre-probiotic cohort had bacteraemia from the probiotic organisms, compared with three infants in the routine probiotic cohort (Table 2). The incidence of bacteraemia with a probiotic organism in the routine probiotic cohort was 0.2%.

There was no significant reduction in NEC for infants administered probiotics. Late-onset sepsis rates were higher in the routine probiotic cohort. Rates of full or exclusive breast feeding on discharge were lower in the routine probiotic cohort. There was no difference in mortality or survival free of neurosensory impairment. However, there was a reduction for infants with Bayley-III motor score <85 in the routine probiotic group.

Table 2 Primary and Secondary Outcomes for Infants in the No Probiotic and Routine Probiotic Cohorts

	No probiotics N = 1536	Routine probiotics N = 1298	Odds ratio/mean difference (95% CI)	P value	Adjusted* odds ratio/mean difference (95% CI)	Adjusted* P value
Bacteraemia cases	0	3	-	-	-	-
NEC						
Clinical	38 (2.5)	21 (1.62)	0.65 (0.37 – 1.11)	0.109	0.62 (0.36 – 1.07)	0.09
Histological	27 (1.8)	15 (1.2)	0.7 (0.3 – 1.2)	0.18	0.62 (0.33 – 1.195)	0.17
Late onset sepsis	112 (7.3)	126 (9.7)	1.4 (1.05 – 1.8)	0.02	1.4 (1.1 – 1.9)	0.02
Death before 36 weeks	82 (5.3)	58 (4.5)	0.8 (0.6 – 1.2)	0.29	0.8 (0.56 – 1.14)	0.22
Death before discharge	107 (7.0)	75 (5.8)	0.8 (0.6 – 1.1)	0.19	0.79 (0.58 – 1.09)	0.15
Fully breast feeding on discharge	1050 (68.4)	773 (59.6)	0.9 (0.6 – 0.8)	<0.0001	0.68 (0.58 – 0.79)	<0.001
Length of stay (days) **	58.2 (35.7)	64.8 (34.5)	6.6 (3.9 – 9.3)	<0.0001	3.1 (2.2 – 3.97)	<0.001

Data are mean (SD), median (IQR) or n (%) as appropriate
NEC = necrotising enterocolitis

*Adjusted for gestational age and birth weight z score.
**Data missing for length of stay, number missing in no probiotic cohort = 114, in routine probiotic cohort = 92

Table 3 Neurodevelopmental Outcomes for Infants in the No Probiotic and Routine Probiotic Cohorts

	No probiotics N = 797	Routine probiotics N=600	Odds ratio/mean difference (95% CI)	P value	Adjusted[1] odds ratio/mean difference (95% CI)	Adjusted[1] P value
Survival free of neurosensory impairment at 2 years	662 (83)	512 (85)	1.2 (0.9 – 1.6)	0.25	1.20 (0.89 – 1.62)	0.22
Cerebral palsy	19 (2.38)	16 (2.67)	1.12 (0.57 – 2.2)	0.74	1.13 (0.57 – 2.22)	0.73
Deafness	0	2				
Blindness	0	0				
Bayley cognitive score ^<85 [2,3]	63 (9.63)	53 (9.55)	1.0 (0.68 – 1.48)	0.96	1.02 (0.69 – 1.50)	0.91
Bayley* motor score <85 [2]	66 (10.09)	36 (6.5)	1.61 (1.05 – 2.47)	0.02	1.64 (1.07 – 2.52)	0.02
Bayley language score <85 [4]	87 (19.42)	102 (18.48)	1.06 (0.77 – 1.46)	0.14	1.07 (0.78 – 1.47)	0.69

Data are n (%) as appropriate

[1] Adjusted for gestational age and birth weight z score.

[2] Data missing for BSID resulting in reduced numbers than for other neurosensory outcomes. Number in each cohort; probiotic cohort N = 654, routine probiotic cohort N = 554

[3] BSID 2 did not have a separate language component, hence cognitive score for period of use of BSID 2 represents both cognitive and language assessment.

[4] Bayley language score has reduced numbers than other Bayley composite scores due to not being a component in BSID 2. No probiotic cohort n=448, routine probiotic cohort n=552

Table 4 Details on the Cases of Bacteraemia with a Probiotic Organism

	GA category (weeks)	Gender	BW category	Probiotic and day commenced	Clinical features of infection	Blood culture isolate	Concurrent infection concerns	Antibiotic used	Outcome
Case 1	23 – 24	Male	500 - 749g	Infloran™ day 1	Feed intolerance, abdominal distension, periumbilical erythema. Elevated inflammatory markers*. Distended bowel loops on AXR.	Bifidobacterium species from peripheral blood culture on day 14 (isolated on day 17).	Umbilical swab from day 14 had heavy growth of *Staphylococcus aureus.*	Flucloxacillin, amikacin and nil by mouth for 5 days.	No repeat growth on further cultures. Recovery.
Case 2	27 - 28	Male	750 - 1000g	Labinic™ day 4	Respiratory deterioration requiring intubation, temperature instability, distended abdomen with periumbilical erythema. Elevated inflammatory markers. Distended bowel loops on AXR. Diagnosed with suspected NEC.	Bifidobacterium species from peripheral blood culture on day 16 (isolated on day 19).	NEC from day 16.	Amikacin, flucloxacillin, metronidazole, cefotaxime and nil by mouth for 10 days.	No repeat growth on further cultures. Recovery.
Case 3	23 - 24	Male	500 - 749g	Infloran™ day 3	Increased lability, omphalitis and elevated inflammatory markers.	*Bifidobacterium breve* from umbilical arterial catheter culture on day 8 (isolated on day 11).	Recurrent coagulase negative staphylococci from day 8.	Amikacin and flucloxacillin day 8 to 10. Cefotaxime and vancomycin day 10 to 13. Ceftazidime, vancomycin and metronidazole day 13 to 22. Vancomycin and clindamycin day 22 to 26.	No repeat growth on further cultures. Recovery.

*Inflammatory markers include full blood count, left shift and c-reactive protein.

GA = Gestational age

BW = Birth weight

4.4 Discussion

Bacteraemia and sepsis with probiotic organisms are the most serious risks associated with probiotic use in very preterm neonates. This observational study found that prior to the introduction of routine probiotics, bacteraemia due to probiotic organisms was not detected in any babies. However, following the introduction of routine probiotics there were three babies with Bifidobacterium bacteraemia. All three cases were in extremely preterm infants, none of whom had repeat growth on subsequent cultures and all had a full recovery with antibiotics.

While still rare, three cases of bacteraemia are significantly more than the absence of any cases found in the systematic reviews on the effect of probiotics for reducing NEC in VLBW infants, the most recent review of which contained 10,812 infants (Sharif et al., 2020). Though less than the 2% of cases found in a recent review (Sakurai et al., 2022). Therefore, this indicates that probiotic bacteraemia is, while rare, is a potential risk of probiotic use in very preterm infants. There may by differences between probiotic use under research conditions compared to clinical conditions i.e. in our NICU the probiotic capsules are opened and added to the milk in the patient room rather than a separate room as was done in the trial. This preparing of the probiotic in the patient room could potentially lead to increased colonisation of the neonatal environment and hence a source of nosocomial infection.

All three infants in our study had their bacteraemia in the context of gastrointestinal signs with abdominal distension and erythema noted on the day of obtaining the blood culture. This does suggest the possibility that there may have been gastrointestinal disturbance and hence vulnerability of the gastrointestinal mucosa which could have led to transmural spread as the source of infection. This trend is consistent with other case reports which have mostly been in infants either extremely premature or with gastrointestinal malformations or recent gastrointestinal illnesses (Bertelli et al., 2014; Brecht et al., 2016; Chiang et al., 2021; Dani et al., 2016; Esaiassen et al., 2016; Guenther et al., 2010; Jenke et al., 2012; Kunz et al., 2004; Ohishi et al., 2010; Zbinden et al., 2015). Another study has reported that endoscopy carried out in the context of a current Lactobacillus bacteraemia illness showed a friable intestine, also supporting the potential of transmural spread as the source of infection (Kunz et al., 2004).

The bifidobacteraemia cases found likely represented true infection rather than blood culture contamination. This is suggested by their clear clinical deterioration prompting the blood culture collection, and their treatment with a full course of antibiotics. The absence of an

international consensus on diagnosis or severity grading of neonatal sepsis (McGovern et al., 2020) makes it difficult to report the severity of their infection. However, it is reassuring that despite all three cases of probiotic bacteraemia occurring in this vulnerable extremely preterm demographic, all resolved with a course of antibiotics, without any repeat growth on subsequent blood cultures.

Other studies on the impact of probiotics on the incidence of NEC have been limited by the ability for inter-clinician variation in interpretation of the NEC diagnostic criteria. The most common criteria used is the Modified Bell Stage (M. C. Walsh & Kliegman, 1986) and while this has the benefit of being widely used over a significant time, it does allow a degree of variation of interpretation. Therefore, to improve accuracy in the diagnosis of NEC, we chose to not only report the incidence when defined by the modified bell criteria, but also using the definition set out by Battersby et al. where a diagnosis of NEC required either tissue evidence or the primary cause of death on the death certificate (Battersby et al., 2016). Our study found no difference in incidence of NEC between the two cohorts. In prior studies however, probiotics have been shown to reduce NEC in VLBW infants with a similar risk reduction in both randomised controlled trials and observational studies (Meyer et al., 2020; Sharif et al., 2020). Therefore, the lack of reduction observed with probiotic use in our study is likely due to our study not being powered to obtain a statistically significant outcome given the low incidence of NEC in the study centre. Given the potentially devastating impact of NEC in preterm infants, and the low incidence of the probiotic bacteraemia cases found in our study, our results support the continued use of probiotics for preventing necrotising enterocolitis in VLBW infants.

No difference was found between the cohorts for survival free of neurosensory impairment at 2 years corrected age. An improvement in BSID composite motor score was observed in the routine probiotic group, with no difference between the groups for cerebral palsy, BSID cognitive or language scores. A relationship between probiotics and improved neurodevelopmental outcome could be explained through either a reduction in the incidence of NEC which is associated with neurodevelopmental impairment in survivors (Schulzke et al., 2007), or, due to a direct effect on the brain-gut microbiome axis (Niemarkt et al., 2019). Despite this plausible link, other studies have not shown an improvement in neurodevelopmental outcome from probiotic use in VLBW infants (Jacobs et al., 2017b; Sharif et al., 2020). The improvement we observed in BSID motor composite outcome is an

interesting correlation however, further studies would be needed before any causal link could be attributed, particularly in the setting of the cerebral palsy incidence not improving.

The incidence of late onset sepsis was higher in the routine probiotic group. This is not consistent with evidence from both observational studies and randomized controlled trials on probiotics which show a reduction in late-onset sepsis with probiotic use (Meyer et al., 2020; Rao et al., 2016; Sharif et al., 2020)The proportion of fully breast-fed infants was reduced in the routine probiotic cohort. Breast feeding is protective against neonatal sepsis and therefore the observed reduction in fully breast-fed infants may have contributed to the increased rates of late-onset sepsis (Carbone, Montecucco, & Sahebkar, 2020).

This study has several limitations. There was a change in 2013 in the hospital laboratory from using Rapid ID 32 A to MALDI-TOF mass spectrometry. This may have may it more likely to be able to identify probiotic bacteraemia as it is a faster and more accurate test (Barba et al., 2014) and has been shown to be able to reliably identify probiotic organisms (Angelakis, Million, Henry, & Raoult, 2011; Mohar Lorbeg, Golob, Kramer, Treven, & Bogovič Matijašić, 2021). MALDI-TOFF mass spectrometry has been shown to be more accurate at identifying organisms to species level (Barba et al., 2014), though given our study only required isolation to the genus level this difference is unlikely causal for the complete lack of cases in the no probiotic cohort compared to the three cases in the routine probiotic cohort.

Another limitation of our study was not doing genetic identification testing on the Bifidobacteremia cases. The lack of genetic testing to confirm an organism match with that found in the probiotic leaves the potential that these may have been unrelated organisms. Future cases of bacteraemia with organisms potentially in the probiotic should all undergo comparative genomic testing to determine if they are a match.

Assessment of neurodevelopmental outcomes was limited by several factors. The change from using BSID II to BSID III part way through the first cohort caused difficulty in comparing scores between the two cohorts. BSID II combined cognitive and language scores, whereas in BSID III these were separate outcomes, infants also tend to score higher on the BSID III than BSID II (Johnson, Moore, & Marlow, 2014). Also, many infants did not have neurodevelopmental outcome data. Reasons for this include the criteria for neurodevelopmental follow up being different to the inclusion criteria, meaning that very preterm infants with birth weight over 1500g did not qualify for follow-up, some infants being

lost to follow up, and some infants not yet being old enough to have had their two-year follow-up assessment.

4.4.1 Conclusion

Three cases of probiotic bacteraemia were observed in the routine probiotic cohort, all of which responded to antibiotic therapy. These results show that while probiotic bacteraemia is a very rare side effect of probiotic use, it likely occurs more often than suggested by the handful of case reports in the literature.

5 Probiotic Contamination of the Neonatal Intensive Care Unit: Study Protocol

Initially intended to be part of the thesis, this study has been delayed due to COVID-19 related restrictions. Despite the study protocol and funding being finalised, the COVID-19 pandemic and resultant standstill of research in the study centre resulted in a pause in this study. As of March 2022, research is now able to be resumed and the study is expected to be completed in the upcoming months. Please see the study protocol below.

5.1 Introduction

Prophylactic probiotic use in premature infants halves the incidence of necrotising enterocolitis (NEC), and potentially reduces mortality, and late-onset sepsis (Sharif et al., 2020). Due to these benefits, many neonatal units routinely administer prophylactic probiotics for very low birth weight (VLBW), very preterm infants (Deshpande et al., 2011). Prebiotics are defined as 'a substrate that is selectively utilized by host microorganisms conferring a health benefit' (Gibson et al., 2017) their use in very preterm infants has been shown to increase gastrointestinal colonisation of the organisms used in probiotics (Srinivasjois et al., 2013), and show promising beneficial immune properties (Kona & Matlock, 2018) including possible reduction of late-onset sepsis (Chi et al., 2019; Tarnow-Mordi et al., 2020) though not reduction of necrotising enterocolitis (Tarnow-Mordi et al., 2020).

Although probiotics have been shown to have significant beneficial effects, they are not without potential harm. There are multiple case reports of blood culture-proven sepsis from a probiotic organisms in neonates (Table 1, Chapter 1.4.6.1). Probiotic organisms can be resistant to commonly used antibiotics (Temmerman et al., 2003), and outbreaks of resistant organisms have occurred in neonatal units during probiotic use (Topcuoglu et al., 2015). Probiotic product contents are often different from their label claims (Aureli et al., 2010; Drago et al., 2004; Drisko et al., 2005; Elliot & Teversham, 2004; Fasoli et al., 2003; Huys et al., 2006; Temmerman et al., 2003; Theunissen et al., 2005; Vermeulen et al., 2020), both in number and type of organisms present, which can include contamination by pathogenic organisms with potentially lethal consequences (Centers for Disease Control and Prevention, 2015).

Probiotic use can result in contamination both of the Neonatal Intensive Care Unit (NICU) environment (Hickey, Garland, Jacobs, O'Donnell, & Tabrizi, 2014) and of infants in the NICU

who are not being administered a probiotic (Costeloe et al., 2016). The PiPs trial, a RCT on probiotic use in VLBW infants, reported 49% of infants in the control group were colonised with the probiotic organism despite the probiotic being prepared in a milk room separate from the patient's room (Costeloe et al., 2016). There has only been one study (Hickey et al., 2014) that reviewed contamination of the neonatal unit environment when using a bacterial probiotic product. The probiotic contained *Bifidobacterium infantis*, *Bifidobacterium lactis*, and Streptococcus and was prepared by a pharmacist in a distant room. Faecal samples and environmental swabs were analysed by PCR. They found 7.9% of faecal samples from infants not taking probiotics had gastrointestinal colonisation with the probiotic organisms. 21% of swabs taken from the infant's room showed contamination. In both these studies, the gastrointestinal and environmental contamination were despite the probiotic being prepared under trial conditions in a separate room. No studies have investigated rates of environmental contamination when bacterial probiotic products are stored and prepared in the patient room, which occurs in clinical practice.

Bacterial colonisation of the hospital environment can be a source of nosocomial infection (Boyce, 2007; Talon, 1999). While this is less studied for probiotic organisms than for pathogens such as *Staphylococcus aureus* the same potential exists for the environment to be a source of probiotic organisms for infants not intentionally being administered a probiotic, and therefore these infants being unintentionally exposed to the potential risks of probiotic organisms. Conversely, it is also plausible that contamination of equipment used in collecting blood cultures could cause falsely positive blood cultures.

5.1.1 Aims and hypothesis

Primary aim

To determine if the NICU environment is contaminated with organisms from the prophylactic probiotic routinely administered to very low birth weight infants for prevention of necrotising enterocolitis.

Secondary aims:

To determine if:

- Nurse's hands are contaminated with probiotic organisms after preparing probiotics.

- There is airborne spread of probiotic organisms from powder and liquid probiotic products.
- There is probiotic contamination of the external surfaces of in vivo central intravenous catheters.

Hypothesis

The organisms used in probiotics have colonised the NICU environment.

5.1.2 Study design and study centre

Study design

Observational study

Study centre:

The study will be multi-centre, in both Auckland NICU, and Middlemore NICU. The characteristics of these centres are discussed further in chapter 2.1.

5.1.3 Outcomes

Primary outcome

Environmental contamination, defined as the detection of DNA from at least one of the probiotic organisms on an environmental swab.

5.2 Method

5.2.1 Swab collection

5.2.1.1 Environmental contamination

To assess for environmental contamination swabs will be taken both from rooms containing infants currently receiving probiotics, and rooms that have not had an infant receiving probiotics since the last full clean. Swabs will be taken from the following surfaces inside the room;

Cots/incubators and immediate surrounds;
Door handle of the incubator
Door handle of the side drawer

Interior surface, on the inside left corner at the head end
Sheet the infant is lying on, swabbed on the left corner of the head end
Touch screen of bedside monitors
Floor, swab taken from adjacent to the cot/incubator leg at the left head end
Diaphragm of stethoscopes hung at the infant's cot side

On the infant;
Hub of in vivo nasogastric tubes
Hub of in vivo central lines
Axilla of the infant.

Fridge;
Handle of fridge doors
EBM and the outside of the container the EBM is stored in.
Exterior of the probiotic containers, both the exterior of the plastic casing and the exterior of the box.

Other in the room;
Lid of blood culture bottles
External packaging of needles and syringes used in obtaining blood cultures.
Inside door handle
Light switch
Computer keyboard and mouse
Outer surface of gloves contained in an opened packet
Benchtop, at the junction of the benchtop with the wall in the corner closest to the sink.
Sink plug holes
Telephone receiver.

In addition to the above surfaces in patient rooms, swabs will be taken from the medication room where probiotics are stored. This will include the external packaging of the probiotic, and the shelf that probiotics are stored on. As a negative control an education room inside the neonatal unit will be swabbed (the inside door handle, light switch, computer keyboard and mouse). For a positive control a swab will be taken from a sample of formula milk that has had probiotic mixed in with it.

5.2.1.2 Airborne spread

To determine if opening and preparing probiotic products causes airborne spread of the probiotic organisms, swabs will be taken at incremental distances (immediately adjacent, 10cm, 20cm, 50cm, 1m, 1.5m, 2m, 3m) from a person opening the probiotic product and preparing it as it is prepared in the unit. Including opening the product, pouring it into a syringe containing formula, then mixing in the probiotic. This will be conducted in a laboratory. The swabs will be collected 6 hours after opening the capsule and processed by PCR to detect DNA from the organisms in the probiotic. This method will be done with Infloran™, Dicoflor™ (powder probiotic products), and Labinic™ (a liquid probiotic product). For a negative control the process will be done without addition of a probiotic to the formula. For a positive control formula with probiotic added to it will be swabbed.

5.2.1.3 Hand contamination

To assess if nurses' hands become contaminated with probiotic organisms during the preparation and administration of probiotics, we will swab the palm of nurses' hands throughout this process. This will include prior to starting, after preparation and administration but before cleaning, and again after hand cleaning. We will record if the nurse wears gloves or not, and if they wash their hands with hand wash or alcohol gel, this will be the decision of the nurse and we will recommend they do their usual practice. We will also document if the probiotic is being mixed with EBM or formula.

5.2.1.4 Central line contamination

To determine if there is probiotic contamination of the external surfaces of in vivo central intravenous catheters, we will swab the hub of central lines one day after insertion and on the day of removal. This will include umbilical catheters, peripherally inserted central catheters, and extended dwell peripheral intravenous catheters. Half of these will be infants being administered probiotics, and half infants not on probiotics.

5.2.2 Detection of DNA from the probiotic organisms

Swabs taken from Middlemore NICU and Auckland NICU will both be tested for the same five organisms, *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium bifidum, Bifidobacterium breve* and *Bifidobacterium infantis* using MassArray technology, iPLEX

chemistry, as described further in chapter 2.2. The primer sequences (forward and reverse) for each organism are listed in the table below.

Table 1 Forward and Reverse Primer Sequences Used for Each Probiotic Organism

Target	Primers
Lactobacillus acidophilus	F: CCTTTCTAAGGAAGCGAAGGAT R: ACGCTTGGTATTCCAAATCGC
Lactobacillus rhamnosus	F: GCCGATCGTTGACGTTAGTTGG R: CAGCGGTTATGCGATGCGAAT
Bifidobacterium bifidum	F: CTGGCAGCCGTGACACTACT R: TGAACTGGCCGTTACGGTCT
Bifidobacterium breve	F: TCATCACGGCAAGGTCAAGA R: GGCCAGAACAGCTGGAACAA
Bifidobacterium infantis	F: ATGATGCGCTGCCACTGTTA R: CGGTGAGCGTCAATGTATCT

F = forward
R = reverse

5.2.3 Numbers

Ten rooms in each hospital will be swabbed, for a combined total of twenty rooms. The ten per hospital will comprise of five rooms containing infants currently receiving probiotics, and five rooms that have not contained an infant receiving probiotics since the last room clean. As per the Environmental Contamination method section there are 33 sites to swab per room, for all twenty rooms this will be a total of 660 swabs. Ten nurses in each hospital will have their hands swabbed, for a combined total of twenty nurses (and 60 total swabs). Ten central lines from each hospital will be swabbed, for a total of twenty central lines (and 40 total swabs). In each hospital half of the central lines will be in infants being administered a probiotic, and half in infants not being administered a probiotic. One sample of each probiotic product, plus our positive and negative controls will be tested to assess for airborne spread, for a total of 28 swabs. This totals to 788 swabs.

5.2.4 Data management

A document will record the details of the swabs. This will include the following information;

Surfaces:

Swab ID, date, time, hospital site, room number, if the room contains an infant receiving a probiotic, length of time from the last clean, surface swabbed and number of infants in the room. For the infants in the rooms the following information will be obtained to assess for possible risk factors and significance of probiotic colonisation including national health index numer, gestational age, birth weight, ethnicity, number of days they have been administered probiotics, if they have had an episode of NEC or late onset sepsis, and if the infant's parent takes a probiotic supplement. After the above information about the infant has been collected from the clinical records, the data will be de-identified with only a study number matched to the patient data. A separate document will have a list showing which study numbers correspond to which NHI numbers to allow the clinical records to be re-reviewed if required. Each document will be stored in a different folder, with a different name. Both documents will be password protected, each with a different password.

Airborne spread:

Swab ID, date, time, probiotic product used, distance of the sample from the preparation of the product.

Nurses' hands:

Swab ID, date, time, when the swab was taken in relation to preparation and cleaning, if gloves were used, if formula or expressed breast milk was given, if hands were cleaned with alcohol gel or hand wash. We will also record the following details of the nurse: age, gender, if takes a probiotic supplement (if yes, which one), if has a diagnosed skin condition (yes or no).

Central lines:

The same information as for surfaces, in addition type of central line, duration of insertion and if before or after swabbed with an alcohol wipe.

5.3 Ethics, culture support, and safety considerations

5.3.1 Ethics

This study poses minimal risk to the staff or infants involved. Informed consent will be obtained from all participants (for infants, consent will be obtained from their parent/caregiver). Participants would have the study explained to them, be provided with

written information (see appendix 8.1) and, be given an appropriate length of time to make a fully informed voluntary decision prior to signing the consent form and partaking in the study. Nurse identity would not be included in the data collection, staff would only be able to participate in the study one time.

Ethics approval has been obtained from Auckland Health Research Ethics Committee, approval number AH3385.

5.3.2 Cultural support

The participant information sheet has contact details for Māori and Pacific cultural support.

5.3.3 Participant safety information

Nursing staff and patients involved in the study will not be exposed to any risk of harm. Staff already prepare and administer the probiotic routinely in the patient's room. As the swab method is PCR no other organisms will be detected. Staff will not be told of their individual swab results. Infants whose central line hubs are colonised with probiotic organisms will have this result reported to their lead clinician.

5.4 Budget

Participants would not be paid to partake in the study. Funding has been obtained to provide for research nurses in both study centres, and to cover for the cost of swab processing. Funding bodies include the Kaakano Fund CM Health Tupu funding Kaakano grant, and the University of Auckland, School of Medicine performance based research fund.

6 Discussion

NEC is a potentially devastating disease in very preterm infants. Probiotics are used to reduce this risk, however there is currently a knowledge gap about the safety of probiotic use in this vulnerable population. This thesis set out to investigate various safety elements of probiotic use, including the reliability and safety of the contents of a probiotic commonly used in neonatal units, the risk of infection from probiotic use as measured by the incidence of bacteraemia associated with routine probiotic use in preterm infants, and if neonatal units are being colonised with probiotic bacteria.

NEC is characterised by necrosis of the gastrointestinal tract, it is notable for its variable presentation, and is estimated to have an incidence of 2 - 7% in very preterm infants (Battersby et al., 2018).

The disease has a 28% mortality (Hull et al., 2014), with complications in survivors including recurrence, strictures, intestinal failure (Hau et al., 2019), and increased risk of neurodevelopmental impairment (Shah et al., 2011). There is no universal prevention or cure for NEC, with the most effective prevention found thus far being probiotics which have been shown to half the risk of developing NEC in very preterm infants (Sharif et al., 2020). The mass of evidence showing the effectiveness of probiotics in preventing NEC which has developed over the past decade has resulted in the introduction of routine probiotic use for very preterm infants in New Zealand. Despite multiple randomised controlled trials showing the reduction in of the risk NEC in preterm infants, there is still a lack of knowledge of the risks of probiotic use. The biggest concerns are regarding the consistency of the probiotic product, and the risk of infection from the probiotic organism (Poindexter et al., 2021). We have explored these concerns further throughout this thesis. The probiotic product contents were shown to not correlate with label claims, with the proportion that did correlate reducing significantly over time. This finding on probiotic product contents raises concern and suggests the requirement for ongoing monitoring at the least. Our research into the risk of infection from a probiotic organism has shown that the routine use of probiotics is associated with a risk of bacteraemia due to probiotic organisms, and while this is rare, it is more frequent than suggested by the absence of cases in the most recent Cochrane review (Sharif et al., 2020).

6.1 Progression from culture based to molecular testing over the studies

The three studies discussed in this thesis show a progressive advancement in the methods of laboratory testing, corresponding to the dates that the data was obtained. The study discussed in chapter 3 reviewed data on the contents of the probiotic Infloran which started to be collected in 2012. The techniques used in quantifying the probiotic organisms were mostly internationally standardized, culture-based methods. The study discussed in chapter 4 began in 2004 and initially the laboratory method for identifying the organisms used Rapid ID 32A, however midway through the study this was changed to MALDI-TOF mass spectrometry, a faster and more accurate test (Barba et al., 2014). The final study discussed in this thesis, in chapter 5, was designed in 2020 and uses MassArray technology, iPLEX chemistry, which is again a more advanced technology than used in the earlier studies. Therefore, we can see the movement over the time these studies have evolved in, away from traditional culture-based techniques to more sophisticated methods which incorporate PCR and advanced molecular technologies.

While there are benefits and limitations of both culture dependent and independent methods, the more recent molecular based methods are overall more reliable and faster. Cell culture-based methods such as the ISO standards discussed in chapter 2.4 have the benefit of being a standardized method for testing probiotic organisms and being cheaper. They also report the concentration in colony forming units per gram, which is the same as both that reported on the label and how the dose is reported in clinical trials, allowing direct comparison of advertised and evidence-based dosing. However, there are several downsides. They do not produce as accurate a colony count, as they only measure cells that are in an active state of replication in the laboratory conditions. They are slower, more labor intensive, and do not accurately differentiate between closely related species (Davis, 2014). The methods are often complex and vulnerable to inter-laboratory variation in procedure, resulting in varying reported final concentration (Jackson et al., 2019). Only measuring cells in an active state of replies may not be appropriate when dormant and dead probiotic cells can also have a physiological effect (Fiore, Arioli, & Guglielmetti, 2020). Culture independent methods have the main advantage of being fast and able to accurately enumerate cells in all metabolic states (Vinderola, Reinheimer, & Salminen, 2019) . These methods are more expensive and historically have been limited by the number of sites that have the appropriate equipment and staff but are becoming more widespread.

6.2 Risk factors for developing probiotic bacteraemia

Comparing the features of the infants with probiotic bacteraemia from our study with those in the literature (Table 1, Chapter 1.4.6.1) allows us to determine potential risk factors and assess if those found in our three infants are consistent with other cases. Our three cases suggested that being extremely premature, male, and presenting with gastrointestinal concerns were all common features.

All our cases, and many of the cases found in our literature review, occurred in extremely preterm infants. Given the population of extremely preterm infants is outnumbered by those of a more mature gestation, having half of the infants in this demographic suggests extremely preterm infants are more susceptible to probiotic bacteraemia than babies of older gestations.

While all three of our case reports were male, this finding was not consistent across the existing case reports in the literature suggesting that male sex is not a risk factor. Similarly, despite all our cases presenting with abdominal concerns on the day of their positive blood culture, this was not a consistent finding in the literature. Interestingly, one study did report two infants with probiotic bacteraemia who had friable intestinal mucosa seen on endoscopy after resolution of sepsis, supporting the potential theory of transmural infection (Kunzet al., 2004).

6.3 Probiotic regulation

There is a requirement for increased regulation and monitoring of probiotics used in preterm infants as shown by the highly variable concentration of organisms present in probiotics, detection of a contaminated sample, reduction in concentration over time, and the association of probiotic use with bacteraemia from the probiotic organism. In New Zealand, probiotics are regulated as dietary supplements which requires the label to list the ingredients, use-by date, recommended dose, weight, manufacturer name, address, and the batch number (Beattie, 2016). Globally, probiotics are classed under different categories with differing regulatory bodies, although most also do not have the stringent monitoring requirements that medications do, including no compulsory adverse event reporting or safety monitoring (Arora & Baldi, 2015). Our concerning findings on the contents of probiotic products show that this is inadequate, particularly when they are being used for the vulnerable very preterm population.

While regulations remain unchanged, monitoring of probiotics should be done at a national level in New Zealand. There are several factors about New Zealand that make this both

appropriate and achievable. We have the benefit of being a small country with only six tertiary neonatal units, all bar one of which use the same probiotic product, Infloran™, (Meyer et al., 2020). This allows central monitoring of Infloran™, with results being available to all centers. Ideally each batch of Infloran™ arriving in New Zealand would be screened to determine if the concentration was appropriate and assess for potential contamination. If results were not reassuring this would allow for all product from the specific batch to not be used, and hence avoid the potential for either using a product that had concentration too low to achieve therapeutic benefit, or a product that had risk of potential harm either from concentration too high or due to containing contamination. This would require agreement from the units involved on their acceptable dose range, a pragmatic solution while we are awaiting further research on the ideal dose range, is that units accept samples within the range used in the majority of trials in the most recent Cochrane review, that being between 108 to 109 CFU/dose (Sharif et al., 2020), this is in keeping with the recommended doses in the recent European position paper (van den Akker et al., 2020). A potential downside of this strategy that would need to be considered is what to do when there were no available probiotic products in the acceptable range, otherwise this could have the potential downside of infants at times not receiving a probiotic. The monitoring by a central location with results being reported at the time to the other units allows coordinated timely safety monitoring and prevents multiple units needing to monitor probiotics in their own centres.

An internationally consistent approach to probiotic regulation would allay many of the current challenges with probiotic legislation. As discussed above, in New Zealand probiotics are regulated as a dietary supplement (Beattie, 2016), this is not consistent globally though with various international approaches including; Australia regulating them as a complementary medicine (Health Products Regulation Group, 2019), whereas the United States of America having different regulation requirements of probiotic products according to whether they will be used as a medication or a dietary supplement (Venugopalan, Shriner, & Wong-Beringer, 2010). This introduces confusion on multiple fronts. In many cases the probiotic products are produced in a country foreign to that of where they are being used, meaning the requirements for probiotics in that country are likely different. These requirements can include what information is needed on the label and what safety monitoring is required and it is often not made clear to consumers that legislation is variable (Arora & Baldi, 2015).

Our research has shown the importance of several key features in what would be an ideal regulation framework for probiotic use. The considerable variation in content concentration

and the potential for contamination makes the requirement for reporting of content surveillance from the producer and from a local regulatory body advisable. Additionally, there is the need for compulsory adverse event monitoring given probiotic use carries the risk of bacteraemia from the probiotic organism or from a contaminant. This system for compulsory/routine reporting of adverse events should be at a national level with the information being available to end users. Regulations also need to be clear on whether claims made by probiotic producers require an evidence base. Given the widely expanding probiotic market and multiple different uses of probiotic products it would be reasonable to have a multi-tiered approach to regulation. The current regulation systems in both the United States of America and Japan have adopted a multi-tiered framework whereby probiotics are regulated depending on whether their intended use is for a specific health claim ie similar to a medication, or if they have a more nutritional/functional claim (Koirala & Anal, 2021). This tiered framework for regulation is pragmatic in such a varied market where clearly products such as yoghurt sold in the supermarket that claim they contain probiotic organisms do not require as much safety monitoring as medications like Infloran^{TM} that are used for preventing disease in a very vulnerable population.

6.4 Probiotic dosing

The ideal dose of probiotics to administer to preterm babies as a prophylaxis against NEC is unknown. To date there have been no randomized controlled trials to determine the safety and efficacy of lower dose compared with a higher dose of probiotics (Sharif et al., 2020). Currently, guidelines recommending probiotic doses are based on the doses used in clinical trials compared to placebo, with a wide variation of doses, mostly in the range of 10^8 to 10^9 (van den Akker et al., 2020). One of the key challenges have been the different probiotic formulations used in the various clinical trials. The most recent meta-analysis included 56 trials with variable probiotic regimes (Sharif et al., 2020). Twenty-three of the trials used multi-genus probiotic combinations and thirty-three used single-genus probiotics, which also varied in their species and strain. This makes determining the ideal dose range difficult as it may not be the same for different organisms. Furthermore, our research has also shown the marked variation in concentration of the probiotic products and the inconsistency of this with label claims, so we cannot assume that the infants in these trials were being delivered the advertised dose.

Our research has shown that the concentration of organisms in probiotic products, and hence the dose being administered to preterm infants, varies considerably, from none to ten billion colony forming units per gram. Presumably there is a lower dose limit at which probiotics are effective at preventing NEC. Moreover, we have shown that probiotic use carries a rare risk of bacteraemia from a probiotic organism and it is plausible that this risk of infection is higher if the dose of probiotic organisms administered to the patient is higher. This risk of infection associated with bacterial load has not been studied for probiotic organisms, however it has biological plausibility, and the risk of bacterial transmission in neonates has been shown to be related to the load of bacterial exposure for other organisms (Seedat et al., 2018). Currently, the upper limit of a safe dose is unknown, once this has been determined it will allow for probiotic batches screened as having a concentration over this upper limit to not be used, reducing the risk of infection in this vulnerable population.

While the benefits in determining the optimal dose range for probiotics are clear, this is not an easy task. A factor compounding the difficulty of determining the ideal dose is the contamination of the control group with the probiotic organisms, as was demonstrated to occur in the PIPs trial (Costeloe et al., 2016). This contamination between infants would raise the potential that if infants in the same neonatal unit were given different doses, the infants randomised to the lower dose may receive more probiotic organisms than expected. Therefore, ideally a study on dose determination would be a multi-site trial, where units were randomised to a specific dose with clinicians blinded to the actual dose.

Future research also needs to be targeted at closing the knowledge gap in extremely preterm infants. There is currently minimal evidence for probiotic use in extremely premature infants (Sharif et al., 2020), this is particularly concerning when we have shown that this demographic is the most at risk of bacteraemia from the probiotic organism. These vulnerable infants have the highest incidence of NEC (Battersby et al., 2018) and therefore potentially the most to gain from probiotics. However, currently we are assuming that this benefit applies by either relying on low quality evidence grade data or extrapolating data from less premature gestations which may not be appropriate in a disease as multifactorial as NEC. As extremely preterm infants have the most to gain while also being at the highest risk of bacteraemia, there is a need for increased evidence in this demographic. Currently probiotics are routinely used in this cohort in New Zealand. This is appropriate given the information of benefits shown in the meta-analysis, and low risk of harm shown in our research, suggesting that overall, the benefits for extremely preterm infants of probiotics in reducing NEC outweigh the risk of bacteraemia from

a probiotic organism. This does mean, however, that it would not be ethical to with-hold probiotic therapy for a group of infants to conduct a RCT in New Zealand. There is the potential for a RCT to be conducted on extremely preterm infants in a country that does not routinely use probiotics, such as in the United States.

6.5 Limitations of this thesis

The main limitations of the research discussed in this thesis center around the retrospective design of the first two studies. With the studies being retrospective and data having been collected over such long time periods, there were changes in both definitions and laboratory techniques throughout the time that data was collected. An example of this was our study in chapter 4 where the laboratory equipment for identifying blood culture isolates was updated part way through the study from Rapid-ID 32A to MALDI-TOFF mass spectroscopy, which being a faster and more accurate test (Barba et al., 2014) may have made the second cohort more likely to have probiotic organisms identified. Likewise, in the same study, the assessment of neurodevelopmental outcomes changed from BSID II to BSID III part way through the study, causing difficulty in comparing the neurodevelopmental outcomes between cohorts.

Another limitation of the study discussed in chapter 4 was not doing comparative genomic testing to confirm the organisms isolated in the blood culture were a genetic match to those in the probiotic product. This introduces the possibility that the organisms found did not originate from the probiotic that the infants were being administered. While a significant limitation, this is unlikely to explain the different outcomes between the two groups given the complete absence of organisms from lactobacillus or bifidobacteria species detected in the no probiotic cohort. Following the research findings, the study center will now do comparative genomic testing on any future blood culture isolates that are identified as potentially originating from the probiotic products.

Whilst the extended time frames and retrospective nature of the studies have the limitations as discussed above, they also had the benefit of allowing us to collect a substantial amount of data. The study discussed in chapter 3 provided over a decade of information on the contents of the probiotic product, and the study in chapter 4 provided fourteen years of surveillance for probiotic bacteraemia, both of which are substantially longer time periods that other studies on these topics.

7 Conclusion

NEC is a potentially devastating disease of the gastrointestinal tract, affecting around 3 – 7% of very preterm infants (Battersby et al., 2018). Meta-analysis has shown that probiotics reduce the risk of NEC and may reduce the risk of mortality and late-onset sepsis (Sharif et al., 2020). Therefore, probiotics prophylaxis is now routine throughout Australasia and some other parts of the world. Despite their widespread use there was an uncertainty on the reliability of the contents of the probiotic products, and the risk of bacteraemia from a probiotic organism. Throughout this thesis we have explored these concerns. The contents of a probiotic product commonly used in neonatal medicine was shown to not correlate with label claims, and concerningly the concentration of organisms present is reducing over time. Routine probiotic use in very preterm infants was associated with a rare risk of bacteraemia, with all identified cases undergoing a full recovery. While these findings are concerning and warrant further investigation, they do not outweigh the significant benefits that probiotics have been shown to have. Similarly with our last study on probiotic contamination of the NICU, if the results show that there is significant contamination, this will support a change in how the probiotics are prepared or administered rather than suggesting that they should no longer be used. Overall, the concerns about probiotics which we have explored in this thesis is not enough to override the cumulative evidence showing benefit of probiotic use in very preterm infants for preventing NEC.

8 Appendix

8.1 Probiotic Contamination of the Neonatal Intensive Care Unit; Information Sheets and Consent Forms

Probiotic Contamination of the Neonatal Intensive Care Unit

Nursing Staff Information Sheet

My name is Dr Sophie Springer, I am a research masters student with the University of Auckland and a neonatal fellow at Auckland District Health Board. My supervisor is Associate Professor Jane Alsweiler.

You are invited to take part in a research study to determine if neonatal nurses' hands become contaminated with probiotic bacteria when they give probiotics to preterm babies. Whether or not you take part in the study is your choice and will not affect your employment. If you choose to not take part in this study you do not need to give a reason. You can withdraw from the study at any time for any reason if you initially decide to partake and then later change your mind.
Before deciding if you want to be part of the study or not, you need to understand why the research is being done and what it would involve for you. Please take the time to read the following four pages carefully and ask questions if anything you read is not clear or if you would like more information.
If you do agree to take part in the study you will be asked to sign the consent form on the last page of this document. You will be given a copy of this information sheet and the consent form.

What is the purpose of this study?
Probiotics are microbial supplements (usually bacteria) that are used for their beneficial effects. Probiotics are routinely given to infants born very preterm or with very low birth weight because they reduce mortality, necrotising enterocolitis, and late-onset sepsis. A concern of probiotic use is the potential for the probiotic organisms to colonise the neonatal unit, exposing other infants and staff to these bacteria. The purpose of this study is to determine if the bacteria used in probiotics are colonising the neonatal intensive care unit environment, including nurses' hands. To investigate this we will swab areas in the neonatal unit to check for the presence of the probiotic bacteria, including many areas in the rooms such as benchtops, fridges, and the cot/incubator. We are also plan to swab the hands of neonatal nurses before and after preparing and administering probiotics to determine if their hands become contaminated with probiotic bacteria during this process.

What will taking part involve?
If you agree to take part in the study, we would swab your hands several times while you are routinely preparing and administering probiotics. This will include swabbing your hands before starting, after preparation and administration of the probiotic but before cleaning your hands, and again after cleaning your hands. We would ask that you follow your usual practice, and we will record if you were wearing gloves and if you wash your hands with soap and water or with alcohol gel. We will also document if the probiotic is being mixed with EBM or formula. The swabs taken will be processed to detect the bacteria that are contained in the probiotic product. They will not detect any other organisms that may be present.

Why have you been invited to take part?
All nursing staff working in level three of the neonatal intensive care unit are being asked to participate in the study. This is voluntary and will not adversely affect you if you choose to not participate in the study.

Do you have to take part?
Participation in this study is voluntary. You have the right to refuse participation in the study, and if you agree now you have the right to change your mind later without needing to explain why and without any consequences.

What if I agree to participate now and then later decide I do not want to?
Participation in this study is voluntary. Even if you initially decide to take part, you can change your mind later without needing to explain yourself. If you want to change your decision please contact one of the study team (contact details on the next page).

What are the possible risks of taking part?
Taking a swab from your hand is safe, it will not cause pain or other harm. The swab will only be processed to detect the probiotic bacteria, other organisms present will not be detected.

Will taking part be confidential? How will information you provide be recorded, stored and protected?
The information will be de-identified. No name, national health index number, or date of birth, will be obtained from you. The only information obtained from staff who have their hands swabbed will be their age, gender, if they have a diagnosed skin condition (yes or no), and if s/he takes a probiotic supplement (if so which one). No identifying information will be included in the write up of the study. Results of individual hand swabs will not be communicated back to the nursing staff.

What if something goes wrong?
In the unlikely scenario that you were injured while partaking in this study you would be eligible to apply for ACC compensation just as you would be for any other accident in the workplace. This does not guarantee that your claim would be accepted, a claim would have to be lodged through the usual process. If you have health or life insurance you may wish to check with your insurer that taking part in this study won't affect your cover.

Who pays for the study?
This study is funded by the University of Auckland. There are no financial costs associated with participation in this study.

What will happen to the results of the study?
Information from this study will be used to contribute towards a Master's qualification. We also hope to publish the results of this study in an international paediatric journal. Identity of study participants in the research will not be possible. There is the possibility that de-identified study data would be made available to other researchers for approved future research.
If you would like, when the study is completed we can write to you outlining the main results of the study.

Who can you contact for further information?
At any stage if you have concerns, complaints, or questions about the study these can be discussed with the study research team;

Dr Sophie Springer
Neonatal Fellow
sspring@adhb.govt.nz
09 367 0000 ext 25365

Dr Jane Alsweiler
Neonatal Paediatrician
j.alsweiler@auckland.ac.nz
09 373 7599 ext 87766

To discuss with someone who is not involved in this study contact options include;

Healthy and Disability Advocate
0800 555 050
Fax 0800 2 SUPPORT (0800 2787 7678)
advocacy@hdc.org.nz

For concerns of an ethical nature, you can contact the Chair of the Auckland Health Research Ethics Committee at ahrec@auckland.ac.nz or at 373 7599 x 83711, or at Auckland Health Research Ethics Committee, The University of Auckland, Private Bag 92019, Auckland 1142.

Who can I contact for cultural support?
For participants working in Auckland Neonatal Intensive Care Unit: If you require Māori cultural support please contact the administrator for He Kamaka Waiora (Māori Health Team) by telephoning 09 486 8324 ext 42324. If you require Pacific cultural support please contact a Pacific family support worker on phone 367 0000 ext 29500.
For participants in Middlemore Neonatal Intensive Care Unit: If you require cultural supports please contact the social worker, in the first instance, on (09)276 0044 ext 50865.

Thank you for taking the time to read this information sheet. We acknowledge that working in the neonatal intensive care unit can be busy and stressful, and we appreciate you considering this study.

Approved by the Auckland Health Research Ethics Committee on [24/06/21] for three years. Reference number AH3385.

Consent Form: Nursing Staff

THIS FORM WILL BE HELD FOR A PERIOD OF 10 YEARS

Project title: Probiotic Contamination of the Neonatal Intensive Care Unit

Research team:

Dr Sophie Springer
Neonatal Fellow
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09 367 0000 ext 25365

Associate Professor Jane Alsweiler
Neonatal Paediatrician
j.alsweiler@auckland.ac.nz
09 373 7599 ext 87766

Dr Michael Meyer
Honorary Associate professor
Neonatal Unit
Middlemore Hospital
mmeyer@middlemore.co.nz
09 276 0044 ext 58872

I have read, or have had read to me in my first language, the Participant Information Sheet. I have been given sufficient time to consider whether or not to participate in this study and to ask questions, and was offered support from whānau/family or a friend to help me understand what the study involves. I am satisfied with the answers given to me, I understand the nature of the research and why I have been invited to participate.

I agree to take part in this research.

- I understand my participation is voluntary.
- I understand I am free to withdraw any data traceable to me up to one week after consenting without giving a reason.
- I understand that my participation in this study is confidential and that no material which could identify me personally will be used in any reports on this study.
- I understand that de-identified data will be kept for 10 years and separate from the Consent Forms, after which they will be destroyed.

- I agree / do not agree that information collected about me up to the point when I withdraw may continue to be processed if I decide to withdraw from the study (please circle one).
- I consent to the research staff collecting and processing my information, including information about my health.
- I wish / do not wish to receive the summary of findings (please circle one).
  Email/postal address: _____
- I know who to contact if I have any questions about the study in general.

Print Name of Participant_____

Signature of Participant _____

Date _____

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

Swab the hand of the nurse during the preparation and administration of a probiotic. This will include prior to starting, after preparation and administration but before cleaning, and again after hand cleaning.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this consent form has been provided to the participant.

Print Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

Approved by the Auckland Health Research Ethics Committee on 24/06/21 for three years. Reference number AH3385.

Probiotic Contamination of the Neonatal Intensive Care Unit

Parent Information Sheet

My name is Dr Sophie Springer, I am a research masters student with the University of Auckland and a neonatal fellow at Auckland District Health Board. My supervisor is Associate Professor Jane Alsweiler.

Your baby is invited to take part in a study to see if the living bacteria given to very preterm infants to improve their outcomes can also spread to other babies who were not supposed to be getting them. Whether or not you take part in the study is your choice and will not affect the care of your infant. If you choose to not take part in this study you do not need to give a reason. You can withdraw from the study at any time for any reason if you initially decide to take part and then later change your mind.
Before deciding if you want to be part of the study or not, you need to understand why the research is being done and what it would involve for you. Please take the time to read the following four pages carefully and ask questions if anything you read is not clear or if you would like more information.
If you do agree to your baby taking part in the study you will be asked to sign the consent form on the last page of this document. You will be given a copy of this information sheet and the consent form.

What is the purpose of this study?
Probiotics are living bacteria given as supplements that are used for their helpful effects. Probiotics are routinely given to infants born very preterm or with very low birth weight because they reduce death, a disease of the bowel called necrotising enterocolitis, and infections. A concern of probiotic use is that the living organisms can spread to other infants. The purpose of this study is to see if the bacteria used in probiotics can survive on surfaces in the neonatal intensive care unit and even be found in other babies who were not planned to be getting them. To check this we will swab areas in the neonatal unit to check for the organisms, including many areas in the rooms such as benchtops, fridges, and the cot/incubator. We are also plan to swab the axilla (armpit) of babies, and if they have one, their nasogastric tube and central line, to see if the organisms are present in these areas.

What will taking part involve?
If you agree to your infant taking part in the study, we would swab your baby's armpit, and if they already have one then also their nasogastric tube and central line. The swabs taken will be checked for the probiotic organisms. They will not detect any other organisms that may be present.

Why have you been invited to take part?
All infants admitted in level three of the neonatal intensive care unit are being invited to take part. This is voluntary and if you choose for them not to take part they will not be worse off.

Do you have to take part?
Taking part is voluntary. You have the right to refuse your infant taking part, and if you agree now you can change your mind later without needing to explain why and your baby will not be worse off.

What if I agree to participate now and then later decide I do not want to?
Taking part is optional. Even if you decide to take part, you can change your mind later without needing to explain yourself. If you wish to withdraw from the study please just let any of the research team know (see contact details below), or, you can ask your infants nurse to contact the research team for you

What are the possible risks of taking part?
Taking a swab from your infant is safe, it will not cause pain or other harm. The swab will only be used to look for probiotic bacteria, other organisms present will not be picked up.

Will taking part be confidential? How will information you provide be recorded, stored and protected?
If you agree to your infant taking part in this study, we will get basic medical information about them to look for things that can cause spread of probiotic organisms. This information will be stored in password protected files in a de-identified way for 26 years. This will include your infants national health index number (NHI), gestational age, birth weight, ethnicity, number of days they have been given probiotics, if they have had necrotising enterocolitis or infection, and if the infant's parent takes a probiotic too. Nothing that can identify you or baby will be included in the write up of the study. Results of your baby's swabs will not be given back to you, but if you would like, after the study we can write to you and tell you the main findingsWhat if something goes wrong?
It is unlikely your infant could be injured as part of study, but you would be able to apply for ACC compensation just as you would be for any other accident. This does not guarantee that your claim would be accepted, a claim would have to go through the usual process. If you have health or life insurance you may wish to check with your insurer that taking part in this study won't affect your cover.

Who pays for the study?
This study is funded by the University of Auckland and Counties Manukau DHB. There are no financial costs for being in the study.

What will happen to the results of the study?
Information from this study will be used to contribute towards a Master's qualification. We also hope to publish the results of this study in an international journal and present at conferences. Identity of study participants in the research will not be possible. It is possible that data could be made available to other researchers for approved future research but no personal information that could identify you or your baby would be made available.
If you would like, when the study is finished we can write to you and explain the main results.

Who can you contact for further information?
At any stage if you have concerns, complaints, or questions about the study these can be discussed with the study research team;

Dr Sophie Springer
Neonatal Fellow
sspring@adhb.govt.nz
09 367 0000 ext 25365

Associate Professor Jane Alsweiler
Neonatal Paediatrician
j.alsweiler@auckland.ac.nz
09 373 7599 ext 87766

Co-investigator:
Dr Michael Meyer
Honorary Associate professor
Neonatal Unit
Middlemore Hospital
mmeyer@middlemore.co.nz
09 276 0044 ext 58872

To discuss with someone who is not involved in this study contact options include;

Healthy and Disability Advocate
0800 555 050
Fax 0800 2 SUPPORT (0800 2787 7678)
advocacy@hdc.org.nz

For concerns of an ethical nature, you can contact the Chair of the Auckland Health Research Ethics Committee at ahrec@auckland.ac.nz or at 373 7599 x 83711, or at Auckland Health Research Ethics Committee, The University of Auckland, Private Bag 92019, Auckland 1142.

Who can I contact for cultural support?
For participants in Auckland Neonatal Intensive Care Unit: If you require Māori cultural support please contact the administrator for He Kamaka Waiora (Māori Health Team) by telephoning 09 486 8324 ext 42324. If you require Pacific cultural support please contact a Pacific family support worker on phone 367 0000 ext 29500.
For participants in Middlemore Neonatal Intensive Care Unit: If you require cultural supports please contact the social worker, in the first instance, on (09)276 0044 ext 50865.

Approved by the Auckland Health Research Ethics Committee on [24/06/21] for three years. Reference number AH3385.

Consent Form: Parent/Caregiver of the Infant

THIS FORM WILL BE HELD FOR A PERIOD OF 26 YEARS

Project title: Probiotic Contamination of the Neonatal Intensive Care Unit

Research team:

Dr Sophie Springer
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Associate Professor Jane Alsweiler
Neonatal Paediatrician
j.alsweiler@auckland.ac.nz
021526363

Dr Michael Meyer
Honorary Associate professor
Neonatal Unit
Middlemore Hospital
mmeyer@middlemore.co.nz
0211903608

I have read or, have had read to me in my first language, the Participant Information Sheet. I have been given sufficient time to consider whether or not for my infant to participate in this study and to ask questions, and was offered support from whānau/family or a friend to help me understand what the study involves. I am satisfied with the answers given to me, I understand the nature of the research and why my infant has been invited to participate.

I agree to my infant taking part in this research.

- I understand participation of my infant is voluntary.
- I understand I am free to withdraw any data traceable to my infant up to one week after consenting without giving a reason.
- I understand that my infant's participation in this study is confidential and that no material which could identify them personally will be used in any reports on this study.

- I understand that de-identified data will be kept for 26 years and separate from the Consent Forms, after which they will be destroyed.
- I agree / do not agree that information collected about my infant up to the point when I withdraw may continue to be processed if I decide to withdraw from the study (please circle one).
- I consent to the research staff collecting and processing my infant's information, including information about my health.
- I wish / do not wish to receive the summary of findings (please circle one).

  Email/postal address: _____
- I know who to contact if I have any questions about the study in general.

Print Name of Parent/Caregiver_____

Signature of Parent/Caregiver _____

Print Name of Infant Participant_____

Date _____

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant's parent/caregiver, and to the best of my ability made sure that they understand that the following will be done to their infant:

Swab the axilla (arm pit), and if the infant already has one then also their central line and nasogastric tube.

I confirm that the participant's parent/caregiver was given an opportunity to ask questions about the study, and all the questions asked have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent for their infant, and the consent has been given freely and voluntarily.

A copy of this consent form has been provided to the participants parent/caregiver.

Print Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

Approved by the Auckland Health Research Ethics Committee on [24/06/21] for three years. Reference number AH3385.

References

AG Pharma S.r.l. (2020). Dicoflor 60. Retrieved September 27, 2020, from https://www.agpharma.eu/en/product/dicoflor-60/

Agena Bioscience Inc. (2019). *MassARRAY System*. San Diego. Retrieved from https://www.agenabio.com/wp-content/uploads/2015/05/MassARRAY-System-Brochure-WEB-SYS0003-04.pdf

Akar, M., Eras, Z., Oncel, M. Y., Arayici, S., Guzoglu, N., Canpolat, F. E., … Oguz, S. S. (2016). Impact of Oral Probiotics on Neurodevelopmental Outcomes in Preterm Infants. *Https://Doi.Org/10.1080/14767058.2016.1174683*, *30*, 411–415.

Alfaleh, K., & Anabrees, J. (2014). Probiotics for Prevention of Necrotizing Enterocolitis in Preterm Infants. *Cochrane Database of Systematic Reviews*.

Andrews, W., Wang, H., Jacobson, A., Ge, B., Zhang, G., & Hammack, T. (1998). BAM Chapter 5: Salmonella | FDA. In *Bacteriological Analytical Manual* (Eighth). Retrieved from https://www.fda.gov/food/laboratory-methods-food/bam-chapter-5-salmonella

Angelakis, E., Million, M., Henry, M., & Raoult, D. (2011). Rapid and Accurate Bacterial Identification in Probiotics and Yoghurts by MALDI-TOF Mass Spectrometry. *Journal of Food Science*, *76*. https://doi.org/10.1111/J.1750-3841.2011.02369.X

Arboleya, S., Binetti, A., Salazar, N., Fernández, N., Solís, G., Hernández-Barranco, A., … Gueimonde, M. (2012). Establishment and Development of Intestinal Microbiota in Preterm Neonates. *FEMS Microbiology Ecology*, *79*, 763–772.

Arora, M., & Baldi, A. (2015). Regulatory Categories of Probiotics Across the Globe: A Review Representing Existing and Recommended Categorization. *Indian Journal of Medical Microbiology*, *33*, S2–S10.

Aureli, P., Fiore, A., Scalfaro, C., Casale, M., & Franciosa, G. (2010). National Survey Outcomes on Commercial Probiotic Food Supplements in Italy. *International Journal of Food Microbiology*, *137*, 265–273.

Australian and New Zealand Neonatal Network. (2022). *ANZNN data dictionary*. Retrieved from https://anznn.net/dataresources/datadictionaries

Balasundaram, P., & Avulakunta, I. D. (2020). Bayley Scales Of Infant and Toddler Development. *StatPearls*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK567715/

Barba, M. J., Fernández, A., Oviaño, M., Fernández, B., Velasco, D., & Bou, G. (2014). Evaluation of MALDI-TOF Mass Spectrometry for Identification of Anaerobic Bacteria. *Anaerobe*, *30*, 126–128.

Barbian, M. E., Buckle, R., Denning, P. W., & Patel, R. M. (2019). To start or not: Factors to consider when implementing routine probiotic use in the NICU. *Early Human Development*, *135*, 66–71.

Barnes, J. (2018). *Developments in Regulations for Complementary Medicines*. Auckland. Retrieved from http://www.naturalproductsnz.org/cms/wp-

Bartle, D., Knight, C., & Cairns, P. (2017). *South West Neonatal Network Guideline The Use of Probiotics in Preterm Babies*.

Battersby, C., Santhalingam, T., Costeloe, K., & Modi, N. (2018). Incidence of Neonatal Necrotising Enterocolitis in High-Income Countries: A Systematic Review. *Archives of Disease in Childhood - Fetal and Neonatal Edition*, *103*, F182–F189.

Battersby, Longford, Mandalia, Costeloe, & Modi. (2016). Incidence and Enteral Feed Antecedents of Severe Neonatal Necrotising Enterocolitis Across Neonatal Networks in England, 2012–13: A Whole-Population Surveillance Study. *Lancet Gastroenterol Hepatol*, *2*, 43–51.

Bayley, N. (2006). *Bayley Scales of Infant and Toddler Development–Third Edition: Technical Manual* (Third). San Antonio, TX: Harcourt Assessment.

Bayley, Nancy. (1993). *Bayley Scales of Infant Development* (Second). San Antonio, TX: The Psychological Corporation.

Beattie, D. (2016, March 1). *Dietary Supplements Regulations 1985*. New Zealand. Retrieved from https://www.legislation.govt.nz/regulation/public/1985/0208/latest/DLM102109.html

Bennett, R. W., Hait, J. M., & Tallent, S. M. (2015). Staphylococcus aureus and Staphylococcal Enterotoxins. In Yvonne Salfinger & M. lou Tortorello (Eds.), *Compendium of Methods for the Microbiological Examination of Foods* (Fifth, pp. 509–526). American Public Health Association.

Bennett, R. W., Tallent, S. M., & Hait, J. M. (2015). Bacillus cereus and Bacillus cereus Toxins. In Yvonne Salfinger & M. lou Tortorello (Eds.), *Compendium of Methods for the Microbiological Examination of Foods* (Fifth, pp. 378–380). American Public Health Association.

Bergmann, K. R., Liu, S. X. L., Tian, R., Kushnir, A., Turner, J. R., Li, H. L., … de Plaen, I. G. (2013). Bifidobacteria Stabilize Claudins at Tight Junctions and Prevent Intestinal Barrier Dysfunction in Mouse Necrotizing Enterocolitis. *American Journal of Pathology*, *182*, 1595–1606.

Bertelli, C., Pillonel, T., Torregrossa, A., Prod, G., Fischer, C. J., Greub, G., & Giannoni, E. (2014). Bifidobacterium longum Bacteremia in Preterm Infants Receiving Probiotics. *Clinical Infectious Diseases*, *60*, 924–927.

Bhutta, A. T., Cleves, M. A., Casey, P. H., Cradock, M. M., & Anand, K. J. S. (2002). Cognitive and Behavioral Outcomes of School-Aged Children Who Were Born Preterm: A Meta-Analysis. *Journal of the American Medical Association*, *288*, 728–737.

Biofloratech Ltd. (2016). Labinic Drops - Information for Health Professionals. Retrieved January 4, 2022, from https://www.biofloratech.com/Health_Professionals.html#modal-one

bioMérieux SA. (2006). *rapid ID 32 A*. Retrieved from https://kaldur.landspitali.is/focal/gaedahandbaekur/gnhsykla.nsf/5e27f2e5a88c898e00256500003c98c2/516b46e98bbdc38600256da300405e64/$FILE/Rapid%20ID%2032%20A.pdf

Black, R. E., Cousens, S., Johnson, H. L., Lawn, J. E., Rudan, I., Bassani, D. G., … Mathers, C. (2010). Global, Regional, and National Causes of Child Mortality in 2008: A Systematic Analysis. *The Lancet*, *375*, 1969–1987.

Blakely, M. L., Tyson, J. E., Lally, K. P., McDonald, S., Stoll, B. J., Stevenson, D. K., … Higgins, R. D. (2006). Laparotomy Versus Peritoneal Drainage for Necrotizing Enterocolitis or Isolated Intestinal Perforation in Extremely Low Birth Weight Infants: Outcomes Through 18 Months Adjusted Age. *Pediatrics*, *117*, e680–e687.

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., … Lawn, J. E. (2012). National, Regional, and Worldwide Estimates of Preterm Birth Rates in the Year 2010 With Time Trends Since 1990 for Selected Countries: A Systematic Analysis and Implications. *The Lancet*, *379*, 2162–2172.

Boonma, P., Spinler, J. K., Venable, S. F., Versalovic, J., & Tumwasorn, S. (2014). Lactobacillus rhamnosus L34 and Lactobacillus casei L39 Suppress Clostridium difficile Induced IL-8 Production by Colonic Epithelial Cells. *BMC Microbiology*, *14*. https://doi.org/10.1186/1471-2180-14-177

Boundy, E. O., Dastjerdi, R., Spiegelman, D., Fawzi, W. W., Missmer, S. A., Lieberman, E., … Chan, G. J. (2016). Kangaroo Mother Care and Neonatal Outcomes: A Meta-Analysis. *Pediatrics*, *137*. https://doi.org/10.1542/peds.2015-2238

Boyce, J. M. (2007). Environmental Contamination Makes An Important Contribution to Hospital Infection. *Journal of Hospital Infection*, 50–54.

Boyle, R. J., Robins-Browne, R. M., & Tang, M. L. K. (2006). Probiotic Use in Clinical Practice: What are the Risks? *American Journal of Clinical Nutrition*, *83*, 1256–1264.

Brecht, M., Garg, A., Longstaff, K., Cooper, C., & Andersen, C. (2016). Lactobacillus Sepsis Following a Laparotomy in a Preterm Infant: A Note of Caution. *Neonatology*, *109*, 186–189.

Brigidi, P., Swennen, E., Vitali, B., Rossi, M., & Matteuzzi, D. (2003). PCR Detection of Bifidobacterium Strains and Streptococcus thermophilus in feces of Human Subjects After Oral Bacteriotherapy and Yoghurt Consumption. *International Journal of Food Microbiology*, *81*, 203–209.

Brook, I. (2008). Microbiology and Management of Neonatal Necrotizing Enterocolitis. *American Journal of Perinatology*, *25*, 111–118.

Brooks, B., Firek, B. A., Miller, C. S., Sharon, I., Thomas, B. C., Baker, R., … Banfield, J. F. (2014). Microbes in the Neonatal Intensive Care Unit Resemble Those Found in the Gut of Ppremature Infants. *Microbiome*, *2*, 1–16.

Bushman, F. D. (2019). De-Discovery of the Placenta Microbiome. *American Journal of Obstetrics & Gynecology*, *220*, 213–214.

Callen, J., & Pinelli, J. (2005). A Review of the Literature Examining the Benefits and Challenges, Incidence and Duration, and Barriers to Breastfeeding in Preterm Infants. *Advances in Neonatal Care*, *5*, 72–88.

Caplan, M. S., Miller-Catchpole, R., Kaup, S., Russell, T., Lickerman, M., Amer, M., … Thomson R., J. (1999). Bifidobacterial Supplementation Reduces the Incidence of Necrotizing Enterocolitis in a Neonatal Rat Model. *Gastroenterology*, *117*, 577–583.

Carbone, F., Montecucco, F., & Sahebkar, A. (2020). Current and Emerging Treatments for Neonatal Sepsis. *Https://Doi-Org.Ezproxy.Auckland.Ac.Nz/10.1080/14656566.2020.1721464*. https://doi.org/10.1080/14656566.2020.1721464

Cavicchiolo, M. E., Magnani, M., Calgaro, S., Bonadies, L., Castagliulo, I., Morelli, L., … Baraldi, E. (2019). Neonatal Sepsis Associated with Lactobacillus Supplementation. *Journal of Perinatal Medicine*, *48*, 87–88.

Centers for Disease Control and Prevention, N. C. for E. and Z. I. D. (NCEZID), D. of F. W. and E. D. (DFWED). (2015, February 20). Fatal Gastrointestinal Mucormycosis in an Infant Following Use of Contaminated ABC Dophilus Powder From Solgar Inc. | Fungal Diseases | CDC. Retrieved April 25, 2020, from https://www.cdc.gov/fungal/outbreaks/rhizopus-investigation.html

Chang, H. Y., Chen, J. H., Chang, J. H., Lin, H. C., Lin, C. Y., & Peng, C. C. (2017). Multiple Strains Probiotics Appear to be the Most Effective Probiotics in the Prevention of Necrotizing Enterocolitis and Mortality: An Updated Meta-Analysis. *PLoS ONE*, *12*. https://doi.org/10.1371/journal.pone.0171579

Chi, C., Buys, N., Li, C., Sun, J., & Yin, C. (2019). Effects of Prebiotics on Sepsis, Necrotizing Enterocolitis, Mortality, Feeding Intolerance, Time to Full Enteral Feeding, Length of Hospital Stay, and Stool Frequency in Preterm Infants: a Meta-Analysis. *European Journal of Clinical Nutrition*, *73*, 657–670.

Chiang, M. C., Chen, C. L., Feng, Y., Chen, C. C., Lien, R., & Chiu, C. H. (2021). Lactobacillus rhamnosus Sepsis Associated With Probiotic Therapy in an Extremely Preterm Infant: Pathogenesis and a Review for Clinicians. *Journal of Microbiology, Immunology and Infection*, *54*, 575–580.

Chollat, C., Sentilhes, L., & Marret, S. (2019). Protection of Brain Development by Antenatal Magnesium Sulphate for Infants Born Preterm. *Developmental Medicine & Child Neurology*, *61*, 25–30.

Clark, A. E., Kaleta, E. J., Arora, A., & Wolk, D. M. (2013). Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry: a Fundamental Shift in the Routine Practice of Clinical Microbiology. *Clinical Microbiology Reviews*, *26*, 547.

Cockburn, D. W., & Koropatkin, N. M. (2016). Polysaccharide Degradation by the Intestinal Microbiota and Its Influence on Human Health and Disease. *Journal of Molecular Biology*, *428*, 3230–3252.

Coppa, G. v., Bruni, S., Morelli, L., Soldi, S., & Gabrielli, O. (2004). The First Prebiotics in Humans: Human Milk Oligosaccharides. *Journal of Clinical Gastroenterology*, *38*. https://doi.org/10.1097/01.MCG.0000128926.14285.25

Costeloe, K., Hardy, P., Juszczak, E., Wilks, M., & Millar, M. R. (2016). Bifidobacterium breve BBG-001 in Very Preterm Infants: A Randomised Controlled Phase 3 Trial. *The Lancet*, *387*, 649–660.

Course, C., & Chakraborty, M. (2016). Respiratory Support for Preterm Infants - the Cochrane Evidence and Beyond. *Paediatrics and Child Health (United Kingdom)*, *26*, 147–151.

Dani, C., Coviello, C., Corsini, I., Arena, F., Antonelli, A., & Rossolini, G. M. (2016). Lactobacillus Sepsis and Probiotic Therapy in Newborns: Two New Cases and Literature Review. *American Journal of Perinatology Reports*, *6*, 25–29.

Dasgupta, S., Erturk-Hasdemir, D., Ochoa-Reparaz, J., Reinecker, H. C., & Kasper, D. L. (2014). Plasmacytoid Dendritic Cells Mediate Anti-Inflammatory Responses to a Gut Commensal Molecule via Both Innate and Adaptive Mechanisms. *Cell Host and Microbe*, *15*, 413–423.

Davidoff, M. J., Dias, T., Damus, K., Russell, R., Bettegowda, V. R., Dolan, S., … Petrini, J. (2006). Changes in the Gestational Age Distribution Among U.S. Singleton Births: Impact on Rates of Late Preterm Birth, 1992 to 2002. *Seminars in Perinatology*, *30*, 8–15.

Davidson, L., & Berkelhamer, S. (2017). Bronchopulmonary Dysplasia: Chronic Lung Disease of Infancy and Long-Term Pulmonary Outcomes. *Journal of Clinical Medicine*, *6*, 4.

Davis, C. (2014). Enumeration of Probiotic Strains: Review of Culture-Dependent and Alternative Techniques to Quantify Viable Bacteria. *Journal of Microbiological Methods*, Vol. 103, pp. 9–17. Elsevier.

de Simone, C. (2019). The Unregulated Probiotic Market. *Clinical Gastroenterology and Hepatology*, *17*, 809–817.

Deshmukh, M., & Patole, S. (2021a). Current Status of Probiotics for Preterm Infants. *Indian Journal of Pediatrics 2021 88:7*, *88*, 703–708.

Deshmukh, M., & Patole, S. (2021b). Prophylactic Probiotic Supplementation for Preterm Neonates - A Systematic Review and Meta-Analysis of Nonrandomized Studies. *Advances in Nutrition*, *12*, 1411–1423.

Deshpande, G. C., Rao, S. C., Keil, A. D., & Patole, S. K. (2011). Evidence-Based Guidelines for Use of Probiotics in Preterm Neonates. *BMC Medicine*, *9*, 1–13.

Dickson, R. P., Erb-Downward, J. R., Freeman, C. M., McCloskey, L., Falkowski, N. R., Huffnagle, G. B., & Curtis, J. L. (2017). Bacterial Topography of the Healthy Human Lower Respiratory Tract. *MBio*, *8*. https://doi.org/10.1128/MBIO.02287-16

Didari, T., Mozaffari, S., Nikfar, S., & Abdollahi, M. (2015). Effectiveness of Probiotics in Irritable Bowel Syndrome: Updated Systematic Review with Meta-Analysis. *World Journal of Gastroenterology : WJG*, *21*, 3072.

Dixon, A., Robertson, K., Yung, A., Que, M., Randall, H., Wellalagodage, D., … Sun, J. (2020). Efficacy of Probiotics in Patients of Cardiovascular Disease Risk: a Systematic Review and Meta-analysis. *Current Hypertension Reports 2020 22:9*, *22*, 1–27.

Drago, L., de vecchi, E., Nicola, L., Colombo, A., & Gismondo, M. (2004). Microbiological Evaluation of Commercial Probiotic Products Available in Italy. *Journal of Chemotherapy*, *16*, 463–467.

Drago, L., Rodighiero, V., Celeste, T., Rovetto, L., & de Vecchi, E. (2010). Microbiological Evaluation of Commercial Probiotic Products Available in the USA in 2009. *Journal of Chemotherapy*, *22*, 373–377.

Drisko, J., Bischoff, B., Giles, C., Adelson, M., Rao, R.-V. S., & Mccallum, R. (2005). Evaluation of Five Probiotic Products for Label Claims by DNA Extraction and Polymerase Chain Reaction Analysis. *Digestive Diseases and Sciences*, *50*, 1113–1117.

Egan, C. E., Sodhi, C. P., Good, M., Lin, J., Jia, H., Yamaguchi, Y., … Hackam, D. J. (2016). Toll-like Receptor 4-Mediated Lymphocyte Influx Induces Neonatal Necrotizing Enterocolitis. *The Journal of Clinical Investigation*, *126*, 495–508.

Elliot, E., & Teversham, K. (2004). An Evaluation of Nine Probiotics Available in South Africa, August 2003. *South African Medical Journal = Suid-Afrikaanse Tydskrif Vir Geneeskunde*, *94*, 121–124.

Ely, D. M., & Driscoll, A. K. (2019). *Infant Mortality in the United States, 2017: Data From the Period Linked Birth/Infant Death File* (Vol. 68). Retrieved from https://www.cdc.gov/nchs/products/index.htm.

Esaiassen, E., Cavanagh, P., Hjerde, E., Simonsen, G. S., Støen, R., & Klingenberg, C. (2016). Bifidobacterium longum subspecies infantis Bacteremia in 3 Extremely Preterm Infants Receiving Probiotics. *Emerging Infectious Diseases*, *22*, 1664–1666.

Esaiassen E, Cavanagh P, Hjerde E, Simonsen GS, Støen R, K. C. B. longum S. infantis B. in 3 E. P. I. R. Probiotics. E. I. Dis. 2016;22(9):1664–1666. doi:10. 3201/eid2209. 160033. (2016). Bifidobacterium longum subspecies infantis bacteremia in 3 extremely preterm infants receiving probiotics. *Emerging Infectious Diseases*, *22*, 1664–1666.

Eurofins Scientific. (2021). Scientific Laboratory - Lab Testing Services - NZ| Eurofins. Retrieved April 16, 2020, from https://www.eurofins.co.nz/

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., … Doré, J. (2010). Intestinal Microbiota of 6-Week-Old Infants Across Europe: Geographic Influence Beyond Delivery Mode, Breast-Feeding, and Antibiotics. *Journal of Pediatric Gastroenterology and Nutrition*, *51*, 77–84.

Fasoli, S., Marzotto, M., Rizzotti, L., Rossi, F., Dellaglio, F., & Torriani, S. (2003). Bacterial Composition of Commercial Probiotic Products as Evaluated by PCR-DGGE Analysis. *International Journal of Food Microbiology*, *82*, 59–70.

Fenster, K., Freeburg, B., Hollard, C., Wong, C., Laursen, R. R., & Ouwehand, A. C. (2019). The Production and Delivery of Probiotics: A Review of a Practical Approach. *Microorganisms 2019, Vol. 7, Page 83*, *7*, 83.

Fenton, T. R. (2003). A New Growth Chart for Preterm Babies: Babson and Benda's Chart Updated with Recent Data and a New Format. *BMC Pediatrics*, *3*, 1–10.

Fiore, W., Arioli, S., & Guglielmetti, S. (2020). The Neglected Microbial Components of Commercial Probiotic Formulations. *Microorganisms 2020, Vol. 8, Page 1177*, *8*, 1177.

Fleming, P. F., Berrington, J. E., & Jacobs, S. E. (2019). Addressing Safety Concerns of Probiotic Use in Preterm Babies. *Early Human Development*, *135*, 72–74.

Fogarty, M., Osborn, D. A., Askie, L., Seidler, A. L., Hunter, K., Lui, K., … Tarnow-Mordi, W. (2018). Delayed vs Early Umbilical Cord Clamping for Preterm Infants: A Systematic Review and Meta-Analysis. *American Journal of Obstetrics and Gynecology*, *218*, 1–18.

Fouhy, F., Guinane, C. M., Hussey, S., Wall, R., Ryan, C. A., Dempsey, E. M., … Cotter, P. D. (2012). High-Throughput Sequencing Reveals the Incomplete, Short-Term Recovery of Infant Gut Microbiota Following Parenteral Antibiotic Treatment with Ampicillin and Gentamicin. *Antimicrobial Agents and Chemotherapy*, *56*, 5811–5820.

Frei, R., Akdis, M., & O'Mahony, L. (2015). Prebiotics, Probiotics, Synbiotics, and the Immune System: Experimental Data and Clinical Evidence. *Current Opinion in Gastroenterology*, *31*, 153–158.

Gabriel, I., Olejek, A., Stencel-Gabriel, K., & Wielgoś, M. (2018). The Influence of Maternal Vaginal Flora on the Intestinal Colonization in Newborns and 3-Month-Old Infants. *The Journal of Maternal-Fetal & Neonatal Medicine : The Official Journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians*, *31*, 1448–1453.

Gale, C., Modi, N., Jawad, S., Culshaw, L., Dorling, J., Bowler, U., … Juszczak, E. (2019). The WHEAT pilot trial - WithHolding Enteral feeds around packed red cell Transfusion to prevent necrotising enterocolitis in preterm neonates: A multicentre, electronic patient record (EPR), randomised controlled point-of-care pilot trial. *BMJ Open*, *9*. https://doi.org/10.1136/bmjopen-2019-033543

GAMA Healthcare Ltd. (2021). Clinell | Universal Wipes 200. Retrieved November 24, 2021, from https://gamahealthcare.com/products/universal-wipes-200

Ganguly, N. K., Bhattacharya, S. K., Sesikeran, B., Nair, G. B., Ramakrishna, B. S., Sachdev, H. P. S., … Hemalatha, R. (2011). ICMR-DBT Guidelines for Evaluation of Probiotics in Food. *Indian Journal of Medical Research*, *134*, 22–25.

Garg, P. M., Paschal, J. L., Zhang, M., Pippins, M., Matthews, A., Adams, K., … Hillegass, W. B. (2021). Brain Injury in Preterm Infants with Surgical Necrotizing Enterocolitis: Clinical and Bowel Pathological Correlates. *Pediatric Research 2021*, 1–14.

Gengaimuthu, K. (2018). The Cross Contamination (Cross Colonization) Phenomenon of Probiotic Use in Neonatal Intensive Care Units: Putative Mechanisms and Clinical and Research Implications. *Cureus*, *10*. https://doi.org/10.7759/cureus.2691

Gephart, S. M., McGrath, J. M., Effken, J. A., & Halpern, M. D. (2012). Necrotizing Enterocolitis Risk State of the Science. *Advances in Neonatal Care*, *12*, 77–87.

Gibbons, S. M., & Gilbert, J. A. (2015). Microbial Diversity — Exploration of Natural Ecosystems and Microbiomes. *Current Opinion in Genetics & Development*, *35*, 66–72.

Gibson, G., Hutkins, R., Sanders, M., Prescott, S., Reimer, R., Salminen, S., … Reid, G. (2017). Expert Consensus Document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) Consensus Statement on the Definition and Scope of Prebiotics. *Nature Reviews Gastroenterology & Hepatology*, *14*, 493–493.

Glassner, K. L., Abraham, B. P., & Quigley, E. M. M. (2020). The Microbiome and Inflammatory Bowel Disease. *Journal of Allergy and Clinical Immunology*, *145*, 16–27.

Goldenberg, J. Z., Yap, C., Lytvyn, L., Lo, C. K. F., Beardsley, J., Mertz, D., & Johnston, B. C. (2017). Probiotics for the Prevention of Clostridium difficile-Associated Diarrhea in Adults and Children. *Cochrane Database of Systematic Reviews*, *2017*. https://doi.org/10.1002/14651858.CD006095.PUB4/MEDIA/CDSR/CD006095/IMAGE_N/NCD006095-CMP-001-48.PNG

Goldenberg, R. L., Culhane, J. F., Iams, J. D., & Romero, R. (2008). Epidemiology and Causes of Preterm Birth. *The Lancet*, *371*, 75–84.

Good, M., Sodhi, C. P., Egan, C. E., Afrazi, A., Jia, H., Yamaguchi, Y., … Hackam, D. J. (2015). Breast Milk Protects Against the Development of Necrotizing Enterocolitis Through Inhibition of Toll-Like Receptor 4 in the Intestinal Epithelium via Activation of the Epidermal Growth Factor Receptor. *Mucosal Immunology 2015 8:5*, *8*, 1166–1179.

Granger, C. L., Embleton, N. D., Palmer, J. M., Lamb, C. A., Berrington, J. E., & Stewart, C. J. (2021). Maternal Breastmilk, Infant Gut Microbiome and the Impact on Preterm Infant Health. *Acta Paediatrica*, *110*, 450–457.

Guenther, K., Straube, E., Pfister, W., Guenther, A., & Huebler, A. (2010). Severe Sepsis After Probiotic Treatment With Escherichia coli NISSLE 1917. *The Pediatric Infectious Disease Journal*, *29*, 188–189.

Guo, Q., Goldenberg, J. Z., Humphrey, C., el Dib, R., & Johnston, B. C. (2019). Probiotics for the Prevention of Pediatric Antibiotic-Associated Diarrhea. *Cochrane Database of Systematic Reviews*. https://doi.org/10.1002/14651858.CD004827.PUB5/MEDIA/CDSR/CD004827/IMAGE_N/NCD004827-CMP-001-18.PNG

Habil, N., Abate, W., Beal, J., & Foey, A. D. (2014). Heat-killed Probiotic Bacteria Differentially Regulate Colonic Epithelial Cell Production of Human β-defensin-2: Dependence on Inflammatory Cytokines. *Beneficial Microbes*, *5*, 483–495.

Hackam, D. J., Good, M., & Sodhi, C. P. (2013). Mechanisms of Gut Barrier Failure in the Pathogenesis of Necrotizing Enterocolitis: Toll-like Receptors Throw the Switch. *Seminars in Pediatric Surgery*, *22*, 76–82.

Hackam, D. J., & Sodhi, C. P. (2022). Bench to Bedside - New Insights into the Pathogenesis of Necrotizing Enterocolitis. *Nature Reviews. Gastroenterology & Hepatology*. https://doi.org/10.1038/S41575-022-00594-X

Halloran, K., & Underwood, M. A. (2019). Probiotic Mechanisms of Action. *Early Human Development*, *135*, 58–65.

Halpern, M. D., & Denning, P. W. (2015). The Role of Intestinal Epithelial Barrier Function in the Development of NEC. *Tissue Barriers*, *3*. https://doi.org/10.1080/21688370.2014.1000707

Hammack, T., Lampel, K., & Chen, Y. (1998). BAM Chapter 29: Cronobacter | FDA. In *Bacteriological Analytical Manual* (Eighth). Retrieved from https://www.fda.gov/food/laboratory-methods-food/bam-chapter-29-cronobacter

Hamrick, S. E. G., & Hansmann, G. (2010). Patent Ductus Arteriosus of the Preterm Infant. *Pediatrics*, *125*, 1020–1030.

Härtel, C., Pagel, J., Rupp, J., Bendiks, M., Guthmann, F., Rieger-Fackeldey, E., … Göpel, W. (2014). Prophylactic Use of Lactobacillus acidophilus/Bifidobacterium infantis Probiotics and Outcome in Very Low Birth Weight Infants. *The Journal of Pediatrics*, *165*. https://doi.org/10.1016/J.JPEDS.2014.04.029

Hau, E. M., Meyer, S. C., Berger, S., Goutaki, M., Kordasz, M., & Kessler, U. (2019). Gastrointestinal Sequelae After Surgery for Necrotising Enterocolitis: A Systematic Review and Meta-Analysis. *Archives of Disease in Childhood: Fetal and Neonatal Edition*, *104*, F265–F273.

Health Products Regulation Group. (2019, December 13). Australian Regulatory Guidelines for Advertising Therapeutic Goods (ARGATG): Advertising interface products | Therapeutic Goods Administration (TGA). Retrieved April 16, 2020, from https://www.tga.gov.au/book-page/advertising-interface-products

Henderickx, J. G. E., Zwittink, R. D., van Lingen, R. A., Knol, J., & Belzer, C. (2019a). The Preterm Gut Microbiota: An Inconspicuous Challenge in Nutritional Neonatal Care. *Frontiers in Cellular and Infection Microbiology*, *9*, 85.

Henderickx, J. G. E., Zwittink, R. D., van Lingen, R. A., Knol, J., & Belzer, C. (2019b). The Preterm Gut Microbiota: An Inconspicuous Challenge in Nutritional Neonatal Care. *Frontiers in Cellular and Infection Microbiology*, *9*. https://doi.org/10.3389/fcimb.2019.00085

Hennequin, C., Kauffmann-Lacroix, C., Jobert, A., Viard, J. P., Ricour, C., Jacquemin, J. L., & Berche, P. (2000). Possible Role of Catheters in Saccharomyces boulardii Fungemia. *Eur J Clin Microbiol Infect Dis*, *19*, 16–20.

Hickey, L., Garland, S. M., Jacobs, S. E., O'Donnell, C. P. F., & Tabrizi, S. N. (2014). Cross-Colonization of Infants with Probiotic Organisms in a Neonatal Unit. *Journal of Hospital Infection*, *88*, 226–229.

Hilgendorff, A., Reiss, I., Ehrhardt, H., Eickelberg, O., & Alvira, C. M. (2014). Chronic Lung Disease in the Preterm Infant: Lessons Learned from Animal Models. *American Journal of Respiratory Cell and Molecular Biology*, *50*, 233–245.

Hilty, M., Burke, C., Pedro, H., Cardenas, P., Bush, A., Bossley, C., … Cookson, W. O. C. (2010). Disordered Microbial Communities in Asthmatic Airways. *PLoS ONE*, *5*. https://doi.org/10.1371/JOURNAL.PONE.0008578

Himpens, E., van den Broeck, C., Oostra, A., Calders, P., & Vanhaesebrouck, P. (2008). Prevalence, Type, Distribution, and Severity of Cerebral Palsy in Relation to Gestational Age: A Meta-Analytic Review. *Developmental Medicine and Child Neurology*, *50*, 334–340.

Holscher, H. D. (2017). Dietary Fiber and Prebiotics and the Gastrointestinal Microbiota. *Https://Doi.Org/10.1080/19490976.2017.1290756*, *8*, 172–184.

Hoyos, A. B. (1999). Reduced Incidence of Necrotizing Enterocolitis Associated with Enteral Administration of Lactobacillus acidophilus and Bifidobacterium infantis to Neonates in an Intensive Care Unit. *International Journal of Infectious Diseases*, *3*, 197–202.

Hull, M. A., Fisher, J. G., Gutierrez, I. M., Jones, B. A., Kang, K. H., Kenny, M., … Jaksic, T. (2014). Mortality and Management of Surgical Necrotizing Enterocolitis in Very Low Birth Weight Neonates: A Prospective Cohort Study. *Journal of the American College of Surgeons*, *218*, 1148–1155.

Hummel, S., Veltman, K., Cichon, C., Sonnenborn, U., & Schmidt, M. A. (2012). Differential Targeting of the E-Cadherin/β-Catenin Complex by Gram-Positive Probiotic Lactobacilli Improves Epithelial Barrier Function. *Applied and Environmental Microbiology*, *78*, 1140–1147.

Huys, G., Vancanneyt, M., D'Haene, K., Vankerckhoven, V., Goossens, H., & Swings, J. (2006). Accuracy of Species Identity of Commercial Bacterial Cultures Intended for Probiotic or Nutritional Use. *Research in Microbiology*, *157*, 803–810.

Imberty, A., Lortat-Jacob, H., & Pérez, S. (2007). Structural View of Glycosaminoglycan–Protein Interactions. *Carbohydrate Research*, *342*, 430–439.

International Organization for Standardization. (2006). ISO - ISO 20128:2006 - Milk Products — Enumeration of Presumptive Lactobacillus acidophilus on a Selective Medium — Colony-Count Technique at 37 Degrees C.

International Organization for Standardization. (2017a). ISO - ISO 6579-1:2017 - Microbiology of the Food Chain — Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella — Part 1: Detection of Salmonella spp.

International Organization for Standardization. (2017b). ISO - ISO 11290-1:2017 - Microbiology of the Food Chain — Horizontal Method for the Detection and Enumeration of Listeria monocytogenes and of Listeria spp. — Part 1: Detection Method.

Jackson, S. A., Schoeni, J. L., Vegge, C., Pane, M., Stahl, B., Bradley, M., … Sanders, M. E. (2019). Improving End-User Trust in the Quality of Commercial Probiotic Products. *Frontiers in Microbiology*, *10*, 739.

Jacobs, S. E., Hickey, L., Donath, S., Opie, G. F., Anderson, P. J., Garland, S. M., … Buksh, M. J. (2017a). Probiotics, Prematurity and Neurodevelopment: Follow-Up of a Randomised Trial. *BMJ Paediatrics Open*, *1*, e000176.

Jacobs, S. E., Hickey, L., Donath, S., Opie, G. F., Anderson, P. J., Garland, S. M., … Buksh, M. J. (2017b). Probiotics, Prematurity and Neurodevelopment: Follow-Up of a Randomised Trial. *BMJ Paediatrics Open*, *1*. https://doi.org/10.1136/BMJPO-2017-000176

Jacobs, S. E., Tobin, J. M., Opie, G. F., Donath, S., Tabrizi, S. N., Pirotta, M., … Garland, S. M. (2013, December). Probiotic Effects on Late-Onset Sepsis in Very Preterm Infants: A Randomized Controlled Trial. *Pediatrics*, Vol. 132, pp. 1055–1062.

Jahanshahi, M., Maleki Dana, P., Badehnoosh, B., Asemi, Z., Hallajzadeh, J., Mansournia, M. A., … Chaichian, S. (2020). Anti-Tumor Activities of Probiotics in Cervical Cancer. *Journal of Ovarian Research 2020 13:1*, *13*, 1–11.

Janvier, A., Malo, J., & Barrington, K. J. (2014). Cohort Study of Probiotics in a North American Neonatal Intensive Care Unit. *The Journal of Pediatrics*, *164*, 980–985.

Jenke, Ruf, Hoppe, Heldmann, & Wirth. (2012). Bifidobacterium Septicaemia in an Extremely Low-Birthweight Infant Under Probiotic Therapy. *Archives of Disease in Childhood. Fetal and Neonatal Edition*, *97*. https://doi.org/10.1136/ARCHDISCHILD-2011-300838

Jilling, T., Simon, D., Lu, J., Meng, F. J., Li, D., Schy, R., … Caplan, M. S. (2006). The Roles of Bacteria and TLR4 in Rat and Murine Models of Necrotizing Enterocolitis. *Journal of Immunology (Baltimore, Md. : 1950)*, *177*, 3273.

Johnson, S., Moore, T., & Marlow, N. (2014). Using the Bayley-III To Assess Neurodevelopmental Delay: Which Cut-Off Should Be Used? *Pediatric Research*, *75*, 670–674.

Jones, L. J., Craven, P. D., Attia, J., Thakkinstian, A., & Wright, I. (2011). Network Meta-Analysis of Indomethacin versus Ibuprofen versus Placebo for PDA in Preterm Infants. *Archives of Disease in Childhood: Fetal and Neonatal Edition*, *96*, F45–F52.

Karimi, K., Kandiah, N., Chau, J., Bienenstock, J., & Forsythe, P. (2012). A Lactobacillus rhamnosus Strain Induces a Heme Oxygenase Dependent Increase in Foxp3+ Regulatory T Cells. *PLoS ONE*, *7*, 47556.

Kim, S. O., Ah, Y. M., Yu, Y. M., Choi, K. H., Shin, W. G., & Lee, J. Y. (2014). Effects of Probiotics for the Treatment of Atopic Dermatitis: A Meta-Analysis of Randomized Controlled Trials. *Annals of Allergy, Asthma & Immunology*, *113*, 217–226.

King, A., & Phillips, I. (1996). Evaluation of the Rapid ID 32A System for the Identification of the Bacteroides fragilis Group. *Clinical Microbiology and Infection*, *2*, 115–122.

Koch, R. (1890). An Address on Bacteriological Research. *British Medical Journal*, *2*, 380.

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., … Ley, R. E. (2011). Succession of Microbial Consortia in the Developing Infant Gut Microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *108 Suppl 1*, 4578–4585.

Koirala, S., & Anal, A. K. (2021). Probiotics-Based Foods and Beverages as Future Foods and their Overall Safety and Regulatory Claims. *Future Foods*, *3*, 100013.

Kona, S. K., & Matlock, D. N. (2018). Probiotics, Prebiotics, and Synbiotics for Preterm Neonates. *NeoReviews*, *19*, e654–e663.

Konieczna, P., Ferstl, R., Ziegler, M., Frei, R., Nehrbass, D., Lauener, R. P., … O'Mahony, L. (2013). Immunomodulation by Bifidobacterium infantis 35624 in the Murine Lamina Propria Requires Retinoic Acid-Dependent and Independent Mechanisms. *PLoS ONE*, *8*, 62617.

Kornacki, J. L., Gurtler, J. B., & Stawick, B. A. (2015a). Enterobacteriaceae, Coliforms, and Escherichia coli as Quality and Safety Indicators . In Yvonne Salfinger & M. lou Tortorello

(Eds.), *Compendium of Methods for the Microbiological Examination of Foods* (Fifth, pp. 110–116). American Public Health Association.

Kornacki, J. L., Gurtler, J. B., & Stawick, B. A. (2015b). Enterococcus species. In Yvonne Salfinger & M. lou Tortorella (Eds.), *Compendium of Methods for the Microbiological Examination of Foods* (Fifth, pp. 124–124). American Public Health Association.

Korpela, K., Blakstad, E. W., Moltu, S. J., Strømmen, K., Nakstad, B., Rønnestad, A. E., … de Vos, W. (2018). Intestinal Microbiota Development and Gestational Age in Preterm Neonates. *Scientific Reports 2018 8:1*, *8*, 1–9.

Kovler, M. L., Gonzalez Salazar, A. J., Fulton, W. B., Lu, P., Yamaguchi, Y., Zhou, Q., … Hackam, D. J. (2021). Toll-like Receptor 4-Mediated Enteric Glia Loss is Critical for the Development of Necrotizing Enterocolitis. *Science Translational Medicine*, *13*. https://doi.org/10.1126/SCITRANSLMED.ABG3459

Kulkarni, T., Majarikar, S., Deshmukh, M., Ananthan, A., Balasubramanian, H., Keil, A., & Patole, S. (2022). Probiotic Sepsis in Preterm Neonates—A Systematic Review. *European Journal of Pediatrics 2022*, 1–14.

Kunz, A., Noel, J., & Fairchok, M. (2004). Two Cases of Lactobacillus Bacteremia During Probiotic Treatment of Short Gut Syndrome. *Journal of Pediatric Gastroenterology and Nutrition*, *38*, 457–458.

Laboratorio Farmaceutico SIT S.r.l. (2019, January 18). *Infloran Product Information Sheet*. Retrieved from https://www.infloran.com.au/wp-content/uploads/2019/04/F_Infloran_Australia-PIL.pdf

Lacroix, C., & Yildirim, S. (2007). Fermentation Technologies for the Production of Probiotics with High Viability and Functionality. *Current Opinion in Biotechnology*, *18*, 176–183.

Land, M. H., Rouster-Stevens, K., Woods, C. R., Cannon, M. L., Cnota, J., & Shetty, A. K. (2005). Lactobacillus sepsis associated with probiotic therapy. *Pediatrics*, *115*, 178–181.

Laptook, A. R., Bell, E. F., Shankaran, S., Boghossian, N. S., Wyckoff, M. H., Kandefer, S., … Wiggins, S. A. (2018). Admission Temperature and Associated Mortality and Morbidity among Moderately and Extremely Preterm Infants. *Journal of Pediatrics*, *192*, 53-59.e2.

Laptook, A. R., Salhab, W., & Bhaskar, B. (2007). Admission Temperature of Low Birth Weight Infants: Predictors and Associated Morbidities. *Pediatrics*, *119*, e643–e649.

Larroque, B., Ancel, P. Y., Marret, S., Marchand, L., André, M., Arnaud, C., … Kaminski, M. (2008). Neurodevelopmental Disabilities and Special Care of 5-year-old Children Born Before 33 weeks of Gestation (the EPIPAGE study): a Longitudinal Cohort Study. *The Lancet*, *371*, 813–820.

Lau, C., Smith, E., & Schanler, R. (2007). Coordination of Suck-Swallow and Swallow Respiration in Preterm Infants. *Acta Paediatrica*, *92*, 721–727.

Laughon, M., O'Shea, M. T., Allred, E. N., Bose, C., Kuban, K., van Marter, L. J., … Leviton, A. (2009). Chronic Lung Disease and Developmental Delay at 2 Years of Age in Children Born Before 28 Weeks' Gestation. *Pediatrics*, *124*, 637–648.

Leaphart, C. L., Cavallo, J., Gribar, S. C., Cetin, S., Li, J., Branca, M. F., … Hackam, D. J. (2007). A Critical Role for TLR4 in the Pathogenesis of Necrotizing Enterocolitis by Modulating Intestinal Injury and Repair. *The Journal of Immunology*, *179*, 4808–4820.

Lee, J. S., & Polin, R. A. (2003). Treatment and Prevention of Necrotizing Enterocolitis. *Seminars in Neonatology*, *8*, 449–459.

Lewis, Z. T., Shani, G., Masarweh, C. F., Popovic, M., Frese, S. A., Sela, D. A., … Mills, D. A. (2015). Validating Bifidobacterial Species and Subspecies Identity in Commercial Probiotic Products. *Pediatric Research 2016 79:3*, *79*, 445–452.

Lin, P. W., & Stoll, B. J. (2006). Necrotising Enterocolitis. *Lancet*, *368*, 1271–1283.

Liu, H., Wang, B., Lu, T., & Pei, Y. (2022). Safety and Efficacy of Probiotics in the Prevention of Necrotizing Enterocolitis in Premature and/or Low-Birthweight Infants: A Systematic Review and Meta-Analysis. *Translational Pediatrics*, *11*, 249–259.

Lokku, A., Mirea, L., Lee, S. K., & Shah, P. S. (2017). Trends and Outcomes of Patent Ductus Arteriosus Treatment in Very Preterm Infants in Canada. *American Journal of Perinatology*, *34*, 441–450.

Lungarotti, M. S., Mezzetti, D., & Radicioni, M. (2003). Methaemoglobinaemia with Concurrent Blood Isolation of Saccharomyces and Candida. *Archives of Disease in Childhood. Fetal and Neonatal Edition*, *88*. https://doi.org/10.1136/FN.88.5.F446

Lyu, Y., Shah, P. S., Ye, X. Y., Warre, R., Piedboeuf, B., Deshpandey, A., … Kajetanowicz, A. (2015). Association Between Admission Temperature and Mortality and Major Morbidity in Preterm Infants Born at Fewer Than 33weeks' Gestation. *JAMA Pediatrics*, *169*, 150277.

Maffei, D., & Schanler, R. J. (2017). Human Milk is the Feeding Strategy to Prevent Necrotizing Enterocolitis! *Seminars in Perinatology*, *41*, 36–40.

Maheshwari, A., Patel, R. M., & Christensen, R. D. (2018). Anemia, Red Blood Cell Transfusions, and Necrotizing Enterocolitis. *Seminars in Pediatric Surgery*, *27*, 47–51.

Manning, T. S., & Gibson, G. R. (2004). Prebiotics. *Best Practice and Research: Clinical Gastroenterology*, *18*, 287–298.

Marchesi, J. R., & Ravel, J. (2015). The Vocabulary of Microbiome Research: A Proposal. *Microbiome 2015 3:1*, *3*, 1–3.

Markopoulou, P., Papanikolaou, E., Analytis, A., Zoumakis, E., & Siahanidou, T. (2019). Preterm Birth as a Risk Factor for Metabolic Syndrome and Cardiovascular Disease in Adult Life: A Systematic Review and Meta-Analysis. *The Journal of Pediatrics*, *210*, 69-80.e5.

Markowiak-Kopeć, P., & Śliżewska, K. (2020). The Effect of Probiotics on the Production of Short-Chain Fatty Acids by Human Intestinal Microbiome. *Nutrients 2020, Vol. 12, Page 1107*, *12*, 1107.

Martin, S., Duke, T., & Davis, P. (2014). Efficacy and Safety of Bubble CPAP in Neonatal Care in Low and Middle Income Countries: A Systematic Review. *Archives of Disease in Childhood: Fetal and Neonatal Edition*, *99*, F495–F504.

McCrea, H. J., & Ment, L. R. (2008). The Diagnosis, Management, and Postnatal Prevention of Intraventricular Hemorrhage in the Preterm Neonate. *Clinics in Perinatology*, *35*, 777–792.

McGoldrick, E., Stewart, F., Parker, R., & Dalziel, S. R. (2020). Antenatal Corticosteroids for Accelerating Fetal Lung Maturation for Women at Risk of Preterm Birth. *Cochrane Database of Systematic Reviews*, *2021*. https://doi.org/10.1002/14651858.CD004454.PUB4/MEDIA/CDSR/CD004454/IMAGE_N/NCD004454-CMP-002.03.SVG

McGovern, M., Giannoni, E., Kuester, H., Turner, M. A., van den Hoogen, A., Bliss, J. M., … Molloy, E. J. (2020). Challenges in Developing a Consensus Definition of Neonatal Sepsis. *Pediatric Research 2020 88:1*, *88*, 14–26.

Meyer, M. P., Chow, S. S. W., Alsweiler, J., Bourchier, D., Broadbent, R., Knight, D., … Patel, H. (2020). Probiotics for Prevention of Severe Necrotizing Enterocolitis: Experience of New Zealand Neonatal Intensive Care Units. *Frontiers in Pediatrics*, *8*, 119.

Millar, M., & Wilks, M. (2003). Probiotics for Preterm Infants? *Arch Dis Child Fetal Neonatal Ed*, *88*, 354–358.

Ministry of Health. (2019). *Report on maternity 2017*. Wellington. Retrieved from https://www.health.govt.nz/system/files/documents/publications/report-maternity-2017-may19.pdf

Ministry of Health. (2022, March 22). Natural Health Products. Retrieved April 16, 2020, from https://www.health.govt.nz/our-work/regulation-health-and-disability-system/natural-health-products

Mitra, S., Florez, I. D., Tamayo, M. E., Mbuagbaw, L., Vanniyasingam, T., Veroniki, A. A., … Thabane, L. (2018). Association of Placebo, Indomethacin, Ibuprofen, and Acetaminophen With Closure of Hemodynamically Significant Patent Ductus Arteriosus in Preterm Infants a Systematic Review and Meta-Analysis. *JAMA - Journal of the American Medical Association*, *319*, 1221–1238.

Moffatt, M. F., & Cookson, W. O. C. M. (2017). The Lung Microbiome in Health and Disease. *Clinical Medicine*, *17*, 525.

Mohanty, D., Misra, S., Mohapatra, S., & Sahu, P. S. (2018). Prebiotics and Synbiotics: Recent Concepts in Nutrition. *Food Bioscience*, *26*, 152–160.

Mohar Lorbeg, P., Golob, M., Kramer, M., Treven, P., & Bogovič Matijašić, B. (2021). Evaluation of Dietary Supplements Containing Viable Bacteria by Cultivation/MALDI-TOF Mass Spectrometry and PCR Identification. *Frontiers in Microbiology*, *12*, 1824.

Moore, R. E., & Townsend, S. D. (2019). Temporal Development of the Infant Gut Microbiome. *Open Biology*, *9*, 190128.

Moossavi, S., Sepehri, S., Robertson, B., Bode, L., Goruk, S., Field, C. J., … Azad, M. B. (2019). Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors. *Cell Host & Microbe*, *25*, 324-335.e4.

Morgan, R. L., Preidis, G. A., Kashyap, P. C., Weizman, A. v., Sadeghirad, B., Chang, Y., … Zeraatkar, D. (2020). Probiotics Reduce Mortality and Morbidity in Preterm, Low-Birth-Weight Infants: A Systematic Review and Network Meta-analysis of Randomized Trials. *Gastroenterology*, *159*, 467–480.

Mueller, N. T., Bakacs, E., Combellick, J., Grigoryan, Z., & Dominguez-Bello, M. G. (2015). The Infant Microbiome Development: Mom Matters. *Trends in Molecular Medicine*, *21*, 109–117.

Neal, M. D., Sodhi, C. P., Dyer, M., Craig, B. T., Good, M., Jia, H., … Hackam, D. J. (2013). A Critical Role for TLR4 Induction of Autophagy in the Regulation of Enterocyte Migration and the Pathogenesis of Necrotizing Enterocolitis. *Journal of Immunology (Baltimore, Md. : 1950)*, *190*, 3541–3551.

Neal, M. D., Sodhi, C. P., Jia, H., Dyer, M., Egan, C. E., Yazji, I., … Hackam, D. J. (2012). Toll-like Receptor 4 is Expressed on Intestinal Stem Cells and Regulates Their Proliferation and Apoptosis via The p53 Up-Regulated Modulator of Apoptosis. *The Journal of Biological Chemistry*, *287*, 37296–37308.

Newborn Services Clinical Practice Committee, & Alsweiler, J. (2020, November 26). Probiotics (Infloran). Retrieved January 6, 2020, from https://www.starship.org.nz/guidelines/probiotics-infloran/

NICHD Neonatal Research Network. (2012). Transfusion of Prematures Trial - Full Text View - ClinicalTrials.gov. Retrieved July 8, 2020, from https://www.clinicaltrials.gov/ct2/show/NCT01702805

Niemarkt, H. J., de Meij, T. G., van Ganzewinkel, C. J., de Boer, N. K. H., Andriessen, P., Hütten, M. C., & Kramer, B. W. (2019). Necrotizing Enterocolitis, Gut Microbiota, and Brain Development: Role of the Brain-Gut Axis. *Neonatology*, *115*, 423–431.

Nolan, L. S., Goree, M., & Good, M. (2021). An Updated Overview of the Medical Management of Necrotizing Enterocolitis. In D. Hackam (Ed.), *Necrotizing Enterocolitis* (1st Edition, pp. 8–11). CRC Press.

Nongena, P., Ederies, A., Azzopardi, D. v., & Edwards, A. D. (2010). Confidence in the Prediction of Neurodevelopmental Outcome by Cranial Ultrasound and MRI in Preterm Infants. *Archives of Disease in Childhood: Fetal and Neonatal Edition*, *95*, F388–F390.

Ohishi, A., Takahashi, S., Ito, Y., Ohishi, Y., Tsukamoto, K., Nanba, Y., … Nakamura, T. (2010). Bifidobacterium Septicemia Associated With Postoperative Probiotic Therapy in a Neonate With Omphalocele. *Journal of Pediatrics*, *156*, 679–681.

Ohland, C. L., & MacNaughton, W. K. (2010). Probiotic Bacteria and Intestinal Epithelial Barrier Function. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *298*. https://doi.org/10.1152/AJPGI.00243.2009

Olszak, T., An, D., Zeissig, S., Vera, M. P., Richter, J., Franke, A., … Blumberg, R. S. (2012). Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function. *Science*, *336*, 489–493.

Pammi, M., & Abrams, S. A. (2019). Enteral Lactoferrin for the Treatment of Sepsis and Necrotizing Enterocolitis in Neonates. *Cochrane Database of Systematic Reviews*, *2019*, 1–16.

Pannaraj, P. S., Li, F., Cerini, C., Bender, J. M., Yang, S., Rollie, A., … Aldrovandi, G. M. (2017). Association Between Breast Milk Bacterial Communities and Establishment and Development of the Infant Gut Microbiome. *JAMA Pediatrics*, *171*, 647–654.

Pascal, A., Govaert, P., Oostra, A., Naulaers, G., Ortibus, E., & van den Broeck, C. (2018). Neurodevelopmental Outcome in Very Preterm and Very-Low-Birthweight Infants Born Over the Past Decade: a Meta-Analytic Review. *Developmental Medicine and Child Neurology*, *60*, 342–355.

Perez-Muñoz, M. E., Arrieta, M. C., Ramer-Tait, A. E., & Walter, J. (2017). A Critical Assessment of the "Sterile Womb" and "In Utero Colonization" Hypotheses: Implications for Research on the Pioneer Infant Microbiome. *Microbiome 2017 5:1*, *5*, 1–19.

Pillai, A., Tan, J., Paquette, V., & Panczuk, J. (2020). Does Probiotic Bacteremia in Premature Infants Impact Clinically Relevant Outcomes? A case report and updated review of literature. *Clinical Nutrition ESPEN*, *39*, 255–259.

Plummer, E. L., Chebar Lozinsky, A., Tobin, J. M., Uebergang, J. B., Axelrad, C., Garland, S. M., … Tang, M. L. K. (2020). Postnatal Probiotics and Allergic Disease in Very Preterm infants: Sub-Study to the ProPrems Randomized Trial. *Allergy*, *75*, 127–136.

Poindexter, B., Cummings, J., Hand, I., Adams-Chapman, I., Aucott, S. W., Puopolo, K. M., … Mowitz, M. (2021). Use of Probiotics in Preterm Infants. *Pediatrics*, *147*. https://doi.org/10.1542/PEDS.2021-051485/180282

Proctor, L. M. (2016). The National Institutes of Health Human Microbiome Project. *Seminars in Fetal and Neonatal Medicine*, *21*, 368–372.

Quigley, M., Embleton, N. D., & McGuire, W. (2019). Formula Versus Donor Breast Milk for Feeding Preterm or Low Birth Weight Infants. *Cochrane Database of Systematic Reviews*, *2019*. https://doi.org/10.1002/14651858.CD002971.PUB5/MEDIA/CDSR/CD002971/IMAGE_N/NCD002971-CMP-002-18.PNG

Rabe, H., Gyte, G. M. L., Díaz-Rossello, J. L., & Duley, L. (2019). Effect of Timing of Umbilical Cord Clamping and Other Strategies to Influence Placental Transfusion at Preterm Birth on Maternal and Infant Outcomes. *Cochrane Database of Systematic Reviews*, *2019*. https://doi.org/10.1002/14651858.CD003248.PUB4/MEDIA/CDSR/CD003248/IMAGE_N/NCD003248-CMP-003-31.PNG

Rao, S. C., Athalye-Jape, G. K., Deshpande, G. C., Simmer, K. N., & Patole, S. K. (2016). Probiotic Supplementation and Late-Onset Sepsis in Preterm Infants: A Meta-Analysis. *Pediatrics*, *137*. https://doi.org/10.1542/peds.2015-3684

Rao, S. C., Basani, L., Simmer, K., Samnakay, N., & Deshpande, G. (2011). Peritoneal Drainage Versus Laparotomy as Initial Surgical Treatment for Perforated Necrotizing Enterocolitis or Spontaneous Intestinal Perforation in Preterm Low Birth Weight Infants. *Cochrane Database of Systematic Reviews*. https://doi.org/10.1002/14651858.cd006182.pub2

Rerksuppaphol, S., & Rerksuppaphol, L. (2010). Lactobacillus acidophilus and Bifidobacterium bifidum Stored at Ambient Temperature are Effective in the Treatment of Acute Diarrhoea. *Annals of Tropical Paediatrics*, *30*, 299–304.

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C. G., & Salazar, N. (2016). Intestinal Short Chain Fatty Acids and Their Link with Diet and Human Health. *Frontiers in Microbiology*, *7*, 185.

Rossi, M., Mirbagheri, S. e y e d S., Keshavarzian, A., & Bishehsari, F. (2018). Nutraceuticals in Colorectal Cancer: A Mechanistic Approach. *European Journal of Pharmacology*, *833*, 396–402.

Roy, U., Jessani, L. G., Rudramurthy, S. M., Gopalakrishnan, R., Dutta, S., Chakravarty, C., … Chakrabarti, A. (2017). Seven Cases of Saccharomyces fungaemia Related to Use of Probiotics. *Mycoses*, *60*, 375–380.

Sakurai, Y., Watanabe, T., Miura, Y., Uchida, T., Suda, N., Yoshida, M., & Nawa, T. (2022). Clinical and Bacteriologic Characteristics of Six Cases of Bifidobacterium breve Bacteremia Due to Probiotic Administration in the Neonatal Intensive Care Unit. *Pediatric Infectious Disease Journal*, *41*, 62–65.

Salfinger, Y, & Tortorello, M. (2015). Compendium of Methods for the Microbiological Examination of Foods. In Yvonne Salfinger & M. lou Tortorello (Eds.), *Compendium of Methods for the Microbiological Examination of Foods* (Fifth). American Public Health Association.

Sanders, M. E., Benson, A., Lebeer, S., Merenstein, D. J., & Klaenhammer, T. R. (2018). Shared Mechanisms Among Probiotic Taxa: Implications for General Probiotic Claims. *Current Opinion in Biotechnology*, *49*, 207–216.

Sanz, Y., Olivares, M., Moya-Pérez, Á., & Agostoni, C. (2014). Understanding the Role of Gut Microbiome in Metabolic Disease Risk. *Pediatric Research*, *77*, 236–244.

Sato, S., Uchida, T., Kuwana, S., Sasaki, K., Watanabe, T., Saito, J., & Kawaji, T. (2016). Bacteremia Induced by Bifidobacterium breve in a Newborn with Cloacal Exstrophy. *Pediatrics International : Official Journal of the Japan Pediatric Society*, *58*, 1226–1228.

Schloss, P. D., & Handelsman, J. (2004). Status of the Microbial Census. *Microbiology and Molecular Biology Reviews*, *68*, 686–691.

Schmölzer, G. M., Kumar, M., Pichler, G., Aziz, K., O'Reilly, M., & Cheung, P. Y. (2013). Non-invasive Versus Invasive Respiratory Support in Preterm Infants at Birth: Systematic Review and Meta-Analysis. *BMJ (Online)*, *347*. https://doi.org/10.1136/bmj.f5980

Schulzke, S. M., Deshpande, G. C., & Patole, S. K. (2007, June 1). Neurodevelopmental Outcomes of Very Low-Birth-Weight Infants With Necrotizing Enterocolitis: A Systematic Review of Observational Studies. *Archives of Pediatrics and Adolescent Medicine*, Vol. 161, pp. 583–590. American Medical Association.

Schwiertz, A., Gruhl, B., Löbnitz, M., Michel, P., Radke, M., & Blaut, M. (2003). Development of the Intestinal Bacterial Composition in Hospitalized Preterm Infants in Comparison with Breast-Fed, Full-Term Infants. *Pediatric Research*, *54*, 393–399.

Seedat, F., Brown, C. S., Stinton, C., Patterson, J., Geppert, J., Freeman, K., … Taylor-Phillips, S. (2018). Bacterial Load and Molecular Markers Associated with Early-onset Group B Streptococcus: A Systematic Review and Meta-analysis. *Pediatric Infectious Disease Journal*, *37*, E306–E314.

Sestito, S., D'Auria, E., Baldassarre, M. E., Salvatore, S., Tallarico, V., Stefanelli, E., … Pensabene, L. (2020). The Role of Prebiotics and Probiotics in Prevention of Allergic Diseases in Infants. *Frontiers in Pediatrics*, *8*, 870.

Shah, T. A., Meinzen-Derr, J., Gratton, T., Steichen, J., Donovan, E. F., Yolton, K., … Schibler, K. R. (2011). Hospital and Neurodevelopmental Outcomes of Extremely Low-Birth-Weight Infants With Necrotizing Enterocolitis and Spontaneous Intestinal Perforation. *Journal of Perinatology 2012 32:7*, *32*, 552–558.

Shao, Y., Forster, S. C., Tsaliki, E., Vervier, K., Strang, A., Simpson, N., … Lawley, T. D. (2019). Stunted Microbiota and Opportunistic Pathogen Colonization in Caesarean-Section Birth. *Nature*, *574*, 117–121.

Sharif, S., Meader, N., Oddie, S. J., Rojas-Reyes, M. X., & McGuire, W. (2020). Probiotics To Prevent Necrotising Enterocolitis in Very Preterm or Very Low Birth Weight Infants. *The Cochrane Database of Systematic Reviews*, *10*, CD005496.

Shaw, A. G., Sim, K., Rose, G., Wooldridge, D. J., Li, M. S., Misra, R. v., … Kroll, J. S. (2021). Premature Neonatal Gut Microbial Community Patterns Supporting an Epithelial TLR-mediated Pathway for Necrotizing Enterocolitis. *BMC Microbiology*, *21*, 1–11.

Shoaf, K., Mulvey, G. L., Armstrong, G. D., & Hutkins, R. W. (2006). Prebiotic Galactooligosaccharides Reduce Adherence of Enteropathogenic Escherichia coli to Tissue Culture Cells. *Infection and Immunity*, *74*, 6920–6928.

Silverman, W. A., Fertig, J. W., & Berger, A. P. (1958). The Influence of the Thermal Environment Upon the Survival of Newly Born Premature Infants. *Pediatrics*, *22*, 876–886.

Singh, B., Shah, P. S., Afifi, J., Simpson, C. D., Mitra, S., Dow, K., & El-Naggar, W. (2019). Probiotics for Preterm Infants: A National Retrospective Cohort Study. *Journal of Perinatology : Official Journal of the California Perinatal Association*, *39*, 533–539.

Soll, R., & Özek, E. (1997). Prophylactic Animal Derived Surfactant Extract for Preventing Morbidity and Mortality in Preterm Infants. *Cochrane Database of Systematic Reviews*, *1997*. https://doi.org/10.1002/14651858.cd000511

Srinivasjois, R., Rao, S., & Patole, S. (2013). Probiotics, prebiotics, and synbiotics for preterm neonates. *Clinical Nutrition*, *32*, 958–965.

Stewart, D., Benitz, W., Watterberg, K. L., Cummings, J. J., Benitz, W. E., Eichenwald, E. C., … Couto, J. R. (2016). Umbilical Cord Care in the Newborn Infant. *Pediatrics*, *138*. https://doi.org/10.1542/peds.2016-2149

Stinson, L. F., Boyce, M. C., Payne, M. S., & Keelan, J. A. (2019). The Not-So-Sterile Womb: Evidence that the Human Fetus is Exposed to Bacteria Prior to Birth. *Frontiers in Microbiology*, *10*, 1124.

Stinson, L. F., Payne, M. S., & Keelan, J. A. (2018). A Critical Review of the Bacterial Baptism Hypothesis and the Impact of Cesarean Delivery on the Infant Microbiome. *Frontiers in Medicine*, *5*, 1.

Stoll, B. J., Hansen, N., Fanaroff, A. A., Wright, L. L., Carlo, W. A., Ehrenkranz, R. A., … Kenneth Poole, W. (2002). Late-Onset Sepsis in Very Low Birth Weight Neonates: The Experience of the NICHD Neonatal Research Network. *Pediatrics*, *110*, 285–291.

Stoll, B. J., Hansen, N. I., Bell, E. F., Shankaran, S., Laptook, A. R., Walsh, M. C., … Higgins, R. D. (2010). Neonatal Outcomes of Extremely Preterm Infants from the NICHD Neonatal Research Network. *Pediatrics*, *126*, 443–456.

Stratiki, Z., Costalos, C., Sevastiadou, S., Kastanidou, O., Skouroliakou, M., Giakoumatou, A., & Petrohilou, V. (2007). The Effect of a Bifidobacter Supplemented Bovine Milk on Intestinal Permeability of Preterm Infants. *Early Human Development*, *83*, 575–579.

Strauss, R. G. (2010). Anaemia of prematurity: Pathophysiology and treatment. *Blood Reviews*, *24*, 221–225.

Swanson, K. S., Gibson, G. R., Hutkins, R., Reimer, R. A., Reid, G., Verbeke, K., … Sanders, M. E. (2020). The International Scientific Association for Probiotics and Prebiotics (ISAPP) Consensus Statement on the Definition and Scope of Synbiotics. *Nature Reviews Gastroenterology & Hepatology 2020 17:11*, *17*, 687–701.

Sweet, D. G., Carnielli, V., Greisen, G., Hallman, M., Ozek, E., te Pas, A., … Halliday, H. L. (2019). European Consensus Guidelines on the Management of Respiratory Distress Syndrome - 2019 Update. In *Neonatology* (Vol. 115). S. Karger AG.

Symonds, E. L., O'Mahony, C., Lapthorne, S., O'Mahony, D., Sharry, J. mac, O'Mahony, L., & Shanahan, F. (2012). Bifidobacterium infantis 35624 Protects Against Salmonella-Induced Reductions in Digestive Enzyme Activity in Mice by Attenuation of the Host Inflammatory Response. *Clinical and Translational Gastroenterology*, *3*, e15.

Talon, D. (1999). The Role of the Hospital Environment in the Epidemiology of Multi-Resistant Bacteria. *Journal of Hospital Infection*, *43*, 13–17.

Tanabe, S. (2013). The Effect of Probiotics and Gut Microbiota on Th17 Cells. *International Reviews of Immunology*, *32*, 511–525.

Tanaka, S., Kobayashi, T., Songjinda, P., Tateyama, A., Tsubouchi, M., Kiyohara, C., … Nakayama, J. (2009). Influence of Antibiotic Exposure in the Early Postnatal Period on the Development of Intestinal Microbiota. *FEMS Immunology & Medical Microbiology*, *56*, 80–87.

Tan-Lim, C. S. C., & Esteban-Ipac, N. A. R. (2018). Probiotics as Treatment for Food Allergies Among Pediatric Patients: A Meta-Analysis. *World Allergy Organization Journal*, *11*, 1–13.

Tarnow-Mordi, W. O., Abdel-Latif, M. E., Martin, A., Pammi, M., Robledo, K., Manzoni, P., … Espinoza, D. (2020). The Effect of Lactoferrin Supplementation on Death or Major Morbidity in Very Low Birthweight Infants (LIFT): A Multicentre, Double-Blind, Randomised Controlled Trial. *The Lancet Child and Adolescent Health*, *4*, 444–454.

Temmerman, R., Pot, B., Huys, G., & Swings, J. (2003). Identification and Antibiotic Susceptibility of Bacterial Isolates from Probiotic Products. *International Journal of Food Microbiology*, *81*, 1–10.

Theunissen, J., Britz, T. J., Torriani, S., & Witthuhn, R. C. (2005). Identification of Probiotic Microorganisms in South African Products using PCR-based DGGE analysis. *International Journal of Food Microbiology*, *98*, 11–21.

Topcuoglu, S., Gursoy, T., Ovali, F., Serce, O., & Karatekin, G. (2015). A New Risk Factor for Neonatal Vancomycin-Resistant Enterococcus Colonisation: Bacterial Probiotics. *Journal of Maternal-Fetal and Neonatal Medicine*, *28*, 1491–1494.

Tseng, C. H., & Wu, C. Y. (2019). The Gut Microbiome in Obesity. *Journal of the Formosan Medical Association*, *118*, S3–S9.

Vallabhaneni, S., Walker, T., Lockhart, S., Ng, D., Infectious Diseases Pathology Branch, Chiller, T., … Smith, R. (2015). Notes from the Field: Fatal Gastrointestinal Mucormycosis in a Premature Infant Associated with a Contaminated Dietary Supplement — Connecticut, 2014. *Morbidity and Mortality Weekly Report (MMWR)*, *64*, 155–156.

van Bueren, A. L., Saraf, A., Martens, E. C., & Dijkhuizena, L. (2015). Differential Metabolism of Exopolysaccharides from Probiotic Lactobacilli by the Human Gut Symbiont Bacteroides thetaiotaomicron. *Applied and Environmental Microbiology*, *81*, 3973–3983.

van de Casteele, S., Vanheuverzwijn, T., Ruyssen, T., van Assche, P., Swings, J., & Huys, G. (2006). Evaluation of Culture Media for Selective Enumeration of Probiotic Strains of lactobacilli and bifidobacteria in Combination with Yoghurt or Cheese Starters. *International Dairy Journal*, *16*, 1470–1476.

van den Akker, C. H. P., van Goudoever, J. B., Shamir, R., Domellöf, M., Embleton, N. D., Hojsak, I., … Szajewska, H. (2020). Probiotics and Preterm Infants: A Position Paper by the European Society for Paediatric Gastroenterology Hepatology and Nutrition Committee on Nutrition and the European Society for Paediatric Gastroenterology Hepatology and Nutrition Working Group for Pr. *Journal of Pediatric Gastroenterology and Nutrition*, *70*, 664–680.

van den Akker, C. H. P., van Goudoever, J. B., Szajewska, H., Embleton, N. D., Hojsak, I., Reid, D., & Shamir, R. (2018). Probiotics for Preterm Infants: A Strain-Specific Systematic Review and Network Meta-analysis. *Journal of Pediatric Gastroenterology and Nutrition*, *67*, 103–122.

van den Berg, A., Fetter, W. P. F., Westerbeek, E. A. M., van der Vegt, I. M., van der Molen, H. R. A., & van Elburg, R. M. (2006). The Effect of Glutamine-Enriched Enteral Nutrition on Intestinal Permeability in Very-Low-Birth-Weight Infants: A Randomized Controlled Trial. *Journal of Parenteral and Enteral Nutrition*, *30*, 408–414.

van der Beek, C. M., Bloemen, J. G., van den Broek, M. A., Lenaerts, K., Venema, K., Buurman, W. A., & Dejong, C. H. (2015). Hepatic Uptake of Rectally Administered Butyrate Prevents an Increase in Systemic Butyrate Concentrations in Humans. *The Journal of Nutrition*, *145*, 2019–2024.

Venugopalan, V., Shriner, K. A., & Wong-Beringer, A. (2010). Regulatory Oversight and Safety of Probiotic Use. *Emerging Infectious Diseases*, *16*, 1661.

Vermeulen, M. J., Luijendijk, A., Toledo, L., Kaam, A. H., & Reiss, I. K. M. (2020). Quality of Probiotic Products for Preterm Infants: Contamination and Missing Strains. *Acta Paediatrica*, *109*, 276–279.

Vinderola, G., Reinheimer, J., & Salminen, S. (2019). The Enumeration of Probiotic Issues: From Unavailable Standardised Culture Media to a Recommended Procedure? *International Dairy Journal*, *96*, 58–65.

Walsh, K., Whiteman, C., Battin, M., McDougall, J., Harilall, M., O'Brien, M., & McCowan, L. (2011). National Women's Annual Clinical Report 2011. In *2011*. Retrieved from https://drive.google.com/file/d/1u0vN3AOAe8z3mTny4beqT4k9-mEo03fE/view

Walsh, M. C., & Kliegman, R. M. (1986). Necrotizing Enterocolitis: Treatment Based on Staging Criteria. *Pediatric Clinics of North America*, *33*, 179–201.

Wang, L., Cao, H., Liu, L., Wang, B., Walker, W. A., Acra, S. A., & Yan, F. (2014). Activation of Epidermal Growth Factor Receptor Mediates Mucin Production Stimulated by p40, a Lactobacillus rhamnosus GG-Derived Protein. *Journal of Biological Chemistry*, *289*, 20234–20244.

Warner, B. B., & Tarr, P. I. (2016). Necrotizing Enterocolitis and Preterm Infant Gut Bacteria. *Seminars in Fetal and Neonatal Medicine*, *21*, 394–399.

Wassenaar, T. M., Gu¨, G., & Klein, G. (2008). Safety Aspects and Implications of Regulation of Probiotic Bacteria in Food and Food Supplements. *Journal of Food Protection*, *71*, 1734–1741.

Widström, A. M., Brimdyr, K., Svensson, K., Cadwell, K., & Nissen, E. (2019). Skin-to-Skin Contact the First Hour after Birth, Underlying Implications and Clinical Practice. *Acta Paediatrica*, *108*, 1192–1204.

Willemsen, L. E. M., Koetsier, M. A., van Deventer, S. J. H., & van Tol, E. A. F. (2003). Short Chain Fatty Acids Stimulate Epithelial Mucin 2 Expression Through Differential Effects on Prostaglandin E1 and E2 Production by Intestinal Myofibroblasts. *Gut*, *52*, 1442–1447.

Wong, J. M., de Souza, R., Kendall, C., Emam, A., & Jenkins, D. (2006). Colonic Health: Fermentation and Short Chain Fatty Acids. *Journal of Clinical Gastroenterology*, *40*, 235–243.

World Health Organization. (1970). The Prevention of Perinatal Mortality and Morbidity. In *World Health Organization - Technical Report Series* (Vol. 457). Retrieved from https://apps.who.int/iris/bitstream/handle/10665/40797/WHO_TRS_457.pdf?sequence=1&isAllowed=y

World Health Organization. (2012). *The Global Action Report on Preterm Birth Born Too Soon*. Retrieved from https://apps.who.int/iris/bitstream/handle/10665/44864/9789241503433_eng.pdf;jsessionid=F8BB3262B6B435D5E7B9835F0F83F7FC?sequence=1

World Health Organization. (2018, February 19). Preterm birth. Retrieved June 28, 2020, from https://www.who.int/en/news-room/fact-sheets/detail/preterm-birth

Wu, Y. W., Croen, L. A., Shah, S. J., Newman, T. B., & Najjar, D. v. (2006). Cerebral Palsy in a Term Population: Risk Factors and Neuroimaging Findings. *Pediatrics*, *118*, 690–697.

Yates, R., Treyvaud, K., Doyle, L. W., Ure, A., Cheong, J. L. Y., Lee, K. J., … Anderson, P. J. (2020). Rates and Stability of Mental Health Disorders in Children Born Very Preterm at 7 and 13 Years. *Pediatrics*, *145*, 20192699.

Yazji, I., Sodhi, C. P., Lee, E. K., Good, M., Egan, C. E., Afrazi, A., … Hackam, D. J. (2013). Endothelial TLR4 Activation Impairs Intestinal Microcirculatory Perfusion in Necrotizing Enterocolitis via eNOS-NO-nitrite Signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 9451–9456.

Yee, W. H., Soraisham, A. S., Shah, V. S., Aziz, K., Yoon, W., Lee, S. K., … Synnes, A. (2012). Incidence and Timing of Presentation of Necrotizing Enterocolitis in Preterm Infants. *Pediatrics*, *129*, e298–e304.

Zbinden, A., Zbinden, R., Berger, C., & Arlettaz, R. (2015). Case Series of Bifidobacterium longum Bacteremia in Three Preterm Infants on Probiotic Therapy. *Neonatology*, *107*, 56–59.

Zhang, G. Q., Hu, H. J., Liu, C. Y., Zhang, Q., Shakya, S., & Li, Z. Y. (2016). Probiotics for Prevention of Atopy and Food Hypersensitivity in Early Childhood: A PRISMA-Compliant Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Medicine*, *95*. https://doi.org/10.1097/MD.0000000000002562

Zhao, L. (2013). The Gut Microbiota and Obesity: From Correlation to Causality. *Nature Reviews Microbiology 2013 11:9*, *11*, 639–647.