INTRODUCTION

The purpose of this literature review is to explore normal human neonatal microbial colonization and the usage of probiotics specifically in the prevention of necrotizing enterocolitis (NEC) in preterm neonates. Efficacies of various probiotic treatments along with analyses of the microorganisms used within the studies will be reviewed.

DEFINITION OF A "PROBIOTIC"

Throughout the last century, the term "probiotic" has been defined many ways by different authors and groups (Azizpour et al. 2009). Although the verbiage differs slightly between definitions, all focus on microorganisms conferring benefits to the consumer (Azizpour et al. 2009). In 2001, the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) released the now widely accepted definition of the term "probiotic" as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). However, in 2014, The International Scientific Association for Probiotics and Prebiotics agreed that the term "probiotic" was being misused for certain products because specific quality requirement were not being met and suggested that lack of oversight, regulation and consensus on what exactly a probiotic is can be blamed (Hill et al. 2014). This same report also notes that both Italy and Canada have defined guidelines for what a probiotic is whereas many countries do not (Hill et al. 2014). Currently, the Food and Drug Administration (FDA) does not regulate the quality or purity of almost all probiotics because most are only considered dietary supplements (Thomas and Greer 2010). Only probiotics that are specifically being used to treat or prevent disease are under strict FDA regulation (Thomas and Greer 2010).

Although there is some confusion on what a probiotic is, this review will uphold the aforementioned FAO/WHO definition of a probiotic throughout and will focus on their use in medical treatments to promote the health and welfare of preterm neonates.

HISTORICAL PERSPECTIVE ON THE USAGE OF BENEFICIAL MICROORGANISMS

For centuries, different cultures have utilized fermented milk products to promote human health without realizing that they were implementing functional foods containing probiotics (Azizpour et al. 2009; Gogineni et al. 2013; Guo et al. 2014; McFarland 2015). Although Louis Pasteur implicated microorganisms in the process of fermentation, he did not conclude that these same organisms might also convey benefit to the consumer (Barnett 2000). This paradigm was then shifted by Elie Metchnikoff in 1905 when he proposed that the health benefit of a fermented milk product was derived from the Lactobacilli within (Metchnikoff 2004). A year later, fermented products claiming to contain bacteria were commercialized; however, the live content of these products cannot be confirmed (Gogineni et al. 2013).

Some of the first observations into the microorganisms present within infant stool were conducted in 1885 by Theodor Escherich, for whom *Escherichia coli* is now named after (Blum-Oehler and Hacker 2007). In 1932, Kopeloff et al. completed what appears to be the first clinical study of the possible correlation of the presence of gut bacteria (*Lactobacillus acidophilus)* in patients with mental disease and constipation. Shortly after, focus shifted away from the medical use of microorganisms and onto World War II and the start of the "Golden Age" of antibiotic discovery (Azizpour et al. 2009; Gogineni et al. 2013). Beginning in the 1980s, interest was eventually renewed in beneficial bacteria for numerous reasons including the high costs of drug development and the growing problem of non-effective antibiotics (Gogineni et al. 2013). Interest in the use of beneficial microorganisms has continued to grow ever since. A search for the term "probiotic" using the PubMed database shows continual growth in the amount of publications that include this topic (Figure 1). Additionally, a recent review from Di Cerbo and Palmieri (2015) indicates that it is widely believed that the consumer spending will continually increase within the probiotic market. In summary, probiotic products have been utilized for many years but the current explosion of interest in their medical usage could have future health and socioeconomic implications.

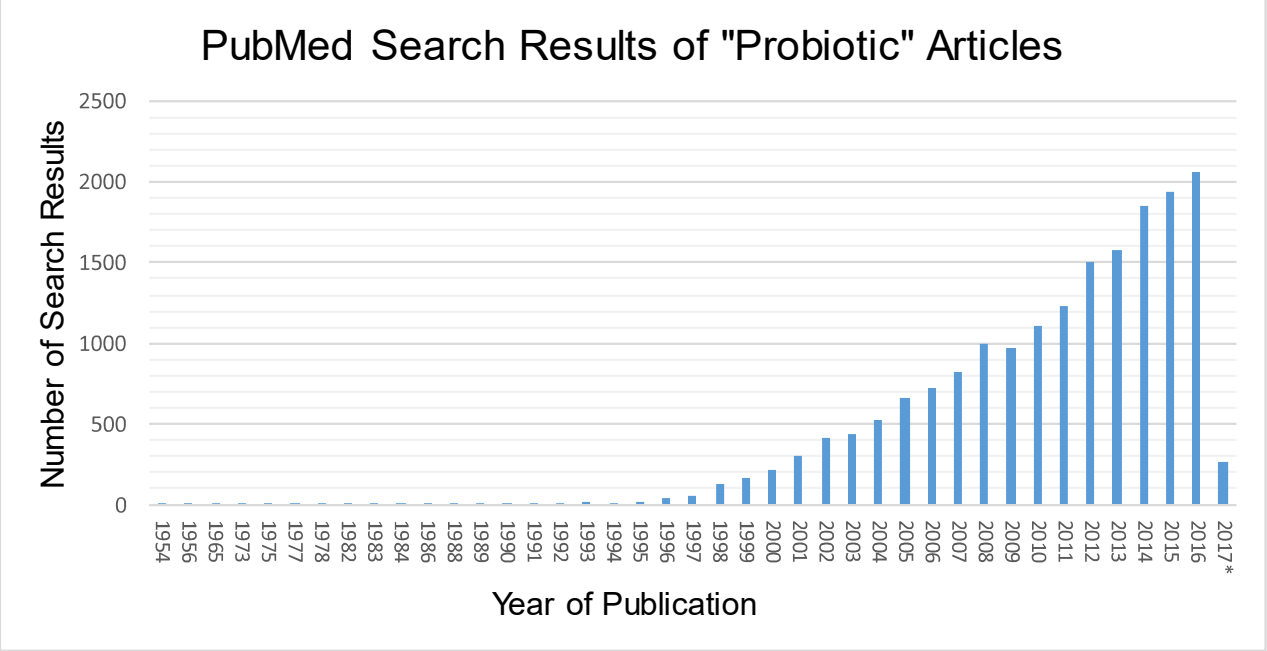

Figure 1. The amount of publications within the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed) that include the term "probiotics" from 1954-2017. *As of February 1st, 2017.

NEONATAL MICROBIAL COLONIZATION

There is strong consensus that a complex interplay of factors impacts the normal colonization of the early childhood gut. There is growing evidence that dysbiosis, which will be defined as a deviation from the normal microbiota even when specific pathogens

are absent (Sartor 2001), can lead to health-related issues. Normal gut colonization is a process that takes several years after the time of birth (Mackie et al. 1999; Koenig et al. 2011; Wopereis et al. 2014; Cheng et al. 2016). Mackie et al. (1999) reported that there is a sequential colonization of the human gut which typically begins with colonization from lactic acid bacteria. Additionally, a metagenomic study by Koenig et al. (2011) tracked the changes within the gut microbiome through the first two and a half years of the life of an infant and determined that there was both a gradual growth in species diversity over time and that there can be major fluctuations to this diversity caused by events such as antibiotic usage and diet changes. Although the authors Mackie et al. (1999) and Koenig et al. (2011) agreed that the microbiome of the human gut 2-3 years after birth is very similar to that of an adult, recent work completed by Cheng et al. (2016) disagreed with this conclusion. Instead, Cheng and colleagues concluded that the gut microbiome of the early child is not developed until after five years of age (Cheng et al. 2016).

Premature birth can alter the microbes present within the neonatal intestines (Magne et al. 2006; Westerbeek et al. 2006; Butel et al. 2007; Rougé et al. 2010). Work from Magne et al. (2006) utilized 16S rRNA analysis to determine that the gut of preterm neonates mainly contained various species of *Streptococcus, Staphylococcus*, and *Enterococcus*. Both Butel et al. (2007) and Rougé et al. (2010) found lower than normal gut biodiversity within preterm neonates which lacked many of the organisms present in a full-term neonate such as various species of *Bifidobacterium* (Harmsen et al. 2000).

Disruption of the normal colonization process can be caused by many additional factors leading as far back as the health of the expecting mother. Studies have implicated maternal weight gain (Collado et al. 2010), stress (Zijlmans et al. 2015), prophylactic antibiotic usage by the mother during birth (Aloisio et al. 2014), cesarean section versus vaginal birth (Penders et al. 2006; Nagpal et al. 2016), genetics (Goodrich et al. 2014), and a formula diet (Penders et al. 2006; Meinzen-Derr 2009) to impact the normal colonization process of neonates. Reviews by Li et al. (2014) and Wang et al. (2016) both implicated certain changes in the gut microbiota to asthma, eczema, allergies, NEC, inflammatory bowel disease and irritable bowel syndrome- all of which are disorders with strong immune system involvement.

In summary, the colonization process of the human gut can be broken into multiple different stages that take several years but begins at, or potentially before, birth. Although dysbioses caused by different factors can be implicated within a variety of diseases, the use of probiotics as a prophylactic treatment for the prevention of NEC in premature (preterm) neonates will be the narrowed focus of this review.

NECROTIZING ENTEROCOLITIS IN PRETERM NEONATES

NEC is one of the most frequent medical emergencies impacting between 3% to 7% of premature, very low birth weight (VLBW) neonates (Henry and Moss 2008) and is characterized by damage to the intestinal mucosa. A study from Guthrie et al. (2003) found a mortality rate of 12% in neonates diagnosed with NEC in comparison to 4% in neonates without NEC.

Damage to the intestinal mucosa can range from limited inflammation all the way to necrosis and death (Zubarioglu et al. 2017). Currently, studies indicate that this disease is caused by a variety of factors. Both Gribar et al. (2009) and Lu et al. (2014) concluded that Toll-like receptor 4 (TLR-4) activation caused a large amount of the inflammation associated with NEC while Meng et al. (2015) found that there were alterations in both the expression and location of TLR-4 during the early stages of development which might play a role in the development of NEC. Sharma et al. (2007) found that neonates with NEC initially had elevated levels of IL-6 along with a possible decrease in barrier strength of the intestinal lumen.

Altered gene expression within the epithelium of the intestines was found in neonates with NEC (Nanthakumar et al. 2011) while a very recent study from Tian et al. (2017) revealed that certain infants with a single nucleotide polymorphism in the *IL17* gene (*IL17F)* were more likely to develop NEC. A review of studies from Hunter et al. (2008) concluded that there is likely no specific causative agent in all cases of NEC but instead a cascade of events that trigger the inflammatory response (involving TLR-4) which is typical of this disease.

Other factors such as type of food given to the neonate (Meinzen-Derr 2009), purity of the formula/milk (van Acker et al. 2001; Boo 2016) and prolonged antibiotic usage (Cotten et al. 2009) can be suggested or directly implicated in the development and prevention of NEC. Additionally, it was documented in a study from Mai et al. (2011) that there was a shift within the gut microbiome of neonates anywhere from a week to three days before NEC was diagnosed. This shift included an increase in the amount of *Proteobacteria* found, although the cause(s) were not suggested and are currently unknown (Mai et al. 2011).

Currently, there are very limited preventative measures to prophylactically prevent NEC (Patole et al. 2016) but the use of probiotics to do so is an active area of research. However, a 2009 meeting of scientists and clinicians concluded that there is much that is still not well understood about the role of probiotics in the prevention of disease (Rowland et al. 2010). Contributing to confusion could be the seemingly multi-factorial nature of NEC. In conclusion, studies indicate that NEC is a severe disease that impacts a large population of neonates and that a variety of factors including alterations in TLR-4, gene expression, antibiotic usage, or the neonatal diet are involved in its onset and progression.

SPECIES AND STRAIN-SPECIFIC PROBIOTIC IMPACT ON THE DEVELOPMENT OF NEC

There is agreement that the impacts of probiotics need to be examined at the level of the bacterial strain instead of just the species (Salminen 2001; Rowland et al. 2010). This section will review the usage of specific strains of probiotics in the prevention of NEC within humans. When strain-specific data is not available, the efficacy of individual species will be reviewed instead. Species were chosen for

inclusion within this section only if they were utilized as a single-strain probiotic or as a common constituent of a multi-strain probiotic.

Bifidobacterium breve M-16V

A clinical study from Patole et al. (2016) indicated that there is a significant reduction in frequency of neonatal NEC with supplementation of only *B. breve* M-16V in neonates who were <34 weeks (NEC ≥ stage II = 3% in no probiotic group vs. 1% in probiotic group, $P = 0.019$) but also concluded that the usage of this strain in neonates <28 weeks did not show statistical benefit. In slight contrast, Li et al. (2004) and Patole et al. (2014) observed that the early supplementation of *B. breve* M-16V did colonize the neonate intestine which could promote health. Patole et al. (2014) did not have a large enough sample size of patients with NEC to show if prophylactic supplementation of this probiotic had any impact while Li et al. (2004) found no reduction in the frequency of NEC. Although NEC was not a focus of the study completed by Hikaru et al. (2010), these authors found a decrease in neonatal infections (possibly related to NEC) when this strain of probiotic was utilized.

Bifidobacterium breve BBG-01

B. breve BBG-01 has been used for decades within Japan to prevent or treat a variety of early childhood illnesses including NEC (Kitajima and Hirano 2016). Early work from Kitajima et al. (1997) supported the usage of *Bifidobacterium breve* YIT4010 (the authors state that this strain is considered "BBG" but no explanation was included nor could be found within the literature) in neonates to prevent NEC and promote the population of gut microbiota that are deemed healthy. A clinical survey completed by Kitajima and Hirano (2016) explored the effectiveness of this strain in the prevention of NEC and found that eighty-six clinicians responded that the usage of this strain in preterm infants led to "mildly good" results (and no "bad" results) in the prevention of NEC. No specific clinical studies focusing on NEC prevention utilizing only this strain could be found within the literature.

Bifidobacterium breve BBG-001

In a landmark three-year study, Costeloe et al. (2016) assessed the efficacy of *B. breve* BBG-001 in the prevention of NEC in preterm infants. This large, randomized study (now known as the PiPS trial) included 1,310 infants split between probiotic versus placebo usage and found no evidence for the prevention of NEC when using *B. breve* BBG-001 (NEC ≥ stage II = 10% in placebo group vs. 9% in probiotic group, adjusted risk ratio = 0.93) (Costeloe et al. 2016). Interestingly, these authors concluded that the routine usage of probiotics to prevent NEC was not supported (Costeloe et al. 2016). Within the same journal publication, arguments from Abrahamsson (2016) included that the PiPS trial did not choose an appropriate strain that had previously

been shown to have a benefit in the prevention of NEC. Later publications included arguments from McKinlay et al. (2016) who stated that the results of this study were "imprecise" and not strong enough to draw specific conclusions. Further arguments from Deshpande et al. (2016) refuted the dosage utilized within the PiPS trial and indicated that it was too low. Of note, one of the co-authors of the Deshpande et al. (2016) rebuttal of the PiPS trial was Sanjay Patole who had previously published work in support of the usage of *B. breve* M-16V in the prevention of NEC (Patole et al. 2016).

Bifidobacterium lactis BB12

Although the amount of studies using *B. lactis* single-species probiotic in the prevention of NEC are limited, a large clinical study from Dilli et al. (2015) indicated that its usage decreased the frequency of NEC in VLBW infants although the specific strain of *B. lactis* was not specified (NEC ≥ stage Ⅱ = 18% in placebo group vs. 2% in probiotic group, $P < .001$). However, in contrast to the study from Dilli et al. (2015), Mihatsch et al. (2010) had previously found that the usage of *B. lactis* BB12 had no statistically significant impact on the prevention of NEC; although, these authors note that only 183 patients were included within the study (NEC ≥ stage Ⅱ = 4.4% in placebo group vs. 2.1% in probiotic group). It should be included that Mihatsch et al. (2010) only observed the development and prevention of NEC as a secondary outcome and was not the focus of the study. Additionally, significant CFU dosage differences existed between these two studies. Dilli et al. (2015) used a probiotic composed of $5x10^9$ CFU while Mihatsch et al. (2010) used a probiotic composed of $2.0\text{-}2.3x10^{10}$ CFU/gram although Dilli et al. (2015) was not clear if this was the dosage given per kilogram of bodyweight or was the composition of the probiotic powder per gram.

Bifidobacterium longum (infantis) and *Bifidobacterium bifidum*

No human studies that examined the efficacy of the single-strain usages of *B. infantis* or *B. bifidum* in the prevention of NEC could be found. A large percentage of probiotics used prophylactically such as Align (Procter and Gamble, Cincinnati, OH) include *B. infantis* which was shown to successfully colonize the gut of preterm infants (Lievin et al. 2000; Underwood et al. 2013). Additionally, Totsu et al. (2014) demonstrated that neonates supplemented with *B. bifidum* established enteral feeding faster than those who were not supplemented with this probiotic.

Bacillus coagulans (Lactobacillus sporogenes)

Although there is a great deal of confusion about the naming of this organism (De Vecchi and Drago 2006), the Bergey's Manual of Determinative Bacteriology (Holt and Bergey 1994) officially recognized this organism as *B. coagulans* although it is still commonly called *L. sporogenes* in many probiotic products. Very few studies have examined the efficacy of this organism as a prophylactic treatment in the prevention of

NEC. At the time of this review, only one study from Sari et al. (2011) has explored the probiotic usage of this organism and found a statistically insignificant decrease in the development of NEC in 221 VLBW infants (NEC ≥ stage Ⅱ = 9% in placebo group vs. 5.5% in probiotic group, *P* = 0.447).

Lactobacillus acidophilus

After a comprehensive literature search, no results of clinical trials or human neonatal studies related to NEC could be found that used a single-strain probiotic only containing *L. acidophilus.* Precedent for possible efficacy in the prevention of neonatal NEC was established by Gonçalves et al. (2015) who demonstrated that prophylactic supplementation of *L. acidophilus* offered some protection from this disease through the improvement of the intestinal integrity in newborn rats. Additionally, Lorca et al. (2001) found that *L. acidophilus* can display bactericidal effects to some pathogenic bacteria through the production of autolysins.

Lactobacillus reuteri DSM 17938

There are mixed conclusions of the efficacy of *L. reuteri* DSM 17938 in the prevention of neonatal NEC. A retrospective study from Hunter et al. (2012) observed that the frequency of NEC was 12.6% lower in ≤ 1,000 gram neonates who received this strain as a prophylactic supplement (NEC = 15.1% in no probiotic group vs. 2.5% in probiotic group, *P* = 0.0475). In contrast to the results from Hunter et al. (2012), Oncel et al. (2014) and Rojas et al. (2012) concluded that the usage of *L. reuteri* DSM 17938 did not decrease the frequency of NEC in ≤ 1,500 gram and ≤ 2,000 gram neonates, respectively (NEC ≥ stage Ⅱ = 5% in placebo group vs. 4% in probiotic group, *P* = 0.63 in the study from Oncel et al. [2014] and NEC ≥ stage Ⅱ = 4.0% in placebo group vs. 2.4%, *P* = 0.23 in probiotic group in the study from Rojas et al. [2012]). A statistical analysis completed by Urbańska and Szajewska (2014) which pooled and compared the results from Oncel et al. (2014) and Rojas et al. (2012) also concluded that *L. reuteri* DSM 17938 did not decrease NEC (risk ratio = 0.69) but failed to include the aforementioned study from Hunter et al. (2012). An additional review from Athalye-Jape et al. (2016) analyzed the usage of this strain to prevent NEC and found a decrease in frequency but this review included studies using *Lactobacillus reuteri* ATCC 55730, the mother strain of *L. reuteri* DSM 17938.

Lactobacillus rhamnosus GG

A retrospective study from Luoto et al. (2010) included 20 years of medical data and compared the incidence of NEC before and after the usage of *L. rhamnosus* GG within five NICUs. This study did not find that the probiotic implementation of *L. rhamnosus* GG decreased the frequency of NEC (NEC ≥ stage Ⅱ = 3.2% in no probiotic group vs. 4.6% in probiotic group) and included that the neonatal unit that

prophylactically utilized this strain had the highest frequency of NEC (Luoto et al. 2010). Studies completed by both Dani et al. (2002) and Manzoni et al. (2006) recorded that the prophylactic usage of *L. rhamnosus* GG decreased the frequency of NEC in VLBW neonates (NEC ≥ stage II = 2.7% in control group vs. 1.4 in probiotic group for the study from Dani et al. [2002] and NEC ≥ stage II = 5% in control group vs. 2.5% in probiotic group for the study from Manzoni et al. [2006]). However, Dani et al. (2002) concluded that this difference was not statistically significant while the prevention of NEC was not the focus of the study from Manzoni et al. (2006).

Bacillus clausii

Although the authors did not specifically record a strain, Tewari et al. (2015) utilized *B. clausii* as a prophylactic treatment in preterm neonates and found no impact on the frequency of NEC. However, NEC was not the primary outcome of this study and no neonates with NEC ≥ stage II were recorded in either the control or probiotic groups. No other studies were found that examined the efficacy this strain for the prevention of NEC in neonates.

Saccharomyces boulardii

In contrast to many bacterial probiotics used within studies, *S. boulardii* is a yeast. A very early study which explored the usage of this yeast in the prevention of NEC was completed by Costalos et al. (2003) which found a slight decrease in NEC frequency (NEC = 16% in control group vs. 9.8% in probiotic group, $P = 0.5$) but this difference was not statistically significant and only included 87 patients. Additional studies to explore the efficacy of *S. boulardii* in larger sample sizes (n = 271) demonstrated that it did not significantly help prevent NEC in VLBW neonates (NEC ≥ stage II = 5.1% in control group vs. 4.4% in probiotic group, $P = 1$) (Demirel et al. 2013). Further studies completed by Serce et al. (2013) corroborate with this result (NEC ≥ stage II = 6.7% in control group vs. 6.7% in probiotic group, $P = 1$) although the dosage utilized within the study was less than half of that used by Demirel et al. (2013). Costalos et al. (2003), Demirel et al. (2013), and Serce et al. (2013) neglected to report if a specific strain of *S. boulardii* was utilized within their studies.

Conclusions from the usage of single-strain probiotics

There are only modest amounts of research into the use of single-strain probiotics in the prevention of NEC in human neonates. These studies tend to have mixed results which are seemingly strain-dependent. Additionally, efficacies can vary within specific probiotic strains possibly due to variations in dosages, location, and feeding practices unique to each study.

MULTI-STRAIN PROBIOTIC IMPACT ON THE DEVELOPMENT OF NEC

Understanding the impact of a single-strain probiotic on the prevention of NEC allows the fundamental groundwork to be laid in order to better understand if multi-species probiotics might be effective. Although some studies have been completed that implemented multi-strain probiotics that contain >4 species/strains, only specific studies limited to probiotics with ≤4 species/strains will be reviewed in order to limit the scope of this report.

Bifidobacterium longum (*infantis*) and *Lactobacillus acidophilus*

As previously mentioned within this review, no studies could be found that utilized only *B. infantis* or *L. acidophilus* as a single-strain probiotic in neonates to prevent NEC; however, both species have been documented to colonize the gut epithelium of neonates (Lievin et al. 2000; Underwood et al. 2013).

Large-scale observational studies have been conducted by Denkel et al. (2016) (n = 10,890) and Härtel et al. (2014) (n = 5,351) that examined the efficacy of a dual-strain probiotic which contained both *B. infantis* and *L. acidophilus* in the prevention of NEC. While both studies showed a decrease in NEC and mortality after probiotic usage, key similarities between them should be reviewed. The study completed by Denkel et al. (2016) included almost double the amount of neonates versus the study from Härtel et al. (2014) but the prophylactic implementation and target groups were virtually identical. Both studies used the dual-strain probiotic Infloran (Laboratorio Farmaceutico, Mede, Italy and Berna, Berne, Switzerland, respectively) which features both *B. infantis* and *L. acidophilus* and was given to German neonates under 1,500 g. Both large-scale studies had a decrease in the frequency of NEC (NEC = 3.4% in no probiotic group vs. 1.7% in probiotic group, $P < 0.001$ for the study from Denkel et al. [2016] and NEC ≥ stage II = 2.6% in no probiotic group vs. 4.2%, $P = 0.028$ in probiotic group for the study from Härtel et al. [2014]).

Other studies from Hoyos 1999 and Lin et al. 2005 that also implemented the probiotic product Infloran showed a decrease in NEC (NEC = 6.6% in no probiotic group vs. 2.7% in probiotic group, $P < 0.0002$ in the study from Hoyos [1999] and NEC ≥ stage II = 5.3% in no probiotic group vs. 1.1% in probiotic group, $P < 0.04$ in the study from Lin et al. [2005]). However, a study from Saengtawesin et al. (2014) which also used Inforan showed no decrease in NEC (NEC ≥ stage II = 3.4% in no probiotic group vs. 3.2% in probiotic group, $P = 0.74$) but only included 60 neonates, notably smaller than the other studies from Lin et al. (2005) and Hoyos (1999) which featured 367 and 1,237 neonates, respectively. A unique study completed by Repa et al. (2015) concluded that the use of Infloran only decreased the frequency of NEC in VLBW infants when utilized with breast milk (NEC = 11.2% in no probiotic group vs. 5.5% in probiotic group, $P = 0.027$) and had no statistically significant benefit when utilized with formula (NEC = 7.4% in no probiotic group vs. 13.6% in probiotic group, $P = 0.345$). Repa et al. (2015) also inferred a possible population bias in other studies using this same probiotic. The

study from Lin et al. (2005) which showed a decrease in the frequency of NEC took place within Asia while this study was the first of its kind within a European population.

Other probiotic products that contain both *B. infantis* and *L. acidophilus* have also had success in the reduction of NEC; however, these studies utilized probiotics containing additional strains of bacteria. In a study completed by Samanta et al. (2009), 274 neonates in Kolkata, India were prophylactically supplemented with a probiotic containing *B. infantis* and *L. acidophilus* along with *B. bifidum* and *B. longum* (strain not stated). According to the authors of this study, the mixture of these four bacterial species was used because it better represented what could be found within natural breast milk (Samanta et al. 2009). Although the results of this study showed a decrease in NEC (NEC ≥ stage II = 1.1% in no probiotic group vs. 15.8% in probiotic group, P = 0.042), the authors also indicated that probiotic supplementation did not decrease the severity of NEC (Samanta et al. 2009). Samanta et al. (2009) point out that this is in contrast to a study completed by Bin-Nun et al. (2005) which showed a decrease in severity in NEC. However, Bin-Nun et al. (2005) used a probiotic that also contained *Streptococcus thermophilus* in addition to *B. infantis* and *B. bifidum.*

Bacillus longum (infantis) and *Lactobacillus rhamnosis* GG

As previously reviewed, studies that utilized the single-strain probiotic *L. rhamnosis* questioned the effectiveness of this strain in preventing NEC (Dani et al. 2002; Manzoni et al. 2006; Luoto et al. 2010). The lack of studies utilizing only *B. infantis* has also already been reviewed within this report. Two studies utilized both strains by mixing together the consumer-grade products Align and Culturelle (Culturelle, Amerifit Brand, Cromwell, CT, USA) to create a multi-strain probiotic (Al-Hosni et al. 2012; Dang et al. 2015). Although both of these studies included different target groups of patients (Dang et al. [2015] target group was VLBW neonates while Al-Hosni et al. [2012] target group was extremely low birth weight neonates) both studies utilized the same concentration of probiotics ($1x10^9$ CFU) and found no statistically significant reduction in the frequency of NEC (NEC = 0.139 odds ratio, P = 0.07 in the study from Dang et al. [2015] and NEC = 8% in no probiotic group vs. 6% in probiotic group in the study from Al-Hosni et al. [2012]) . In contrast, a trial completed by Van Niekerk et al. (2015) did find a reduction in the frequency of NEC in VLBW neonates (NEC = 6% in no probiotic group vs. 3% in probiotic group, P = 0.029). Of interest, Van Niekerk et al. (2015) did not mix two single-strain probiotics but utilized the consumer-grade product Pro-B2 (C Pharm, Cape Town, South Africa) which, per these authors, contains $0.35x10^9$ CFUs of each strain.

Bacillus longum BB536 and *Lactobacillus rhamnosis* GG

Although closely related to the previous studies, it appears that *B. longum* BB536 is a unique strain different from *B. longum* (*infantis*). Currently, three biotypes of *B. longum* are recognized- type infantis, type longum, and type suis (Sakata et al. 2002)

and it appears that strain BB536 is a unique strain from Morinaga Milk Industry Co, Ltd (Tokyo, Japan) and belongs to type longum. A study from Rougé et al. (2009) utilized *B. longum* BB536 along with *L. rhamnosis* GG and found inconclusive results based on the few instances of NEC that entered the NICU where the study was conducted (NEC = 2% in no probiotic group vs. 1% in probiotic group, P = 0.51). No other studies measuring the frequency of NEC while prophylactically implementing only these two strains could be found.

Bacillus bifidum and *Lactobacillus acidophilus*

Although both *L. acidophilus* (Lievin et al. 2000) and *B. bifidum* (Underwood et al. 2013) are known colonizers of the neonatal gut, neither has been studied as a single-strain probiotic to determine efficacy in the prevention of NEC in preterm neonates. However, two clinical trials from Lin et al. (2008) and Samuels et al. (2016) investigated the efficacy of a dual-strain probiotic containing both strains. Lin et al. (2008) concluded that, when administered together, these two strains can significantly reduce the frequency of NEC in VLBW neonates (NEC ≥ stage II = 6.45% in no probiotic group vs. 1.84% in probiotic group, *P* = .02). In contrast, although the study from Samuels et al. (2016) did show a decrease in the frequency of NEC, it was only statistically significant in neonates who were solely fed breastmilk (NEC = 0.43 odds ratio, P = 0.03). These two studies both used the same probiotic from the same manufacturer (Infloran, SIT Laboratorio Farmaceutico, Mede, Italy) and included VLBW infants but came to different conclusions. The only major difference between these studies included location and the timing of the supplementation given to the neonates (the study from Lin et al. [2008] took place in Taiwan and utilized 125mg twice daily while the study from Samuels et al. [2016] took place in the Netherlands and utilized 250mg once daily).

Bacillus breve and *Lactobacillus casei* (Shirota strain)

A controlled trial from Braga et al. (2011) investigated the usage of both *B. breve* (strain never specified) and *L. casei* (Shirota strain) for the prevention of NEC in 231 VLBW preterm neonates. These authors found a decrease in the frequency of NEC but statistical significance was not established (zero cases of NEC in those using the probiotic supplement and four cases of NEC in those using a placebo) (Braga et al. 2011). It should be noted that this study utilized a large range of dosages from $3.5x10^7$ to $3.5x10^9$ CFU (Braga et al. 2011).

Other multi-strain probiotics containing greater than two species

Although there are a variety of studies that utilize >2 species within the probiotic implemented, variations such as included strains and dosages utilized limit the ability to compare them to each other. Because of this, only two studies will be reviewed within this section. Both studies implemented *Streptococcus thermophilus* Th-4, *Bacillus*

infantis BB-02, and *B. lactis* BB12 (ABC Dophilus, Solgar, Leonia, New Jersey) as prophylactic treatment in VLBW neonates and found a reduction in the frequency of NEC (Bin-Nun et al. 2005; Jacobs et al. 2013). Although the dosage and probiotic used were consistent between these two studies, the locations and sizes of the studies were significantly different. The study from Bin-Nun et al. (2005) included 145 neonates residing within only one hospital in Israel whereas the study from Jacobs et al. (2013) included 1,099 neonates distributed between ten hospitals in both Australia and New Zealand. Although both studies found a decrease in the frequency of NEC, Bin-Nun et al. (2005) observed a 12.4% risk reduction (NEC = 16.4% in no probiotic group vs. 4% in probiotic group, P = 0.03) while Jacobs et al. (2013) only observed a 2.4% risk reduction (NEC ≥ stage Ⅱ = 4.4% in no probiotic group vs. 2.0% in probiotic group, P = .03).

Conclusions from the usage of multi-strain probiotics

In summary, there are variations between studies utilizing probiotics that contain multiple strains of bacteria. Multi-strain probiotics have been successfully utilized to decrease the frequency of NEC in preterm neonates although some studies have questioned their effectiveness. The strains utilized within multi-strain probiotics along with their concentrations relative to each other are unique challenges within this group of prophylactic treatment.

META-ANALYTICAL ANALYSES OF PROBITOIC USAGE IN NEC PREVENTION

Meta-analytical studies have been completed which examined the efficacy of many varieties of probiotics in the prevention of NEC, sepsis and mortality (both sepsis and mortality are directly related to the progression of NEC). For this review, six total meta-analyses were explored for similarities and differences within their findings. Although the required factors for a study to be included within each meta-analysis were slightly different, most of these meta-analyses included the same clinical studies. Out of the seven meta-analyses included within this review, two stood apart as being the most comprehensive. The first of these was an analysis completed by AlFaleh and Anabrees (2014) which examined 24 different studies from all over the world and included almost 5,000 neonates. The second meta-analysis contained 12 different studies (none of which were in common with the analysis from AlFaleh and Anabrees [2014]) and contained over 10,000 neonates (Olsen et al. 2016). Both comprehensive meta-analyses concluded that the prophylactic supplementation of probiotics reduced the frequency of NEC (NEC ≥ stage Ⅱ = 0.43 relative risk in the meta-analysis from AlFaleh and Anabrees [2014] and NEC ≥ stage Ⅱ = 0.55 relative risk in the meta-analysis from Olsen et al. [2016]) and overall mortality (overall mortality = 0.65 relative risk in the meta-analysis from AlFaleh and Anabrees [2014] and overall mortality = 0.72 relative risk in the meta-analysis from Olsen et al. [2016]. However, no statistically significant reduction in the frequency of sepsis was found (sepsis = 0.91 risk ratio from AlFaleh and Anabrees [2014] and sepsis = 0.86 risk ratio from Olsen et al. [2016]).

Although the other four meta-analyses contained many of the same clinical studies analyzed by Olsen et al. (2016) and AlFaleh and Anabrees (2014), each contained unique clinical studies and derived conclusions that should not be ignored. Meta-analyses completed by Baucells et al. (2016), and Wang et al. (2012) also concluded that prophylactic supplementation of probiotics reduced the frequency of NEC with risk ratios = 0.39 and 0.33, respectively. A meta-analysis from Mihatsch et al. (2012) found that prophylactic probiotic usage in neonates only sometimes reduced the frequency of NEC depending on the strain utilized. It is important to note that most of the clinical studies included within these meta-analyses used different species (and/or strains) of bacteria within the supplemented probiotic along with different dosages. Mihatsch et al. (2012) includes that this variety and lack of consistency within the clinical implementation of probiotics should be considered when drawing conclusions about probiotic efficacy.

In a deviation from the previous style of meta-analyses which compared overall efficacy of all probiotics in the prevention of NEC, two recent meta-analyses from Aceti et al. (2015) and Chang et al. (2017) examined the efficacy of single-strain versus multi-strain probiotics in the prevention of NEC. Aceti et al. (2015) examined a total of 26 trials which featured 6,605 infants while Chang et al. (2017) examined a total of 25 trials which featured 7,345 infants. It is important to note that because of the extremely limited amount of research examining the efficacy of single-strain probiotics, both meta-analyses analyzed many of the same studies (three studies were unique within the meta-analysis from Aceti et al. [2015] and three studies were unique within the meta-analysis from Chang et al. [2017], two of which were published at the same time or after the publication from Aceti et al. [2015]). These two meta-analyses contained almost identical studies but their conclusions had both similarities and differences. While Aceti et al. (2015) concluded that single-strain probiotic usage of *Lactobacillus* did not reduce the risk of NEC (risk ratio = 0.62), Chang et al. (2017) concluded that usage of *Lactobacillus* had a minor impact in the prevention of NEC (odds ratio = 0.60). Additionally, Aceti et al. (2015) used pooled data from different *Bifidobacterium* species to conclude that its usage showed a significant decrease in NEC (risk ratio = 0.24) while Chang et al. (2017) concluded that there was no significant benefit (odds ratio = 0.85). Both meta-analyses agreed that *S. boulardii* had no benefit (risk ratio = 0.81 from Aceti et al. [2015] and odds ratio = 0.80 from Chang et al. [2017]) and that multi-strain probiotics decreased the frequency of NEC better than single-strain probiotics (Aceti et al. 2015, Chang et al. 2017).

In conclusion, a variety of meta-analyses have been completed exploring the effectiveness of probiotics for the prevention of NEC in neonates. A limited amount of clinical trials are utilized within most meta-analyses. The meta-analyses included in this review concluded that certain probiotics can reduce the frequency of NEC in neonates. Additionally, it was concluded that multi-strain probiotics decrease the frequency of NEC better than single-strain probiotics.

POTENTIAL AND DEMONSTRATED PROBIOTIC MECHANISMS OF ACTION

There is little empirical evidence for how the prophylactic implementation of probiotics may decrease the frequency of NEC in neonates but some literature suggests mechanisms of how probiotics may inhibit the development of some diseases related to the gastrointestinal tract. A review of literature exploring how probiotics may maintain intestinal health, and therefore reduce the frequency of NEC, will be reviewed within this section.

There is evidence that the normal colonization of the early childhood gut promotes the establishment of a protective chemical barrier against potential pathogens (van Limpt et al. 2004) and prepares the immune system to defend against pathogenic strains of bacteria (Salzman 2014; Wopereis et al. 2014). As previously reviewed, the preterm neonate gut is not colonized in the same way, or as quickly, as a full-term neonate (Magne et al. 2006; Westerbeek et al. 2006; Butel et al. 2007; Rougé et al. 2010). One of the main overall purposes of prophylactic probiotic supplementation in preterm neonates is to establish gut microflora that mimics what should be there if they were full-term and healthy. In a recent study from Denkel et al. (2016), which utilized the Firmicute *L. acidophilus,* the authors suggested the shift of microbiome away from Firmicutes prior to NEC (reported by Mai et al. [2011]) was possibly prevented by establishing a healthy gut at an earlier stage. Although specific mechanisms to prevent NEC were not suggested or explored by Mohan et al. (2006), this study concluded that *B. lactis* BB12 supplementation encouraged the growth of this species within the gut of neonates and suppressed the growth of certain species of *Clostridia* which have been implicated in some cases of NEC (Cassir et al. 2015).

Butyric acid can increase the production of IL-8 which can bring additional neutrophils to the area, as commonly seen in NEC. Wang et al. (2007) found that the amount of butyric acid within neonatal fecal material significantly decreased ($P < 0.05$) after administration of *B. breve* M-16V while del Mar Rigo-Adrover et al. (2016) found that this same strain promoted the production of IgA within the intestines, both of which could offer some protection from NEC. Work from del Mar Rigo-Adrover et al. (2016) also found differences in TLR-4 proportions, the migration of T-lymphocytes, and a 2-fold increased production of IgA which the authors suggested as being mechanisms to improve early mucosal health.

Although human neonatal studies examining probiotic mechanisms of action are relatively rare, animal studies completed by Khailova et al. (2009) showed a significant, and drastic, decrease in the frequency of NEC when *B. bifidum* was supplemented to mice (NEC = 57% in no probiotic group vs. 17% in probiotic group, $P \leq 0.01$). These authors found that the mice given this probiotic had decreased levels of the proinflammatory cytokine IL-6 and enhanced production of proteins that contribute to cellular junctions (Khailova et al. 2009). These results from Khailova et al. (2009) agreed with prior work completed by Madsen et al. (2001) and Stratiki et al. (2007) who both found that probiotics that contain *Bifidobacterium* decreased the permeability of the

intestinal lumen. It should be noted that the probiotic used by Madsen et al. (2001) contained numerous other species including *Lactobacilli* and *Streptococcus*.

Certain strains of bacteria found within probiotics can inhibit the growth, or kill, other species that are potentially pathogenic. As previously included in this review, Lorca et al. (2001) found that *L. acidophilus* can produce autolysins and lactic acid- both of which inhibited the gut pathogen *Helicobacter pylori*. Similar conclusions were made by Lee et al. (2003) and Collado et al. (2005) who both found that certain strains of bacteria commonly found within probiotics had antimicrobial activity.

Meta-analyses from Aceti et al. (2015) and Chang et al. (2017) concluded that multi-strain probiotics were superior to single-strain probiotics. Chang et al. (2017) suggested that multi-strain probiotics may confer greater benefit that single-strain probiotics by offering greater biodiversity which may have a synergistic impact when together while Aceti et al. (2015) contributed the benefit to the formation of a better "ecological barrier". Some authors suggested that certain strains of probiotics can offer synergistic effects when supplemented together (Timmerman et al. 2004) or with breastmilk (Lin et al. 2008). Empirical evidence for a synergistic relationship existing between microorganisms utilized within some probiotics was investigated by Ouwehand et al. (2000) who found that the *in vitro* binding (a vital first step of colonization) of *B. lactis* BB12 to ileostomy glycoproteins went from 18% (control) to 44% ($P < 0.05$) when in the presence of *L. rhamnosis* GG.

In conclusion, it has been found that certain strains of bacteria found within probiotics can help establish a protective barrier to prevent pathogenic bacterial growth. Additionally, some probiotic strains can have an antagonistic impact on pathogenic bacteria and/or synergistic effects with other probiotic strains. In the same way that NEC is a multifactorial disease, probiotics impact the gut in a variety of ways.

POTENTIAL DRAWBACKS OF PROBIOTIC USAGE

None of the studies included within this review found that neonatal prophylactic probiotic supplementation was dangerous to their patients but probiotic usage is not without risk. Literature reviewed within this section will examine the potential drawbacks to probiotic usage as well as common concerns from various authors.

Rare cases of bacteremia after probiotic usage have been reported from several authors. Jenke et al. (2012), Zbinden et al. (2015) and Bertelli et al. (2015) all reported cases in which *B. infantis* was found within the preterm neonatal patient's blood after daily usage of Infloran. Utilizing modern molecular methods, all studies confirmed that the *B. infantis* found within the neonate's blood were from the probiotic being utilized. Additional authors have published separate cases of septicemia when *B. breve* BBG-01 has been utilized as a probiotic (Ohishi 2010; Kitajima and Hirano 2016). All authors note the rarity of these findings but urge caution when utilizing probiotics.

Some authors have raised questions about the scientific quality of some probiotic studies. Although the meta-analysis from Aceti et al. (2015) was written to analyze the efficacy of certain probiotics in the prevention of NEC, these authors indicated that

many probiotic studies failed to identify the actual strain of probiotic used. This creates inherent confusion when attempting to determine optimal strains to utilize within probiotics. The importance of strain-specificity can be seen in a study from Lievin et al. (2000) which verified that different strains of *Bifidobacterium* isolated from infants demonstrated different antimicrobial properties. Additionally, the obligation to provide the highest quality care to all neonates should not be forgotten. In the study completed by Bin-Nun et al. (2005), all deaths related to NEC were within the control group not given probiotics.

The purity of the probiotic employed has implications in the accuracy and safety of the prophylactic treatment. Few studies included within this review utilized modern techniques to examine the purity and accuracy of the probiotic product implemented. A notable exception to this observation is the highly referenced study from Jacobs et al. (2013) which did use real-time polymerase chain reaction (PCR) to examine the purity and concentration of the triple-strain probiotic ABC Dophilus before usage of each product batch. However, in 2014 the FDA concluded that a preterm neonate death was caused by this same product which was found to be contaminated with the fungus *Rhizopus oryzae* (US Food and Drug Administration 2014). In a study conducted by Marcobal et al. (2008) the purity of 13 out of 14 commercial probiotics tested were not found to contain exactly what the manufacturer claimed was within the product. Out of the 14 commercial products tested, 7 contained additional species that were not included on the manufacturers label and 5 of them contained fewer species that were included on the label (some species were "present" but in extremely low amounts only detectable through PCR). These results correlate with previous work from Canganella et al. (1997) and Theunissen et al. (2005) which both found inaccuracies in what was claimed by the manufacturer. Although the study from Canganella et al. (1997) is relatively old, these authors did find that single-strain probiotic contents were usually more accurate that multi-strain products.

The differences of dosage amounts (CFUs) within studies that utilize the same strains of probiotics further complicates effectiveness. The study conducted by Repa et al. (2015) utilized higher concentrations of probiotic but demonstrated less efficacy than studies which utilized the exact same probiotic in lower concentrations (Hoyos 1999; Lin et al. 2005). Additionally, these authors suggested that lower dosages of probiotics might be effective in the treatment of NEC (Repa et al. 2015). Evidence supporting this hypothesis was recently established by Baglatzi et al. (2016) who completed a study that focused on the usage of probiotics to decrease neonatal diarrhea. These authors demonstrated that a low dosage of *Bifidobacterium lactis* CNCM-I3446 (10^4 CFU/g of probiotic) had similar outcomes ($P = 0.70$) to a "regular" dosage of the same strain (10^7 CFU/g of probiotic) (Baglatzi et al. 2016).

There can be a great deal of confusion within the literature in the naming of some probiotic bacterial strains utilized. Lin et al. (2008) reported that the supplier of the probiotic used in their previous studies had changed the formulation from originally containing *B. infantis*) to *B. bifidum* but the same product name "Infloran" was kept. It is

important to note that these two species (*B. infantis* and *B. bifidum*) are not closely related (Felis and Dellaglio 2007).

Reviewed studies within this report indicated great variation in the method that neonatal intensive care units (NICU) utilize probiotics, if utilized at all. A 2016 Viswanathan et al. survey found that only 14.0% of 500 NICUs were utilizing probiotics in VLBW neonates. Additionally, this paper included that the probiotic product Culturelle (which is a single-strain *Lactobacillus* GG probiotic) was the most commonly used probiotic in surveyed NICUs (Viswanathan et al. 2016) although the efficacy of this single-strain probiotic has mixed results (Dani et al. 2002; Manzoni et al. 2006; Luoto et al. 2010). Viswanathan et al. (2016) suggested that NICUs pick what probiotic to implement based upon price and availability instead of the empirical evidence of efficacy. Additionally, a study from Anderson (2016) found that although caretakers stated that they were likely to prescribe probiotics in the prevention of NEC, none of them did so. Anderson (2016) also suggested that although the caretakers claimed to have a good understanding of probiotic usage, many of them probably lacked a thorough understanding of current research within the area. One of the surveyed caretakers within this study referenced the deficiency of FDA oversight into the quality of probiotics as a reason for the lack of implementation (Anderson 2016).

In conclusion, there are various reasons that probiotics should be used with caution. Although rare, there is still a small risk of bacteremia and septicemia when probiotics are utilized. The quality of scientific studies, purity of the probiotic being utilized, and dosages given have direct impacts on the health of the neonate. NICUs need to be well-informed on current research within the field to make judgements on probiotic implementation and usage.

CONCLUSION

Although probiotics have been utilized for many years, their usefulness within the medical field is just now being explored in great depth. Studies examining their usefulness are becoming more common although human trials are still somewhat limited. Lack of standardization and oversight of their production and usage further complicates their implementation. Some countries have established strict guidelines for their naming and usage but the United States has much room to improve within this area.

The bacterial colonization of the human gut is important for short-term and long-term health. This multi-year sequential process begins extremely early in life but is disrupted by premature birth and other factors. Often, premature neonates do not have the same biodiversity of microbiota within their gut leading to dysbiosis. These differences in the microbiota can be implicated in certain immune-related diseases such as NEC. Currently, there are very few preventative measures that can be taken to limit this disease. A large variety of prophylactic probiotic treatments are now being utilized in clinical studies and NICUs in the attempt to better mimic the natural microbiota of full-term neonates, possibly prevent colonization from pathogens, and modulate the immune response. Single-strain probiotics are used within the medical and commercial

markets but limited studies on each strain show variations in their effectiveness. Additionally, multi-strain probiotics are also being utilized, and favored, in the prevention of NEC but complexities in understanding the possible synergetic relationships between the included strains is not understood. Differences in the geography of the preterm neonates and the local practices within the NICUs treating them need to be considered when studying the efficacy of any probiotic treatment regimen. The safety of probiotic usage is firmly established but small risks do exist when utilized and should not be ignored. The oversight of purity and strain concentrations within every probiotic product should be established to standardize implementation.

In conclusion, probiotics offer a safe and possibly effective route of prophylactic prevention of NEC. Although there is still much that needs to be learned about the causes of this disease as well as strain-specific interactions and multi-strain synergy, research in the area is bringing scientists and doctors closer to preventing this devastating disease.

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