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An Update on the Gut Microbiome and the Use of Probiotics for Disease Prevention in Preterm Infants

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Abstract

Very low birth weight infants (VLBWIs) are at high risk for inflammatory diseases including necrotizing enterocolitis (NEC) or neonatal sepsis, which are primary causes of neonatal mortality. The intestinal microbiota plays an essential role in maintaining local immune homeostasis and enhancing the intestinal barrier in preterm infants; however, appropriate intestinal colonization with normal flora after birth is interrupted by immature gastrointestinal tract, intestinal mucosal damage, insufficient nutrient transport, or formation of abnormal intestinal flora due to the use of antimicrobials in VLBWIs. Large randomized controlled trials and meta-analyses have highlighted the potential benefits of the clinical use of probiotics on NEC or neonatal sepsis for immunologically immature VLBWIs. However, standardized guidelines for the optimum strain, combination of strains, dosage, timing, and duration of probiotics are unknown for the routine application of probiotics in VLBWIs. Here, we review the results of previous studies on the effects of probiotics in preventing morbidity, NEC, or neonatal sepsis in VLBWIs with the administration of single-strain or multi-strain probiotics. Future clinical trials should address the safety of each probiotic strain and the potential efficacy of strain combinations for the routine use of probiotics in preterm infants.

The key findings of the manuscript: This study reviewed the focus on the efficacy of probiotics for the prevention of sepsis and necrotizing enterocolitis in preterm infants weighing less than 1,500 g at birth according to single-strain probiotics or multi-strain probiotics.

Keywords: Infants; Probiotics; Inflammatory

Introduction

In recent years, the survival rate of very low birth weight infants (VLBWIs) has increased due to rapid developments in medical technology. Despite such improvements, resulting complications including necrotizing enterocolitis (NEC) and neonatal sepsis, which are prevalent diseases in the neonatal intensive care unit (NICU), are the primary cause of neonatal death and adverse long-term neurodevelopmental outcomes [1]. Because the gastrointestinal tract makes up a large portion of the body surface area, it is most often exposed to various antigens and microbes that can cause damage to the intestinal mucosal barrier. Infants with a weak immune system are often later diagnosed with neurodevelopmental deficits after surviving treatment for an infection [2]. The formation of an intestinal mucus membrane with normal flora in the intestine after birth is important for maintaining normal physiologic homeostasis. Typical growth of the intestine is stalled in VLBWIs, who require neonatal intensive care and are susceptible to intestinal mucosa damage from the formation of intestinal flora, insufficient nutrient transport due to an incomplete gastrointestinal (GI) tract, insertion of a nasogastric tube, or formation of abnormal intestinal flora due to the use of antimicrobials [3]. Furthermore, other necessary treatments can destroy the normal intestinal mucus membrane, allowing invasive infection to proceed.

Although minimizing invasive treatment is the optimum solution to reduce the possibility of complications in VLBWIs, preventing the abnormal establishment of intestinal pathogenic bacteria that can cause postnatal sepsis or NEC is also a method of reducing the complications of infection. Despite high morbidity and mortality rate in preterm infants due to frequent occurrence of NEC, effective treatment has not been suggested. Recently, growing evidence has indicated that the intestinal flora plays a pivotal role in brain development affecting future cognitive functioning and behavior through brain-gut communication [4]. Here, probiotics, which have a beneficial effect on health, have been suggested as a method to establish positive changes in the intestinal flora of the host [5,6]. It is increasingly clear that probiotics lower the incidence of NEC and the infant mortality rate [7-9]. This review aims to introduce formation and establishment of gut microbiome at birth as well as its role in preterm infants with NEC and sepsis by comparing effectiveness of single strain and multi-strain probiotics supplement.

Formation of the Intestinal Microbiota in Preterm Infants

The GI tract plays a pivotal role as an interface between the host and the environment. Three important factors for GI immunity include the intestinal microbiota, gastrointestinal surface protection, and local immune mechanisms such as gut-associated lymphoid tissue (GALT). M cells are specialized intestinal epithelial cells in lymphoid follicles that display a local immune mechanism as an important GALT. Intestinal microbiota activate the local immune response by interacting with the host to maintain local immune homeostasis and enhance the intestinal barrier. In vivo studies have suggested a critical role of the gut microbiota in secondary lymphoid tissues (Peyer's patches and

mesenteric lymph nodes) and tertiary lymphoid structures (isolated lymphoid follicle or cryptopatches) that are mediated by dendritic cells, T cells, and B cells [10,11].

Infants born via cesarean section without rupture of the amniotic membrane are at risk of infection from the amniotic fluid as bacteria begin to colonize the intestine quickly after birth. In fact, components of the maternal flora affect the passive transfer of neonatal microbiota to colonize the gut, supporting potential postnatal development of the immune system. Intestinal microbiota are transferred through the maternal vagina during delivery and also following exposure to the external environment, such as by breastfeeding or oral ingestion [12]. The settling period and composition of the intestinal flora are determined by gestational age, delivery method, feeding, antibiotic intake, probiotics, and additional factors of the surrounding environment including the NICU [13,14]. Human breast milk has attracted considerable attention as a source of intestinal colonization in normal gut microbial development resulting in bacterial diversity in the infant gut. Furthermore, maternal IgA hinders microbial attachment by binding nutritional antigens and controlling excessive immune activation [15]. Human milk oligosaccharide consumption by gut microbes indicates that human milk oligosaccharides contribute to the infant intestinal microbiota, which are important components of the intestines of breastfed infants [16,17]. Many factors in milk, including N-acetylglucosamine, glucose, lactoferrin, galactose, and fructose, select for *Bifidobacterium* species [18].

A previous study based on 16S ribosomal RNA pyrosequencing highlighted the diversity of stool microbiota in the meconium that depends on prenatal and postnatal factors in infants with a gestational age <32 weeks at birth [19]. The VLBWIs of a <30 week gestational age demonstrated a decreased number and decreased diversity of intestinal flora compared with infants with a >30 week gestational age. The impacts of intestinal flora were confirmed to differ with gestational age based on the sequences of causative organisms, such as *Citrobacter*, *Enterococcus*, and *Klebsiella*, which are reported to be causative organisms of both NEC and sepsis.

The Role of Intestinal Microbiota in Preterm Infants

The intestinal mucus contains intestinal commensal flora, and great numbers of bacteria are found in the intestines. Intestinal flora can be categorized into either primary flora [$>10^9$ colony-forming units (CFU)/g] or secondary flora [$<10^6$-10^9 CFU/g]. Although the intestines must distinguish between symbiotic microbiota and external pathogens, little is known about the mechanism of differentiation between the different species. Despite not understanding the mechanism, it is widely accepted that intestinal commensal flora is helpful in the host defense against external pathogens. The intestinal microbiota affects intestinal organ development by maintaining a symbiotic relationship with the host, activating intestinal cells, and controlling the structure of vessels in the intestinal villi, enhancing tight junctions between the cells, and increasing the secretion of mucus.

The dysbiosis of microbial colonization in VLBWIs tends to increase the risk of infections and inflammatory processes. NEC is a major threat that primarily affects preterm neonates and typically occurs in the first few weeks after birth [18]. Previous studies based on the analysis of microbiota from the feces of NEC patients and control patients have shown that unusual intestinal microbial species and an overall reduction in diversity of the microbiota are related with NEC [19,20]. Inappropriate early microbial colonization can be an injury-causing factor in VLBWIs with immature intestinal function, and the associated immune defense mechanism is susceptible to intestinal damage [21,22]. In short, VLBWIs displayed reduced levels of protective Bifidobacterium and a high prevalence of facultative anaerobic microorganisms such as *Staphylococcus*, *Enterobacteriaceae*, and *Enterococcaceae* [23,24].

Colonized intestinal flora can also protect against external pathogens. Crosstalk between the intestinal flora and epithelial cells regulates intestinal inflammation by interacting with the epithelium, endothelial cells, and lymphocytes across the mucus layer. Toll-like receptors (TLRs) are known to play a central role in this action. TLRs are a major focus of neonatal immunological research due to the wide range of basic science knowledge in this area [15]. Intestinal microflora that causes bacterial translocation in VLBWIs is associated with excessive TLR-4 signaling, which produces an inflammatory cascade and necrosis characteristic of NEC [25]. The direct activation of TLRs leads to the activation of M cells and dendritic cells that balance intestinal immunity, but this is skewed toward T helper type 2 cells via T helper type 1 cells, which also control other inflammatory responses [26].

Preterm infants encounter several challenges to intestinal microbiota formation after birth. Compared with normal infants, VLBWIs show downregulated variation in intestinal microbiota and attenuated TLR function [12]. This reduction in intestinal microbiota diversity allows pathogenic bacteria to develop into the primary flora at a decreased degree of intestinal maturity, increasing the risk and incidence of sepsis or NEC. VLBWIs are susceptible to pathogenic bacteria due to an incomplete innate immune response and a downregulated immune response. Furthermore, the use of histamine-2 blockers, steroids, or opioids can impact the formation of intestinal flora with such vulnerabilities in VLBWIs [15,27]. Therefore, emerging studies have focused on the use of probiotics to encourage the formation of healthy intestinal flora and to prevent inflammatory GI disorders in VLBWIs with vulnerable intestinal immunity [28,29].

Types and Roles of Probiotics

The World Health Organization (WHO) defines probiotics as live microorganisms that, when consumed in adequate amounts, confer a health benefit on the host by balancing the intestinal flora. The most important species are *Lactobacillus* and *Bifidobacterium* which are both present in dietary and fermented dairy products. In contrast, prebiotics are substances that cannot be digested and therefore improve the health of the host by influencing the growth and activity of the intestinal flora. Insulin, fructo-oligosaccharides, and galacto-oligosaccharides are examples of prebiotics. A vast number of studies previously identified the beneficial effects of the administration of specific probiotic strains, including enhancing the intestinal barrier, increasing the systemic immune response, and aiding in the formation of normal intestinal flora in preterm infants (Figure 1) [8,28,30]. Probiotics enhance intestinal epithelial cells and form a barrier that resists the invasion of pathogens and accelerates the secretion of mucin to impede the adherence and colonization of pathogens to epithelial cells. Probiotics are known to increase the mucus barrier by thickening the mucosa with induced mucin mRNA to prevent adhesion of pathogenic microbes, such as enteropathogenic *Escherichia coli*. They are also known to enhance the mucosal barrier by increasing the level of secretory IgA while also augmenting tight junctions and preventing hypoxic damage in vitro by decreasing vessel resistance through the

production of nitric oxide [31]. Increasing immunity through a controlled immune response and induced cytoprotective responses is another benefit of probiotics [32,33]. The specific probiotics used in studies for preterm infants are usually *Lactobacillus* and *Bifidobacterium*, which secrete lactic acid, acetic acid, and butyric acid, inhibiting the growth of pathogenic microbes [34]. In addition, the microbiota plays a pivotal role in alleviating stress caused by invasive and/or antibiotic treatments along with the physical and emotional stress that result from separation from the mother. Probiotics also influence the long-term intestinal environment via the brain-gut-microbiota signaling system [4].

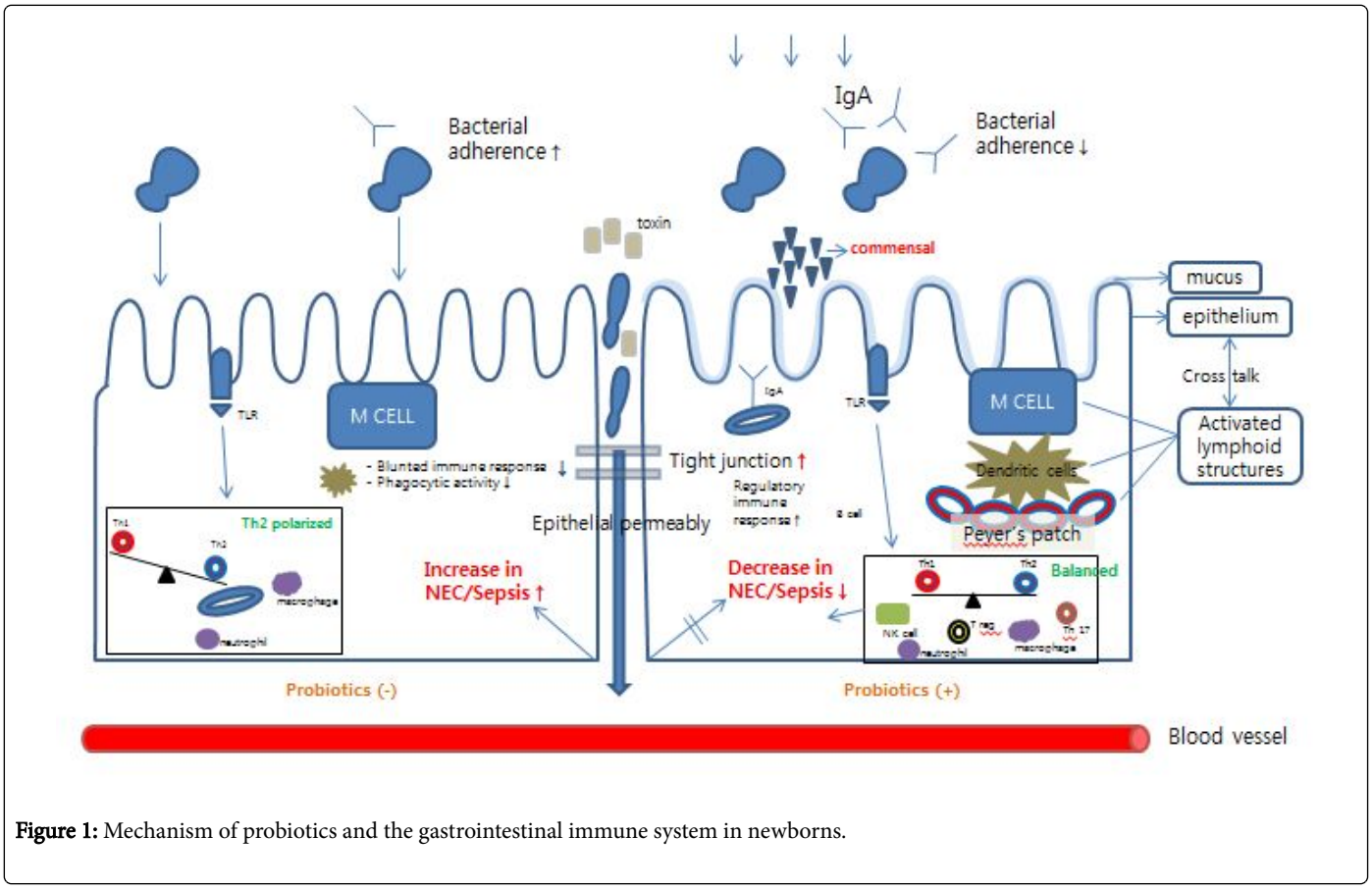

Figure 1: Mechanism of probiotics and the gastrointestinal immune system in newborns.

Although *Lactobacilli* (*L. acidophilus*, *L. casei*, *L. rhamnosus* GG, *L. reuteri*, *L. bulgaricus*, *L. plantarum*) and *Bifidobacterium* (*B. bifidum*, *B. longum*, *B. infantis*, *B. lactis*, *B. breve*) are used as a primary strain of probiotics, Streptococcus thermophiles and Saccharomyces boulardii are also employed as strains. To be used as an effective probiotic, microbes should be non-pathogenic and must reach the intestine in a live form after direct ingestion. Commonly, combinations of [*L. GG* + *B. longum*], [*L. acidophilus* + *B. bifidum*], [*L. acidophilus* + *B. infantis*], or [*L. casei* + *B. breve*] are used. For two or more mixtures, [*B. bifidum* (± *B. lactis*) + *B. infantis* + *L. acidophilus*], [*Lactobacillus* (*acidophilus* + *rhamnosus GG* + *casei* + *plantarum*) + *B infantis* + *Streptococcus thermophilus*] or [*B. infantis* + *B. lactis* + *Streptococcus thermophilus*] are commonly used.

The Effects of Probiotics and Their Prevention of NEC, Morbidity, and Sepsis in VLBWIs with the Administration of Single-strain or Multi-strain Probiotics

Recently, increasing numbers of studies have focused on the effects of probiotics, and meta-analyses have been performed to identify the clinical effects of probiotics in preterm infants [35-38]. Although there are differences among these analyses, a vast number of reports suggest that supplementation with probiotics prevents NEC and mortality in preterm infants. Mihatsch et al. [5] conducted a systematic review that identified the beneficial effects of some probiotics in preterm infants <37 weeks of gestational age with a significant decrease in the severity of NEC. Furthermore, a Cochrane Database review that included 37 randomized trials also reported a significant decrease in the risk of late-onset sepsis following administration of probiotics in preterm infants; however, these results were only seen when they excluded studies that had risk of bias [36]. A different Cochrane Database review of 24 randomized studies showed inconsistent results for nosocomial sepsis in preterm infants weighing less than 2,500 g at birth, but the small sample size was inadequate to prove significant benefit for sepsis [37]. Although the outcome suggested that the administration of probiotics was related to a decrease in NEC or mortality rate, there was no significant difference in the incidence of nosocomial sepsis. The most recent meta-analysis conducted in 2016 amongst 5,033 infants weighing less than 2,500 g and with a gestational age of 37 weeks also reported significant decrease in severe NEC and all-cause mortality in a group given a probiotic, but not for a culture-proven sepsis group [38]. The VLBWIs are predisposed to the development of dysbiosis of the gut microbiome and are a research

priority group to study the effect of probiotics. "Dysbiosis" can also facilitate bacterial translocation through the intestinal mucosa barrier.

Of single-strain studies, a multicenter study conducted in Taiwan compared the outcomes in 217 VLBWIs assigned to either a control group or intervention groups given *B. bifidum* and *L. acidophilus* along with probiotics [39]. In their investigation, the beneficial effects of probiotics were clearly noted along with a significant reduction in NEC (1.8% vs. 6.5%, respectively, p=0.02) and in the instance of NEC or the all-cause mortality rate (1.8% vs. 9.2%, respectively, p=0.02). In 2012, Wang et al. [40] reported that administration of probiotics decreased NEC (RR 0.33, 95% CI 0.24 to 0.46) and mortality rate (RR 0.56, 95% CI 0.43 to 0.73) in VLBWIs based on a published meta-analysis that used data from 20 other studies. In 2014, Oncel et al. [41] conducted a randomized controlled study to evaluate the effect of oral Lactobacillus reuteri on severity of NEC and on sepsis in preterm infants <32 weeks of gestational age. Although beneficial effect was not observed in incidence of NEC, significant reduction in sepsis was noted (6.5% vs. 12.5%). Similarly, investigation conducted by Demirel et al. [42] demonstrated that positive effects of probiotics were shown with decrease in clinical sepsis with slight difference (34.8% vs. 47.8%: control vs. probiotics), but were not observed in mortality from NEC using *S. boulardii*. In 2007, Stratiki et al. [43] reported that VLBWIs with *B. lactis* (2 × 10(7) cfu/g of dry milk) showed decreased intestinal permeability during a sugar absorption test and displayed increased head circumference despite no significant differences in NEC, sepsis, or mortality rate between the two groups compared to the control group. An Italian study reported low NEC and all-cause mortality in VLBWIs that received L. GG but did not show a statistically meaningful clinical difference in any subgroup [44]. The PiPS trial of probiotic efficacy did not support the routine administration of *B. breve* and found no evidence of benefit for prevention of NEC [45]. Studies conducted by Mihatsch et al. [35] using *B. lactis* and Sari et al. [46] with L. sporogenes both demonstrated a positive effect in improving the feeding tolerance with probiotics during breastfeeding, but there was no significant variation in NEC, sepsis, or mortality rate between the two groups (Table 1).

Study	Inclusion Criteria		Number randomized in each group	Probiotic Species	Total Dose (cfu/day)	Duration	Decrease in NEC	Decrease in Sepsis
	Gestational age	Birth weight						
Stratiki et al., 2007 [43]	27-37 weeks	None	Probiotic: 41 Control: 34	*B. lactis*	0.2 billion/kg	Not stated	No effect	31.7% vs. 69.4%
Mihatsch et al., 2010 [35]	Less than 30 weeks	Less than 1,500 g	Probiotic: 93 Control: 90	*B. lactis*	12 billion /kg	28 days or more	No effect	No effect
Sari et al., 2011 [46]	Less than 33 weeks	Less than 1,500 g	Probiotic: 110 Control: 111	*L. sporogenes*	0.35 billion	28 days or more	5.5% vs. 9% (stage ≥ 2)	No effect
Demirel et al., 2013 [42]	Less than 32 weeks	1,500 g or less	Probiotic: 135 Control: 136	*S. boulardii* (Reflor®)	5 billion	28 days or more	No effect	34.8% vs. 47.8% (clinical sepsis)
Oncel et al., 2014 [41]	32 weeks or less	1,500 g or less	Probiotic: 200 Control: 200	*L. reuteri*	0.1 billion	28 days or more	No effect	6.5% vs. 12.5%
Costeloe et al., 2016 [45] (the PiPS Trial)	23-30 weeks	None	Probiotic: 650 Control: 660	*B. breve* BBG	0.2-0.53 billion	28 days or more	No effect	No effect

Table 1: Characteristics of studies with single-strain probiotics in very low birth weight infants.

Detailed characteristics of multi-strain probiotics supplement in VLBWIs were also reviewed (Table 2). Recently, Jacobs et al. [47] conducted a randomized trial (ProPrems) in Australia and New Zealand that investigated VLBWIs at a gestational age <32 weeks using a preparation of three different strains (*B. infantis* (3 × 108) + *Streptococcus thermophilus* (3.5 × 108) + *B. lactis* (3.5 × 108)). There were no significant differences in sepsis (23% vs. 26%: probiotics vs. control) or mortality (4.9% vs. 5.1%: probiotics vs. control) rates between the two groups, but the infants who were given a compound strain of 109 until discharge showed a decreased rate of NEC (2% vs. 4.4%, p=0.03). However, the incidence of late-onset sepsis after administration of probiotics differed according to gestational age and showed a decrease in incidence in infants who were >28 weeks of gestational age. Previous studies showed that supplementation with probiotics reduced the incidence of feeding intolerance and sepsis in VLBWIs [41,42]. An investigation conducted in Mexico in 2013 analyzed the positive effects of probiotics in VLBWIs and showed statistically significant decrease in NEC and in the overall morbidity rate (9% vs. 25%, respectively, p=0.015) [48]. Studies were further conducted by Lin et al. [29,39] both using *L. acidophilus* and *B. infantis* in VLBWIs. While both study results demonstrated positive effects of multi-strain probiotics in decreasing NEC, trials tested at birth showed no effect in improving sepsis. However, probiotics groups administered at <34 weeks experienced alleviated severity in NEC (1.1% vs. 5.3%: probiotics vs. control). Results of administration of multiple strains were statistically significant in decreasing incidence of

NEC, but the beneficial effects noted in NEC were not investigated in sepsis groups, showing either no effect or mild improvement. Of these results, studies conducted by Braga and Ren et al. [8,9] demonstrated meaningful data in NEC using multiple strains, but showed no effect in alleviating sepsis. Furthermore, Rapa et al. [21] reported that the compound administration of probiotics with *L. acidophilus* and *B. infantis* showed a meaningful alleviation in the incidence of NEC in breastfed VLBWIs, but did not demonstrate any difference in formula-fed VLBW Is. The studies that utilize probiotic mixtures are still needed to prove their significant outcome in infants with birth weight less than 1,000 g (Table 2). Despite the controversy regarding a beneficial effect of probiotics on the incidence of NEC and sepsis, multi-strain probiotics seem to be a reasonable choice with their impact on reducing rates of NEC [44].

Study	Inclusion Criteria		Number randomized in each group	Probiotic Species	Total Dose (cfu/day)	Duration	Decrease in NEC	Decrease in Sepsis
	Gestational age	Birth weight						
Lin et al., 2005 [29]	None	Less than 1,500 g	Probiotic: 180	*L. acidophilus*	1 billion/kg	28 days or more	1.8% vs. 9.2%	No effect
			Control: 187	*B. infantis*				
Lin et al., 2008 [39]	Less than 34 weeks	Less than 1,500 g	Probiotic: 222	*L. acidophilus*	1 billion/kg	6 weeks	1.1% vs. 5.3%	12.2% vs. 19.3%
			Control: 221	*B. infantis*				
Ren, 2010 [9]	28-33 Weeks	1,000–1,800 g	Probiotic: 80	*B. infantis*	0.016 billion	Up to 13 days	3.7% vs. 7.1%	No effect
				L. acidophilus				
			Control: 70	*E. faecalis*				
				Bacillus cereus (B. tetravaccine)				
Braga et al., 2011 [8]	None	750-1,499 g	Probiotic: 119	*L. casei*	0.035 - 3.5 billion	28 days or more	0% vs. 3.6% (stage ≥ 2)	No effect
			Control: 112	*B. breve*				
Jacobs, 2013 [47] (The ProPrems trial)	Less than 32 weeks	Less than 1,500 g	Probiotic: 548	*B. infantis*	1 billion	28 days or more	2.0% vs. 4.4% (stage ≥ 2)	5.5% vs. 10.8% (subgroup analysis)
				B. bifidum				
			Control: 551	*S. thermophilus*				
Fernandez-Carrocera et al., 2013 [48]	"preterm"	Less than 1,500 g	Probiotic: 75	*L. acidophilus*	2.65 billion	28 days or more	9.3 vs. 25.3% (NEC or death)	No effect
				L. rhamnosus				
				L. casei				
			Control: 75	*L. plantarum*				
				B. infantis				
				S. thermophillus				

Table 2: Characteristics of studies with multi-strain probiotics in very low birth weight infants.

Limitations of Probiotics in Preterm Infants and Future Directions

The perinatal and early postnatal periods are often called a "window of vulnerability" for microbiota establishment because of their putative role in producing an immune-modulator with potentially life-long consequences. In contrast, infants with poor immunity were found to be susceptible to infections caused by probiotics. Though such infections caused by administered probiotics were more frequently found in adults, late-onset sepsis due to infection from identical strain (*L. rhamnosus* strain GG) was noticed in VLBWIs with underlying diseases and incomplete immunity [49,50]. Probiotics are live microorganisms that may impact patients with incomplete immunity and intestinal integrity with diverse effects due to the characteristics. Careful attention is needed, especially clear identification of relation between probiotics and sepsis is yet to be established in cases of VLBWIs with severe disease where intestinal integrity is threatened. A larger trial is needed to examine the long-term effects of probiotics, especially since preterm infants are exposed to risk of infection due to having an incomplete immune system. Several studies have identified the benefits of probiotics in reducing the rates of NEC and morbidity in VLBW preterm infants; however, the clinical use of probiotics remains unclear without standard guidelines. Therefore, multilateral approaches including the type, amount, and period of administration will be needed to safely and efficiently use probiotics in preterm infants. Currently, data regarding long-term follow-up on probiotic administration is sparse. In order to establish standardized guidelines, appropriate strain should be selected from VLBWIs and breastfeeding

should be encouraged to develop normal intestinal flora and barrier. Recent studies support that multiple strains probiotics is the most promising therapy to prevent NEC and mortality in VLBWIs, but identical effectiveness was relatively found less in sepsis. Beneficial effects on NEC discovered in multi-strain probiotics were marginal in single strain probiotic. Further studies on the optimal combination of species, influence of probiotics on neurodevelopment, long-term immunity and sepsis are needed to decrease the incidence of NEC and promote intestinal integrity in VLBWIs as a preventive strategy.

Competing Interests

The authors declare that they have no competing interest.

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