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Probiotics and Innate and Adaptive Immune Responses in Premature Infants

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Abstract

Premature infants are at increased risk for morbidity and mortality due to necrotizing enterocolitis (NEC) and sepsis. Probiotics decrease the risk of NEC and death in premature infants; however, mechanisms of action are unclear. A wide variety of probiotic species have been evaluated for potential beneficial properties in vitro, in animal models, and in clinical trials of premature infants. Although there is variation by species and even strain, common mechanisms of protection include attenuation of intestinal inflammation, apoptosis, dysmotility, permeability, supplanting other gut microbes through production of bacteriocins, and more effective use of available nutrients. Here, we review the most promising probiotics and what is known about their impact on the innate and adaptive immune response.

Keywords

bifidobacteria; lactobacilli; necrotizing enterocolitis; probiotic; sepsis

I. INTRODUCTION

Initial colonization of the intestinal tract begins in utero but is heavily influenced by timing of rupture of the fetal membranes, mode of delivery, initial feeding mode, gestational age, exposure to antibiotics and other medications, and length of stay in a hospital setting. This initial colonization appears to have long-term consequences on the composition of the adult intestinal microbiota and risk for developing a variety of diseases, likely due to interactions between gut microbes and the host immune system.¹ The concept of dysbiosis (alteration in the microbiota of a specific anatomic niche associated with disease) is perhaps most immediately evident in the premature infant. This population is particularly vulnerable in the short term to diseases related to the composition of the intestinal microbiota, including sepsis and necrotizing enterocolitis (NEC),² and to long-term consequences of premature delivery, including neurodevelopmental delays and chronic lung disease, both of which appear to be related to inflammation triggered by an immature immune response.^{3,4}

Probiotics are variably described as food products, dietary supplements, or drugs that contain live microbes designed to be ingested to improve health outcomes. This review

briefly summarizes the evidence for benefit of probiotics in preventing sepsis and NEC in premature infants (Tables 1 and 2) and considers potential mechanisms by which common probiotic species influence innate and adaptive immune responses of the premature infant.

II. NEONATAL SEPSIS

Sepsis during the neonatal period is generally divided into early onset (first 48–72 h of life) and late onset (first 48–72 h to 28 d of life). This classification is somewhat artificial but is meant to distinguish sepsis directly related to perinatal infection (early) versus nosocomial infection (late). Evidence that preterm labor and early-onset sepsis are both influenced by the composition of the maternal microbiota has affected current guidelines regarding administration of antibiotics to women with vaginal dysbiosis,⁵ preterm premature rupture of membranes,⁶ and women colonized with group B streptococcus⁷ and suggests that maternal probiotic administration to decrease risk of preterm labor may be helpful.⁸ Late-onset sepsis in premature infants is common and likely influenced by immaturity and microbiota in both the skin and gastrointestinal tract. The hypothesis that altering intestinal microbiota in the premature infant would decrease the risk of translocation of intestinal pathogens and therefore decrease the risk of gut-related sepsis is compelling. Several clinical trials of probiotics in premature infants have included late-onset sepsis as a primary or secondary outcome. The largest study to date found no significant difference between the probiotic and the placebo group; however, the subgroup of infants with gestational age ≥ 28 weeks showed a decrease in culture-proven late-onset sepsis with probiotic treatment (18 of 327 infants in the probiotic group vs 34 of 345 infants in the placebo group; relative risk [RR] 0.51, 95% confidence interval [CI] 0.29–0.88).⁹ A recent meta-analysis of blinded randomized placebo-controlled trials found no difference in nosocomial sepsis (typical RR 0.91, 95% CI 0.80–1.03; 19 studies, 5338 infants).¹⁰

III. NEC

NEC is a common and devastating disease that predominantly affects premature infants. Risk factors include degree of prematurity (the youngest preterm infants are at the highest risk), enteral feeding (particularly with infant formula),¹¹ and exposure to antibiotics^{12,13} or acid-blocking agents.¹⁴ The extent to which intestinal hypoxia and ischemia have a role in NEC in the premature infant is unclear. Intestinal dysbiosis has recently been proposed as an important factor in the pathogenesis of NEC. This hypothesis is consistent with four key observations: (1) dysbiosis is common in premature infants and changes with development,¹⁵ (2) antibiotics and acid-blocking agents are both associated with NEC and with intestinal dysbiosis,¹⁶ (3) feeding human milk to premature infants reduces the risk of NEC¹¹ and influences the intestinal microbiota,¹⁷ and (4) dysbiosis has been demonstrated in several animal models of NEC.^{18–20} Several small but carefully performed studies of fecal specimens obtained before the onset of NEC symptoms suggest that a bloom of Gram-negative proteobacteria (family Enterobacteriaceae)^{21–23} or Gram-positive firmicutes (family Clostridiaceae)^{23–25} may be an early marker or trigger of NEC.²

The first demonstration of a decrease in the incidence of NEC was in a cohort study performed in Colombia.²⁶ Since that time, multiple randomized clinical trials and cohort

studies have been performed, many of which confirmed a decreased risk for developing NEC in premature infants receiving probiotic products. A recent meta-analysis of blinded randomized placebo-controlled trials found the relative risk of NEC to be 0.43 (95% CI 0.33–0.56, 5529 infants, 20 studies), compared to that of infants receiving placebo.¹⁰

IV. ADAPTIVE AND INNATE IMMUNITY IN THE PREMATURE INFANT

The host immune response in mammals includes innate immunity, characterized by rapid onset, limited specificity, and lack of memory, and adaptive immunity, characterized by slower onset of response to initial exposure plus a memory response allowing a rapid and highly specific response to future exposures. These two responses are tightly integrated with some overlap, but in general, innate immunity includes barriers, cellular components (e.g., neutrophils, macrophages, Paneth cells, NK cells, and dendritic cells), sensors (e.g., Toll-like receptors [TLRs], nucleotide-binding oligomerization domain–like receptors, and soluble CD14), and secreted molecules (e.g., antimicrobial peptides and proteins, complement proteins, and interferons), whereas adaptive immunity includes T and B cells with their associated antibodies, cytokines, and chemokines. Premature infants are particularly vulnerable, because the adaptive immune response is severely limited by lack of exposure and highly dependent on maternal antibodies transferred through the placenta and/or human milk (both of which are disrupted by premature delivery). In addition, essentially every aspect of the innate immune system studied to date shows some degree of dysfunction in the premature infant. This is particularly true of the premature gastrointestinal tract, which has decreased gastric acid production, altered gastric emptying, decreased Paneth cell function, altered intestinal motility, abnormalities in intestinal mucus production, increased permeability of the intestinal monolayer, and a poorly regulated inflammatory response, all of which increase the potential for bacterial stasis, dysbiosis, bacterial translocation, sepsis, and NEC.²⁷

V. IMPACT OF PROBIOTIC MICROBES ON THE ADAPTIVE AND INNATE IMMUNE SYSTEMS OF PREMATURE INFANTS

A. Bifidobacteria

1. Bifidobacterium longum ssp. infantis—*Bifidobacterium longum* ssp. *infantis* (BLI) is appealing as a probiotic for infants for the following reasons: (1) In healthy breast-fed infants in developing countries, BLI is the dominant gut microbe, and increased numbers of BLI in the feces are associated with improved growth, thymus size, and vaccine response.²⁸ (2) The bacterial genomes of several strains of BLI have been sequenced and demonstrate a conserved group of genes essential for the capture, internalization, and complete digestion of human milk oligosaccharides (HMOs).²⁹ (3) BLI shows more dramatic growth in culture media wherein HMOs are the only carbon source than other bifidobacteria.³⁰ These observations, together with the discovery that the human intestine is unable to digest HMOs, suggest that human milk and BLI (either by design or coevolution) work synergistically to shape the intestinal microbiota of the neonate at significant cost to the mother.^{31,32} Furthermore, when grown in the presence of HMOs, BLI becomes “activated,” expressing genes that are not expressed by the same strain grown in the presence of lactose.³³

Comparisons among bifidobacterial species have yielded valuable insights: BLI is better able to colonize the intestinal tract of the premature infant than *Bifidobacterium animalis* ssp. *lactis* (BAL);³⁴ BLI is able to use the full range of HMOs as a nutrient source, whereas *Bifidobacterium bifidum* (BBi) is unable to use fucosylated and sialylated HMOs,³⁵ and *Bifidobacterium breve* (BBr) predominantly uses neutral HMOs, with its capacity to use fucosylated and sialylated HMOs varying by strain;³⁶ and BLI binds to Caco-2 cells in vitro more effectively than BBr.³³ BLI has been administered in several clinical trials and cohort studies to premature infants (Tables 1 and 2). A single bacteriocin produced by BLI has been identified with activity against other lactic acid bacilli as well as staphylococci, streptococci, *Salmonella*, and *Escherichia coli*.³⁷ In addition to advantages in colonizing the neonatal intestine in the presence of human milk, BLI has anti-inflammatory properties that are likely due to products secreted by the microbe. In vitro, activated BLI down-regulates a variety of genes important in chemokine and cytokine signaling.³³ In human immature xenografts, both BLI and the culture medium in which BLI was grown down-regulate interleukin (IL)1 β -induced IL8 and IL6 expression, whereas this effect is not seen in mature human xenografts (suggesting selective anti-inflammatory activity in the premature intestinal tract).³⁸ In the rat model of NEC, BLI decreased incidence and severity of NEC and decreased expression of IL6, IL8, tumor necrosis factor α (TNF α), IL23, and inducible nitric oxide synthase (iNOS).²⁰ In the mouse, culture medium from BLI decreases the severity of infection with *Cronobacter sakazakii* with attenuation of three key processes induced by this pathogen: induction of IL1 β and TNF α , decrease in mucin production, and increase in apoptosis.³⁹

2. *Bifidobacterium bifidum*—BBi is commonly included in commercial probiotic products and has been studied in several clinical trials and cohort studies in premature infants. Two broad-spectrum bacteriocins have been identified, with production highly strain specific.³⁷ In vitro studies in cultures of feces from premature infants suggest that strains of BBi induce higher production of short-chain fatty acids than several other tested strains of bifidobacteria.⁴⁰ In the rat model of NEC, BBi decreases the incidence and severity of NEC, attenuates induction of IL6, attenuates induction of trefoil factor 3 and several antimicrobial peptides (lysozyme, secretory phospholipase A2, and pancreatic-associated proteins 1 and 3), improves barrier function, activates TLR2, and decreases apoptosis through alterations in cyclooxygenase-2 signaling.^{41–43}

3. *Bifidobacterium breve*—BBr is found in several foods and dietary supplements and has been administered to premature infants in clinical trials and cohort studies. In the rat model of NEC, BBr decreased incidence and severity of NEC, attenuated TLR4 signaling, increased TLR2 signaling, and suppressed inflammation.⁴⁴ In weanling rat pups, BBr ameliorated dextran sulfate sodium (DSS)-induced colitis with attenuations in DSS-induced elevations in IL1 α , IL1 β , IL10, TNF α , and transforming growth factor β (TGF β) in the colon.⁴⁵ In HT-29 cell culture, BBr showed strain-specific induction of a variety of TNF α -induced genes.⁴⁶ In the ovalbumin sensitized mouse, administration of BBr suppressed T-helper type-2 responses and immunoglobulin E production, suggesting a potential role in allergy prevention.⁴⁷ In premature infants, BBr decreased fecal butyric acid levels after 4 wk of administration⁴⁸ and in a small study increased serum levels of TGF β and the TGF β

signaling molecule Smad2 but did not alter serum levels of IL4 (IL5, IL6, TNF α , and interferon γ [IFN γ] were undetected in the serum).⁴⁹

4. *Bifidobacterium animalis* ssp. *lactis*—BAL is commonly added to foods and dietary supplements and has been studied in several clinical trials and cohort studies in premature infants. A single bacteriocin has been identified with activity against a variety of gut-related pathogens.³⁷ Recently, a bacteriocin-like substance with activity against *Listeria* was identified from a strain of BAL.⁵⁰ In vitro, BAL strengthens tight junctions, as measured by increased transepithelial electrical resistance; however, this appears to be strain specific.⁵¹ BAL has not been studied in animal models of NEC, but in adult animal models of colitis, BAL attenuates weight loss, improves histologic scores, decreases expression of IL6 and TNF α , decreases infiltration of inflammatory T cells, and increases numbers of regulatory T cells in the gut.⁵² Through alteration in the cecal pH and production of short-chain fatty acids, BAL has been shown in the adult mouse to create a niche that is inhospitable to colitogenic bacteria.⁵³ As noted above, BAL is unable to use HMOs as a nutrient source and cannot colonize the intestinal tract of the premature infant even at higher doses;³⁴ however, at least one study demonstrated a significant reduction in NEC with administration of BAL, suggesting that colonization may not be essential to disease prevention.⁵⁴

B. Lactobacilli

1. *Lactobacillus acidophilus*—*Lactobacillus acidophilus* (LA) is a common food additive, facilitating fermentation of a wide variety of foods, including yogurt and other dairy products. It is stable across a range of pH and temperature. Administration of LA strains to premature infants in clinical trials is common. Studied mechanisms of action include the following. (1) LA provides direct antimicrobial activity against other gut microbes through production of a wide variety of bacteriocins.⁵⁵ Activity against pathogens associated with sepsis and NEC (e.g., *C. sakazakii*) may be of particular relevance in premature infants.⁵⁶ A recent review summarized 27 bacteriocins produced by strains of LA with a broad range of antimicrobial activity against other lactobacilli, Enterobacteriaceae, *Bacillus* sp., *Corynebacterium* sp., *Vibrio* sp., and Staphylococci.⁵⁷ (2) LA and the supernatant from cultured LA have direct anti-inflammatory effects in vitro. Both inhibit the induction of nuclear factor- κ B (NF- κ B) and IL8 by platelet activating factor, an inflammatory pathway important in NEC. The response to the supernatant is similar to that of live LA (but not seen with killed LA), suggesting that a secreted molecule is central to this mechanism. Cell-culture studies suggest two possible pathways: modulation of signaling events associated with activation of the inhibitor of NF- κ B kinase complex⁵⁸ and induction of TGF- β /microRNA 21 (MIR21) expression and down-regulation of SMAD7 expression.⁵⁹ (3) Colonocytes use butyric acid produced by gut microbes as their primary nutrient source. Some gut pathogens (e.g., enteropathogenic *E. coli* [EPEC]) inhibit uptake of butyrate by the host. LA and its supernatant (but not killed LA) facilitate host butyrate uptake by increasing expression of monocarboxylate transporter 1 on the apical surface of the colonocyte and inhibit EPEC-induced disruption of this process.⁶⁰ (4) LA induces production of reactive oxygen species (ROS) in the host epithelial cell. ROS appear to be important signaling molecules for inducing epithelial cell proliferation.⁶¹ (5) Of particular relevance to

premature infants, a recent study of alteration in gene expression in immature human fetal intestinal epithelial cells demonstrated marked changes after exposure to conditioned media from LA in hundreds of genes involved in the immune response, apoptosis and cell survival, cell adhesion, the cell cycle, development, and angiogenesis.⁶²

2. *Lactobacillus reuteri*—*Lactobacillus reuteri* (LRe) is among the most common of the lactic acid bacilli, and varying strains are found in the intestinal tract and feces of a wide variety of animals. LRe has been administered to premature infants in clinical trials and cohort studies and has shown direct antimicrobial activity through secretion of at least one bacteriocin (reuterin) and an antibiotic (reutericyclin).^{63,64} In vitro studies have demonstrated strain-specific anti-inflammatory effects via suppression of TNF in activated human macrophages. The likely mechanism is inhibited activation of mitogen-activated protein kinase-regulated c-Jun and the transcription factor AP-1.⁶⁵ In animal models, several strains of LRe show variable suppression of gut inflammation induced by LPS administration (decreased IFN γ and IL8) or by cow-milk feeding (decreased IFN γ and IL13).⁶⁶ In a rat model of NEC, LRe strains DSM 17938 and ATCC phosphate acetyltransferase (PTA) 4659 decreased the incidence and severity of NEC; decreased messenger RNA expression of IL6, TNF α , TLR4, and NF- κ B and protein expression of TLR4, TNF α , and IL1 β ;⁶⁷ and increased numbers of regulatory T cells in the ileum (particularly in dam-fed animals).⁶⁸

LRe also impacts intestinal motility, which may be of importance in the premature infant given immaturity of peristaltic activity. Rats fed LRe had increased excitability of sensory neurons in the myenteric plexus (decreased threshold for activation plus an increase in action potentials generated) with the likely mechanism inhibition of a calcium-dependent potassium channel.⁶⁹ *Ex vivo* spatio-temporal mapping of the intestinal tract demonstrated strain- and region-specific changes in gut motility. LRe strain DSM 17938 decreased migrating motor complex (MMC) frequency and velocity in the jejunum but increased both in the colon, whereas LRe strain PTA showed no effect in either location.⁷⁰

3. *Lactobacillus rhamnosus*—(LRh) can be found in the intestinal tract and genitourinary tract of healthy adults. A popular strain, LRh GG ATCC 53103, is perhaps the most studied commercially available probiotic. LRh GG has antimicrobial activity that appears to be predominately related to reducing luminal pH through production of lactic acid and other bacterial products.⁷¹ LRh GG also has anti-inflammatory activity; up-regulation of the IL10R2 receptor subunit appears to be the key mechanism by which this microbe decreases expression of TNF α and MIP2 and increases expression of the suppressors of cytokine signaling family.⁷² In neonatal mice, LRh GG facilitates maturation of intestinal barrier function, likely through a combination of stimulation of antiapoptotic and cytoprotective responses⁷³ including up-regulation of the tight junction protein claudin 3⁷⁴ and inhibition of apoptosis by an LRh GG-derived soluble protein p40.⁷⁵ A different strain of LRh (HN001) decreased incidence and severity of NEC in both the piglet and the mouse and appears to influence TLR9 signaling because the benefit was not seen in mice with selective knockout of TLR9 in the intestinal tract.⁷⁶ It is noteworthy that the benefits of LRh HN001 were also apparent with administration of killed organisms. LRh strain JB1 altered

intestinal motility in the ex vivo model by decreasing MMC frequency and velocity (the opposite pattern from that seen with LRe).⁷⁰

C. Combination Products

One of the challenges in this field is that many commercial probiotic products contain several strains of bacteria, and interactions between strains may influence efficacy. The culture media from the combination of BLI and LA was evaluated in the previously mentioned study of alteration in gene expression in immature human fetal intestinal epithelial cells and demonstrated more profound changes than either BLI media or LA media alone.⁶² Adherence of pathogenic bacteria to the intestinal mucosa may be an important step in NEC pathogenesis and was better prevented by a combination of two probiotics (BAL plus LRh) than either probiotic alone.⁷⁷ Perhaps the most compelling data suggesting synergy between probiotic strains are seen in animal studies that demonstrate improved protection against NEC from either combination probiotic products (e.g. BBi plus *Bifidobacterium longum*)⁷⁸ or from conditioned media from multiple probiotic organisms (e.g., LA plus BLI plus *Lactobacillus plantarum*).⁷⁹ In premature infants, there are only a few clinical trials directly comparing probiotics containing a single organism and a combination of organisms. A cohort study comparing administration of a single species (BBr) to three species (BBr, BLI, and *Bifidobacterium longum* ssp. *longum*) to premature infants demonstrated that the three-microbe combination resulted in higher and more sustained levels of bifidobacteria in the feces (though some of the difference may have been the result of a dose effect) and that BBr and BLI were more effective colonizers than *B. longum*. Animal studies suggest decreased risk of NEC with multimicrobe cocktails compared to single probiotic regimens.⁷⁸ A meta-analysis of randomized controlled trials suggested no difference in the relative risk of NEC between single probiotic microbe trial and those with combination probiotics.⁸⁰ Probiotics are commonly used in food animal production. A probiotic containing four lactobacilli (LA, *L. fermentum*, LRe, *L. salivarius*) provided to broiler chicks inhibited colonization by *Salmonella* enteritidis, decreased proinflammatory cytokines (IL1 β , IL6, IL10, and LPS-induced TNF α factor), and increased TLR2 expression.⁸¹ A cocktail of four probiotic strains improved colitis in the adult DSS colitis mouse model, with attenuation of TLR4 signaling and decreases in iNOS and NF- κ B expression.⁸²

VI. CONCLUSION AND FUTURE DIRECTIONS

The premature infant is at a particularly high risk of disease related to the intestinal microbiota due in part to prolonged hospitalizations; frequent exposure to antibiotics; and, most importantly, immaturity of the intestinal tract and the innate and adaptive immune systems. Probiotics decrease the risk of NEC and death in premature infants, but a trend toward decreased risk of sepsis does not reach statistical significance. The degree of risk reduction is similar between randomized controlled trials and cohort studies. Effects of various probiotic microbes on the innate and adaptive immune systems are highly species and even strain specific. From available studies, the most promising probiotic microbes for this population include BLI, BAL, BBi, LA, LRe, and LRh. Future studies of probiotics in premature infants should include products of demonstrated purity and viability, specification

of the strain administered, and inclusion of subgroup analyses by feeding type and gestational age or birth weight. Comparisons of promising probiotic microbes in large cluster-randomized clinical trials will be necessary to determine the most effective organisms or combinations.

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ABBREVIATIONS

ATCC	American Type Culture Collection
BLI	<i>Bifidobacterium longum</i> ssp. <i>infantis</i>
BBi	<i>Bifidobacterium bifidum</i>
BBr	<i>Bifidobacterium breve</i>
BAL	<i>Bifidobacterium animalis</i> ssp. <i>lactis</i>
DSM	Deutsche Sammlung von Mikroorganismen (German collection of microorganisms)
HMO	human milk oligosaccharide
LA	<i>Lactobacillus acidophilus</i>
LRe	<i>Lactobacillus reuteri</i>
LRh	<i>Lactobacillus rhamnosus</i>
NEC	necrotizing enterocolitis
SMAD7	mothers against decapentaplegic homolog 7
TNF	tumor necrosis factor

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Thirty-five randomized controlled trials of probiotics published in English, including at least one of the following clinical outcomes: NEC stage 2 or greater, culture-positive sepsis, or death

TABLE 1

Authors	Country	Year	Probiotic species	Number enrolled		NEC cases, stages 2, 3		Culture + sepsis cases		Deaths	
				Pro	Con	Pro	Con	Pro	Con	Pro	Con
Costeloe et al. ⁸³	UK	2015	BBr	650	660	62	66	73	77	54	56
Dilli et al. ⁵⁴	Turkey	2015	BAL or BAL + inulin	200	100	6	18	16	13	6	12
Saengtaewasin et al. ⁸⁴	Thailand	2015	LA + <i>Bifidobacterium bifida</i>	31	29	1	1	2	1	0	0
Tewari et al. ⁸⁵	India	2015	<i>Bacillus clausii</i>	123	121	0	0	20	25	12	14
Hays et al. ⁸⁶	France	2015	BAL or <i>Bifidobacterium longum</i> or BAL + <i>B. longum</i>	147	52	8	3	25	10	5	1
Van Niekirk et al. ⁸⁷	South Africa	2015	BLI + LRh	91	93	0	4	15	10	5	7
Dutta et al. ⁸⁸	India	2015	LA + LRh + <i>B. longum</i> + <i>Saccharomyces boulardii</i>	114	35	6	0	10	6	8	2
Patole et al. ⁸⁹	Australia	2014	BBr	79	80	0	1	17	12	0	0
Oncel et al. ⁹⁰	Turkey	2014	LRh	200	200	8	10	13	25	15	20
Totsu et al. ⁹¹	Japan ^a	2014	BBi	153	130	0	0	6	10	2	0
Manzoni et al. ⁹²	Italy + NZ	2014	LRh + bovine lactoferrin	238	258	0	14			9	26
Jacobs et al. ⁹	Australia + NZ	2013	BLI + <i>Saccharomyces thermophilus</i> + BAL	548	551	11	24	72	89	27	28
Serce et al. ⁹³	Turkey	2013	<i>S. boulardii</i>	104	104	7	7	19	25	5	4
Demirel et al. ⁹⁴	Turkey	2013	<i>S. boulardii</i>	135	136	6	7	20	21	5	5
Rojas et al. ⁹⁵	Colombia	2012	LRh	372	378	9	15	24	17	22	28
Fernandez-Carrocera et al. ⁹⁶	Mexico	2012	LA + LRh + <i>Lactobacillus casei</i> + <i>Lactobacillus plantarum</i> + BLI + <i>S. thermophilus</i>	75	75	6	12			1	7
Al-Hosni et al. ⁹⁷	US	2012	BLI + LRh	50	50	2	2	13	16	3	4
Sari et al. ⁹⁸	Turkey	2011	<i>Lactobacillus sporogenes</i>	110	111	6	10			3	4
Braga et al. ⁹⁹	Brazil	2011	L. casei + BBr	119	112	0	4			26	27
Romeo et al. ¹⁰⁰	Italy	2011	LRh or LRh	166	83			3	9		
Awad et al. ¹⁰¹	Egypt	2010	LRh	60	30	0	5				
Mihatsch et al. ¹⁰²	Germany	2010	BAL	91	89	2	4	28	29	2	1

Authors	Country	Year	Probiotic species	Number enrolled		NEC cases, stages 2, 3		Culture + sepsis cases		Deaths	
				Pro	Con	Pro	Con	Pro	Con	Pro	Con
Underwood et al. ¹⁰³	US	2009	LA + <i>B. longum</i> + BLI + BBi or LRh	61	29	2	1	8	5	0	0
Samanta et al. ¹⁰⁴	India	2009	<i>B. longum</i> + BLI + BBi + LA	91	95	5	15	13	2	4	14
Rouge et al. ¹⁰⁵	France	2009	<i>B. longum</i> + LRh	45	49	2	1	15	13	2	4
Lin et al. ¹⁰⁶	Taiwan	2008	LA + BBi	217	217	4	14	40	24	2	9
Stratiki et al. ¹⁰⁷	Greece	2007	BAL	41	36	0	3	0	3		
Wang et al. ⁴⁸	Japan ^b	2007	BBr	33	33	0	0	0	0		
Manzoni et al. ¹⁰⁸	Italy	2006	LRh	39	41	1	3	19	22	5	6
Fuji et al. ⁴⁹	Japan	2006	BBr	11	8	0	0	1	1		
Lin et al. ¹⁰⁹	Taiwan	2005	LA + BLI	180	187	2	10	22	36	7	20
Bin-Nun et al. ¹¹⁰	Israel	2005	BLI + <i>S. thermophilus</i> + BAL	72	72	1	10			3	8
Costalos et al. ¹¹¹	Greece	2003	<i>S. boulardii</i>	51	36	5	6	3	3		
Dani et al. ¹¹²	Italy	2002	LRh	295	290	4	8	14	12	0	2
Reuman et al. ¹¹³	US	1986	LA	15	15					1	3
Totals				5007	4585	166	278	511	542	234	256
Percentage (among those reporting the outcome)						3.4	6.2	12	14	5.0	7.2

^aCluster-randomized trial of 19 neonatal intensive-care units.

^bNumber of NEC cases obtained directly from the author.

BAL: *Bifidobacterium animalis* ssp. *lactis*; BBi: *Bifidobacterium bifidum*; BBr: *Bifidobacterium breve*; BLI: *Bifidobacterium longum* ssp. *infantis*; Con, control; LA: *Lactobacillus acidophilus*; LRe: *Lactobacillus reuteri*; LRh: *Lactobacillus rhamnosus*; NEC, necrotizing enterocolitis; NZ, New Zealand; pro, probiotic; UK, United Kingdom; US, United States.

TABLE 2

Eight cohort studies reporting at least one of the clinical outcomes

Authors	Country	Year	Probiotic species	Number enrolled		NEC cases, stages 2, 3		Culture + sepsis cases		Deaths	
				Pro	Con	Pro	Con	Pro	Con	Pro	Con
Repa et al. ¹¹⁴	Austria	2015	LA + BLI	230	233	16	24	60	78	16	30
Hartel et al. ¹¹⁵	Germany	2014	LA + BLI	3789	1562	116	76	428	195	292	160
Janvier et al. ¹¹⁶	Canada	2014	BBi + BBr + BLI + <i>Bifidobacterium longum</i> + LRh	294	317	16	31	54	57	20	31
Hunter et al. ¹¹⁷	US	2012	LRh	79	232	2	35	19	72		
Li et al. ¹¹⁸	US	2013	BBi + BLI + <i>Saccharomyces thermophilus</i>	291	289	7	8			4	3
Bonsante et al. ¹¹⁹	France	2013	LRh	347	783	4	41	37	130	8	38
Manzoni et al. ¹²⁰	Italy ^a	2011	LRh	743		5					
Hoyos et al. ²⁶	Colombia	1999	LA + BLI	1237	1282	34	85	69	70	137	140
Totals				7010	4698	200	300	667	602	477	402
Percentage (among those reporting the outcome)						2.8	6.4	11	14	7.7	9.0

^aLikely some overlap with Table 1.