REVIEW

Prophylactic Probiotic Supplementation for Preterm Neonates—A Systematic Review and Meta-Analysis of Nonrandomized Studies

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ABSTRACT

Systematic review and meta-analyses of randomized controlled trials (RCTs) show that probiotics reduce the risk of necrotizing enterocolitis (NEC ≥ Stage II), late onset sepsis (LOS), all-cause mortality, and feeding intolerance in preterm neonates. Data from observational studies is important to confirm probiotic effects in clinical practice. We aimed to compare outcomes before and after implementing routine probiotic supplementation (RPS) in preterm neonates (<37 weeks of gestation) by performing a systematic review of non-RCTs using Cochrane methodology. Databases including PubMed, The Cumulative Index to Nursing and Allied Health Literature (CINAHL), Embase, Cochrane Central library, and Google Scholar were searched in May 2020. A meta-analysis was performed using a random effects model. Categorical measure of effect size was expressed as OR and 95% CI. Statistical heterogeneity was assessed by the chi-squared test, I^2 statistic. The level of evidence (LOE) was summarized using GRADE (Grading of Recommendations Assessment, Development, and Evaluation) guidelines. Primary outcomes were NEC ≥ Stage II, LOS, and all-cause mortality. Secondary outcomes included probiotic sepsis. Thirty good-quality non-RCTs ($n = 77,018$) from 18 countries were included. The meta-analysis showed RPS was associated with significantly reduced: *1*) NEC ≥ Stage II (30 studies, $n = 77,018$; OR: 0.60; 95% CI: 0.50, 0.73; $P < 0.00001$, I^2: 65%; LOE: Moderate), *2*) LOS: (21 studies, $n = 65,858$; OR: 0.85; 95% CI: 0.74, 0.97; $P = 0.02$, I^2: 74%; LOE: Low), and *3*) all-cause mortality (27 non-RCTs, $n = 70,977$; OR: 0.77; 95% CI: 0.68, 0.88; $P = 0.0001$, I^2: 49%; LOE: Low). Subgroups: *1*) extremely low birth weight (ELBW: birth weight <1000 g) neonates: RPS was associated with significantly reduced NEC ≥ Stage II (4.5% compared with 7.9%). However, there was no difference in LOS and mortality. *2*) Multistrain RPS was more effective than single strain. One study reported 3 nonfatal cases of probiotic sepsis. In summary, moderate- to low-quality evidence indicates that RPS was associated with significantly reduced NEC ≥ Stage II, LOS, and all-cause mortality in neonates <37 weeks of gestation and NEC ≥ Stage II in ELBW neonates. *Adv Nutr* 2021;12:1411–1423.

Keywords: neonates, necrotizing enterocolitis, outcomes, preterm infant, probiotics, very low birth weight

Background

Necrotizing enterocolitis (NEC) is a devastating condition in preterm neonates with significant mortality (15–30%) and morbidity including long-term neurodevelopmental disability (1). The incidence (2–7%) and mortality of NEC are inversely proportional to gestation and birth weight (2–4). Extremely low birth weight (ELBW; birth weight <1000 g) neonates are most vulnerable with higher incidence (8 to 12%) and mortality (45–100%) (3). NEC is also associated with huge economic burden (1, 5).

The modified Bells criteria are used to classify NEC into 3 stages. Stage 1 includes nonspecific clinical findings such as feeding intolerance, mild abdominal distention, or both. Stage 2 involves abdominal tenderness or cellulites and radiological findings such as pneumatosis intestinalis, portal gas with or without ascites. Stage 3 is severe disease characterized by marked abdominal tenderness, peritonitis,

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Abbreviations used: ELBW, extremely low birth weight; FEM, fixed effect model; GRADE, Grading of Recommendations Assessment, Development, and Evaluation; LOE, level of evidence; LOS, late onset sepsis; MD, mean difference; NEC, necrotizing enterocolitis; RCT, randomized controlled trial; REM, random effects model; ROB, risk of bias; RPS, routine probiotic supplementation; TFF, time to full feeds.

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pneumoperitoneum, perforated viscus, etc. Prematurity is the single most important risk factor for NEC. However, prevention of preterm birth has proved to be a difficult task. Until recently, antenatal glucocorticoids, trophic feeds, exclusive human milk diet, and standardized feeding protocol (consistency in starting trophic and nutritional feeds, daily increments, and criteria for withholding and restarting feeds to minimize variation in practice) have been the only prophylactic strategies for minimizing the risk of NEC. Based on the systematic reviews and meta-analyses of randomized controlled trials (RCTs) and non-RCTs in preterm neonates, probiotic supplementation has emerged as another option for reducing the risk of NEC $\geq$ Stage II, late onset sepsis (LOS), all-cause mortality, and feeding intolerance (6–8). Subsequently many neonatal units have adopted probiotics as a standard prophylaxis for preterm neonates. However, despite its benefits, routine probiotic supplementation (RPS) for preterm neonates remains controversial. The reasons for the continued controversy include lack of clarity on optimal probiotic strains and protocol, risk of probiotic sepsis, and inadequate data in ELBW neonates (9–11). The debate about strain-specific effects of probiotics has been addressed in a recent review suggesting that commonly used strains of the genera *Bifidobacterium* and *Lactobacillus* share many beneficial mechanisms (12). These findings support the meta-analysis of studies assessing such probiotic genera/species. However, the concerns about inadequate data on ELBW neonates continue to prevent optimal uptake of this intervention for this most vulnerable population (13). We hence aimed to systematically review the current evidence from non-RCTs reporting on benefits of RPS for reducing the risk of NEC, LOS, and all-cause mortality in preterm neonates. Our emphasis was ELBW neonates, as previous reviews have not provided adequate data in this important subgroup (6). Our results, based on real-life scenario in clinical practice, will guide research and clinical practice in this field.

Methods

The Cochrane methodology and MOOSE guidelines (Meta-analysis of Observational Studies in Epidemiology) were followed for conducting and reporting this systematic review (14–16). Ethics approval was not required.

Selection criteria and search strategy

Types of studies.

Only observational, before and after, cohort, and case-control studies (non-RCTs) were eligible for inclusion. Reviews and commentaries were excluded but read to identify other potential studies.

Participants.

(*1*) Inclusion criteria: Neonates born at <37 weeks gestation; (*2*) exclusion criteria: major chromosomal and congenital anomalies.

Type of intervention.

Oral probiotic (any strain, dose, or duration) with (synbiotic) or without a prebiotic oligosaccharide as an adjuvant to standard treatment compared with control as placebo or standard treatment alone. Studies investigating other interventions (e.g. lactoferrin, human milk fortifier) with probiotics were excluded.

Types of outcomes.

Primary. 1) NEC $\geq$ Stage II (17); *2*) LOS: positive blood/cerebrospinal fluid (CSF) culture with onset of symptoms of infection at $\geq$72 h of age; and *3*) all-cause mortality: death before discharge during first admission after birth.

Secondary. 1) Time to full feeds (TFF: 140 mL/kg/d or as defined by individual study authors); *2*) length of stay: during first admission; and *3*) adverse effects including probiotic sepsis.

Search strategy.

We searched Medical Literature Analysis and Retrieval System Online (MEDLINE) (from 1946), Embase (from 1974), CINAHL, and the Cochrane Central register of Controlled Trials initially in December 2019 and May 2020. We used the following search terms in various combinations: *1*) population: neonate(s), newborn(s), infant*, premature, extremely low birth weight, neonatal intensive care, *2*) intervention: probiotic, probiotics, *Lactobacillus, Bifidobacterium, Saccharomyces, 3*) outcome: necrotizing enterocolitis, late onset sepsis, sepsis, mortality, adverse effects, and *4*) publication type: observational, cohort, case control, cross sectional studies, retrospective, prospective studies, "non randomized" controlled trial. Online abstracts of Pediatric Academic Society (PAS) meetings were reviewed from 2002. Abstracts of conference proceedings including Perinatal Society of Australia and New Zealand (PSANZ) and European Academy of Paediatric Societies were searched in Embase. Google Scholar was searched for articles that might not have been cited in standard medical databases. The reference lists of identified studies and review articles were searched to identify additional eligible studies. We searched www.clinicaltrials.gov and the Australian New Zealand trial registry (www.anzctr.org.au) for ongoing studies. No language restriction was applied. Reviewers MD, SP, and librarian (RM) conducted the literature search independently.

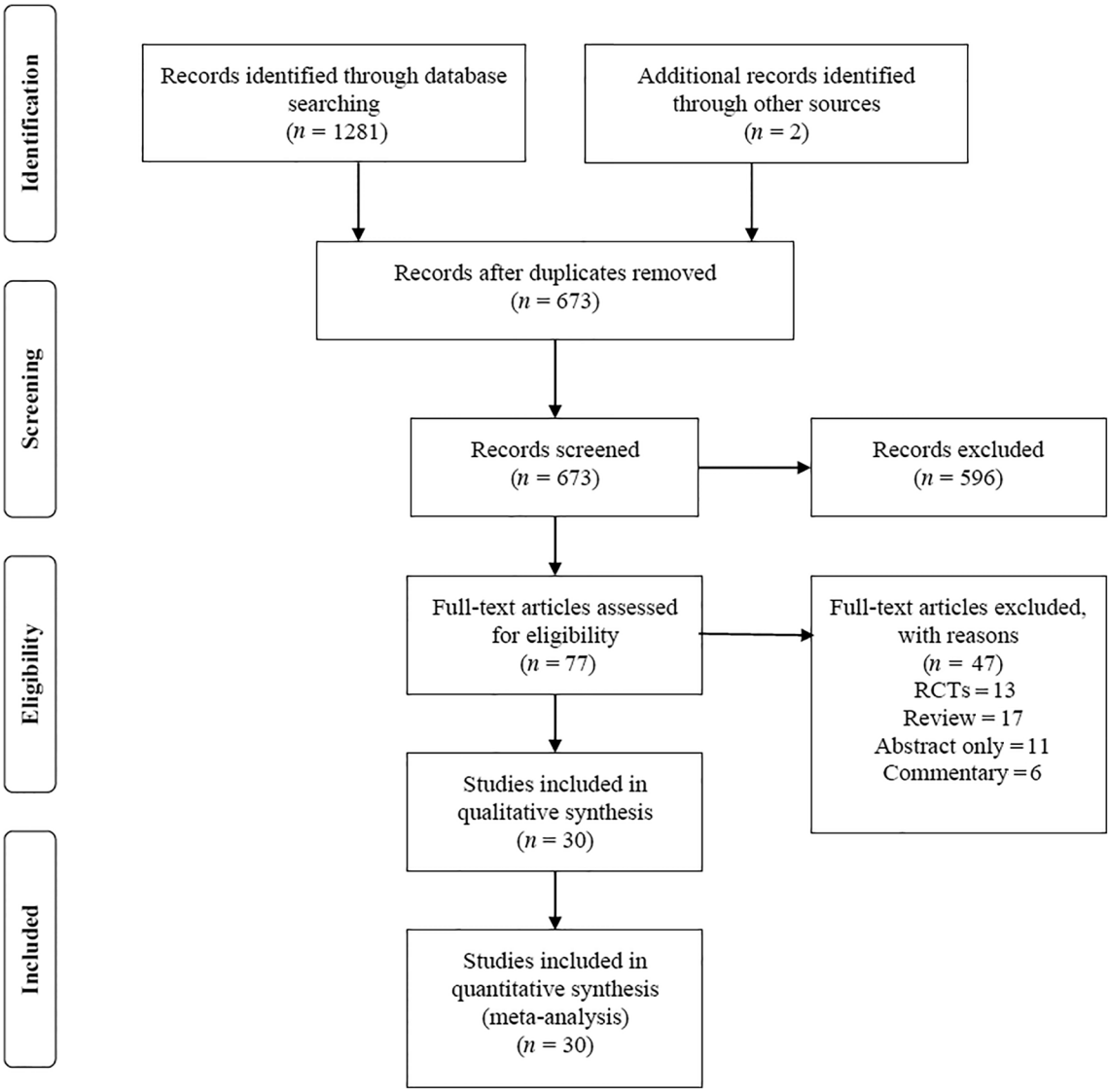

FIGURE 1 Flow chart of study selection process after screening of electronic search.

Study selection.
Reviewers MD and SP independently read abstracts of citations obtained from the initial broad search to identify potentially eligible studies. We independently assessed the full-text articles of these studies for eligibility using the predefined eligibility criteria. Differences in opinion were resolved by joint discussion to reach consensus. Multiple publications of the same study were excluded.

Data extraction.
Reviewers MD and SP extracted the data independently, using the data collection form designed for this review. For dichotomous outcomes, the number of patients with the event and the number analyzed in each treatment group of each study were recorded. For continuous outcomes, we entered the mean and SD. Both reviewers verified information about study design and outcomes. When necessary, we contacted the lead authors of studies for information not available in published articles. We derived the mean and SD from median and range and from median and IQR using the Hozo et al. (18) and Wan et al. (19) formulas, respectively. Discrepancies during data extraction were resolved by joint discussion to reach consensus.

Risk of bias assessment.
The quantitative scoring tool, Newcastle-Ottawa Scale (NOS) was used for evaluating the methodological quality of included non-RCTs (20). This scale contains 3 major domains: selection of subjects, comparability between groups, and

outcome measures. The maximum score for each domain is 4, 2, and 3 points, respectively. A total score ≤3 indicates low methodological quality.

Data synthesis.

The meta-analysis was performed using Review manager 5.3 (Cochrane Collaboration, Nordic Cochrane Centre) if pooling of data was possible and justified with intention to treat analysis. We used the random effects model (REM) assuming heterogeneity. For categorical outcomes, the effect size was expressed as OR (Mantel Haenszel method). For continuous outcomes, we used the mean difference (MD) (Inverse Variance method). Statistical heterogeneity was assessed using the chi-squared test, I^2 statistic, and visual inspection of the forest plot (overlap of CIs). Validity of REM results was crosschecked by comparing them with fixed effect model (FEM) meta-analysis.

Subgroup analyses.

The subgroups were based on *1*) birth weight <1000 g (i.e. ELBW neonates) and 2) single compared with multistrain probiotic for routine supplementation. Separate analyses were performed using the data from studies reporting the prestated outcomes in these 2 subgroups.

Publication bias.

This was assessed by a funnel plot and a statistical test if required. The typical symmetrical funnel plot shows studies with larger sample size at the top clustering around the mean effect size (midline), whereas those with smaller sample size spread around the broad range of values (21). A visual inspection of a funnel plot is an unreliable method for judging the probability of publication bias especially if the number of included studies is low. A statistical test for assessing publication bias is therefore advisable. We chose Peters' test for dichotomous outcomes as it has appropriate type I error rates regardless of the degree of heterogeneity and magnitude of effect size (22). The Egger's test was used to assess small-study effects for a continuous outcome.

Summary of findings.

The data on quality of evidence, magnitude of intervention effect, and the sum of available data on main outcomes, are presented in the "Summary of findings table" as per GRADE (Grading of Recommendations Assessment, Development, and Evaluation) guidelines (23). We used probiotics compared with placebo/control as the comparison, and included key outcomes in the summary of findings table. We graded the evidence in the following domains: risk of bias (ROB), inconsistency indirectness, imprecision, and publication bias. The evidence was downgraded 1 level for serious and 2 levels for very serious limitation (23).

Results

The literature search retrieved 1283 potentially relevant citations (MEDLINE: 364, Embase: 626, Emcare: 215, Cochrane: 36, gray literature: 40, others: 2). After carefully reviewing their abstracts and titles, 1206 citations were excluded because they were either duplicates (n = 610) or not relevant (n = 596). After reading the remaining 77 in detail, another 47 citations were excluded for various reasons (**Figure 1**). Meyer and Alexander 2017 (Lactoferrin) (24) and Sato et al. (human milk fortifier) (25) were excluded as the cointerventions might have an effect on primary outcomes. Finally, 30 high-quality non-RCTs (n = 77,018; Probiotics: 21,008, Control: 56,010) from 18 countries were included in the systematic review (26–55).

All studies reported on NEC ≥ Stage II; 21 reported on LOS, and 27 reported on all-cause mortality. The majority (21/30) of studies used multistrain whereas 9 used single-strain probiotics (*Lactobacillus*: 6, *Bifidobacterium*: 2, *Saccharomyces*: 1). Data from Gray et al. (54) couldn't be used for subgroup analyses as it was not stratified (single compared with multistrain RPS) and had a high risk of confounding (ELBW). Except for Raguž et al. (47) and Nepean (34), all studies reported data adjusted for maternal and neonatal variables (e.g. multiple pregnancy, gestation, small for gestational age). The characteristics and quality of these studies are presented in **Table 1**.

Primary outcomes

The number of studies reporting the primary outcomes and the corresponding total sample sizes were as follows: *1*) NEC ≥ Stage II: 30 studies (n = 77,018; Probiotic: 21,008, Control: 56,010) (26–55), *2*) LOS: 21 studies (n = 65,858; Probiotic: 15,893, Control: 49,965) (26–29, 31, 32, 34, 35, 38, 39, 41–43, 45–48, 51–55), *3*) all-cause mortality: 27 studies (n = 70,977; Probiotic: 18,016, Control: 52,961) (26–46, 48, 50, 51, 53–55).

RPS was associated with a significant reduction in: *1*) NEC ≥ Stage II: Probiotic: 4.2% compared with Control: 6.8%, (OR: 0.60; 95% CI: 0.50, 0.73; P < 0.00001; heterogeneity: chi^2 = 83.39, I^2 = 65%) (**Figure 2**), *2*) LOS: Probiotic: 9.5% compared with Control: 11%, (OR: 0.85; 95% CI: 0.74, 0.97; P = 0.02; heterogeneity: chi^2 = 74.56, I^2 = 73%) (**Figure 3**), and *3*) all-cause mortality: Probiotic: 4.6% compared with Control: 5.9%; (OR: 0.77; 95% CI: 0.68, 0.88, P = 0.0001; heterogeneity: chi^2 = 50.51, I^2 = 49%) (**Figure 4**). The number needed to treat (NNT) to prevent 1 case of NEC ≥ Stage II, LOS, and all-cause mortality by RPS was 39, 68, and 77, respectively.

Secondary outcomes

TFF and duration of hospital stay was reported in 15 (27–30, 32–34, 41, 44, 46, 48, 50, 51, 53) and 10 studies (26, 27, 29, 30, 33, 41, 48, 51, 53, 55), respectively. The corresponding total sample sizes for these outcomes were 14,215 (Probiotic: 5580, Control: 8635) and 10,289

TABLE 1 Characteristics and quality of included studies on effects of probiotics in preterm neonates

	Inclusion				Quality of study NOS		
Study	Period	Location	GA/BW	Probiotic	Selection (maximum 4 stars)	Comparability (maximum 2 stars)	Outcome (maximum 3 stars)
Bonsante 2013 (53)	C: 2003–08 P: 2008–11	France	24–31wk	*L. rhamnosus* (2×10^8 CFU BD until CGA 36 wk)	***	*	***
Dang 2015 (44)	C: 2010–11 P: 2012	USA	<28 wk, <1250 g	*LGG* + *B. infantis* (1×10^9 CFU/d until CGA 34 wk)	****	*	***
Guthmann 2016 (40)	C: 2005 P: 2007–08	Switzerland, Germany	<32 wk, <1500 g	*L. acidophilus* + *B. infantis* (1×10^9 CFU/d for 10–14 d)	****	**	***
Hoyos 1999 (39)	C: 1993–94 P: 1994–95	Colombia	<1500 g	*L. acidophilus* + *B. infantis* (5×10^8/d) until discharge	****	*	**
Hunter 2012 (52)	C: 2004–09 P: 2009–11	USA	<1000 g	*L. reuteri* (5.5×10^7 CFU/d until CGA 40 wk)	****	*	***
Janvier 2014 (38)	C: 2009–10 P: 2011–12	Canada	<32 wk	*Bifidobacterium* + *Lactobacillus* (2×10^9 CFU/d until CGA 34 wk)	***	**	***
Lambaek 2016 (37)	C: 2006– 09 P: 2010–13	Denmark	<30 wk	*B. lactis BB12* + *LGG* (1×10^8 and 1×10^9 CFU) 2 capsules daily until discharge	***	**	***
Li 2013 (36)	C: 2003– 07 P: 2007–11	USA	<1500 g	*Streptococcus* + *Bifidobacterium* 1×10^9 CFU until CGA 36 wk	***	**	**
Luoto 2010 (49)	C: 1986–96 P: 1997–07	Finland	<30 wk, <1500 g	*LGG* (6×10^9 CFU/d until discharge)	***	**	***
Patole 2016 (48)	C: 2008–10 P: 2012–14	Australia	<34 wk	*B. breve* M-16V ($1.5–3 \times 10^9$ CFU/d) until 37 wk	***	**	***
Repa 2015 (32)	C: 2008–09 P: 2010–12	Austria	<34 wk	*L. acidophilus* + *B. infantis* (2×10^9 BD) 1 tablet BD until CGA 37 wk	***	**	**
Yamashiro 2010 (45)	C:1994–98 P:1999–2003	Japan	<1500 g	*B. breve* (1×10^9/d) until discharge	***	*	**
Rutz 2019 (30)	C: 2011–12 P: 2012–13	Australia	<32 wk	Infloran® (*L. acidophilus*, *B. bifidum*) 1 tablet once daily until CGA 35 wk or discharge	****	**	***
Rolnitsky 2019 (46)	C: 2014 P: 2015–18	Canada	<33 wk	*L. reuteri* dose and duration not reported	****		***
Raguž 2016 (47)	C: 2013 P: 2013–14	Bosnia Herzegovina	30–35 wk	*L. reuteri* 5 drops once daily until discharge	***	*	***
Singh 2019 (27)	C: 2013–14 P: 2013–14	Canada	<29 wk	Florababy® and *L. reuteri* 5 drops once daily until CGA 34 wk	****	**	***
Garg 2017 (41)	C: 2012–13 P: 2013–15	India	<32 wk, <1500 g	Darolac (*L. acidophilus* + *B. longum* + *Saccharomyces*) 1 sachet once daily until CGA 35 wk or discharge	****	**	***
Uberos 2017 (26)	C: 2010–13 P: 2013–16	Spain	32 wk, 1500 g	Bivos® 9 drops daily and Infloran® 1 tablet twice daily until CGA 35 wk	****	*	***
Kane 2018 (51)	C: 2008–14 P: 2014–16	USA	<1500 g	*LGG* $2.5–5 \times 10^9$ CFU/d until 35 wk	****	**	***
Patel 2018 (33)	C: 2013–14 P: 2014–15	India	<35 wk	Prowell (*L. acidophilus* + *B. longum*, *B. bifidum*) 2.5 billion CFU/d until CGA 37 wk	****	**	***
Samuels 2016 (29)	C: 2008–12 P: 2012–14	Netherlands	<32 wk, <1500 g	Infloran® 1 tablet once daily until 35 wk or discharge	****	**	***
Kim 2016 (55)	C: 2009–11 P: 2012–14	S. Korea	<32 wk, <1500 g	*L. plantarum*, *L. rhamnosus*, *B. lactis*, until CGA 35 wk or discharge	***	**	**
Nepean 2013 (34)	C: 2010–11 P: 2012–13	Australia	<32 wk <1500 g	Infloran® not reported	****		***

(*Continued*)

TABLE 1 (*Continued*)

Study	Period	Inclusion		Probiotic	Quality of study NOS		
		Location	GA/BW		Selection (maximum 4 stars)	Comparability (maximum 2 stars)	Outcome (maximum 3 stars)
Escribano 2018 (42)	C: 2005–07 P: 2009–15	Spain	<28 wk	Infloran® 1 tablet once daily for 6 wk or until discharge	****	*	***
Robertson 2020 (31)	C: 2008–12 P: 2013–17	UK	<32 wk, 1500 g	Infloran® ½ tablet twice daily and Labinic® 4 drops daily until 34 wk	****	**	***
Sharpe 2018 (28)	C: 2007–12 P: 2013–14	Australia	<32 wk, 1500 g	Infloran® for 6 wk, dose not reported	****	**	***
Denkel 2016 (43)	C: 2004–10 P: 2010–14	Germany	<1500 g	Infloran® dose and duration not reported	****	*	***
Karthikeyan 2015 (50)	C: 2009–11 P: 2011–12	India	1000–1999 g	*S. boulardii* 1/3 to 1/2 sachet once daily from 4 h of life until day 7 or transfer out of nursery	****	*	***
Meyer 2020 (35)	C: 2007–10 P: 2013–15	New Zealand	<32 wk, <1500 g	Infloran® 1 capsule once daily (5 units) *LGG* + *bovine lactoferrin* (1 unit) until 34–36 wk CGA	*	*	*
Gray 2020 (54)	C: 1997–2006 P: 2006–16	USA	23–29 wk	*Lactobacillus* species (71%), Ultimate flora, ABC Dophilus, Align	****	**	***

1 – Note: Except for Nepean, all were retrospective studies.
2 – BW, birth weight; B, Bifidobacterium; C, Control; CGA, corrected gestational age; GA, Gestational age; LGG, *Lactobacillus rhamnosus GG*; L, *Lactobacillus*; NOS, Newcastle-Ottawa scale; P, Probiotic; S, *Saccharomyces*.
3 – Probiotic products: *1*) ABC Dophilus: Bifidobacterium, Lactobacilli, and Streptococcus species, *2*) Align: Bifidobacterium, *3*) Biogaia® (Ferring Inc.): *L. reuteri* DSM 17,938 (10^8 CFU), *4*) Bivos® (Ferring): *L.rhamnoosus GG* (53,103), *5*) Darolac: containing 0.125×10^9 CFU *L. acidophilus*, *L. rhamnosus*, *B. longum*, and *S. boulardii*, *6*) Florababy® (Review Life Canada): Bifidobacterium species (*B. breve*, *B. bifidum*, *B. infantis*, and *B. longum*) and *L. rhamnosus GG* (2×10^9 CFU/0.5 g), *7*) Infloran® (Berna Biotech): *L acidophilus* and *B. bifidum*, (1×10^9 CFU), *8*) Labinic®: *L. acidophilus*, *B. bifidum*, and *B. longum* subspecies *infantis* (~0.5×10^9 CFU), (9) Prowell: *L. acidophilus* (1.25×10^9 CFU), *B. longum* (0.125×10^9 CFU), *B. bifidum* (0.125×10^9 CFU), and *B. lactis* (1.0×10^9 CFU) per 1-g sachet with inulin 25, (10) Ultimate flora: Bifidobacterium and Lactobacilli species.

(Probiotic: 3694, Control: 6595), respectively. The meta-analysis showed that RPS was associated with a significant reduction in TFF (MD: –1.23; 95% CI: –2.1, –0.37; $P = 0.005$; heterogeneity: $chi^2 = 126.05$, $I^2 = 89\%$) (**Supplemental Figure 1**). However, it was not associated with any effect on hospital stay (MD: 1.77; 95% CI: –1.23, 4.76; $P = 0.25$; heterogeneity: $chi^2 = 56.09$, $I^2 = 84\%$) (**Supplemental Figure 2**).

Adverse effects

Except for 2, none of the included studies reported adverse effects related to RPS (54, 55). Kim et al. (55) reported 3 ELBW neonates with probiotic sepsis including 1 with an ileostomy and another with NEC Stage IIIb. Two were born at 23 and 1 was born at 27 weeks of gestation. The postnatal age at probiotic sepsis was 45, 58, and 122 d. Blood cultures grew *Bifidobacterium* in 2 cases, and *Lactobacillus* species in 1 case. All survived after treatment with Vancomycin (2 cases) and Meropenem and Piperacillin Tazobactam (1 case each). Gray et al. (54) reported an increase in candida infection in Probiotics compared with Control (1% compared with 0.4%).

Prestated subgroup analyses

ELBW neonates: (Figure 5).

NEC Stage ≥ II. The meta-analysis of 10 studies ($n = 8464$, Probiotic: 4499, Control: 3965) (35, 38, 40–43, 48, 51, 52, 55) showed RPS was associated with a significant reduction in NEC Stage ≥ II: Probiotic: 4.5% compared with Control: 7.9% (OR: 0.59; 95% CI: 0.36 ,0.97; $P = 0.04$; $chi^2 = 40.01$, $I^2 = 78\%$).

LOS. The meta-analysis of 9 studies ($n = 7976$; Probiotic: 4261, Control: 3715) (35, 38, 41–43, 48, 51, 52, 55) showed that RPS was not associated with a significant reduction in LOS: Probiotic: 24.1%, compared with Control: 30.9% (OR: 0.67; 95% CI: 0.45, 1.00; $P = 0.05$; $chi^2 = 78.64$, $I^2 = 90\%$).

All-cause mortality. The meta-analysis of 9 studies ($n = 8153$; Probiotics: 4420, Control: 3733) (35, 38, 40–43, 48, 51, 55) showed no significant reduction in all-cause mortality: Probiotics: 12.4% compared with Control: 15.3% (OR: 0.77; 95% CI: 0.53, 1.12; $P = 0.17$; $chi^2 = 43.03$, $I^2 = 81\%$).

Posthoc sensitivity analysis

Results after excluding Kane et al. (51) and Escribano et al. (42) as outliers showed significant benefit of RPS on NEC ≥ Stage II (OR: 0.43; 95% CI: 0.35, 0.54; $P < 0.00001$, $I^2 = 0\%$) and LOS (OR: 0.60; 95% CI: 0.37, 0.99; $P = 0.05$, $I^2 = 92\%$). All-cause mortality remained nonsignificant (OR: 0.66; 95% CI: 0.44 ,1.00; $P = 0.05$, $I^2 = 82\%$).

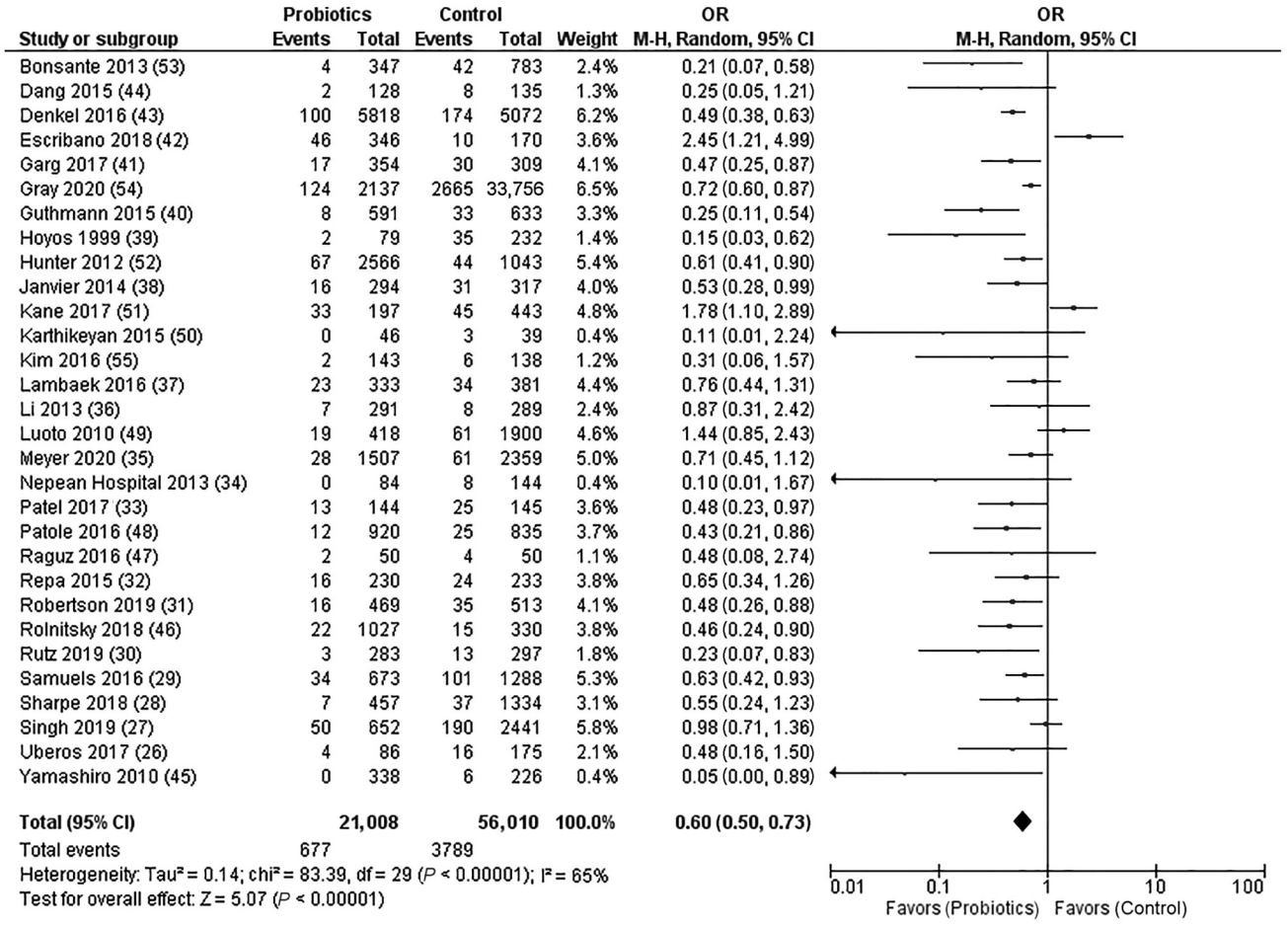

FIGURE 2 Forest plot showing effect of probiotics on NEC ≥ Stage II in preterm neonates. NEC, necrotizing enterocolitis.

Single compared with multistrain probiotics

Multistrain RPS was associated with reduced NEC Stage ≥ II [$n = 29{,}567$; Probiotics: 12,962, Control: 16,605 (Probiotic: 3% compared with Control: 5.3%) OR: 0.58; 95% CI: 0.46, 0.72; $P < 0.00001$, $I^2 = 57\%$] but not LOS [$n = 24{,}020$; Probiotics: 10,758, Control: 13,262 (Probiotic: 17% compared with Control: 18%), OR: 0.87; 95% CI: 0.73, 1.05; $P = 0.14$, $I^2 = 77\%$]. Single-strain RPS was associated with reduced LOS [$n = 5853$; Probiotics: 2957, Control: 2896 (Probiotic: 12% compared with Control: 18%); OR: 0.74; 95% CI: 0.59, 0.94; $P = 0.01$, $I^2 = 50\%$] but not NEC Stage ≥ II [$n = 11{,}558$; Probiotics: 5909, Control: 5649 (Probiotic: 2.7% compared with Control: 4.3%), OR: 0.58; 95% CI: 0.33, 1.01; $P = 0.05$, $I^2 = 77\%$]. Mortality was reduced by both single [$n = 5531$; Probiotics: 2853, Control: 2678 (Probiotic: 5% compared with Control: 7%) OR: 0.68; 95% CI: 0.51, 0.90; $P = 0.008$, $I^2 = 20\%$] and multistrain RPS [$n = 29{,}461$; Probiotics: 12,985, Control: 16,476 (Probiotic: 7% compared with Control: 9%) OR: 0.78; 95% CI: 0.66, 0.91; $P = 0.002$, $I^2 = 53\%$]. The detailed results are shown in **Supplemental Figures 3–5** and **Supplemental Table 1**.

FEM compared with REM meta-analysis

FEM and REM results were comparable for all outcomes (**Supplemental Table 2**).

Publication bias.

The funnel plot for primary outcomes showed asymmetrical distribution of small studies suggesting the possibility of publication bias. However, the Peters' test results showed this was unlikely for NEC ≥ Stage II ($P = 0.1537$) and LOS ($P = 0.2474$). The Peters' test was significant for all-cause mortality ($P = 0.0798$). The Egger's test for TFF was nonsignificant ($P = 0.2702$) (**Supplemental Figures 6–9**) (**Supplemental File 1: Statistical analysis publication bias**).

Grading evidence and summary of findings

The quality of evidence was deemed to be low for all outcomes considering the included studies were non-RCTs. The evidence was upgraded to moderate for outcome of

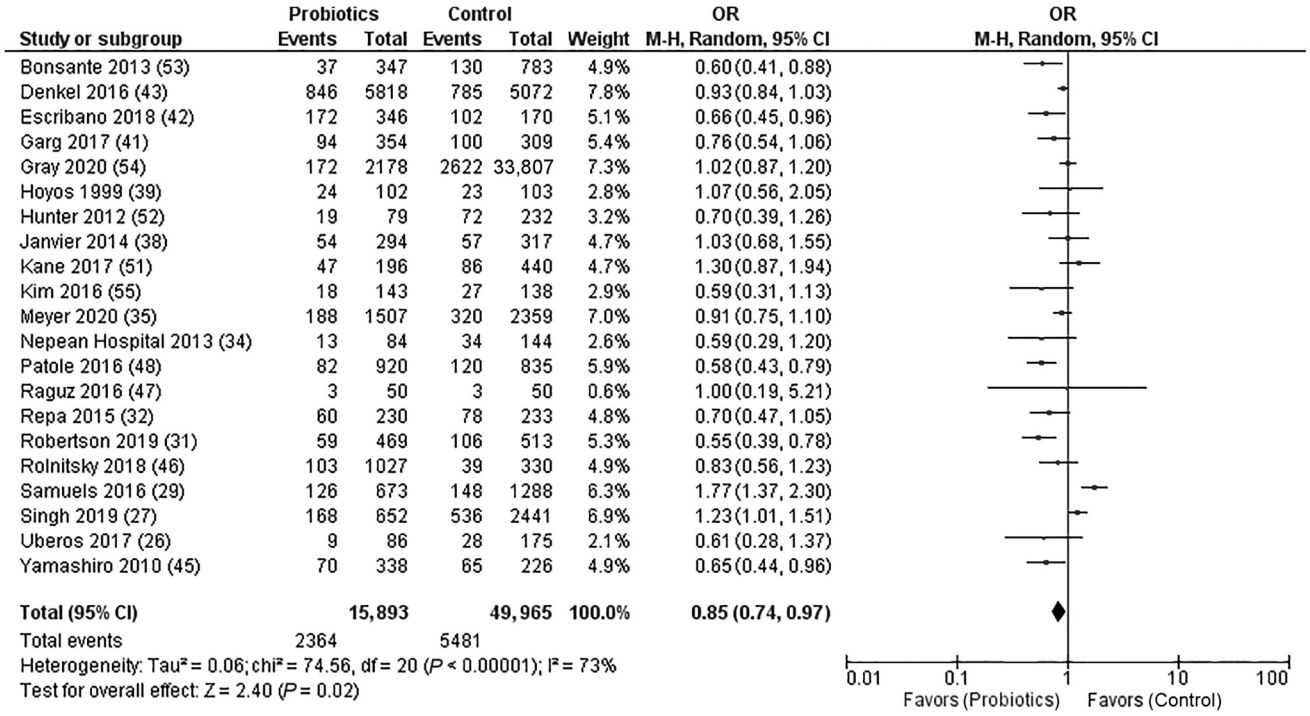

FIGURE 3 Forest plot showing effect of probiotics on late onset sepsis in preterm neonates.

NEC ≥ Stage II given the very large effect size. The grading of evidence is presented in **Table 2**.

Discussion

The results of our systematic review showed that RPS was associated with reduced incidence of NEC ≥ Stage II (from 6.8% to 4.2%), LOS (from 11% to 9.5%), all-cause mortality (from 5.9% to 4.6%), and TFF (−1.23 d) in preterm neonates. Except for the report of 3 nonfatal cases of probiotic sepsis, there were no adverse effects (55). Importantly, in ELBW neonates RPS was associated with improvement in NEC ≥ Stage II (4.5% compared with 7.9%); however, it had no effect on LOS and all-cause mortality. Considering the overall results and biologic plausibility, the small number of studies contributing to this subgroup may explain the lack of "statistical significance" for LOS and mortality in ELBW neonates (56).

Similar to previous reports, multistrain RPS was more effective than single strain (57). The small number of studies made it difficult to conduct further analysis based on probiotic genera. Importantly, none of the included studies provided evidence on synergy or compatibility between different strains in the multistrain products used for RPS (58).

The 2 studies reporting increased incidence of NEC following RPS warrant discussion. Kane et al. (51) included 640 preterm neonates (Probiotic: 175 compared with Control: 465) with median (IQR) gestation of 28.7 (26.3–30.6) wk. NEC increased after RPS using LGG (Probiotic: 16.8% compared with Control: 10.2%) but LOS and mortality remained comparable. Significantly more neonates received prophylactic indomethacin in RPS compared with the non-RPS group (49% compared with 37%) (51). RPS was started on day 6, perhaps too late for optimal benefit (8, 59). The conflicting results were attributed to an unmeasured difference in patient characteristics and clinical practices. Escribano et al. (42) included 516 neonates (Probiotic: 346 compared with Control: 170) with median gestation and birth weight as 26^{+1} wk and 827±177 g compared with 26^{+1} wk and 822±161 g in RPS compared with the control group, respectively. NEC increased (Probiotic: 13.3% compared with Control: 5.9%) after RPS with a dual-strain probiotic. LOS was significantly less in the RPS group whereas mortality was similar in both groups. The incidence of bronchopulmonary dysplasia, a marker of prematurity, was almost 50% and ∼4% neonates in the RPS group received postnatal corticosteroids for it, suggesting a cohort at high risk of NEC (42). The analysis of individual patient data is important to interpret such conflicting results. The results of our posthoc sensitivity analysis excluding these 2 studies showed significant benefits of RPS for NEC and LOS in ELBW neonates.

As for probiotic sepsis, the 3 cases reported by Kim et al. (55) survived after antibiotic therapy. Apart from

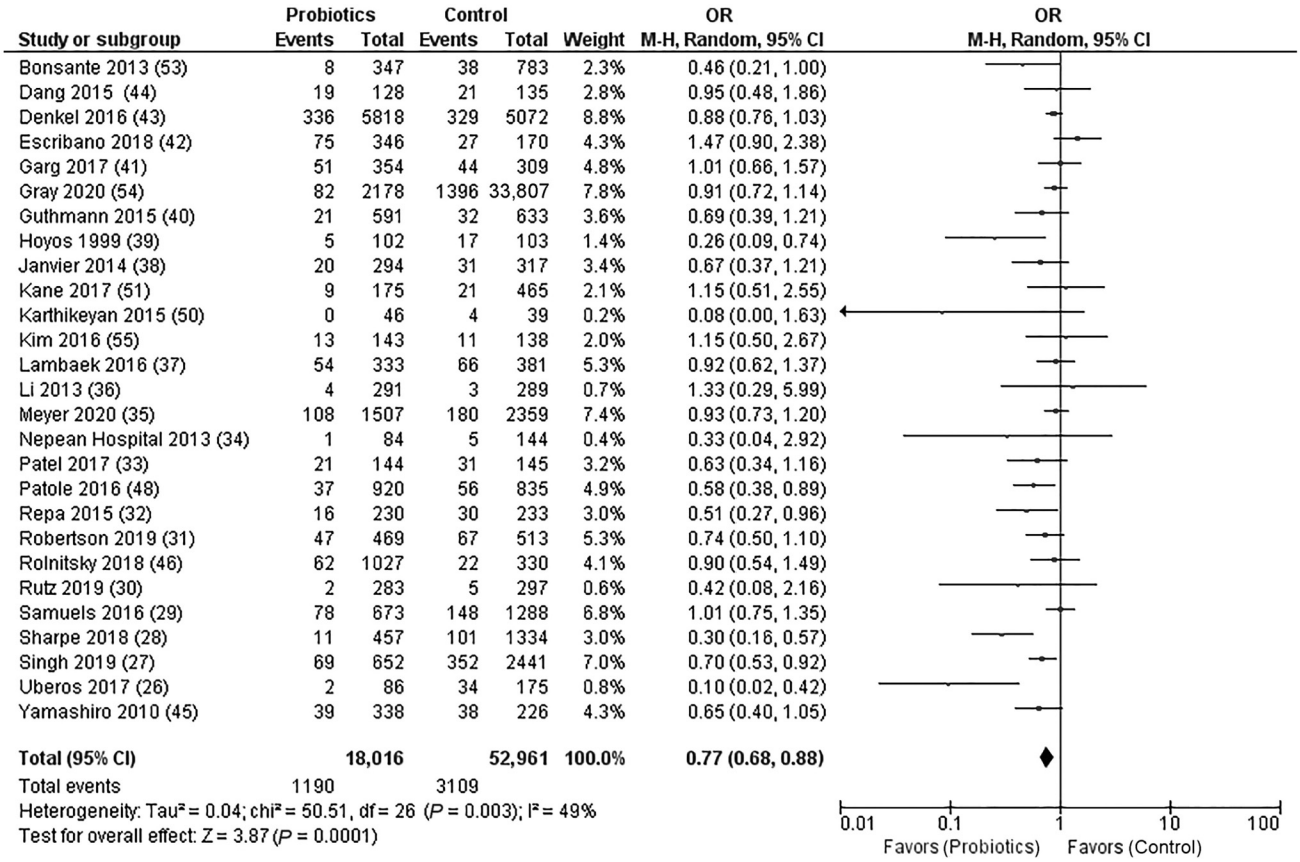

FIGURE 4 Forest plot showing effect of probiotics on all-cause mortality in preterm neonates.

extreme prematurity, the presence of ileostomy and NEC Stage IIb were important risk factors for probiotic translocation followed by sepsis in these cases. The increase in candida infection reported by Gray et al. (54) is unexpected as probiotics are known to reduce this risk (60).

The strength of our review includes its comprehensiveness, robust methodology, large sample size (30 studies from 18 countries), and use of GRADE guidelines for summarizing the level of evidence. Compared with the recent systematic review by Dermyshi et al. (6) (14 non-RCTs; $n = 13{,}779$ including 1216 ELBW neonates), our review provides substantially more data ($n = 77{,}018$ including 8464 ELBW neonates). The precision of our results is supported by the tight CIs for primary outcomes, and small P values. Despite the significant heterogeneity, the validity of our results is supported by comparable findings on meta-analysis by FEM and REM. It is important to note the effect sizes for NEC, LOS, and all-cause mortality are similar to those reported in a recent systematic review of RCTs (61).

The limitations of our review need to be acknowledged. Inclusion of only non-RCTs carries the ROB and overestimation of effect size (62). We only reviewed non-RCTs as they provide real-life data rather than that from the controlled environment of RCTs. Moreover, conventional RCTs carry the risk of crosscolonization of the control arm resulting in underestimation of probiotic effects (63). Considering the strengths and weakness of both study designs, we believe that the guidelines for probiotic supplementation should be based on data from both RCTs and non-RCTs.

The expected variations in RPS protocol (strain, dose, and duration) and baseline risk in included studies may explain the high statistical heterogeneity in our review. We believe that such true heterogeneity is a strength, reflecting external validity in a broad context rather than a limitation considering the consistent direction towards benefits of RPS in 28/30 included studies. As for publication bias, there is no gold standard against which the funnel plot test results can be compared (64). Moreover, it is not the only reason for an asymmetrical funnel plot. True heterogeneity also contributes to the small-study effect. Comparable results of FEM compared with REM meta-analysis are reassuring in this context. Experts suggest that conclusions based solely on asymmetrical funnel plots or statistical tests may discredit valid evidence (64). Considering the span

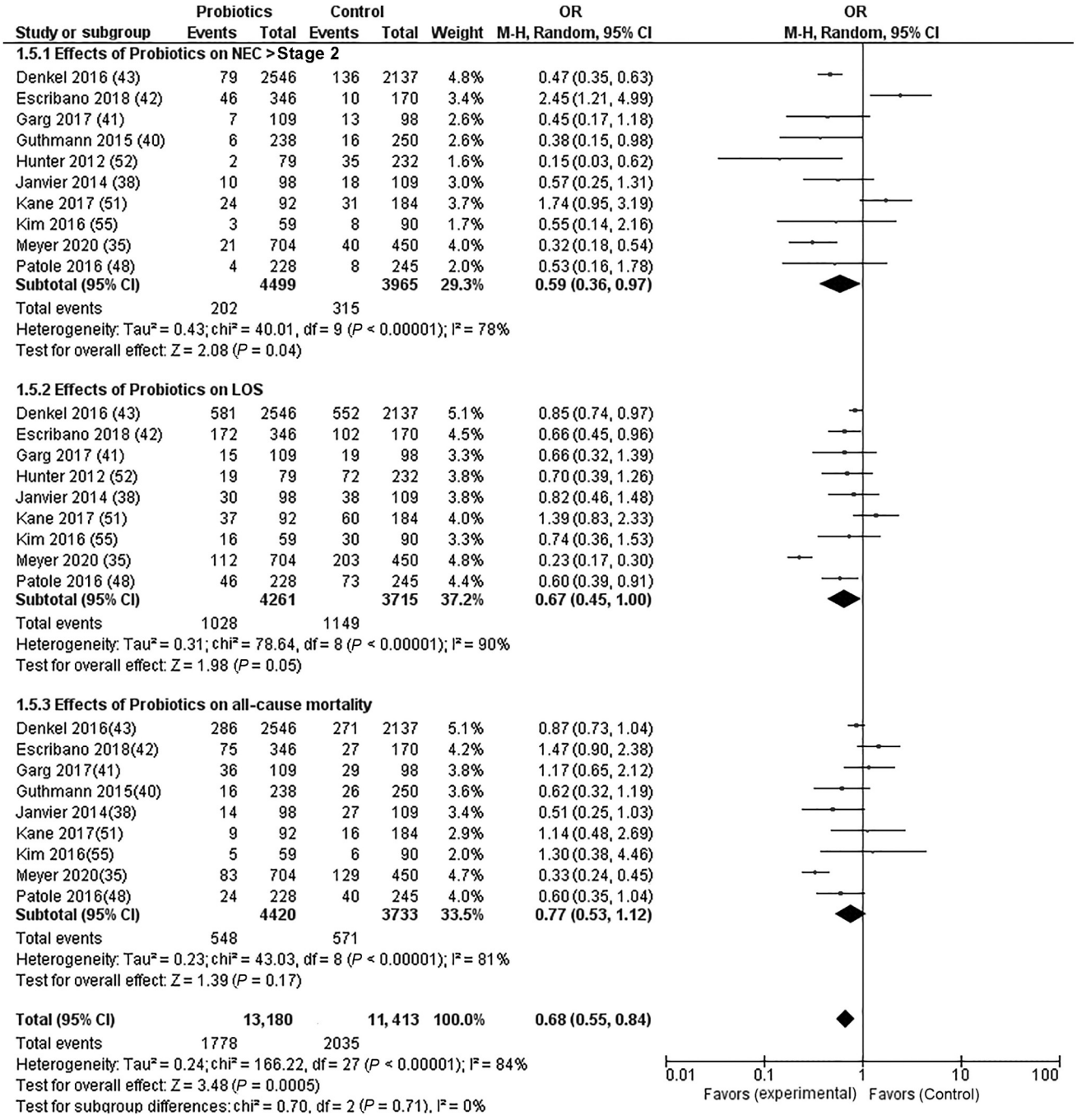

FIGURE 5 Forest plot showing effect of probiotics on ELBW neonates. ELBW, extremely low birth weight; LOS, late onset sepsis; NEC, necrotizing enterocolitis.

(1997–2020) of included studies, our results might be influenced by changes in clinical practice over time. Improved survival of extremely preterm neonates has increased the population at higher risk of NEC (65). The reduction in NEC after RPS in ELBW neonates is thus reassuring.

In summary, moderate- to low-quality evidence indicates that RPS was associated with significantly reduced NEC ≥ Stage II, LOS, mortality, and TFF in preterm neonates without significant adverse effects. Importantly, RPS was associated with significant reduction in NEC ≥ Stage II in ELBW neonates. Our results will

TABLE 2 Summary of finding for pooled data as per GRADE guidelines.

Outcome	Anticipated absolute effect (95% CI)				
Effect of probiotics on	Estimated risk in control group	Corresponding risk in probiotic group	Relative effect OR (95% CI)	Number of participants	Quality of evidence GRADE
NEC ≥ Stage II	68 per 1000	42/1000 (95% CI: 35, 50/1000)	OR: 0.60 (95% CI: 0.50 to 0.73)	77,018 (30 studies)	⊕⊕⊕◯ Moderate[1]
Late onset sepsis (LOS)	110 per 1000	95/1000 (95% CI: 84, 107/1000)	OR: 0.85 (95% CI 0.74 to 0.97)	65,858 (21 studies)	⊕⊕◯◯ Low[2]
All-cause mortality	59 per 1000	46/1000 (95% CI: 41, 52/1000)	OR: 0.77 (95% CI: 0.68 to 0.88)	70,977 (27 studies)	⊕⊕◯◯ Low[3]
Time to full feeds	The mean effect was 0	MD 1.23 lower (95% CI: 2.1 lower to 0.37 lower)	MD: −1.23 (95% CI: −2.1 to −0.37)	14,215 (15 studies)	⊕⊕◯◯ Low[2]
Duration of hospital stay	The mean effect was 0	MD 1.77 higher (95% CI: 1.23 lower to 4.76 higher)	MD: 1.77 (95% CI: −1.23 to 4.76)	10,289 (10 studies)	⊕◯◯◯ Very low[4]
ELBW neonates—NEC ≥ Stage II	79 per 1000	48/1000 (95% CI: 30 to 77/1000)	OR: 0.59 (95% CI: 0.36 to 0.97)	8464 (10 studies)	⊕◯◯◯ Low[2]
ELBW neonates: late onset sepsis	309 per 1000	231/1000 (95% CI: 168, 309/1000)	OR: 0.67 (95% CI: 0.45 to 1.00)	7976 (9 studies)	⊕◯◯◯ Very low[4]
ELBW neonates: all-cause mortality	153 per 1000	122/1000 (95% CI: 87, 168/1000)	OR: 0.77 (95% CI: 0.53 to 1.12)	8153 (9 studies)	⊕◯◯◯ Very low[4]

1 – Grading was started as low due to the observational nature of all included studies, and serious heterogeneity.
2 – Evidence was upgraded as moderate in view of the very large effect size.
3 – ELBW, extremely low birth weight; GRADE, Grading of Recommendations Assessment, Development and Evaluation; MD, mean difference; NEC, necrotizing enterocolitis.
4 – Quality of evidence GRADE.
[1]Moderate: Observational studies; Risk of bias: Low; Inconsistency: Serious; Indirectness: Not serious; Imprecision: Not serious; Effect size: Very large; Publication bias: Not detected.
[2]Low: Observational studies; Risk of bias: Low; Inconsistency: Serious; Indirectness: Not serious; Imprecision: Not serious; Effect size: Large; Publication bias: Not detected.
[3]Low: Observational studies; Risk of bias: Low; Inconsistency: Serious; Indirectness: Not serious; Imprecision: Not serious; Effect size: Very large; Publication bias: detected.
[4]Very low: Observational studies; Risk of bias: Low; Inconsistency: Serious; Indirectness: Not serious; Imprecision: Serious; Effect size: Small; Publication bias: Not detected.

help in guiding research and clinical practice in this area.

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The authors' responsibilities were as follows—MD and SP: design, writing, and final content of the manuscript; MD: conception, design, independent literature search, handling meta-analysis software, writing first and final draft of the manuscript, addressing reviewers' comments; SP: conception, design, independent literature search, updating the first and final draft of the manuscript, addressing reviewers' comments; and both authors: read and approved the final manuscript.

We have used published data for this manuscript which is available in the public domain. We also obtained additional data from Frank Schwab, Michael Meyer, Hari Balakrishnan, Ravi Mangal Patel, and Saeyun Kim. Ethical and legal restrictions prevent us from making the minimal data set publicly available. The contact details of authors who provided additional data are given below. Readers may contact them to request the data which should be available upon request to all interested researchers: *1*) Frank Schwab: frank.schwab@charite.de; *2*) Michael Meyer: Michael.Meyer@middlemore.co.nz; *3*) Hari Balakrishnan: doctorhbk@gmail.com; *4*) Ravi Mangal Patel: rmpatel@emory.edu; *5*) Saeyun Kim: sysmile@gmail.com.

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