Review

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Effects of probiotics on experimental necrotizing enterocolitis: a systematic review and meta-analysis

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BACKGROUND: Meta-analyses of randomized controlled trials (RCTs) suggest that probiotics decrease the risk of necrotizing enterocolitis (NEC) in preterm infants. Many animal RCTs have evaluated probiotics for preventing NEC. We systematically reviewed the literature on this topic.

METHODS: The protocol for systematic review of animal intervention studies (SYRCLE) was followed. Medline, Embase, ISI Web of Science, e-abstracts from the Pediatric Academic Society meetings, and other neonatal conferences were searched in December 2015 and August 2016. RCTs comparing probiotics vs. placebo/no probiotic were included.

RESULTS: A total of 29 RCTs were included (Rats: 16, Mice: 7, Piglets: 3, Quail: 2, Rabbit: 1; $N \sim 2{,}310$), with 21 reporting on histopathologically confirmed NEC; remaining 8 assessed only pathways of probiotic benefits. Twenty of the 21 RCTs showed that probiotics significantly reduced NEC. Pooling of data was possible for 16/21 RCTs. Meta-analysis using random-effects model showed that probiotics significantly decreased the risk of NEC (203/641 (31.7%) vs. 344/571 (60.2%); relative risk: 0.51; 95% confidence interval (CI): 0.42–0.62; $P < 0.00001$; $I2 = 44\%$; number needed to treat: 4; 95% CI: 2.9, 4.3).

CONCLUSION: Probiotics significantly reduced NEC via beneficial effects on immunity, inflammation, tissue injury, gut barrier, and intestinal dysbiosis.

INTRODUCTION

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal emergency in mostly preterm infants (gestation <32 weeks) with significant mortality (25%) and morbidity, including long-term neuro-developmental impairment (1–11). Mortality (45–100%) and morbidity is highest in infants born before 28 weeks of gestation (12–14). The economic burden associated with $\geqslant$ Stage II NEC has been estimated to be as high as one billion dollars per year in United States of America, not accounting for the expenses associated with ongoing care of survivors of NEC with neurodevelopmental impairment (15,16). Despite extensive research over decades, there is currently no cure for the condition because the pathogenesis of NEC is not clearly understood (17,18). Prevention of NEC is hence a priority given the substantial health burden associated with the condition.

Meta-analyses of randomized controlled trials (RCTs) have suggested that probiotic supplementation significantly decreases the risk of $\geqslant$ Stage II NEC in human preterm infants (19–22). The proposed mechanisms for the beneficial effects of probiotics include enhancement of gut barrier, immune response modulation (e.g., Toll-like receptor 4 receptor, nuclear factor-B, inflammatory cytokines), and competitive inhibition of gut colonization by pathogens to limit dysbiosis (23–27).

Studies in animal models are crucial for understanding the mechanisms for the benefits and adverse effects of an intervention selected for potential clinical use (28–30). Investigators have evaluated the effects of probiotics in different animal models of NEC, but the sample sizes of individual animal studies are usually small. Meta-analysis of data from small but comparable individual studies is a valuable method to generate reliable evidence with higher precision and power (31,32). Systematic reviews of animal studies can enable the translation of findings to clinical trials rapidly and appropriately while ensuring effective use of time (33,34). Hence, we decided to systematically review studies in animal models to evaluate the efficacy of probiotic supplementation in decreasing the risk of NEC and to understand the pathways for benefits of probiotics in reducing the incidence and/or severity of the illness.

Aim

To conduct a systematic review of studies assessing the effects of probiotics in animal models of NEC.

METHODS

The SYRCLE protocol for systematic review of animal intervention studies was followed (Systematic Review Protocol for Animal Intervention Studies Format by SYRCLE (WWW.SYRCLE.NL). The PRISMA (preferred reporting items for systematic reviews and meta-analyses) guidelines (35) were followed for reporting this systematic review. Ethics approval was not required. The protocol of this systematic review was registered on the SYRCLE website (https://www.radboudumc.nl/Research/Organisationofresearch/Departments/cdl/SYRCLE/Pages/Protocols).

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Search Strategy
The databases PubMed (http://www-ncbi-nlm-nih-gov, 1966–August 2016), EMBASE (Excerpta Medica dataBASE) via Ovid (http://ovidsp.tx.ovid.com, 1980–August 2016), ISI Web of Science (v.5.21.1), http://webofscience.com, 1900–August 2016), and E-abstracts from the Pediatric Academic Society meetings (www.abstracts2view.com/pasall, 2000–August 2016), SCiELO (Scientific Electronic Library Online), and Lilacs (Literatura Latino Americana em Ciências da Saúde/Latin American database) databases were searched in December 2015 and August 2016. Abstracts of other conference proceedings such as Perinatal Society of Australia and New Zealand (PSANZ) and European Academy of Paediatric Societies (EAPS) were searched in EMBASE. The Animal Welfare Information Centre (http://awic.nal.usda.gov) data were searched in August 2016. Gray literature was searched using the national technical information services (http://www.ntis.gov/), Open Grey (http://www.opengrey.eu/), Grey net International (http://www.greynet.org/greysourceindex), and Trove (http://trove.nla.gov.au/). The reference lists of eligible studies and review articles were searched to identify additional studies. Reviewers GA-J and SR conducted the literature search independently. No language restrictions were applied.

Search Terminology
We searched PubMed using the following terms: ("necrotising enterocolitis" [All Fields] OR "enterocolitis, necrotizing"[MeSH] AND ("models, animal"[MeSH] AND "Probiotics" [Majr]. We also searched for ("Enterocolitis, Necrotizing"[Mesh]) AND "Models, Animal"[Majr], ((necrotizing enterocolitis) AND animal model AND probiotic, necrotizing enterocolitis and animal models, necrotizing enterocolitis and animal models and probiotics, experimental necrotizing enterocolitis and probiotics. The MeSH term "models, animal" was replaced with the following: "Rats"[Mesh], "Mice"[Mesh], "Swine"[Mesh], "Rabbits"[Mesh], and "Quail"[Mesh] for a further detailed search to enable inclusion of different species. Other databases were also searched using similar terms.

Inclusion Criteria
Only RCTs assessing effects of enteral probiotic supplementation (any dose, duration, frequency, type, and combination) vs. placebo/control in validated animal models (rats, mice, piglets, rabbit, quail) of NEC (36) were eligible for inclusion. Studies assessing probiotic enriched formula, killed/inactivated probiotic, probiotic DNA, or probiotic conditioned medium vs. placebo/control/dam-fed animals were also included.

Exclusion Criteria
Studies assessing effects of probiotic supplemental in animal models that are not validated for NEC as it occurs in human preterm infants (e.g., colitis, or ischemia-reperfusion, *in vitro* studies) were excluded. Studies in hamsters, nematodes, and invertebrates were excluded as their relevance to NEC as it occurs in human preterm infants is uncertain (36). Narrative reviews, systematic reviews, case reports, letters, editorials, and commentaries were excluded but read to identify potential additional studies.

Outcomes
Primary outcomes included the incidence and/or severity of NEC. Secondary outcomes included the effects of probiotics or their derivatives on pathways involved in the pathogenesis of NEC, including (1) immunity, inflammation, and tissue injury, (2) intestinal barrier, (3) dysbiosis/gut microbiota, and (4) other mechanisms (e.g., epithelial growth factor, short-chain fatty acids, oligosaccharides, intestinal and liver fatty acid-binding protein, lysozyme and intestinal phospholipases, oxidative stress, plasma endotoxin, and organic acids).

Data Extraction
Authors GA-J and SR extracted the data independently by using a data collection form designed for this review. The number of animals in each group (probiotic, placebo, and control groups) with details of species, protocol for inducing NEC, and outcomes were entered into the form. All authors verified information about the study design and outcomes. Discrepancies during the data extraction process were resolved by discussion and consensus among all authors. We contacted authors for additional information and clarifications when details on incidence and severity of NEC were not available in the published manuscripts.

Assessment of Risk of Bias in the Included Studies
The risk of bias (ROB) was assessed using the SYRCLE "Risk of Bias" tool (37). This tool is based on the Cochrane ROB tool and incorporates aspects of bias that have a specific role in animal intervention studies. Authors GA-J and SR independently assessed the ROB in all domains, including random number generation, allocation concealment, random housing of animals, blinding of intervention and outcome assessors, selectivity of reporting, and other potential sources of bias. For each domain, the risk was assessed as low, high, or unclear based on the SYRCLE guidelines (37).

Data Synthesis
Meta-analysis was conducted in Review Manager 5.3 (Cochrane Collaboration, Nordic Cochrane Centre, Copenhagen, Denmark). A random-effects model (Mantel–Haenszel method) was used. Analysis was also conducted using the fixed-effects model to ensure that the results and conclusions were not influenced by the type of model used for the meta-analysis. Effect size was expressed as relative risk (RR) and 95% confidence interval (CI). Statistical heterogeneity was assessed with I^2 statistic and by visual inspection of the forest plot (overlap of CIs). I^2 statistic values were interpreted according to the Cochrane Handbook guidelines as follows: 0–40%: might not be important; 30–60%: may represent moderate heterogeneity; 50–90%: may represent substantial heterogeneity; and 75–100%: considerable heterogeneity (38). The risk of publication bias was assessed by visual inspection of the funnel plot (39).

Subgroup Analysis
Considering that the effects of an intervention can be animal species specific, a subgroup analysis was conducted for each of the animal species in the included studies.

Sensitivity Analysis
Analyses were conducted by including only studies where Bifidobacterium was present or absent, whether Lactobacillus was present or absent, studies in preterm animals, studies in term animals, and whether studies used single or multiple strain probiotics.

Summary of Findings Table
The key information about the quality of evidence, the magnitude of effect of the intervention, and the sum of available data on the main outcome are presented in the summary of findings table according to the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) guidelines (40,41).

RESULTS
The literature search retrieved 651 potentially relevant citations of which a total of 29 RCTs (4,270) (Rodents: 23, Piglets: 3, Rabbits: 1, Quails: 2, $N \sim 2{,}310$) were considered eligible for inclusion. The flow diagram of study selection process is given in **Supplementary Figure S1**. Eleven studies were conducted in preterm animal models (42–52), 11 in term newborn animals (53–63), and the remaining 7 studied term

animals at 2–4 weeks of age (42,64–69). Twenty-five studies used single-strain probiotics (42,43,46–48,50,52–70) and four used multi-strain probiotics (44,45,49,51). The median sample size in the included studies was 72 (interquartile range: 36–96; range: 16–343). Twenty-one of the 29 trials reported the incidence of histologically proven NEC (42–51,53–59,64–67) (**Supplementary Table S1**). The remaining eight RCTs (52,60–63,68–70) assessed the pathways of benefits of probiotics in reducing NEC but did not give data on the incidence, severity, or histology of NEC (**Supplementary Table S2**). Khailova *et al.* (70) in 2010 and Khailova *et al.* (46) in 2009 were two separate publications from the same RCT with different primary outcomes. A total of 11 studies used Bifidobacteria (43,46–48,50,53,54,57,62,65,68), 9 studies used Lactobacilli (42,55,56,58,59,63,66,67,69), 3 used both Bifidobacteria and Lactobacilli (44,45,51), 3 used Saccharomyces (60,61,64), 1 study used Lactococci (52), and 1 study tested 5 different strains individually or in combination (49). The daily dose of probiotics ranged from 5 million to 24 billion colony-forming units (CFU). The heterogeneous nature of reporting of the dose (CFU/day, CFU/kg, and CFU/ml) made it difficult to estimate the median administered dose. The characteristics of the included studies (e.g., animal model details, probiotic protocol, outcomes) are given in **Supplementary Tables S1 and S2**.

ROB Assessment Based on SYRCLE Guidelines

Only one study reported on the method used for random sequence generation (54) **Supplementary Table S3**. Method used for allocation concealment was unclear in all studies. Only one study reported on the use of placebo (peptone water) (44). Castro *et al.* (59) mentioned that they used placebo; however, the placebo was milk. In 17/29 studies, pathologists who interpreted the intestinal histopathology for NEC were blinded. Only 7/29 studies reported that baseline characteristics were similar in the probiotic and no probiotic groups. None of the studies reported on measures that were used to house the animals randomly within the animal room. All studies reported complete follow-up data. The domain of selective reporting could not be assessed owing to lack of access to the original protocols of the included studies. Only two studies reported on sample size calculation (49,51).

Meta-Analysis

Pooled data from 16/21 RCTs using random-effects model (Mantel–Haenszel) showed that probiotics significantly decreased the risk of NEC (203/641 (31.7%) vs. 344/571 (60.2%); RR: 0.51; 95% CI: 0.42, 0.62; $P<0.00001$; $I^2=44\%$; number needed to treat: 4; 95% CI: 2.9, 4.3) (**Supplementary Figure S2**). The incidence of severe NEC was reduced significantly in the probiotics vs. placebo group (72/331 (21.8%) vs. 156/353 (44.2%); RR: 0.49, 95% CI: 0.36, 0.67; $P<0.00001$, $I^2=20\%$; number needed to treat: 5; 95% CI: 3.4, 6.4) (**Supplementary Figure S3**). The results remained significant on analysis by fixed-effects model (1) NEC: RR: 0.51; 95% CI: 0.44–0.59; $P<0.00001$ and (2) Severe NEC: RR: 0.5, 95% CI: 0.4, 0.64, $P<0.00001$.

Publication Bias

Visual inspection of the funnel plot indicated that publication bias was unlikely (**Supplementary Figure S4**).

Results of Subgroup Analysis

Subgroup analysis showed that probiotics were beneficial in all animal species, except piglets (**Supplementary Figure S2**).

Results of Sensitivity Analysis

Probiotic supplementation was beneficial in preterm as well as term gestation animals (**Supplementary Table S4**). The beneficial effects were noted whether Bifidobacterium was present or absent, Lactobacillus was present or absent, and in single-strain probiotic studies. Studies that used multiple strains showed benefit on fixed-effects model meta-analysis but not on random-effects model; however, the results for this analysis were heavily influenced by the only RCT that found probiotics to be harmful (45).

Mechanisms of Benefits of Probiotics

The mechanisms of benefits of probiotics observed in various studies are summarized in **Supplementary Table S5**. These include the following: modulation of the inflammatory response (Toll-like receptor 4, nuclear factor-κB, reduction of plasma endotoxin levels), enhancement of the gut barrier (mucus production, synthesis of intercellular junction proteins, brush border enzyme activity), competitive inhibition of colonization by pathogens, secretion of antimicrobial peptides, production of short-chain fatty acid, reduction of oxidative stress, regulation of apoptosis and restitution, and modulating Paneth cell function.

GRADE Evidence

The positive aspects of the total evidence were the relatively large cumulative sample size, large effect size of benefit, narrow CIs around the effect size estimate, very low *P*-value for effect size estimate, absence of publication bias, mild statistical heterogeneity, and blinding of outcome assessors (**Supplementary Table S6**). The main limitation was the fact that majority of included studies had unclear ROB in many domains. Hence, overall the evidence was downgraded to moderate.

DISCUSSION

Our systematic review found that probiotic supplementation significantly reduced the incidence and severity of histologically proven NEC in animal models of the illness. The benefits of probiotics were consistent despite the heterogeneity of their strain, formulation, dose, and duration across four different species of term and preterm animals. These findings support the current thinking that, apart from their strain-specific effects, probiotics 'in general' provide a strain nonspecific protection toward NEC probably by sharing different

pathways of benefits (71,72). Sanders (73) suggested that "there may be a spectrum of probiotic functions which are strain specific but there are others, which are more general to larger groups of strains". Even though many meta-analyses of RCTs (20–22,74) as well as non-RCTs (75–77) in human preterm infants have shown the benefits of probiotics, experts are still concerned about the routine use of probiotics in preterm very low birth weight (birth weight $<1{,}500$ g) infants. Some have pointed out that we still do not specifically know how probiotics work (78), some plead the need for much more basic science research in the field of probiotics in preterm infants (79), while others emphasize the need for further studies into the mechanism of actions of specific probiotic strains in models of immature intestine (80). Our systematic review is an attempt to address some of these concerns by synthesizing the evidence on the effects of probiotics in animal models of NEC. Such pathways are difficult to study in human infants, given that it is not feasible to obtain intestinal specimens from live human preterm infants. In addition, the meta-analysis results from animal studies are expected to add strength to the existing evidence and hopefully provide further reassurance to the clinicians.

The results of this meta-analysis are congruent with the multiple meta-analyses of RCTs and non-RCTs from human preterm infants that have found probiotics to reduce the incidence of NEC. These results are also congruent with the results of the ProPrems trial (81) that showed a statistically significant reduction in the incidence of NEC in the probiotic group (2.0% vs. 4.4%; RR 0.46, 95% CI 0.23, 0.93, $P=0.03$; sample size 1,099). However, the other large RCT (PIPS trial) (82) did not show protection of NEC with probiotics (adjusted risk ratio 0·93 (95% CI 0·68, 1·27; $N=1{,}315$). The authors of the PIPS trial reported a very high cross-colonization rate (20% at 2 weeks and 49% by 36 weeks postmenstrual age) in the placebo group in their trial. Such cross-colonization could have reduced the incidence of NEC even in the placebo group, thereby leading to no statistically significant differences between the two groups. Hence the authors of the PIPS trial suggested that any future RCTs should consider cluster RCT design to avoid the problem of cross-contamination (82).

Although the evidence on the beneficial effects of probiotics in preterm infants is mounting, further research is necessary to identify the optimal strain/s, optimal dose, and duration of supplementation. The RCTs addressing these questions do not require the use of placebo; they can be answered with head-to-head comparisons (e.g.,: single strain vs. multiple strain; high dose vs. low dose; short duration vs. long duration of supplementation, bifidobacteria vs. lactobacillus, one type of bifidobacteria vs. other, and many more).

The strengths and limitations of our review need to be discussed. To our knowledge, this is the first systematic review and meta-analysis of studies assessing the effects of probiotics in animal models of NEC. Our meta-analysis of data from animal models adds further strength to the evidence from studies in human preterm infants. The detailed synthesis of evidence regarding the various mechanisms of benefits of probiotics is an additional strength of this review. This is also the first such comprehensive review in neonatal medicine. The validity of its results is high considering the methodology based on the SYRCLE protocol (WWW.SYRCLE.NL), comprehensive literature search, and the use of PRISMA guidelines (35) for reporting. The significance of our results cannot be overemphasized considering the large effect size, extremely low *P*-values that almost rule out the role of chance alone, low risk for publication bias, minimal-to-moderate statistical heterogeneity, and their consistency on analysis by both random-effects model and fixed-effects model.

The limitations of our review include smaller sample size of individual studies, lack of power calculations in majority of the studies, and the fact that most studies carried unclear ROB on various domains. In the context of sample sizes, we do accept the limitations in carrying out adequately powered animal studies considering the costs, logistics, and ethical issues. Our view on this issue is shared by other investigators (83,84). Another limitation of our review was the inability to fully evaluate the safety of probiotics in animal models. However, it is reassuring to note that >40 RCTs ($n\sim11{,}000$) in human preterm infants have not shown increased risk of late-onset sepsis and mortality. If at all, probiotics have significantly reduced the risk of these outcomes (19–22,80,85). The recently published largest RCT (PIPS trial) did not find Bifidobacterium species cultured from any normally sterile site (81). Although these findings are reassuring, one should not be complacent considering the case reports of probiotic sepsis (86,87). Rigorous quality control of the probiotic product and routine monitoring for probiotic sepsis are therefore critical (88).

Extrapolating results from animal models to human preterm infants may not be appropriate as the effects of an intervention could be species specific (82–84,89). For example, intraperitoneal pentoxifylline prevented NEC in a rat model but not in a rabbit model (90,91). Animal models of NEC may not reflect the multifactorial (e.g., intrauterine growth restriction, sepsis, patent ductus arteriosus, indomethacin, no enteral intake, prolonged parenteral nutrition) pathogenesis of classical late-onset NEC in very/extremely preterm infants. The developmental anatomy and physiology of the gastrointestinal tract of piglets is comparable to that of the human preterm infants, but for inducing NEC, the model often includes only formula feeding to which the animals are very sensitive (44,45). The rat pup model utilizes a combination of insults, including hypoxia, hypothermia, and formula feeds (43,46–49,55,57,58), which is not what commonly leads to NEC in human preterm infants (92). However, it is reassuring to know that our findings are similar to the results of systematic reviews of probiotic RCTs and non-RCTs (before vs. after routine use) in human preterm infants (20–22,74–76).

Two of the included studies in our review used probiotic-conditioned medium and found benefits in decreasing NEC (51,62). The benefits of probiotic conditioned medium are

important as this strategy may help in avoiding the risk of probiotic sepsis, antibiotic resistance, and the need for cold chain. Further studies are essential for assessing its advantages and limitations. For example, probiotic conditioned medium may not be as effective as live/killed-inactivated strains, effects of probiotic conditioned medium from different strains and/or combination may differ, and there may be logistical issues related to mass production.

In summary, our systematic review of animal studies provides robust evidence supporting the benefits of probiotics in reducing the risk of NEC in preterm infants. Despite their limitations, the importance of studies in animal models in guiding research and clinical practice in the field of probiotics and NEC is emphasized. Addressing the need for raising the gold standard by improving the design, conduct, and reporting of animal studies is important (93–96). We believe that our results are a significant contribution toward advancing knowledge in the field of probiotic supplementation for reducing the risk of NEC in preterm infants.

SUPPLEMENTARY MATERIAL

Supplementary Information accompanies this paper at http://www.nature.com/pr

Disclosure: The authors declare no conflict of interest.

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