Abstract
Systematic reviews of randomized controlled trials indicate that probiotic supplementation significantly reduces the risk of necrotizing enterocolitis (NEC) without adverse effects in preterm very-low-birthweight neonates. A change in practice in favor of probiotic supplementation is justified considering the health burden of NEC in this population. The reduction in the risk of NEC seems to occur even when the baseline incidence of the illness is as low as 5%. Facilitation of feed tolerance is a significant benefit of probiotics considering that optimizing enteral nutrition is a priority in extremely preterm neonates, including those with intrauterine growth restriction, who are at a higher risk for feed intolerance and NEC. The increasing number of reports on routine use of probiotics indicates that difficulty in accessing clinically proven and safe probiotic products is not a significant barrier towards a change in practice. Strategies to address important gaps in knowledge and the impact of routine use of probiotic supplementation are reviewed to prepare for what lies ahead in this field.

Necrotizing enterocolitis (NEC) is a potentially disastrous illness in preterm very-low-birthweight (VLBW) neonates with significant mortality and morbidity, including long-term neurodevelopmental impairment, especially in extremely low-birthweight neonates requiring surgical intervention for the illness [1]. The incidence, outcomes, and overall health burden of stage 2 NEC have not changed significantly despite the improvements in neonatal intensive care, and extensive research over decades. The poorly understood pathogenesis of the illness has been the main reason for the failure to develop strategies for primary
and early detection. Current evidence indicates that excessive intestinal inflammation due to an immature innate immune response, decreased stability, diversity and complexity of gut flora, and delayed colonization by beneficial microbes (‘dysbiosis’) play an important role in the pathogenesis of NEC [2]. Presence of substrate continues to be an important risk factor as an overwhelming majority (∼90%) of the cases occur in those who have been fed with milk.

Probiotics are defined as live microbial supplements which when administered in adequate amounts, colonize the gut and benefit the host [3]. Probiotics may prevent NEC by enhancing and protecting the gut mucosal barrier, promoting colonization of the gut with beneficial microbes, inhibiting colonization by pathogens, and modulating the immune system to the advantage of the host [4]. Probiotic-conditioned media have recently been shown to modulate enterocyte genes that regulate innate immune-mediated inflammation [5].

Systematic reviews of randomized controlled trials (RCTs) indicate that prophylactic probiotic supplementation significantly reduces the risk of NEC without adverse effects in preterm VLBW neonates [6]. Considering the health burden of NEC in this population, a change in practice in favor of probiotic supplementation is justified if safe and clinically proven products are available. The reduction in the risk of NEC seems to occur even when the baseline incidence of the illness is as low as 5% [7]. The increasing number of reports on routine use of probiotic supplementation indicates that many centers have recently adopted this strategy, and that accessing clinically proven and safe probiotic products is not difficult [8–11]. Currently, 15 tertiary neonatal intensive care units in Australia provide probiotic supplementation as a standard practice for preterm VLBW neonates. Considering the increasing acceptance of probiotic supplementation to prevent NEC in preterm neonates, a review of what lies ahead in this field is important.

**Extremely Preterm (Gestation <28 Weeks) Neonates**

Lack of adequate data specifically on extremely preterm neonates is often quoted as a problem with adopting probiotic supplementation in this population. A placebo-controlled probiotic trial with at least a few thousand extremely preterm neonates would be required to address this issue. Considering the increasing acceptance of probiotics for preterm neonates, feasibility of completing such a trial in a reasonable time frame is very much questionable, especially if the current evidence in totality is to be shared with honesty and transparency with the parents for an informed consent. Extremely preterm neonates are most deserving of probiotic supplementation as their burden of ≥stage 2 NEC is signifi-
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_data on close to 800 extremely preterm neonates from RCTs and reports on routine use of probiotics are assuring with regard to the safety of probiotics in this population. Probiotic sepsis, even if it occurs, is easy to treat compared with sepsis due to other organisms. The efficacy of probiotics in extremely preterm neonates may be suboptimal considering the various factors adversely affecting the gut flora including frequent exposure to antibiotics, suboptimal enteral nutrition due to frequent stoppage of feeds, recurrent episodes of late-onset sepsis, and dependence on parenteral nutrition. Frequent exposure to antibiotics is a significant issue in extremely preterm neonates. Fouhy et al. [12] have reported that the combined use of ampicillin and gentamicin in early life had significant adverse effects on the evolution of the gut microbiota in infants. The gut microbiota of the antibiotic-treated infants had significantly higher proportions of Proteobacteria (p = 0.0049) and significantly lower proportions of Actinobacteria (p = 0.00001) and the associated genus Bifidobacterium (p = 0.0132) as well as genus Lactobacillus (p = 0.0182) than the untreated controls 4 weeks after stopping antibiotics. By week 8, the Proteobacteria levels remained significantly higher in the treated infants (p = 0.0049), but the Actinobacteria, Bifidobacterium, and Lactobacillus levels had recovered and were similar to those in the control samples [12]. Greenwood et al. [13] have recently reported that preterm neonates who received 5–7 days of empiric antimicrobials in the first week had significantly increased relative abundance of Enterobacter (p = 0.016) and lower bacterial diversity in the 2nd and 3rd weeks of life. The frequency of NEC, sepsis, and death was higher in those receiving early antibiotics compared with those not exposed to antibiotics [13]. Other investigators have also reported an association of prolonged exposure to antibiotics with NEC and death in preterm neonates [14]. The importance of early feeding with colostrum followed by breast milk, probiotic supplementation, antibiotic stewardship, and strategies for reducing the risk of late onset sepsis cannot be overemphasized in this context.

_Intrauterine Growth Restriction_

Preterm growth-restricted neonates are at high risk of feed intolerance, NEC, and postnatal growth failure [15]. Dorling et al. [15] have reported a meta-analysis of independent case series (n = 14) comparing NEC rates in neonates who had fetal absent/reversed end diastolic flow (AREDFA) in the umbilical artery with a control group. Nine studies showed an excess of NEC in those with fetal AREDFA. The overall odds ratio for developing NEC was 2.13 (95% CI: 1.49–3.03) compared with controls with forward fetal end diastolic flow [15]. Fre-
quent signs of feed intolerance (e.g. abdominal distension with visible ropy bowel loops, large/colored gastric residuals) and the fear of the higher risk of NEC means it often takes few weeks to get these neonates on enteral feeds of 120–150 ml/kg per day. Kempley et al. [16] have reported a post hoc analysis of data on neonates <29 weeks’ gestation from an RCT comparing benefits of ‘early’ (starting on day 2 after birth) with ‘late’ (starting on day 6) feeds in preterm neonates (gestation <35 weeks) with intrauterine growth restriction (IUGR). Subsequent feed advancement following a regimen should have achieved full feeds by day 16 in the early and day 20 in the late group. Neonates with gestation <29 weeks achieved full feeds significantly later compared with those ≥29 weeks; median (IQR) age: 28 (22–40) versus 19 (17–23) days (HR 0.35, 95% CI: 0.3–0.5). The incidence of NEC was higher in neonates with gestation <29 compared with ≥29 weeks: 32/83 (39%) versus 32/312 (10%), RR 3.7 (95% CI: 2.4–5.7) [16].

Several mechanisms may explain the higher risk of NEC and feed intolerance in preterm neonates with IUGR following fetal umbilical artery AREDF. These include fetal hypoxia, redistribution of the gastrointestinal blood flow to spare the brain of the adverse circulatory effects of AREDF, structural and functional changes in the intestine, and altered gut colonization [17–22]. Fetal hypoxia and increased mesenteric vascular resistance may cause prenatal hypoxic-ischemic injury of the intestine, affecting the development of intestinal motor, secretory, and mucosal function, and increasing its postnatal vulnerability to ileus, abnormal colonization, and bacterial invasion [17–22]. The postprandial rise in superior mesenteric artery (SMA) flow required for digestion is compromised in preterm IUGR neonates [23]. The recovery of the low-baseline SMA flow velocity is slow during the first week of life. On day 7, the values are similar to those in appropriately grown neonates not on feeds [23]. Pseudo-obstruction due to meconium plug is not uncommon, and the incidence of late-onset sepsis, which further compromises the gut, is higher in preterm IUGR neonates [24, 25]. D’Inca et al. [17] have reported that, at birth, intestinal weight and length, ileal and colonic weight per unit of length, and villous sizes were significantly lower in piglets with IUGR compared with same-age controls. These alterations persisted, although less marked at day 5. Counts of bacteria adherent to ileal and colonic mucosa were significantly greater in 2-day-old IUGR piglets compared with same-age controls. Study of the expression of genes involved in proliferation (proliferating cell nuclear antigen, PCNA) and apoptosis (BAX and CASP3) pathways indicated a significantly lower expression of PCNA at birth in IUGR piglets compared with controls, and at day 2 it tended to be significantly lower. BAX expression tended to be significantly greater at day 5 in piglets with IUGR compared with same-age controls. Apoptosis rates tended to be significantly greater in IUGR versus control piglets at birth but not at days 2 and 5 [17]. Fan-
ça-Berthon et al. [18] have studied the effect of IUGR on gut microbiota by comparing the composition and activity of ceccolonic microbiota from birth to adulthood in rats with and without IUGR. Bacterial density was increased at day 5 and decreased at day 12. In adulthood, rats with IUGR still differed from controls, containing fewer *Bifidobacterium* species at day 40 and more bacteria related to *Roseburia intestinalis* at day 100. In vivo, propionate concentration was decreased by IUGR before weaning, whereas the concentrations of other short chain fatty acids (SCFAs) were decreased at day 40, although the in vitro metabolic capability was unaffected overall. These results indicate that IUGR can cause both neonatal and long-term alterations of the intestinal microbiota [18]. Deficits in the bioavailability of butyrate could adversely affect the proliferation of colonocytes and the maintenance of colonic homeostasis by modulating the expression of intestinal transcripts involved in gut barrier function, and reducing mucin secretion [19, 20]. Faña-Berthon et al. (2009) have reported that IUGR impairs mucus barrier development and is associated with long-term alterations of mucin expression [21]. The lack of an efficient colonic barrier induced by IUGR may predispose to colonic injury not only in neonatal life but also in later life [21]. Wang et al. [22] have reported continuous impairment of intestinal development in neonatal piglets with IUGR using temporal proteomics analysis coupled with histological and biochemical studies.

Together, the consequences of pre- and postnatal disturbances of gut perfusion, altered intestinal structure and function, altered gut flora, and increased metabolic demands of enteral feeds may explain the increased risk of NEC and feed intolerance in preterm IUGR neonates. Studies specifically addressing the effects of probiotic supplementation in preterm IUGR neonates are required considering the significance of gut flora in IUGR.

**Focus on Facilitating Enteral Nutrition**

Optimizing enteral nutrition is a priority in extremely preterm neonates, including those with IUGR. Focusing on the benefits of probiotic supplementation on enteral nutrition is thus important. Probiotics may modulate gut motility by their secreted products or products of fermentation, influence on intestinal neuroendocrine factors, or by mediators secreted by the gut as an immune reaction to probiotics [26]. SCFAs are the main end products of colonic fermentation of dietary fiber by gut microbiota that play an important role in maintaining gut function and well-being. Colonic fermentation contributes to regulation of upper gastrointestinal motility, reduction in gastric emptying rate and lower interdigestive acid output through the effects of SCFA [27]. Butyrate plays an impor-
tant role in gastrointestinal homeostasis [28]. Overall, these mechanisms of benefits of the gut flora support the findings from RCTs that probiotics significantly reduce the time to full enteral feeds (120–150 ml/kg per day) in preterm VLBW neonates. Strain selection is an important issue when targeting such specific beneficial effects of probiotic supplementation in preterm neonates. *Lactobacillus reuteri* DSM 17938 has been shown to improve gastric emptying (assessed by gastroelectrography and ultrasound) and feed tolerance in preterm neonates [29]. Awareness of factors affecting gastric motility and function (e.g. formula feeds, high osmotic load of milk due to medications and other additives, feeding position, sepsis, phototherapy) is important as they may reduce/negate the benefits of probiotic supplementation [30].

**Advancing Knowledge in the Field**

Rates of colonization of the gut depend on the probiotic strain properties, and host-related factors such as the gestational and postnatal age in neonates. Evidence indicates that colonization may not be necessary for the beneficial effects of probiotics. Adopting strategies to improve colonization is important if it is indeed required for optimal effects of probiotics. Yamasaki et al. [31] have reported that early administration of *Bifidobacterium bifidum* OLB6378 accelerated enteral feeds and optimized colonization. In their pilot study, 36 preterm VLBW neonates were randomly divided into either group E (*B. bifidum* supplementation started ≤ 48 h of birth) or group L, where it was started >48 h after birth. Group E reached 100 ml/kg per day feeds earlier than group L [median (interquartile): 10 (7–13) vs. 11 (10–15) days, respectively]. The daily weight gain was significantly higher in group E. Fecal *Bifidobacterium* levels were not significantly different between the groups (real-time polymerase chain reaction assay) at 1 and 4 weeks of age. However, the highest colonization rate of *Bifidobacterium* was observed when the supplementation was started between 24 and 48 h after birth [31]. These results need to be confirmed in larger studies, and with different strains.

Limited evidence indicates that the efficacy of multistrain probiotics may be greater than single strains, including strains that are components of the multistrain mixture themselves. It is not clear whether this benefit is due to the synergistic interactions between strains or a consequence of the higher probiotic dose used in such studies. Ishizeki et al. [32] administered a single-strain (*Bifidobacterium breve* M-16V, $5 \times 10^8$) or a multistrain probiotic (*B. breve* M-16V, *Bifidobacterium longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536, $5 \times 10^8$ each strain) daily for 6 weeks in low-birthweight neonates. Detection rates and fecal counts of bifidobacteria increased significantly between weeks 1 and 6.
The proportion of bifidobacteria was significantly higher in the multistrain compared with the single-strain group at weeks 1 and 6. The proportion of infants with bifidobacteria-predominant microbiota was significantly higher in the multistrain than in the control group. The proportions of Enterobacteriaceae were significantly lower in the multistrain group at weeks 4 and 6. *B. breve* M-16V and *B. infantis* M-63 were detected in ≥85% of neonates during the administration period, while *B. longum* BB536 was detected in ≤40%. Administration of three strains of bifidobacteria was thus more beneficial [32]. Awaited results of the large multicenter probiotic trial from the UK will be important to judge whether the effects of single strain (*B. breve*, Yakult) are comparable to those reported in previous RCTs of single- or multistrain products in preterm VLBW neonates. Wu et al. [33] have reported that administration of a mixture of probiotic strains with *B. bifidum* and *B. longum* was most effective in preventing death and NEC in a rat model of NEC. Their observations are helpful in designing clinical trials comparing effects of single- versus multistrain probiotics.

Human milk oligosaccharides (HMO) are the third most abundant class of molecules in breast milk. As infants do not have the enzymes required for milk glycan digestion, HMOs pass undigested to the lower part of the gut, where they can be consumed as substrate by specific gut microbiota. Research on consumption of specific HMOs by different probiotic strains will help in developing optimal pre- and probiotic combinations (synbiotic) [34].

Further research assessing the effects of killed or inactivated versus live probiotic agents will be important considering the potential benefits with regard to probiotic sepsis, development of antibiotic resistance, and need for maintaining a cold chain which have implications for global utility of probiotics [35].

Development of new techniques (e.g. newer methods for encapsulation) for improving the tolerance of probiotic strains to bile, acid, and oxygen exposure is expected to enhance the benefits of probiotics.

In summary, current evidence from within and outside RCTs strongly supports the use of probiotic supplementation to reduce the risk of NEC in preterm VLBW neonates. Probiotic prophylaxis outside the rigid framework of RCTs is expected to provide data on real-life benefits, and importantly, the uncommon/rare adverse effects of this intervention in preterm neonates. Reporting outcomes and safety data following introduction of routine probiotic supplementation is thus important. Strain-specific population data will help in guiding clinical practice. As with any intervention, it is expected that the real-life benefits of probiotic supplementation may not be as dramatic as reported in RCTs. However, even a much smaller reduction in the risk of definite NEC (e.g. 20–25%) may be acceptable considering the overall health burden of the condition and the relatively very low cost of probiotics.
Probiotics will not be a panacea for NEC, which is known to present at different postnatal ages with different triggers and different mode of presentation. Strategies such as maximizing exposure to antenatal glucocorticoids, early feeding with maternal/donor breast milk, and an aggressive approach for prevention and treatment of late-onset sepsis are equally or perhaps more important if a ‘zero tolerance’ to NEC is required. Focusing on nutritional benefits of probiotics is important, especially when further reduction in the low baseline incidence of definite NEC is not a priority or possibility.

Finally, without easy access to safe and clinically proven probiotic products, it will be the case of ‘all dressed but nowhere to go’. Cooperation between various stakeholders including the regulatory authorities and the industry is urgently required to address the issue of quality control and, importantly, decide whether probiotics should be classified as drugs, food supplements, or biotherapeutic agents. Considering that different probiotic strains may benefit by different pathways, and in different ‘doses’, probiotics will not be available as a standardized packaged product like a conventional drug.

Disclosure Statement

The author declares that no financial or other conflict of interest exists in relation to the contents of the chapter.

References