Exploratory Studies from the PiPS Trial fail to find evidence that *Bifidobacterium breve* BBG-001 modifies intestinal barrier function

Cite this article as: Paul Fleming, Mark Wilks, Simon Eaton, Nicola Panton, Richard Hutchinson, Abena Akyempon, Pollyanna Hardy, Michael R. Millar and Kate Costeloe, Exploratory Studies from the PiPS Trial fail to find evidence that *Bifidobacterium breve* BBG-001 modifies intestinal barrier function, *Pediatric Research* doi:10.1038/s41390-020-01135-5

Exploratory Studies from the PiPS Trial fail to find evidence that *Bifidobacterium breve* BBG-001 modifies intestinal barrier function

*Paul Fleming [1,2], Mark Wilks [3,4], Simon Eaton [5], Nicola Panton [4], Richard Hutchinson [2], Abena Akyempon [1], Pollyanna Hardy [6], Michael R Millar [3,4], Kate Costeloe [1,2]

1. Homerton University Hospital, NHS Foundation Trust, London, UK
2. Centre for Genomics and Child Health, Queen Mary University of London, London, UK
3. Department of Infection, Barts Health NHS Trust, London, UK
4. Blizard Institute, Queen Mary University of London, London, UK
5. UCL Great Ormond Street Institute of Child Health, University College London, London, UK
6. Birmingham Clinical Trials Unit, University of Birmingham, Birmingham, UK

***Corresponding Author**

Dr Paul Fleming

Address: Homerton University Hospital, Homerton Row, London E96SR, London, UK

Email: Paulfleming1@nhs.net Phone: +44 208 510 7361

Funding

This study was supported by a strategic Research Grant from Barts Charity (ref: 719/1102).

Ethics and consent

Ethics approval was granted by the South London REC 2 Committee (Ref 10/H0802/40). Participants were recruited following informed written parental consent.

Acknowledgements

Our thanks are extended to Yakult Honsha European Research Centre for Microbiology ESV (YHER), Belgium for analysing faecal samples for both microbiota and short chain fatty acids. YHER had no role in the data analyses and interpretation of the results presented in this manuscript. The interventions for the PiPS trial were provided without charge by Yakult Honsha Co Ltd (Tokyo, Japan) until the point of entry to the UK. The PiPS trial was funded by the UK National Institute for Health Research Health Technology Assessment Programme, project number 05/501/04. Special thanks are extended to Mr Nik Hannay for creating the figure presented in this manuscript.

Word Count

3893

Conflicts of Interest

None declared

Author Contributions PF designed the study, performed laboratory analyses, analysed data and wrote the manuscript; MW designed the study, provided laboratory support and reviewed the data and manuscript; SE performed laboratory analyses and reviewed the manuscript; NP performed laboratory analyses and reviewed the manuscript; RH performed laboratory analyses and reviewed the manuscript; AA assisted with clinical data collection and reviewed the manuscript; PH provided statistical advice and reviewed the manuscript; MM designed the study and co-wrote the manuscript; KC designed the study and co-wrote the manuscript.

Impact

What is the key message of this article?

1. Uncertainty about the therapeutic role of probiotics to prevent necrotising enterocolitis is in part due to the wide range of bacterial strains with no previous evidence of efficacy used in clinical trials.
2. We hypothesised that mechanistic studies embedded in a probiotic trial would provide evidence about which properties of probiotics might be important for NEC prevention.

What does it add and what is the impact?

3. The finding that the probiotic strain tested, *Bifidobacterium breve* BBG-001, showed neither effects on intestinal barrier function nor clinical efficacy supports the possibility that these tests have potential to identify strains to progress to large clinical trials.

Abstract

Background

Uncertainty remains about the role of probiotics to prevent necrotising enterocolitis (NEC) some of which arises from the variety of probiotic interventions used in different trials, many with no prior evidence of potential efficacy. Mechanistic studies of intestinal barrier function embedded in a large probiotic trial could provide evidence about which properties of probiotics might be important for NEC prevention thus facilitating identification of strains with therapeutic potential.

Methods

Intestinal permeability, stool microbiota, SCFAs and mucosal inflammation were assessed from the second postnatal week in babies enrolled to a randomised controlled trial of *B breve* BBG-001 (the PiPS Trial). Results were compared by allocation and by stool colonisation with the probiotic.

Results

Ninety-four preterm babies were recruited across six nested studies. *B breve* BBG-001 content was higher by allocation and colonisation, *Enterobacteriaceae* and acetic acid levels were higher by colonisation. No measure of intestinal barrier function showed differences. The PiPS trial found no evidence of efficacy to reduce NEC.

Conclusion

That the negative results of the PiPS trial were associated with failure of this probiotic to modify intestinal barrier function supports the possibility that the tests described here have potential to identify strains to progress to large clinical trials.

1 Introduction

Necrotising enterocolitis (NEC) is the major gastro-intestinal emergency following pre-term birth - it is a devastating disease carrying high mortality and long-term morbidity [1]. The pathogenesis remains incompletely understood and is complex and heterogeneous involving functional and anatomical immaturity of both the intestinal barrier and gastrointestinal immune systems, abnormal patterns of intestinal microbial colonisation, abnormalities in gastro-intestinal blood flow and a possible genetic predisposition[2] [3]. Figure 1 outlines some of the characteristics of the foetal and preterm neonatal intestine that may predispose preterm babies to the development of NEC.

There is no clear case definition for NEC and whilst the global incidence varies widely, it is estimated to affect between 2-9% of babies born <1500g birthweight [4]. Clinical management involving 'resting' the bowel and giving antibiotics with recourse to surgical intervention if necessary[5] has not changed for decades and there is urgent need for evidence based interventions both to prevent and treat disease.

Over the past twenty years there has been interest in the potential of enteral probiotics to prevent NEC [6-10]. There have been a number of meta-analyses all of which suggest evidence of benefit, one of the most recent including 38 trials involving 10,520 participants [11]. Despite this, clinicians remain divided about the therapeutic role of probiotics. Various aspects of the trials have been criticised, one of the frequent comments being the heterogeneity of the interventions in terms of bacterial strain, dosage and the number of strains given [12]. In the majority of these trials the choice of probiotic appears to be based on availability rather than any pre-clinical evidence of benefit. The largest of the trials included in these meta-analyses, the Probiotics in Preterm Babies trial (PiPS), was a placebo controlled trial of the probiotic *Bifidobacterium breve* BBG-001 in babies born before 31 weeks of gestation[13].

When the PiPS trial was designed the investigators believed it likely that this probiotic would prevent NEC. The current paper reports results of studies embedded within the trial investigating effects of the probiotic *Bifidobacterium breve* BBG-001 on aspects of intestinal barrier function, this being thought to be an important factor in the pathogenesis of NEC. Previous studies have shown increased intestinal permeability in preterm babies both with [14-17] and without NEC [18-22], abnormal patterns of intestinal microbial colonisation, short chain fatty acid and lactic acid excretion preceding NEC [23, 24] and increased intestinal inflammation in association with NEC [25]. Different probiotics have shown potential to modulate gut permeability [26], intestinal microbiota composition [27] and short chain fatty acid production [24, 28] and to reduce intestinal inflammation (albeit in laboratory induced NEC) [29]. The studies reported in this paper, were designed to evaluate whether *Bifidobacterium breve* BBG-001 administration or colonisation influenced these intestinal barrier functions. It was hypothesised that these studies would provide potential evidence about which properties of probiotics might be important for NEC prevention thus facilitating the identification of strains with therapeutic potential.

2 Aims

The aims of this study were to investigate whether randomisation to, or colonisation with, *Bifidobacterium breve* BBG-001 affected: (1) intestinal permeability; (2) intestinal microbiota composition, short chain fatty acid and lactic acid production and; (3) intestinal mucosal inflammation and enterocyte damage; in a subset of babies enrolled to the Probiotics in Preterm Babies (PiPS) trial.

3 Methods

3.1 Study Population, Funding and Ethics

Nested studies were conducted on a subset of participants already enrolled to the PiPS trial at two hospitals in inner East London (The Homerton University Hospital NHS Foundation Trust, Homerton Row, London and The Royal London Hospital NHS Trust, Whitechapel, London). Inclusion and exclusion criteria were the same as those for the PiPS trial [30] with funding and ethics approval provided independently of the trial by Barts Charity (Ref: 719/1102) and the South London REC 2 Committee (Ref 10/H0802/40) respectively. Parents were approached about these additional studies during the second week after the birth of their baby and were recruited following informed written parental consent.

There were six separate studies conducted under three themes outlined in 3.1.1. Because the research ethics committee requested that parents be given the choice about which studies their baby took part in, there are different numbers of babies in each of the studies described in this paper. A schedule of events outlining the postnatal age (pna) at which each study was performed is presented in **Table 1**.

3.1.1 Theme 1: Intestinal Permeability

Assessments of intestinal permeability were divided into tests evaluating (a) afferent movement of substances from within the intestinal lumen into the systemic circulation and (b) efferent movement of substances from the systemic circulation into the intestinal lumen.

Tests evaluating afferent movement included: (1) a sugar absorption test (using lactulose and mannitol) (SAT) conducted at day 14 after birth; and (2) weekly evaluations (for four weeks) of blood and plasma for the presence of bacterial DNA and lipopolysaccharide (LPS) endotoxin. The sugar

absorption test was conducted using the protocol previously published by van Elburg and colleagues[22] and whole blood assessed for the presence of bacterial DNA using the methods described by Jiang [31]. Plasma endotoxin was quantified using the ToxinSensor™ Chromogenic Endotoxin Assay Kit following manufacturer protocols and guidance (http://www.genscript.com).

Tests evaluating efferent movement involved quantifying stool alpha-1-antitrypsin (A1AT) by an enzyme-linked immunoassay (ELISA) on stool samples collected between days 14-21 after delivery. A1AT ELISA kits and protocols were supplied by Immundiagnostik (AG Bensheim, Germany).

3.1.2 Theme 2: Intestinal Microbiota, Short Chain Fatty Acids and Lactic Acid

Stool microbiota were identified and quantified using 16S rRNA gene targeted group and species specific primers. The materials and methods used for the development of primers, extraction of DNA from faecal samples, PCR and short chain fatty acid and lactic acid quantification, have previously been described [32, 33]′ [34]. These tests were performed at the Yakult Honsha European Research Centre for Microbiology ESV (YHER) at their laboratory in Ghent-St-Peters, Belgium.

3.1.3 Theme 3: Intestinal Mucosal Inflammation

Intestinal mucosal inflammation/enterocyte damage was assessed by measuring urinary intestinal fatty acid binding protein ($I\text{-}FABP_u$) by ELISA using a commercial kit and manufacturer protocols provided by Hycult Biotech Ltd. Urine samples were corrected for dilution by normalising to creatinine content, measured using an enzymatic kit (Sentinel Diagnostics). Measurement of $I\text{-}FABP_u$ was performed on urine samples collected for the sugar absorption tests.

3.1.4 Stool colonisation

Stool colonisation with *B breve* strain BBG-001 was determined by culture at two weeks postnatal age using a selective medium provided by Yakult Honsha Co Ltd (Tokyo, Japan) and by PCR for microbiota and SCFAs/lactic acid.

3.2 Statistical Analyses

The results were analysed two ways, firstly by whether the baby was randomised to *B breve* BBG-001 or placebo and secondly by whether or not the stools of babies were colonised with *B breve* BBG-001. All analyses were conducted using GraphPad Prism 6 (© GraphPad Software, Inc.) and IBM SPSS for Windows (version 26). Continuous data are summarised using medians and interquartile ranges and compared by group using the Mann-Whitney U-test (for non-normally distributed data). The Hodges-Lehmann method was used to estimate the median differences with corresponding 95% confidence intervals (CIs). Dichotomous data are presented using frequencies and percentages and compared by group using Fisher's exact test. Differences in proportions are presented with corresponding 95% CIs. Sensitivity analyses were carried out on continuous outcomes using linear regression methods and, assuming the data were normally distributed, adjusted for gestational age at birth.

3.2.1 Sample Size Calculations

Sample size estimates were calculated at a 2-sided 5% level of significance with 80% power. Where possible, sample sizes assumptions were based on previously published works [26, 35] however, for many of the studies undertaken, we lacked robust data in the preterm infant in health and disease and in the context of probiotic administration. Where data for preterm babies did not exist, term infant data or data from adult studies were used [36]′ [25, 37] . The target numbers for each component study were: (a) SAT-16 per group; (b) stool A1AT-19 per group; (c) bacterial translocation-38 per group; (d) stool microbiota and SCFA-11 per group; and (e) IFABP$_u$-14 per group (see Appendix 1).

3.3 Access to Participant Clinical, Trial Allocation and Colonisation Data

All clinical and trial related data, including the allocation to probiotic or placebo and stool colonisation by *Bifidobacterium breve* BBG-001 at two weeks postnatal age, were provided after completion of the PiPS trial by the Clinical Trials Unit at the National Perinatal Epidemiology Unit (NPEU) in Oxford, UK. The NPEU was responsible for the day to day administration of the main PiPS trial. The data were provided with permission granted by the Trial Steering Committee and issued following completion of a data sharing agreement.

4 Results

Between August 2010 and October 2013, ninety-four infants were recruited to these studies. The median [IQR] gestation of all participants was 26.7 weeks [25.43 to 28.18] and median [IQR] birthweight was 848 grams [774 to 1013]. There were 53 males and 41 females. Baseline data, trial allocation to probiotic or placebo and stool colonisation with *B breve* BBG-001 are presented in Table 2. In all six studies, the proportion of participants colonised with *B breve* BBG-001 at two weeks pna is higher than the number allocated to receive it, suggesting that some babies in the placebo group were colonised. Adjustment for gestational age at birth did not change the results.

4.1 Theme 1: Intestinal Permeability

4.1.1 Afferent permeability from within the intestinal lumen to the systemic circulation

Afferent permeability from the intestinal lumen to the systemic circulation was assessed by the sugar absorption test and by bacterial translocation. Table 3 outlines the results of these evaluations.

4.1.2 Efferent permeability from the systemic circulation into the intestinal lumen

Efferent intestinal permeability (i.e. movement from the systemic circulation to the intestinal lumen) was assessed by measuring stool alpha-1-antitrypsin. The median [IQR] stool A1AT among babies randomised to *B breve* BBG-001 was 12.94 [10.36 to 32.58] mg/dl and in babies allocated to placebo was 17.78 [10.95 to 29.71] mg/dl (p=0.49; difference in medians: 1.19; 95% CI -3.95 to 8.5). The median [IQR] stool A1AT in babies colonised with the probiotic was 13.68 [10.56 to 34.39] mg/dl and in babies who were not colonised was 16.59 [10.52 to 23.82] mg/dl (p=0.6; difference in medians: -1.13; 95% CI -11.77 to 4.95).

4.2 Theme 2: Stool Microbiota and Short Chain Fatty Acids

4.2.1 Stool Microbiota

Stool microbiota were identified and quantified using family, genus and species specific primers originally designed to detect the predominant bacteria in adult human faeces [33]. A number of bacteria were not detected in any of the samples (*Clostridium cocoides, Prevotella* and *Atopobium cluster; Lactobacillus casei, L. brevis, L. fermentum, L. fructivorans, L. reuteri, L. sakei and C. difficile*) or were present in a small number of babies precluding comparisons between groups (*Bacteroides fragilis* [1/29 samples]; *C. perfringens* [4/29 samples]; *L. gasseri* [5/29 samples]; *L. plantarum* [3/29 samples]; *L. ruminis* [1/29 samples]; *Streptococcus* [8/29 samples] and *Pseudomonas* [4/29 samples].

Table 4 outlines the median bacterial counts (Log_{10}/g faeces) detected in the stools of babies.

4.2.2 Stool short chain fatty acids and lactic acid

Short chain fatty acid and lactic acid content was also quantified on the same stools as those used to assess bacterial content. High Performance Liquid Chromatography was performed to detect succinic acid, lactic acid, formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid.

Comparisons between groups were limited to succinic acid, lactic acid and acetic acid because of the small proportion of samples in which the other SCFAs were detected. The results of comparisons by randomisation and colonisation to *B breve* BBG-001 are presented in Table 5.

4.3 Theme 3: Intestinal inflammation

Intestinal inflammation/enterocyte damage was assessed by measuring urinary intestinal fatty acid binding protein (I-FABP$_u$) in urine.

The median [IQR] value for I-FABP$_u$ in babies randomised to *B breve* BBG-001 was 5.94pg/mmol [3.3 to 20.6] and was 3.5pg/mmol [1.1 to 8.1] in babies receiving placebo (p=0.14; difference in medians: -3.4; 95% CI -9.2 to 1.1).

By colonisation with *B breve* BBG-001, the median [IQR] I-FABP$_u$ was 6.35pg/mmol [2.4 to 12.02] and in babies who were not colonised with the probiotic was 1.8pg/mmol [0.94 to 9.3] (p=0.16; difference in medians: -2.2; 95% CI -7.3 to 1.5).

5 Discussion

These studies prospectively evaluated mechanisms of action of the probiotic *Bifidobacterium breve* BBG-001 in a subset of babies enrolled in a large randomised controlled trial and found no evidence that *B breve* BBG-001 affected intestinal permeability or intestinal mucosal inflammation. Differences in intestinal microbiota and short chain fatty acid production were observed in babies colonised with the probiotic at two weeks post-natal age. The findings are consistent with the PiPS trial which showed no evidence of benefit for any of its three primary outcomes (NEC, late onset sepsis (LOS) and death) nor for any of the pre-specified secondary outcomes with rates of major neonatal morbidities similar between groups [30].

When the PiPS trial was designed (in 2005), the investigators believed it likely the probiotic would reduce the incidence of NEC. *Bifidobacterium breve* BBG-001 was chosen as the intervention because it was the only product that had been reported at that time to show any benefit, albeit for nutritional outcomes rather than for NEC prevention [27]. One of the key aims underpinning the work reported in this paper was not only to understand the mechanism of any benefit of *B breve* BBG-001 found in the PiPS trial, but also to identify tests that might be useful to evaluate further probiotic strains with therapeutic potential. If these mechanisms had been evaluated prior to commencing the PiPS trial, the findings may have influenced strain selection in the main study. Large randomised controlled trials are not only expensive but are time consuming for staff and potentially impose strain on families and risks for participants; surrogate markers of probiotic mechanisms might be useful in evaluating new or proposed probiotic interventions before progressing to phase 3 clinical trials.

Increased intestinal permeability is frequently cited as a risk factor for NEC and substantial proportions of neonatal septicaemias are associated with gut-derived organisms, suggesting an aetiological role for translocation in LOS[38]. We evaluated intestinal permeability using three different methods. The modified sugar absorption test has previously been used to assess baseline

intestinal permeability in preterm babies [22], effects of feeding type [39] and in examining the effects of probiotics [26]and prebiotics [40] in preterm babies. Babies allocated to and colonised with *B breve* BBG-001 had trends towards reduced permeability by the SAT. However, the SAT is designed to measure small intestinal permeability and one could argue that assessment by this method alone may not truly reflect whole bowel permeability.

To overcome this, we also assessed passive markers of permeability (faecal A1AT) and serum markers (endotoxin and bacterial DNA reflecting whole bowel permeability). Both of these methods are less well reported in preterm babies though both have been evaluated in adult studies[41]. Median faecal A1AT levels among babies were within the normal range (<54mg/dl) so this test may not be useful in assessing interventions that might reduce permeability. In babies allocated to the probiotic intervention, there was a trend towards increased bacterial translocation (p=0.05). Overall high rates of bacterial translocation were found among all babies enrolled to this study with 43/86 (50%) having at least one episode during the study period. This finding has not previously been reported and the contribution of bacterial translocation (especially endotoxinaemia) to episodes of clinically suspected infection in preterm babies requires further evaluation. There are however, limitations to the assays used to detect plasma endotoxin and previous reviews have recommended caution when interpreting results [42].

Stool microbiota were assessed using primers originally designed to detect the predominant bacteria in adult human faeces[32]. Samples processed from preterm babies in our study showed less microbial diversity in comparison to those seen in adults which likely reflects the range of taxa assessed using this primer set and the typical constituents of the preterm gut flora[43, 44]. When analysed by colonisation with *B breve* BBG-001, we saw increased bacterial loads of potentially pathogenic *Enterobacteriaceae* on samples processed at three weeks after birth. This observation has been reported previously[27] and is surprising given that acetic acid levels were also raised in babies colonised with *B breve* BBG-001; we would expect raised acetic acid to inhibit or suppress

colonisation with potentially pathogenic bacteria[45]. In an analysis of the intestinal microbiome of infants recruited to the PiPS trial at 36 weeks post menstrual age, there was no difference in microbial richness and diversity nor of the relative proportion of *Proteobacteria,* predominantly *Enterobacteriaceae,* either by allocation or colonisation. *Enterobacteriaceae* were more dominant in the intestinal microbiome of infants with longer durations of exposure to antibiotics during the first two weeks after birth[46]. There are many factors that might determine the relationship between colonisation with *B breve*, *Enterobacteriaceae* and acetic acid production. These include substrate availability (to produce acetic acid or consume human milk oligosaccharides), antibiotic effects on bacterial metabolism and viability, microbial interactions (including displacement and/or competitive exclusion), the specific strains colonising infants in the comparative groups and the methods of analysis. Higher numbers of *Enterobacteriaceae* by colonisation may also explain trends towards higher levels of I-FABP$_u$.

These contradictory findings (together with wide confidence intervals) may also suggest that the sample sizes were too small. For many of the studies undertaken, we lacked robust data in the preterm infant in health and disease and in the context of probiotic administration, on which to base our sample size calculations. Instead, for many studies, the best we could do was to extrapolate from term infant data or data from adult studies. These patient groups represent different cohorts at different stages of development and with different diseases and the reference ranges obtained from these groups may not be applicable to the preterm baby.

Choosing appropriate targets on which to design mechanistic studies is dependent upon understanding the pathophysiology of the disease for which an intervention is proposed to exert benefit. The hypotheses supporting the use of probiotics to prevent necrotising enterocolitis and septicaemias are that their administration to the preterm infant will strengthen intestinal barrier function, encourage gut microbiota resembling that of the term infant, reduce intestinal inflammation and modulate intestinal immunity [13]. Probiotics work through a diverse range of

biological mechanisms but not all probiotics act similarly. Choosing the right probiotic to prevent important diseases in preterm babies is dependent upon evaluating different probiotic species both at mechanistic levels and in large appropriately designed randomised trials. If intestinal permeability is an important contributor to the development of necrotising enterocolitis and LOS then the results of this study may explain why the probiotic did not influence the clinical outcomes assessed in the main trial. However, there are limited longitudinal data in the preterm baby relating to normal values (both in health and disease) for evaluation of interventions that may influence the intestinal barrier functions we investigated in this study. Without such data, the results we report here could easily reflect a lack of underlying pathology as much as a failure of the probiotic to alter these parameters.

Few previous studies have sought to prospectively evaluate probiotic mechanisms by both randomisation and colonisation across such a diverse range of potential areas for which probiotics might exert benefit. When evaluating subjects enrolled to a study using a live intervention, potential cross colonisation of the placebo group is inevitable and evaluating results by randomisation and colonisation is important when determining the overall effect an intervention might exert.

In 2018, The European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), published a strain specific systematic review and network meta-analysis of probiotic use in preterm babies. Despite including 51 studies and over 11,000 participants, the authors were unable to identify the optimal strain, dose or combination of probiotics to reduce NEC and concluded that clinicians are left using inadequately tested, potentially unsafe and possibly ineffective treatments [47]. More recently, ESPGHAN has produced a further conditional recommendation for four probiotic bacteria that may reduce NEC. However the authors are clear this recommendation comes with a low certainty of evidence [48].

Future studies evaluating specific probiotic bacteria in preterm babies may benefit from undertaking mechanistic evaluations to inform optimum strain selection for testing in larger randomised trials

and we believe the studies outlined here may provide essential data relating to the preterm baby on which to adequately power these studies.

6 References

1. Battersby, C., et al., *Incidence and enteral feed antecedents of severe neonatal necrotising enterocolitis across neonatal networks in England, 2012-13: a whole-population surveillance study.* Lancet Gastroenterol Hepatol, 2017. **2**(1): p. 43-51.
2. Neu, J. and W.A. Walker, *Necrotizing enterocolitis.* N Engl J Med, 2011. **364**(3): p. 255-64.
3. Alganabi, M., C. Lee, E. Bindi, B. Li, and A. Pierro, *Recent advances in understanding necrotizing enterocolitis.* F1000Res, 2019. **8**.
4. Battersby, C., T. Santhalingam, K. Costeloe, and N. Modi, *Incidence of neonatal necrotising enterocolitis in high-income countries: a systematic review.* Arch Dis Child Fetal Neonatal Ed, 2018. **103**(2): p. F182-F189.
5. Hall, N.J., S. Eaton, and A. Pierro, *Royal Australasia of Surgeons Guest Lecture. Necrotizing enterocolitis: prevention, treatment, and outcome.* J Pediatr Surg, 2013. **48**(12): p. 2359-67.
6. Dani, C., R. Biadaioli, G. Bertini, E. Martelli, and F.F. Rubaltelli, *Probiotics feeding in prevention of urinary tract infection, bacterial sepsis and necrotizing enterocolitis in preterm infants. A prospective double-blind study.* Biol Neonate, 2002. **82**(2): p. 103-8.
7. Lin, H.C., et al., *Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants.* Pediatrics, 2005. **115**(1): p. 1-4.
8. Bin-Nun, A., et al., *Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates.* J Pediatr, 2005. **147**(2): p. 192-6.
9. Lin, H.C., et al., *Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial.* Pediatrics, 2008. **122**(4): p. 693-700.
10. Jacobs, S.E., et al., *Probiotic effects on late-onset sepsis in very preterm infants: a randomized controlled trial.* Pediatrics, 2013. **132**(6): p. 1055-62.

11. Sawh, S.C., S. Deshpande, S. Jansen, C.J. Reynaert, and P.M. Jones, *Prevention of necrotizing enterocolitis with probiotics: a systematic review and meta-analysis.* PeerJ, 2016. **4**: p. e2429.

12. Jarrett, P., A. Meczner, K. Costeloe, and P. Fleming, *Historical aspects of probiotic use to prevent necrotising enterocolitis in preterm babies.* Early Hum Dev, 2019.

13. Costeloe, K., et al., *Bifidobacterium breve BBG-001 in very preterm infants: a randomised controlled phase 3 trial.* Lancet, 2016. **387**(10019): p. 649-60.

14. Piena-Spoel, M., M.J. Albers, J. ten Kate, and D. Tibboel, *Intestinal permeability in newborns with necrotizing enterocolitis and controls: Does the sugar absorption test provide guidelines for the time to (re-)introduce enteral nutrition?* J Pediatr Surg, 2001. **36**(4): p. 587-92.

15. Weaver, L.T., M.F. Laker, and R. Nelson, *Enhanced intestinal permeability in preterm babies with bloody stools.* Arch Dis Child, 1984. **59**(3): p. 280-1.

16. Shulman, R.J., G. Buffone, and L. Wise, *Enteric protein loss in necrotizing enterocolitis as measured by fecal alpha 1-antitrypsin excretion.* J Pediatr, 1985. **107**(2): p. 287-9.

17. Sharma, R., et al., *Neonatal gut barrier and multiple organ failure: role of endotoxin and proinflammatory cytokines in sepsis and necrotizing enterocolitis.* J Pediatr Surg, 2007. **42**(3): p. 454-61.

18. Beach, R.C., I.S. Menzies, G.S. Clayden, and J.W. Scopes, *Gastrointestinal permeability changes in the preterm neonate.* Arch Dis Child, 1982. **57**(2): p. 141-5.

19. Weaver, L.T., M.F. Laker, and R. Nelson, *Intestinal permeability in the newborn.* Arch Dis Child, 1984. **59**(3): p. 236-41.

20. Shulman, R.J., et al., *Early feeding, antenatal glucocorticoids, and human milk decrease intestinal permeability in preterm infants.* Pediatr Res, 1998. **44**(4): p. 519-23.

21. Rouwet, E.V., et al., *Intestinal permeability and carrier-mediated monosaccharide absorption in preterm neonates during the early postnatal period.* Pediatr Res, 2002. **51**(1): p. 64-70.

22. van Elburg, R.M., W.P. Fetter, C.M. Bunkers, and H.S. Heymans, *Intestinal permeability in relation to birth weight and gestational and postnatal age.* Arch Dis Child Fetal Neonatal Ed, 2003. **88**(1): p. F52-5.

23. Warner, B.B., et al., *Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study.* Lancet, 2016.

24. Wang, C., et al., *Effects of oral administration of bifidobacterium breve on fecal lactic acid and short-chain fatty acids in low birth weight infants.* J Pediatr Gastroenterol Nutr, 2007. **44**(2): p. 252-7.

25. Thuijls, G., et al., *Non-invasive markers for early diagnosis and determination of the severity of necrotizing enterocolitis.* Ann Surg, 2010. **251**(6): p. 1174-80.

26. Stratiki, Z., et al., *The effect of a bifidobacter supplemented bovine milk on intestinal permeability of preterm infants.* Early Hum Dev, 2007. **83**(9): p. 575-9.

27. Kitajima, H., et al., *Early administration of Bifidobacterium breve to preterm infants: randomised controlled trial.* Arch Dis Child Fetal Neonatal Ed, 1997. **76**(2): p. F101-7.

28. Mohan, R., et al., *Effects of Bifidobacterium lactis Bb12 supplementation on body weight, fecal pH, acetate, lactate, calprotectin, and IgA in preterm infants.* Pediatr Res, 2008. **64**(4): p. 418-22.

29. Goncalves, F.L., et al., *Evaluation of the expression of I-FABP and L-FABP in a necrotizing enterocolitis model after the use of Lactobacillus acidophilus.* J Pediatr Surg, 2015. **50**(4): p. 543-9.

30. Costeloe, K., et al., *A randomised controlled trial of the probiotic Bifidobacterium breve BBG-001 in preterm babies to prevent sepsis, necrotising enterocolitis and death: the Probiotics in Preterm infantS (PiPS) trial.* Health Technol Assess, 2016. **20**(66): p. 1-194.

31. Jiang, W., et al., *Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection.* J Infect Dis, 2009. **199**(8): p. 1177-85.

32. Matsuki, T., et al., *Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces.* Appl Environ Microbiol, 2002. **68**(11): p. 5445-51.

33. Matsuki, T., K. Watanabe, J. Fujimoto, T. Takada, and R. Tanaka, *Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces.* Appl Environ Microbiol, 2004. **70**(12): p. 7220-8.

34. Kanazawa, H., et al., *Synbiotics reduce postoperative infectious complications: a randomized controlled trial in biliary cancer patients undergoing hepatectomy.* Langenbecks Arch Surg, 2005. **390**(2): p. 104-13.

35. Underwood, M.A., et al., *A randomized placebo-controlled comparison of 2 prebiotic/probiotic combinations in preterm infants: impact on weight gain, intestinal microbiota, and fecal short-chain fatty acids.* J Pediatr Gastroenterol Nutr, 2009. **48**(2): p. 216-25.

36. Keller, K.M., R. Knobel, and K. Ewe, *Fecal alpha 1-antitrypsin in newborn infants.* J Pediatr Gastroenterol Nutr, 1997. **24**(3): p. 271-5.

37. Majamaa, H. and E. Isolauri, *Probiotics: a novel approach in the management of food allergy.* J Allergy Clin Immunol, 1997. **99**(2): p. 179-85.

38. Vergnano, S., et al., *Neonatal infections in England: the NeonIN surveillance network.* Arch Dis Child Fetal Neonatal Ed, 2011. **96**(1): p. F9-F14.

39. Taylor, S.N., L.A. Basile, M. Ebeling, and C.L. Wagner, *Intestinal permeability in preterm infants by feeding type: mother's milk versus formula.* Breastfeed Med, 2009. **4**(1): p. 11-5.

40. Westerbeek, E.A., et al., *The effect of enteral supplementation of specific neutral and acidic oligosaccharides on the faecal microbiota and intestinal microenvironment in preterm infants.* Eur J Clin Microbiol Infect Dis, 2013. **32**(2): p. 269-76.

41. Bischoff, S.C., et al., *Intestinal permeability--a new target for disease prevention and therapy.* BMC Gastroenterol, 2014. **14**: p. 189.

42. Munford, R.S., *Endotoxemia-menace, marker, or mistake?* J Leukoc Biol, 2016. **100**(4): p. 687-698.

43. Berrington, J.E., C.J. Stewart, N.D. Embleton, and S.P. Cummings, *Gut microbiota in preterm infants: assessment and relevance to health and disease.* Arch Dis Child Fetal Neonatal Ed, 2013. **98**(4): p. F286-90.

44. Drell, T., et al., *The development of gut microbiota in critically ill extremely low birth weight infants assessed with 16S rRNA gene based sequencing.* Gut Microbes, 2014. **5**(3): p. 304-12.

45. Sorbara, M.T., et al., *Inhibiting antibiotic-resistant Enterobacteriaceae by microbiota-mediated intracellular acidification.* J Exp Med, 2019. **216**(1): p. 84-98.

46. Millar, M., et al., *The Microbiome of Infants Recruited to a Randomised Placebo-controlled Probiotic Trial (PiPS Trial).* EBioMedicine, 2017. **20**: p. 255-262.

47. van den Akker, C.H.P., et al., *Probiotics for Preterm Infants: A Strain-Specific Systematic Review and Network Meta-analysis.* J Pediatr Gastroenterol Nutr, 2018. **67**(1): p. 103-122.

48. van den Akker, C.H.P., et al., *Probiotics and Preterm Infants: A Position Paper by the European Society for Paediatric Gastroenterology Hepatology and Nutrition Committee on Nutrition and the European Society for Paediatric Gastroenterology Hepatology and Nutrition Working Group for Probiotics and Prebiotics.* J Pediatr Gastroenterol Nutr, 2020. **70**(5): p. 664-680.

Legend

Figure1.

Foetal intestine: The intestinal lumen (A) in the foetus is sterile; intestinal mucus (B) is watery; TLR4 receptors (C) are abundantly expressed on enterocytes; large proteins from amniotic fluid (D) are easily absorbed between widely spaced enterocytes (E) and enter the circulation (F).

Preterm neonatal intestine: The intestinal lumen (A) contains a higher proportion of potentially pathogenic (purple) than beneficial (green) bacteria; intestinal mucus is more abundant but still watery (B); potentially pathogenic bacteria are in close proximity to TLR4 receptors (C); in the absence of food the intercellular gap remains wide (D); fluctuations in intestinal blood flow produces vasodilating substances (e.g. nitric oxide) (E).

Necrotising enterocolitis: Potentially pathogenic bacteria (purple) outnumber other bacteria in the intestinal lumen (A); intestinal mucus (B) may be interrupted allowing some bacteria to adhere to TLR4 receptors (C); activation of TLR4 leads to downstream production of IL8 through the NF-κB pathway (D) initiating further inflammation; bacterial translocation occurs through (E) and between (F) enterocytes into the systemic circulation.

6.1.1 Table 1: Summary of Schedule of Events

Theme 1: Intestinal permeability				
	14 days PNA	**21 days PNA**	**28 days PNA**	**35 days PNA**
Urine (SAT)	X			
Blood (16S PCR and LPS)	X	X	X	X
Stool (A1AT)	X			
Theme 2: Intestinal microbiota composition and short chain fatty acid production				
	14 days PNA	**21 days PNA**	**28 days PNA**	**35 days PNA**
Stool (PCR & HPLC)		X		
Theme 3: Intestinal mucosal inflammation				
	14 days PNA	**21 days PNA**	**28 days PNA**	**35 days PNA**
Urine ($IFABP_U$)	X			

Table 1 outlines the postnatal age (PNA) at which each evaluation was conducted. SAT= sugar absorption test; PCR=polymerase chain reaction; HPLC=high performance liquid chromatography; LPS= lipopolysaccharide; A1AT=alpha-1-antitrypsin; $IFABP_U$= urinary intestinal fatty acid binding protein.

Table 2 Patient Characteristics

	Sugar Absorption Test	Bacterial Translocation	Stool alpha-1-antitrypsin	Stool microbiota and short chain fatty acids	Urinary Intestinal Fatty Acid Binding Protein
Number recruited	35	86	45	29	36
Female	20/35 (57%)	38/86 (44%)	24/45 (53%)	16/29 (55%)	20/36 (56%)
Median [IQR] Gestation	27.5 [25.57 to 28.71] weeks	26.93 [25.43 to 28.04] weeks	26.71 [25.43 to 28.36] weeks	26.71 [25.5 to 28.07] weeks	27.5 [25.57 to 28.68] weeks
Median [IQR] Birthweight	900 [770 to 1070] grams	869 [748 to 1021] grams	870 [788 to 1025] grams	890 [792 to 1000] grams	900 [773 to 1068] grams
Randomised to *B breve* BBG-001	12/35 (34%)	42/86 (49%)	20/45 (44%)	13/29 (45%)	13/36 (36%)
Colonised with *B breve* BBG-001	19/32 (59%)	46/80 (58%)	25/44 (57%)	*17/29 (59%)	20/34 (59%)

Table 2: Number of participants enrolled into each component study. Gestation and birthweight are presented as medians [interquartile ranges]. Colonisation with *B breve* BBG-001 refers to colonisation at two weeks postnatal age confirmed by growth on selective media or PCR (*).

Table 3 Evaluations of Intestinal Permeability

Test	Allocated to *B breve* BBG-001	Allocated to Placebo	Effect estimate and 95% CI	P value
SAT Median [IQR]	0.24 [0.05 to 0.53]	0.42 [0.24 to 1.03]	0.17 (-0.08 to 0.43)	0.16
Bacterial translocation (bacterial DNA and LPS) n (%)	26/42 (62%)	17/44 (39%)	23% (2% to 44%)	0.05
Test	**Colonised with *B breve* BBG-001**	**Not colonised with *B breve* BBG-001**	**Effect estimate and 95% CI**	**P value**
SAT Median [IQR]	0.34 [0.1 to 0.46]	0.41 [0.24 to 2.76]	0.18 (-0.1 to 1.4)	0.19
Bacterial translocation (bacterial DNA and LPS) n (%)	24/46 (52%)	16/34 (47%)	5% (-17% to 27%)	0.82

Table 3: Results for the SAT (lactulose/mannitol ratios) are presented as medians with interquartile ranges, difference in medians and 95% confidence intervals and compared by allocation or colonisation using Mann-Whitney U test and Hodges Lehmann methods. Results for bacterial DNA and lipopolysaccharide are presented as a composite number (n) and percent of babies with any episode where bacterial DNA and/or lipopolysaccharide were detected in peripheral blood with differences in percentages and 95% CIs and compared using Fisher's exact test. Colonisation with *B breve* BBG-001 refers to colonisation at two weeks postnatal age confirmed by growth on selective media.

Table 4 Stool Microbiota

	Allocated to *B breve* BBG-001	Allocated to Placebo	Effect estimate and 95% CI	P value
Total Bacteria	9.5 [9.0 to 10.2]	8.6 [7.5 to 9.3]	-0.95 (-1.9 to -0.2)	0.01
C. leptum	6.3 [1.0 to 7.4]	6.0 [1.0 to 7.0]	0.0 (-1.3 to 4.87)	0.9
Enterobacteriaceae	8.4 [6.7 to 9.4]	7.5 [1.0 to 8.9]	0.9 (-6.2 to 0.2)	0.13
Enterococcus	7.5 [1.0 to 8.5]	3.9 [1.0 to 7.4]	-0.29 (-6.1 to 0.2)	0.35
Staphylococcus	6.8 [5.7 to 7.3]	7.3 [5.4 to 7.9]	0.34 (-0.8 to 1.2)	0.42
Bifidobacterium	9.4 [8.5 to 10]	1.0 [1.0 to 8.3]	-8.0 (-8.5 to -0.9)	0.001
***B breve* BBG-001**	9.4 [8.6 to 9.8]	1.0 [1.0 to 5.5]	-8.1 (-8.6 to -5.6)	0.001
	Colonised with *B breve* BBG-001	**Not colonised with *B breve* BBG-001**	**Effect estimate and 95% CI**	**P value**
Total Bacteria	9.5 [9.0 to 10.1]	8.4 [6.6 to 9.0]	-1.2 (-2.4 to -0.5)	0.001
C leptum	6.3 [1.0 to 7.4]	5.6 [1.0 to 6.9]	0.0 (-1.9 to 0.65)	0.46
Enterobacteriaceae	8.4 [7.0 to 9.2]	4.2 [1.0 to 8.5]	-1.92 (-7.1 to -0.07)	0.03
Enterococcus	7.5 [3.8 to 8.5]	1.0 [1.0 to 7.5]	-5.6 (-6.5 to 0.0)	0.09
Staphylococcus	7.1 [6.1 to 7.6]	6.8 [5.2 to 8.1]	-0.15 (-1.58 to 0.83)	0.84
Bifidobacterium	9.5 [8.5 to 9.9]	1.0 [1.0 to 1.0]	-8.3 (-8.8 to -6.7)	<0.0001
***B breve* BBG-001**	9.4 [8.5 to 9.9]	1.0 [1.0 to 1.0]	-8.4 (-8.8 to -7.8)	<0.0001

Table 4: Data are median bacterial counts (log_{10}/g of faeces) with interquartile ranges, difference in medians with 95% confidence intervals and compared by allocation or colonisation using Mann-Whitney U test and Hodges Lehmann methods. Colonisation with *B breve* BBG-001 refers to colonisation by PCR.

Table 5 Stool Short Chain Fatty Acids & Lactic Acid

	Allocated to *B breve* BBG-001	Allocated to Placebo	Effect estimate and 95% CI	P value
Succinic acid	6.75 [3.25 to 16.85]	5.2 [2.6 to 10.60]	-0.7 (-10 to 6.2)	0.77
Lactic acid	4.45 [3.1 to 7.25]	4.6 [3.65 to 5.12]	0.0 (-7.3 to 2.0)	0.99
Acetic acid	27.95 [18.7 to 35.0]	11.7 (5.3 to 30.9)	-12.3 (-22 to 2.4)	0.08
	Colonised with *B breve* BBG-001	**Not colonised with *B breve* BBG-001**	**Effect estimate and 95% CI**	**P value**
Succinic acid	7.5 [3.6 to 14.53]	3.0 [2.2 to 33.9]	-1.55 (-8.4 to 22)	0.37
Lactic acid	4.7 [3.6 to 5.3]	4.5 [2.6 to 5.8]	-0.2 (-7.7 to 2.5)	0.99
Acetic acid	27.95 [18.7 to 35.0]	6.1 [4.2 to 18.5]	-16.2 (-24.8 to -5.5)	0.009

Table 5: Data are median mmol SCFA/g of stool with interquartile ranges, difference in medians with 95% confidence intervals and compared by allocation or colonisation using Mann-Whitney U test and Hodges Lehmann methods. Colonisation with *B breve* BBG-001 refers to colonisation by PCR.

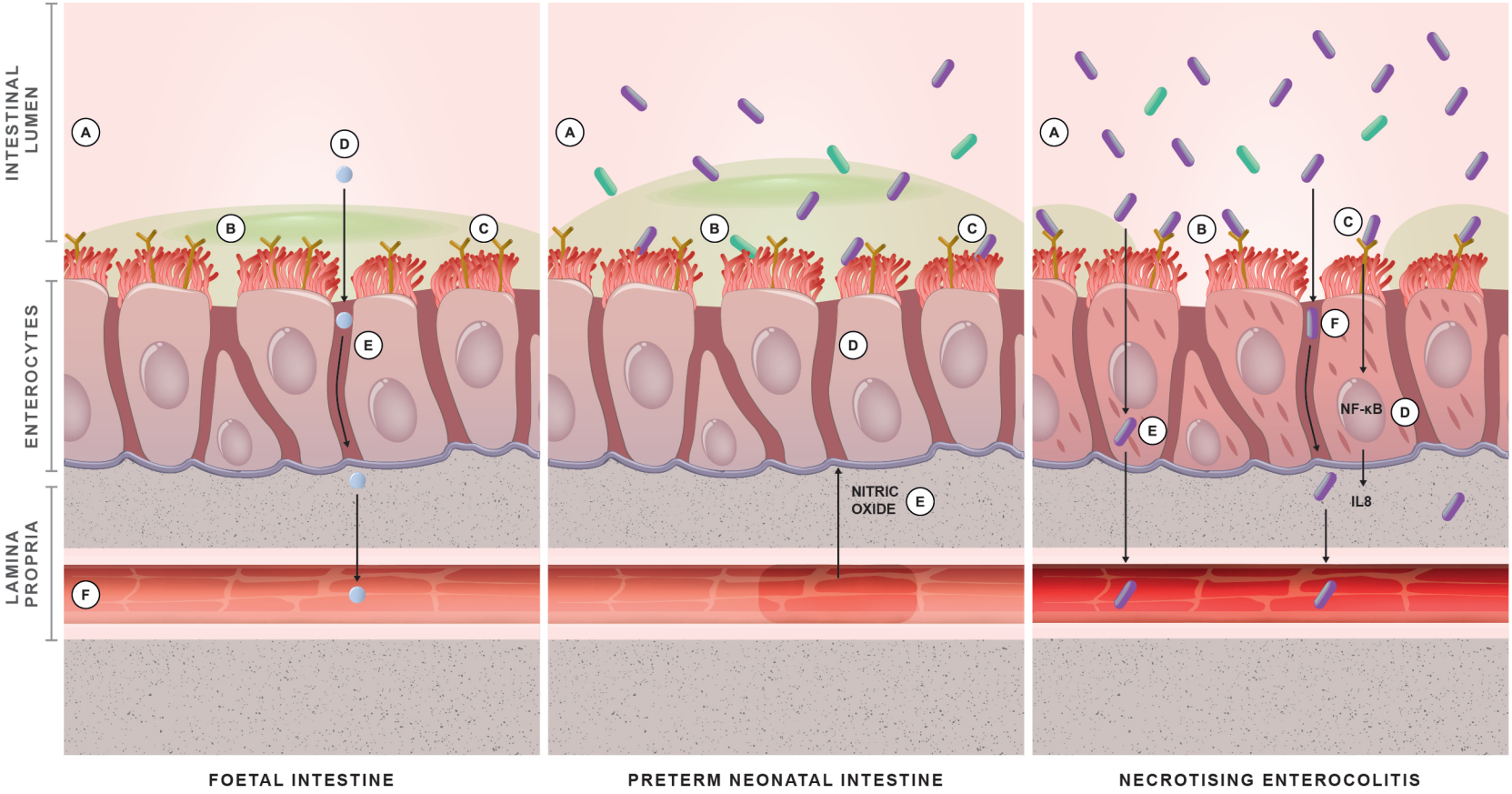

Figure 1.

Foetal intestine: The intestinal lumen (A) in the foetus is sterile; intestinal mucus (B) is watery; TLR4 receptors (C) are abundantly expressed on enterocytes; large proteins from amniotic fluid (D) are easily absorbed between widely spaced enterocytes (E) and enter the circulation (F).

Preterm neonatal intestine: The intestinal lumen (A) contains a higher proportion of potentially pathogenic (purple) than beneficial (green) bacteria; intestinal mucus is more abundant but still watery (B); potentially pathogenic bacteria are in close proximity to TLR4 receptors (C); in the absence of food the intercellular gap remains wide (D); fluctuations in intestinal blood flow produces vasodilating substances (e.g. nitric oxide) (E).

Necrotising enterocolitis: Potentially pathogenic bacteria (purple) outnumber other bacteria in the intestinal lumen (A); intestinal mucus (B) may be interrupted allowing some bacteria to adhere to TLR4 receptors (C); activation of TLR4 leads to downstream production of IL8 through the NF-κB pathway (D) initiating further inflammation; bacterial translocation occurs through (E) and between (F) enterocytes into the systemic circulation.