PiPS:
Trial of probiotic administered early to prevent infection and necrotising enterocolitis

Protocol

Sponsors protocol code number BBG001

Version 6.0

24th July 2012
ISRCTN Number: 05511098
Eudract Number: 2006-003445-17

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1. INTRODUCTION, BACKGROUND AND RATIONALE

1.1 Introduction

This is a multi-centre double blind randomised controlled trial to study possible benefits of the early administration of the probiotic *Bifidobacterium breve* strain BBG (*B breve* BBG) to babies less than 31 weeks of gestation, recruited within 48 hours of birth. The primary endpoints are late onset blood stream infection diagnosed on a sample drawn after 72 hours, necrotising enterocolitis and death. The trial will recruit 1,300 babies over 30 months from approximately 20 UK neonatal units.

1.2 Background and rationale

Hospital acquired infection is reported in around 25% of babies with birthweight <1500g who survive the first 3 days and it contributes to the high mortality and morbidity in this population.

Necrotising enterocolitis (NEC) is the most common serious gastro-intestinal complication of prematurity and itself has high mortality and morbidity; the pathogenesis of NEC is multifactorial and involves bacterial invasion of the intestinal mucosa. Estimates of the incidence of proven NEC in babies less than 1500g birthweight vary between around 6 and 10%.

Healthy breast fed term infants become colonised early in life with a wide range of bacteria dominated by bifidobacteria and lactobacilli acquired during and after birth from close contact with the mother. By comparison, preterm infants nursed on Neonatal Units become colonised with a more limited range of bacteria and fungi. The pattern of colonisation reflects the bacteria and fungi found in the ‘antibiotic-rich’ environment of the neonatal unit and is predominated by species such as *Enterobacteriaceae, Pseudomonas spp.*, enterococci, yeasts, staphylococci, and clostridia that are potentially pathogenic and may cause infection in the colonised infant or may spread and cause disease in other infants.

Little is known of the specific mechanisms by which anaerobic lactobacilli and bifidobacteria protect against infection with pathogenic organisms; these are believed to involve increased secretion of IgA and upregulation of IgA receptor sites, strengthening of epithelial tight junctions, lowering the intraluminal pH through acid fermentation and modification of intestinal inflammatory responses through preferential stimulation of T helper cells, all resulting in reduced bacterial translocation. This subject has been the focus of a number of reviews.

The extent to which early colonisation with pathogenic bacterial species has deleterious effects upon later health is also incompletely understood. Interest in this topic in the newborn has focused on its role in the pathogenesis of NEC in which a number of studies have demonstrated abnormal small intestinal colonisation.
For the context of this trial a probiotic is defined as a live microbial supplement that colonises the gut and improves health.\textsuperscript{13} The extent to which either colonisation or health benefit can be achieved in the preterm newborn baby is unclear. However the concept of active management of the bowel flora to prevent hospital acquired infection and NEC is an attractive therapeutic option because of its likely good safety profile.

In a non-randomised historical cohort study reported from South America there was a reduction from 6.6\% to 3.0\% in the incidence of NEC in a population of 1237 neonatal unit admissions in the year following routine introduction of enteral supplementation with a product with \textit{Lactobacillus acidophilus} and \textit{Bifidobacterium infantis}.\textsuperscript{14}

**Randomised controlled trials of probiotics in preterm infants at the start of recruitment to PiPS**

A randomised controlled trial published in 2002 involving 585 babies below 33 weeks gestational age\textsuperscript{15}, using a product with \textit{Lactobacillus rhamnosus} GG, failed to show a significant reduction in NEC or blood culture positive episodes of sepsis (probiotic v placebo: NEC 1.4\% v 2.7\%; septic episodes 4.1\% v 4.7\%). These results are difficult to interpret as the analysis of this trial was not by intention-to-treat; babies dying in the first 2 weeks were excluded and only septic episodes and episodes of NEC with onset at least 7 days after commencement of the intervention were considered in the analysis. This probably accounts for the low reported rates of adverse outcomes.

Two randomised controlled trials published in 2005 showed a reduction in the incidence of NEC in infants given probiotic mixtures; both studies recruited at a single site. In the first\textsuperscript{16} a mixture of \textit{Lactobacillus acidophilus} and \textit{Bifidobacterium infantis} or placebo was given in breast milk twice daily until discharge from hospital to 367 babies of less than 1500g birthweight who had survived beyond 7 days, who were clinically stable with umbilical lines removed and commencing milk feeds. Reductions in incidence were seen for: NEC from 5.3\% to 1.1\%, (p=0.04); blood culture positive sepsis from 19.3\% to 12.2\%, (p=0.03); death from 10.7\% to 3.9\%, (p=0.009); and for the combined outcome of NEC, sepsis or death from 32.1\% to 17.2\%, (p=0.009). The second study\textsuperscript{17} recruited 145 babies who were randomised to receive a product with 3 probiotic strains: \textit{Bifidobacterium infantis}, \textit{Streptococcus thermophilus} and \textit{Bifidobacterium bifidus}, or placebo started with milk feeds and given once a day. The median age at commencement of the intervention was on the third day after birth and it was continued until 36 weeks post-menstrual age. There was no difference in episodes of blood culture positive infection or of death but NEC appeared to be reduced in the intervention arm (16.4\% to 4.0 \%, p=0.03) and it was reported that there was a reduction in the severity of the illness. The implications of these two studies have been subject to considerable interest and extensive review.\textsuperscript{18-20}

More recently a further two studies have been reported.\textsuperscript{21,22}

One of these is a further single site study\textsuperscript{21} using a combination of \textit{Bifidobacterium infantis}, \textit{Bifidobacterium bifidum}, \textit{Bifidobacterium longum} and \textit{Lactobacillus acidophilus} given with breast milk twice daily to babies below 32 weeks gestation
and 1500g birthweight versus breast milk alone. Participants were clinically stable and as in the previous studies receiving breast feeds. The endpoints included feed tolerance, length of stay and serious neonatal morbidities, babies dying from causes other than sepsis or NEC were excluded; no power calculation is given. 186 babies were randomised. Significantly reduction of time to achieve full feeds and length of stay was reported in association with probiotic use. There was an overall reduction of NEC from 15.8% in the control to 5.5% in the probiotic group, p=0.04 but no significant reduction in NEC with Bell stage ≥2. There was a significant reduction in culture positive sepsis, 29.5% to 14.3%, p=0.02 and of deaths, 14.7% to 4.4%, p=0.04.

The second study recruited a total of 434 babies with birthweight <1500g and gestational age < 34 weeks from 7 centres in Taiwan. A product with Bifidobacterium bifidum and Lactobacillus acidophilus was added to the milk feed; babies entirely fed with formula were excluded as were babies in whom feeds had not been started by 3 weeks of age. There was a composite primary outcome, death or NEC ≥Bell stage 2 which was significantly lower in the intervention group, 1.8% v 9.2%, p=0.002. There were more babies with sepsis in the intervention group but this did not reach statistical significance; the interpretation of these data is confounded because of an imbalance between the groups with excess babies of birthweight <750g in the probiotic group. It was noted that a large number of babies had died (98 of a potential 580 babies) without ever achieving the study entry criteria.

None of these studies reported whether intestinal colonisation with the administered probiotics was achieved, so we do not know to what extent colonisation with the probiotic strains was a determinant of efficacy, nor do we know whether there was cross-colonisation so that babies receiving placebo became colonised as has been reported in other studies. If successful colonisation is important in determining efficacy then the recent studies might underestimate the potential benefit of the probiotic intervention.

It is a common feature of all of these studies that the probiotic intervention is started only after milk feeds are introduced and it is clear from the data that many of the babies at most high risk of NEC are excluded.

Use of the product proposed for the current study, Bifidobacterium breve strain BBG (B breve BBG), was first reported by a Japanese group. Ninety one infants who were less than 1500g birthweight were randomised to receive active product or placebo commenced with milk feeds administered twice daily and continued for 28 days, by which time 82% of the intervention and 28% of the placebo group were colonised with BBG. Clinical outcomes were analysed by whether or not the baby was successfully colonised with the probiotic organism. Improved food tolerance, accelerated time to establish full feeds and increased weight gain that was sustained after discontinuation of administration of probiotic were reported. No other clinical outcomes were published and none is available (personal communication); probiotic use is routine in that investigator’s department.

A single site pilot study using the same product, B breve BBG, was undertaken by the current investigators. B breve BBG was used as it was the only probiotic strain at the time the study was designed to have been reported to confer clinical
benefit in the preterm baby. The design differed from previous studies in that the study product was commenced within 48 hours of birth, whether or not milk feeds had been started. This was in order not to exclude those babies at greatest risk of adverse outcomes and to maximise the possibility of early colonisation with the probiotic organism even in babies from whom the responsible clinician might choose to withhold milk feeds because of a perceived high risk of NEC. In other published clinical studies it had been possible to ‘blind’ the staff by giving the probiotic in milk feeds. When made up in pure water the probiotic organisms render the liquid cloudy. In order to achieve ‘blinding’ the products for the pilot study were prepared in a dilute mixture of the elemental infant formula Neocate.

The primary objectives of the pilot were to study whether the intervention was tolerated this early and to confirm that colonisation was achieved with a once daily dosage regimen. Forty infants less than 1500g birthweight were randomised to receive *B breve* BBG or placebo, both products were well tolerated by all babies. Quantitative microbiology was undertaken on stools. Analysing the stool passed closest to 28 days 79% of the group receiving probiotic and 35% receiving placebo were colonised with *B breve* BBG – this high cross contamination rate is comparable with published experience and is likely to have occurred both in the milk kitchen and between babies in the ward. All babies who commenced enteral feeding did so with maternal breast milk.

Analysed by intention-to-treat, probiotic supplementation was associated with improved feed tolerance and weight gain, and there were fewer babies with episodes of infection at 28 days (23 v 44%). The study was too small to allow any estimate of an impact on the incidence of NEC (9 suspected or proven cases of whom 5 were randomised to receive probiotic and 4 placebo). When outcomes were analysed by whether or not the infant was colonised with the administered probiotic it was found that colonisation was associated with a reduction of the number of babies with any episode of infection over the entire hospital stay from 66% to 24%, p=0.017, and also that fewer colonised babies, 40% v 79%, p=0.038, remained oxygen dependent at 36 weeks post-menstrual age. In addition there was some evidence of increased microbial diversity in the stools of colonised babies although the numbers are small: in particular at 28 days no stool of non-colonised babies was also colonised with anaerobes in contrast with 66% of those colonised with *B breve* BBG; colonisation with Gram negative organisms was high in both groups. When analysed by intention to treat there was no difference in the duration of antibiotic use in the two groups but when analysed by whether there was successful colonisation there was a significant reduction of the number of days on antibiotics over the whole hospital stay from a mean of 39 to 19 days, p=0.04.

In this pilot study the intervention was continued for a shorter time (28 days) than in the more recently reported studies that showed reduction of NEC with probiotic use. Two babies randomised to receive probiotic in the pilot study developed proven NEC that was fatal; one at 29 and one at 30 days (one of these infants was not successfully colonised). It is well recognised in clinical practice that preterm babies at high risk of developing NEC may do so later than 4 weeks post-natal age. Administration of systemic antibiotic may result in loss of successful colonisation. It is therefore proposed that in this new multi-centre study the intervention will be continued until 36 weeks post-menstrual age. To continue the intervention up until discharge from hospital would raise problems because of the likely consternation caused to some parents if the intervention was discontinued.
coincident with the time of discharge. For those few babies in the study discharged before 36 weeks the intervention will need to be stopped; it will be suggested to clinicians that they stop it two or three days before anticipated discharge and discuss this with the parents reassuring them that their baby, who by definition has done well, is not at significant risk at that stage of major acute neonatal complications.

In published commentaries of the two studies reported in 2005\textsuperscript{16, 17} that showed a reduction in NEC, the theory was advanced that a possible reason for these studies being successful in reducing NEC whereas the only previous randomised study\textsuperscript{15} had been unsuccessful, might be because the recent studies had used polymicrobial as opposed to single strain products. However, as discussed, the results of the earlier unsuccessful study have to be regarded with caution because of the study design and the low reported rates of septic episodes and NEC. Those studies that have reported stool colonisation have all indicated that a proportion of infants receiving placebo (up to 44\% six weeks after recruitment into the study\textsuperscript{23}) become colonised. This adds a level of complexity to the interpretation of data from a RCT using a polymicrobial product since it is likely that different components would cross-colonise different babies receiving placebo.

The safety profile of all probiotic preparations appears to be good but since there is a suggestion in the literature that systemic infection with lactobacilli may occasionally occur in the immunocompromised patient we believe, given the encouraging results from our pilot study, that we should fully investigate the efficacy of \textit{B breve} BBG rather than change to a polymicrobial product at this stage.

There have been occasional reports of disseminated infection following enteral supplementation with \textit{Lactobacillus spp}.\textsuperscript{25, 26} Recently there has been what we believe is the first report of an infection associated with administration of a \textit{Bifidobacterium}. This involved a positive blood culture of \textit{B breve} BBG in a fullterm baby recovering after surgery for exomphalous.\textsuperscript{27} The report is from a hospital, which we believe is one of several in Japan, where \textit{B breve} BBG has been given routinely for several years to all babies receiving intensive care. The baby is described as having a mild illness which was treated with standard empirical antibiotic treatment involving ampicillin/sulbactam and amikacin. She made an uneventful recovery. Babies with any gastrointestinal abnormalities known at birth are excluded from this trial. Overall there is little evidence of adverse consequences for preterm infants resulting from probiotic use.\textsuperscript{28}

There has been a recent report\textsuperscript{29} of a randomised placebo controlled trial of a probiotic product with 6 bacterial strains, four of them lactobacilli, in adult patients with acute pancreatitis that reported increased mortality in the intervention arm. In an accompanying commentary\textsuperscript{30} it was suggested that the intervention group already had more multi-organ failure at baseline. This study serves as a reminder that we have not reached a point where it can be assumed that probiotics are safe in any high-risk patient group and highlights the need for further trials particularly emphasising the importance of consideration of the bacterial species given. The trial of patients with acute pancreatitis differs in important ways from the proposed neonatal study: (i) the contrast between the complex mixture of bacterial strains and the single strain \textit{B breve} BBG product to be given to the
babies; (ii) the patients with acute pancreatitis were already extremely ill whereas the proposed study is for prevention; (iii) in the pancreatitis study the probiotics were initially administered directly into the jejunum.

Evidence of efficacy and safety since the start of recruitment to the PiPS trial

Since the beginning of recruitment to the PiPS trial two further RCTs have been published. The first was a single site trial undertaken in Turkey involving administration of a single strain product containing Lactobacillus sporogenes, not tested in other trials, in babies born either below 33 weeks of gestation or with birthweight below 1500g. The event rate of the primary outcome, death or NEC ≥ Bell stage 2, in the control group at 11.7% was below the estimate of 32% used in the power calculation; 221 babies were randomised and there was no statistical difference in the primary outcome nor in sepsis rates between the groups.

The second trial was stopped early when 231 of a target of 564 babies of birthweight 750 – 1499g born in a hospital in Lima, Peru had been recruited. This decision was criticised in an accompanying editorial. The intervention was a mixture of Bifidobacterium breve and Lactobacillus casei and the primary outcome was NEC ≥ Bell stage 2. At the time of stopping recruitment the incidence of the primary outcome in the control group was 3.6% in contrast to the 10% used in the power calculation (3 babies in the intervention and 9 in the control group had died before the intervention had started) and the difference in NEC ≥ Bell stage 2 between the groups using Fisher’s exact test had a significance of 0.054 in favour of the intervention group. As with the previous trials both gave the active intervention in milk feeds, neither encountered any complications nor reported colonisation rates with the administered bacteria.

A third trial as yet only published in abstract recruited 750 larger babies with birthweight below 2kg randomised to receive Lactobacillus reuteri or placebo within 48 hours of birth. There were no statistically significant differences in any of the three reported outcomes death or nosocomial infection, nosocomial pneumonia or NEC.

It remains the case that no complication of probiotic administration has been reported in the context of a clinical trial in the preterm infant. There has been a single recent report of a bacteraemia with Bifidobacteria. The baby was a twin birthweight 600g born at 27 completed weeks of gestation who was given ‘Infloran’ (Bifidobacterium infantis and Lactobacillus acidophilus) from Day 9 who developed an episode of abdominal pathology (not definite NEC) and grew both Bifidobacteria infantis and longum from blood culture, the baby recovered with antibiotics but later developed a large bowel stricture; it is impossible to know whether the organisms grown derived from the administered product or indeed whether they were the cause of the baby’s deterioration.
2. **OBJECTIVE**

To determine whether early administration of *B breve* BBG to preterm infants reduces the incidence of episodes of infection, necrotising enterocolitis and death.

3. **TRIAL DESIGN**

Double-blind placebo-controlled randomised trial.

3.1 **Outcome Measures**

3.1.1 **Primary outcomes**

- Any baby with an episode of blood stream infection, with any organism other than a skin commensal, diagnosed on a sample of blood drawn more than 72 hours after birth and before 46 weeks post-menstrual age, death or discharge from hospital whichever is soonest. Skin commensals include coagulase negative staphylococci (CoNS) and Corynebacteria. Definitions at Appendix 1;

- Necrotising enterocolitis, Bell stage II or III\(^36\) definitions at Appendix 2;

- Death before discharge from hospital.

3.1.2 **Secondary outcomes**

1. Number of babies with the composite outcome of any or a combination of the 3 primary outcomes.

*Microbiological outcomes: definitions at Appendix 1*

Outcomes 2 to 7 are for samples taken more than 72 hours after birth and before 46 weeks post-menstrual age, death or discharge home whichever is soonest:

2. Number of babies with any positive blood culture with an organism recognised as a skin commensal e.g. CoNS or Corynebacteria;

3. Number of babies with blood cultures taken;

4. Number of blood cultures taken per baby;

5. Number of babies with episodes of blood stream infection with organisms other than skin commensals by organism: e.g. *E.Coli, Klebsiella spp*, fungi, and by antibiotic resistance types: specifically MRSA, vancomycin resistant enterococci (VRE) and extended spectrum betalactamase producing Gram negative bacteria (ESBL);

6. Number of babies with isolates of organisms other than skin commensals from a normally sterile site other than blood: e.g. CSF, supra-pubic aspiration of urine, pleural cavity etc.;
7. Number of babies with a positive culture of *B breve* BBG from any normally sterile site;

Also:

8. Total duration of days of antibiotics and/or anti-fungals administered per baby after 72 hours and until 46 weeks post-menstrual age, death or discharge from hospital whichever is soonest for treatment of suspected or proven sepsis i.e. excluding prophylactic use;

9. The number of babies colonised with the administered probiotic strain defined by the isolation of *B breve* BBG from stool samples at 2 weeks post-natal and at 36 weeks post-menstrual age;

10. Stool flora: the number of babies colonised with MRSA, VRE (vancomycin resistant enterococci) or extended spectrum betalactamase producing Gram negative bacteria (ESBL) at 2 weeks post-natal and at 36 weeks post-menstrual age.

**Nutritional and gastroenterological outcomes**

11. Age at achieving full enteral nutrition (defined as 150 ml/kg/day for 1 day);

12. Change of weight Z score from birth to 36 weeks post-menstrual age or discharge from hospital if sooner;

**Other clinical outcomes**

13. Broncho-pulmonary dysplasia: definitions at Appendix 3;

14. Hydrocephalus and / or intraparenchymal cysts confirmed by cerebral ultrasound scan performed during the baby’s in-patient stay.

15. Worst stage of retinopathy of prematurity in either eye at any time before discharge or death;

16. Length of stay in intensive, high dependency and special care (BAPM 2001: definitions at Appendix 4);

**3.2 Details of study design and procedures**

The study is a randomised double-blind placebo-controlled trial. The trial design and procedures are summarised in Figure A and described in more detail in subsequent sections.
Figure A: Trial Design and Procedures

Eligibility Criteria
- ≥ 23w + 0d and ≤ 30w + 6d gestation at birth
- < 48h after birth
- Written informed consent

Exclusion Criteria
- Lethal congenital malformation known at trial entry
- Any known gastro-intestinal malformation

Randomise via NPEU secure website

- Complete baseline data collection
- Administer first dose of intervention as soon as possible after randomisation and continue daily doses until 36w post-menstrual age

- Daily data collection until the age of two weeks
- Data collection for all episodes of suspected NEC

- Data collection at 36 weeks post-menstrual age

- Data collection at discharge or death
3.3 Informed consent

3.3.1 Consent Process

Studies show that the need to seek informed consent is best handled as a staged process rather than a single isolated event.\(^{37}\)

Some preliminary written and oral information will, whenever possible, be offered to the parents prior to birth if the baby is likely to be eligible.

Additional information will be given once the baby has been born. This will be available both at participating centres and at local hospitals that routinely refer babies into the participating centres. Informed written consent will be sought from a parent only after they have been given a full oral and written explanation of the study.

Parents will also be offered an early appointment with the Principal Investigator or delegated deputy who will meet with them during the intervention period to ensure that they understand the trial procedures and continue to consent to participate in the trial. At all stages it will be made clear to the parents that they remain free to withdraw their baby from the study at any time. If they withdraw their baby from further administration of probiotic or placebo we would ask them for consent to complete data collection.

Parents who do not speak English will only be approached if an appropriate adult interpreter is available.

A senior investigator will be available at all times to discuss concerns raised by parents or clinicians during the course of the trial.

Information about the study will continue to be offered to parents after their baby leaves the neonatal unit or dies. A regular newsletter will be produced giving parents up to date information about the study until it has finished. Experience with other studies in this area suggests that parents of babies who die may want to receive these newsletters, and all parents will be offered the opportunity to receive this information if they wish to.

3.4 Study treatment

The intervention under investigation is \textit{B breve} BBG. The product is supplied freeze dried with corn starch. The placebo is freeze dried corn starch alone. Active product or placebo will be given once daily starting as soon as possible after randomisation whether or not the baby has started enteral feeds and continuing until 36 completed weeks (36+0) post-menstrual age or discharge from hospital if sooner.

All other aspects of care will be at the discretion of the responsible neonatal team.
3.5 Randomisation

Randomisation to receive either probiotic or placebo will use a central service (web-based with telephone back-up) based at the National Perinatal Epidemiological Unit (NPEU), University of Oxford. To confirm eligibility investigators will need gestational age, time of birth and signed parental consent. The randomisation program will use minimisation to ensure balance between the groups with respect to the collaborating hospital, sex, gestational age at birth (23, 24, 25, 26/27, 28/29/30 weeks) and whether or not randomisation occurs sooner than 24 hours after birth.

The trial computing staff at the NPEU will write the randomisation program and hold the code. If necessary, the code may be broken for a single participant at the request of the Chief Investigator or their delegated Deputy by a designated independent member of staff at the NPEU.

3.6 Minimisation of bias

The study is randomised and blind to parents and clinicians.

Efforts will be made to ensure high rates of follow-up for the study outcomes and high rates of completion of the case record forms. Experience with other perinatal trials suggests that it will be possible to determine early neonatal events in all babies recruited.

The primary data analysis will be by intention to treat (see section 6).

3.7 Inter-hospital transfers

If a study baby is transferred to another hospital, the Trial Co-ordinating Centre will be informed so that all babies can be followed up until discharge from hospital or death. Each hospital to which the baby is transferred will be asked to provide information relating to any of the study outcomes which may have occurred during the baby’s stay in that hospital.

3.8 Duration of study

1,300 babies will be recruited over 30 months. The trial will have ended when the last recruited baby is discharged from hospital or dies.

3.9 Discontinuation criteria

In accordance with the current revision of the Declaration of Helsinki (amended October 2000, with additional footnotes added 2002 and 2004) and any other applicable regulations, a parent has the right to withdraw their baby from the study at any time and for any reason, without prejudice to the child’s future medical care by the physician or at the institution, and is not obliged to give his or her reasons for doing so.
The attending clinician may withdraw the baby at any time in the interests of the baby’s health and well-being.

### 3.10 Accountability of the study treatment

The study intervention in this trial is *B breve* BBG.

The active product and placebo are manufactured by Yakult Honsya Co. Ltd., Tokyo, Japan. Details of product manufacture, distribution, stability, storage conditions and disposal of unused material are at Appendix 5.

### 3.11 Data collection

All data for trial analysis are routine clinical items that can be obtained from the clinical notes or local microbiological laboratory records.

Clinical information will be collected at the following times:

- At trial entry: data to confirm eligibility and baseline data
- Daily data with details of type of milk given and antibiotics administered until the age of 2 weeks.
- Data until discharge from hospital around suspected or proven episodes of NEC to facilitate classification (definitions at Appendix 2)
- Static data at 36 weeks post-menstrual age or sooner if discharged earlier, and at discharge.

The number of occasions and the reasons for omitting the intervention will be recorded.

A record of all samples taken from normally sterile sites, for microbiological investigation, including the date of the sample and details of organisms grown together with their antibiotic sensitivities, will be obtained from the local microbiological laboratory.

No additional blood samples are required for this study.

Stool samples will be collected as close as possible to 2 weeks post-natal and 36 weeks post-menstrual age. Quantitative microbiological studies will be performed at the Royal London Hospital to determine colonisation by *B breve* BBG and other bowel flora together with antibiotic resistance patterns. Details of handling and investigation of non-routine stool samples are at Appendix 6.

The primary endpoints in this study are of such importance that it is considered that they justify the study without needing formally to demonstrate longer term benefit.

It is planned to take advice as to whether these children should be followed to study any differences with respect to neuro-developmental outcomes and allergic and immunologic characteristics, independent of the main trial outcomes.
A log will be kept on all recruiting units of all babies admitted within 48 hours of birth who are within the target gestational age range for the study, 23w+0d to 30w+6d.

4. SELECTION AND WITHDRAWAL OF TRIAL PARTICIPANTS

4.1 Inclusion criteria

4.1.1 Hospital eligibility

Hospitals with neonatal units admitting around 50 babies or more each year born before 31 completed weeks of gestation (up to and including 30 weeks + 6 days) are eligible to join the study.

4.1.2 Infant eligibility

- Gestational age between or equal to 23 weeks and 0 days and 30 weeks and 6 days by the best estimate of Expected Date of Delivery (usually by first trimester antenatal ultrasound, alternatively by ‘certain’ LMP).
- Less than 48 hours old.
- With written informed parental consent.
- Babies already on antibiotics for suspected or proven infection are eligible for recruitment to the study.

4.2 Exclusion criteria

- A lethal congenital abnormality known at trial entry.
- Any known gastrointestinal malformation.
- No realistic prospect of survival.

4.3 Withdrawal of participants

Babies may be withdrawn for any of the reasons given in Section 3.9 Discontinuation criteria. The reason for withdrawal if it is known, parents not being obliged to give a reason, will be recorded on the data collection form. If the baby is withdrawn due to an adverse event, the investigator will arrange for follow-up until the adverse event has resolved or stabilised.

5. TREATMENT OF TRIAL PARTICIPANTS

5.1 Description of treatments

The investigational product to be tested is Bifidobacterium breve strain BBG (B. breve BBG). The product is supplied freeze dried with corn starch; the placebo is corn starch alone. Both products are manufactured in Japan at the Yakult Fujisusono Pharmaceutical Plant by the Yakult Honsha Co. Ltd., and provided in
identical foil sachets each containing 1 gram of product. The manufacturing process is described in greater detail at Appendix 5.

The foil sachets are packaged in the UK in packages containing 91 sachets; each individual sachet will be identified to indicate that they all belong to the same treatment course by a package number. At randomisation babies will be allocated a study number and a package number, the study number will be added to the label of the allocated package along with the baby’s name and date of birth which is checked before each administration of study product.

The active product is stable within unopened sachets and does not need to be stored at any special temperature.

When the baby has completed its course of treatment, either at 36 completed weeks (36 + 0 weeks) or at death or discharge from hospital if sooner, any unused product should be retained for collection by the study team.

5.2 Preparation of product for administration

The freeze dried powder is suspended, the starch allowed to settle and the supernatant administered to the baby. In order that the active product and placebo cannot be distinguished both are suspended in 3 ml 1/8 strength (1 scoop to 240 ml sterile water) of the elemental infant formula Neocate and allowed to settle for 30 minutes. 1 ml of supernatant is withdrawn to be given to the baby; for the active product this contains $6.7 \times 10^7 - 6.7 \times 10^9$ colony forming organisms. The products are administered via a naso-gastric or oro-gastric tube or, for babies no longer tube fed, directly into the mouth using a syringe.

The intervention will be given once daily starting as soon as possible after randomisation and continuing until 36 completed weeks of post-menstrual age (36 weeks + 0 days) or death or discharge from hospital if sooner. If the baby is transferred between different neonatal units, e.g. transferred back to a local unit when he/she no longer needs intensive care, the aim will be to continue the intervention to complete the course of treatment.

If there is any evidence of intestinal perforation the dose will be omitted; whether doses are omitted at other times when the baby is unwell will be at the discretion of the attending consultant paediatrician. In the pilot study undertaken by the investigators this intervention was well tolerated even in babies who were acutely unwell and very few doses were omitted.

5.2.1 Other clinical management

The study involves no other interventions. All aspects of care, including the timing of the commencement of enteral feeds and the type of feed used, will be left to local discretion. It is, however, hoped that babies will be able to receive maternal breast milk since, in addition to its other advantages, it is believed that this promotes colonisation with probiotic strains.
5.3 Concomitant medication

Throughout the study, the babies may be prescribed concomitant medications deemed necessary to provide adequate supportive care.

6. ASSESSMENT OF EFFICACY

Hospital mortality and other short term outcomes will be assessed from the case notes and local microbiological laboratory records.

7. ASSESSMENT OF SAFETY

Responsibility for pharmacovigilance has been delegated by the Sponsor (Queen Mary, University of London) to the University of Oxford.

Safety will be assessed continuously during each baby’s stay in the neonatal unit. Any adverse events which require expedited reporting will follow the system outlined below.

Other outcomes, which may also be considered safety outcomes, such as death or early neonatal morbidity, but which are anticipated outcomes for this group of very preterm babies, will be captured using the mechanisms described under Section 6 ASSESSMENT OF EFFICACY.

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Expected Adverse Drug Reactions
No adverse drug reactions are expected.

Expected Serious Adverse Events (SAEs)*
The following are serious adverse events that could be reasonably expected for this group of babies during the course of the study:

- Death
- Culture positive infection with organisms other than *Bifidobacterium breve* strain BBG.
- Necrotising enterocolitis or focal intestinal perforation
- Broncho-pulmonary dysplasia
- Intracranial abnormality (haemorrhage or focal white matter damage) on cranial ultrasound scan or other imaging
- Pulmonary haemorrhage
- Patent ductus arteriosus
- Retinopathy of Prematurity requiring retinal surgery

*These SAEs do not require immediate reporting (see 7.2)

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Suspected Unexpected Serious Adverse Reaction (SUSAR)
The only recognised possible adverse reactions associated with probiotic administration are:
• Positive culture of the probiotic organism *Bifidobacterium breve* strain BBG from a normally sterile site – This is a very rare event and with this organism it has only been reported once.\(^{27}\)

• Intestinal obstruction caused by starch – this was reported when this product was first used\(^{21}\) but has not been reported with the product prepared as it will be in this study administering only the supernatant after suspension and allowing the starch to settle.

Neither of these is expected to occur

### Causality Assessment

All cases judged by either the reporting medically qualified person or the Chief Investigator or her delegated deputy, as having a reasonable suspected causal relationship to the treatment qualify as ADRs.

The severity of events will be assessed on the following scale: 1 = mild, 2 = moderate, 3 = severe. The relationship of SAEs to the study medication will be assessed according to the definition provided in Section 7.1.

#### 7.1 Reporting procedures for all serious adverse events (SAEs)

All SAEs occurring during the study observed by the investigator or reported by the participant, whether or not attributed to study medication, will be reported on the data collection form. SAEs considered to be related to the study medication by the investigator will be followed up until resolution or the event is considered stable. The investigator may be asked to provide follow-up information.

All related SAEs that result in a participant’s withdrawal from the study or are present at the end of the study, should be followed up until a satisfactory resolution occurs.

It will be left to the investigator’s clinical judgment whether or not an SAE is of sufficient severity to require the participant’s removal from treatment. A participant may also be voluntarily withdrawn from treatment due to what the attending clinician or the parents perceive to be an intolerable SAE.

#### 7.2 Serious (unexpected) adverse event (SUSAR) reporting procedures

All SUSARs must be reported to the Chief Investigator or their delegated deputy within one working day of discovery or notification of the event. A Standard Operating Procedure (SOP) outlining the reporting procedure for clinicians will be provided on the reverse of the SUSAR form. An SOP will also be available as part of the Trial Specific SOPs which will outline the reporting procedure for the Trial Co-ordinating Centre. All SUSAR information must be recorded on a SUSAR form and faxed to the Chief Investigator or their delegated deputy. Additional information received for a case (follow-up or corrections to the original case) needs to be detailed on a new SUSAR form and faxed to the Chief Investigator or their delegated deputy.
The Trial Co-ordinating Centre will report all suspected adverse reactions which are both serious and unexpected (SUSAR) to the Competent Authorities (MHRA) and the Ethics Committee concerned. Fatal or life-threatening SUSARs must be reported within 7 days and all other SUSARs within 15 days. In addition a copy of the SUSAR will be forwarded to the Chair of the Data Monitoring Committee. The Chair will also be provided with a document detailing all previous SUSAR/SAEs with their unblinded allocation. The Chief Investigator will also inform all investigators concerned of relevant information about SUSAR/SAEs that could adversely affect the safety of participants.

In addition to the expedited reporting above, the Chief Investigator shall submit, once a year throughout the clinical trial, or on request, a safety report to the Competent Authority and Ethics Committee that includes all SUSARs.

8. **STATISTICS**

8.1 **Description of statistical methods**

Comparative analyses will be restricted to babies who are assigned to the active treatment and placebo groups. Specifically, babies will be analysed in the groups to which they are assigned.

For dichotomous outcomes, e.g. whether or not a baby ever had an episode of infection, relative risks and 95% confidence intervals will be calculated. For continuous outcomes, e.g. the number of episodes of infection, differences in means or differences in medians (depending on the distribution of the data) will be calculated with 95% confidence intervals. Analysis of time to event outcomes such as reaching full enteral feeding will use survival analysis techniques.

Subgroup analysis will include an interaction test and, where appropriate, results will be presented as ratios of relative risks with confidence intervals.

The following pre-specified subgroups will be analysed on an intention-to-treat basis:

- Gestational age as per minimisation: 23w, 24w, 25w, 26/27w, 28/29/30w.
- Male v female
- Whether randomised in the 1st or 2nd 24 hours after birth

A secondary analysis of all clinical and microbiological outcomes will be conducted by whether the baby was colonised with *B breve* BBG at 2 weeks post-natal age and 36 weeks post-menstrual age.

Logistic regression analysis will be used to study determinants of successful colonisation with *B breve* BBG in those babies allocated to receive probiotic.

8.2 **Interim analyses**

An independent Data Monitoring Committee (DMC) has been established, whose remit is to review the trial’s progress. The DMC is independent of the trial organisers. Interim analyses will be supplied, in strict confidence, to the DMC, as frequently as its Chair requests. The terms of reference for the DMC were agreed.
at their first meeting. A DMC charter has been completed following the recommendations of the DAMOCLES Study\textsuperscript{38}. Meetings of the committee will be arranged periodically, as considered appropriate by the Chair. In the light of interim data on the trial’s outcomes, adverse event data, accumulating evidence from other trials (see below) and any other relevant evidence (including updated overviews of the relevant randomised controlled trials), the DMC will inform the Trial Steering Committee (TSC) if in their view there is proof beyond reasonable doubt that the data indicate that any part of the protocol under investigation is either clearly indicated or contra-indicated, either for all infants or for a particular subgroup of trial participants. Unless modification or cessation of the trial is recommended by the DMC, the TSC, investigators, collaborators and administrative staff (except those who supply the confidential information) will remain ignorant of the results of the interim analysis. Collaborators and all others associated with the study may write to the DMC via the Trial Co-ordinating Centre, to draw attention to any concern they may have about the possibility of harm arising from the treatment under study. The TSC’s terms of reference were discussed and agreed at the joint DMC/TSC meeting held on 11\textsuperscript{th} November 2008 prior to the start of recruitment.

\textbf{8.3 The number of participants}

Our aim is to recruit 1,300 babies with a gestational age between 23 weeks and 0 days and 30 weeks and 6 days over a 2.5 year period. Balance by gestation will be monitored by the DMC.

\textbf{Neonatal sepsis:} The percentage of babies with blood stream infection in our pilot study was 44\%, this included infection with skin-commensals. The number of babies fulfilling criteria for the primary endpoint in this study will be lower as infections with skin commensals are excluded. A trial of 1,300 babies will have 90\% power to detect a 40\% relative risk reduction from 15\% to 9.1\%; likewise if the incidence is closer to 12\%, a trial of this size will still have 90\% power to be able to detect a 44\% relative risk reduction from 12\% to 6.7\%, and a 44\% reduction from 10\% to 5.6\%.

\textbf{NEC:} The incidence of NEC is estimated to be 15\%. This is based on NEC incidence at the Homerton Hospital over a 3 year period in babies less than 1,000g birthweight. The trial will have 90\% power to be able to detect a 40\% relative risk difference in this outcome from 15\% to 9\%.

\textbf{Death:} The incidence of death is also estimated to be 15\%. This is based on survival of babies below 31 weeks gestational age in London extracted from pan-London data collected by the Thames Regional Perinatal Group. The trial will have 90\% power to be able to detect a 40\% relative risk difference in this outcome from 15\% to 9\%.

We have used a power of 90\% in our calculations because of the need to be certain that if there is a true effect the trial will be sufficiently powered to detect it.

A power of 80\% would result in a slightly smaller trial for the assumptions of the treatment effect described above (a total sample size of approximately 1,000). However, this would mean that if this intervention did work, the trial would run a 1 in five chance of missing it and falsely concluding that there was no effect of this intervention. This lack of ‘sensitivity’ in being able to detect such important effects
with an intervention which appears to have a low probability of serious adverse effects, would, in our view, be unreasonable.

However, if the treatment effect is larger than anticipated, or the incidence of the outcomes are larger than estimated, it may be possible for the trial to stop recruitment earlier, on the advice of the Data Monitoring Committee.

8.4 The level of statistical significance

For the primary outcome a 95% confidence interval will be calculated, but, to take account of their number, 99% confidence intervals will be used for the secondary outcomes.

8.5 Criteria for the termination of the trial

In the light of interim data and other evidence from relevant studies the DMC will inform the TSC if, in their view, there is proof beyond reasonable doubt that the data indicate that the trial should be terminated. A decision to inform the TSC of such a finding will in part be based on statistical considerations.

Appropriate proof beyond reasonable doubt cannot be specified precisely. A difference of at least 3 standard errors in the interim analysis of a major endpoint may be needed to justify halting or modifying the study prematurely.

9. DIRECT ACCESS TO SOURCE DATA/DOCUMENTS

The investigator(s)/institution(s) will permit trial-related monitoring, audits, review by DMC/Independent Ethics Committee, and regulatory inspection(s), providing direct access to source data/documents to authorised study or regulatory personnel.

A random sample of auditing of source data will be undertaken during visits to centres by the Study Research Nurse or the Study Co-ordinator.

- A random selection of key data items will be checked.
- A small random sample of participants will be selected.
- Output from the data held in the Trial Co-ordinating Centre will be provided for the visiting member of staff.
- Any data queries or verification will be dealt with at the same time.
- Permission to inspect the baby’s notes must be obtained from the Principal Investigator in the hospital to be visited.
- Baby’s notes should be requested from the medical records by the visiting staff member.

10. QUALITY CONTROL AND QUALITY ASSURANCE PROCEDURES

Compliance with the protocol will be ensured by a number of procedures:
10.1 Site set-up and training

Start-up visits at each site, including training in trial procedures, will be performed before the first baby is enrolled.

Regular site visits will be made by the study research nurse to ensure adherence to the protocol and to deal with any specific site issues. A major focus of these visits will be to confirm that procedures to minimise the risk of cross contamination with the probiotic organism are followed both in the milk kitchen and in clinical areas.

Nurse study days will be undertaken to ensure that nurses involved with the study are fully apprised of issues such as consent, data collection, follow-up and changing regulations.

An annual meeting for Principal Investigators and nurses will be organised when workshops to discuss protocol issues, data collection issues and study specific procedures are conducted.

10.2 Data collection, processing and monitoring

All study data are:
- Collected using PiPS data collection forms or directly from the local microbiological laboratory.
- Processed and monitored centrally for consistency, viability and quality at the Trial Co-ordinating Centre, NPEU, University of Oxford
- Screened for out-of-range data, with cross-checks for conflicting data within and between data collection forms using computerised logic checking screens
- Referred back to the relevant centre for clarification in the event of missing items or uncertainty
- Processed using a double data-entry system by independent data clerks.

10.3 Monitoring of colonisation with Bifidobacterium breve strain BBG

Those studies that have reported stool colonisation with the active probiotic bacterium, including our pilot, have reported cross contamination of the placebo group. A monitoring system will be established to confirm compliance to procedures to minimise cross contamination involving regular comparison of local and overall colonisation rates. The research nurse will visit hospitals where the rates of colonisation suggest that cross colonisation may be a problem.

10.4 Central statistical monitoring

All data are monitored using central statistical monitoring (at the NPEU) for consistency, viability and quality using bespoke data management systems. Central statistical monitoring is used to monitor patterns of recruitment at sites, disease severity among site populations, time of recruitment, etc. Central statistical monitoring can be utilised ‘for cause’ purposes if necessary.
The trial programmer runs trial-specific programs to extract certain fields from the database (as requested by the Chief Investigator (CI), or Trial Statistician (TS)) and to cross-check certain information. These fields may include measures of eligibility criteria, treatments given after trial entry and compliance but not by allocation.

The trial programmer and Chief Investigator will review the results generated for logic and for any patterns or problems. Outlier data will be investigated.

The Chief Investigator and Trial Statistician will decide if any action needs to be taken.

10.5 On-site monitoring
- A random sample of cases are monitored at source when site visits are performed (Source Document Verification).
- The documents to be verified are randomly selected using computerised study number generation. Any major discrepancies found at a site visit would trigger a more extensive audit of study data at the site involved.

10.6 National registration systems

All surviving babies recruited into PIPS will be 'flagged' after discharge to confirm status using records held and maintained by the Health and Social Care Information Centre (HSCIC) and provided by the Medical Research Information Service (MRIS). MRIS will also verify a baby’s name, area of residence, date of birth, date and cause of death.

10.7 Data Monitoring Committee (DMC)

The DMC will meet regularly throughout the study period to receive and review the progress and accruing data of this trial and provide advice on the conduct of the trial to the Trial Steering Committee (TSC).

The DMC will inform the Chair of the TSC if, in their view the results are likely to convince a broad range of clinicians, including those supporting the trial and the general clinical community, that one trial arm is clearly indicated or contra-indicated, and that there was a reasonable expectation that this new evidence would materially influence patient management.

The DMC will undertake interim review of the trial’s progress including updated figures on recruitment, data quality, and main outcomes and safety data. The role of the DMC is to:
- assess data quality, including completeness (and by so doing encourage collection of high quality data)
- monitor recruitment figures and losses to follow-up
- monitor compliance with the protocol by participants and investigators
- monitor evidence for treatment differences in the main outcome measures
- monitor evidence for treatment harm (e.g. SAEs, SUSARs and deaths)
• decide whether to recommend that the trial continues to recruit participants or whether recruitment should be terminated either for everyone or for some treatment groups and/or some participant subgroups
• suggest additional data analyses
• advise on protocol modifications suggested by investigators or sponsors (e.g. to inclusion criteria, trial outcomes, data collection)
• monitor planned sample size assumptions
• monitor continuing appropriateness of patient information
• monitor compliance with previous DMC recommendations
• consider the ethical implications of any recommendations made by the DMC
• assess the impact and relevance of any external evidence provided

11. ETHICS

11.1 Declaration of Helsinki

The Investigator will ensure that this study is conducted in full conformity with the current revision of the Declaration of Helsinki (last amended October 2000, with additional footnotes added 2002 and 2004).

11.2 MRC Guidelines for Good Clinical Practice (GCP)

The Investigator will ensure that this study is conducted in full conformity with relevant regulations and with the MRC GCP guidelines which are based on ICH Guidelines for GCP (CPMP/ICH/135/95) July 1996.

11.3 Informed consent

Written and verbal versions of informed consent will be presented to the baby’s parent detailing no less than: the exact nature of the study; the implications and constraints of the protocol. It will be clearly stated that the parents are free to withdraw their baby from the study at any time for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal.

One of the aims of this study is to investigate the use of probiotic administered early. We are therefore keen to recruit babies as soon as is reasonable while recognising that some of the babies will have been transferred from another hospital after birth and that the mothers of some of the babies will be at a different site and may themselves be ill. It is hoped that parents will be approached initially within 24 hours of birth and certainly by 36 hours so that parents have at least 12, and usually 24, hours to consider the information and ask questions of the Principal Investigator and other clinical staff to help them to decide whether they will participate in the study before the baby is 48 hours old. Once the baby is randomised, information about the trial will continue to be given as and when parents request.

Written Informed Consent will be obtained by means of dated parental signature and the signature of the person who obtained informed consent; this will be the Principal Investigator (or clinician with delegated authority). A copy of the signed
Informed Consent will be given to the parents. A further copy will be retained in the baby’s medical notes, a copy will be retained by the Principal Investigator and a final copy will be sent to the Trial Co-ordinating Centre.

11.4 Independent Ethics Committee

A copy of the protocol, proposed informed consent form, and written participant information and any proposed advertising material will be submitted to an Independent Ethics Committee for written approval.

The Chief Investigator will submit and, where necessary, obtain approval from the Independent Ethics Committee for all subsequent protocol amendments and changes to the informed consent document.

The Investigator will notify deviations from the protocol or SAEs occurring at the site to the sponsor and will notify the Independent Ethics Committee of these in accordance with local procedures.

11.5 Participant confidentiality

The Chief Investigator will ensure that the baby’s information is kept confidential. The baby will be identified by name (consent will have been given by the parent(s)) and study number on the data collection form. All documents will be stored securely and kept in strict confidence in compliance with the Data Protection Act (1998).

12. DATA HANDLING AND RECORD KEEPING

All data management will be undertaken by the National Perinatal Epidemiological Unit (NPEU).

All study data will be entered into a Microsoft Access database with a Visual Basic interface to restrict access to the raw data.

Data collected on the data collection forms will be stored in an electronic database in which the baby will be identified by a study specific number. The baby’s name and any other identifying detail will be stored in a separate database linked only by the study number. This information will be collected with the parent(s) consent to enable follow-up to be undertaken later should it be necessary.

Stools analysed in the microbiological laboratory at Barts and the London (colonisation by B breve BBG, stool flora and antibiotic resistance patterns) will be identified by study specific number and date of sample. Complete results will be transferred electronically and linked to the main study database at the NPEU.

Data entered will undergo statistical analysis to produce the results of the trial. Coding is required to ensure the consistent and appropriate description of items reported on data collection forms such as concomitant medications, reasons for study entry and other medical terms that may include complications of birth.
Standard operating procedures are in place for the collection and handling of data received at the Trial Co-ordinating Centre.

Storage is on a restricted area of a file server (running Novell Netware). The server is in a secure location and access is restricted to a few named individuals. Access to the building in which NPEU is situated is via an electronic tag and individual rooms are kept locked when unoccupied. Authorisation to access restricted areas of the NPEU network is as described in the NPEU security policy.

Data will be processed on a workstation by authorised staff. The workstations access the network via a login name and password (changed regularly). No data are stored on individual workstations. Backing up is done automatically overnight to an off site storage area. The location of the backup computer is in a separate department which has electronic tag access. Access to the room in which the backup machine is located is via a key pad system.

13. FINANCING AND INSURANCE

The trial is funded by the Department of Health, Health Technology Assessment Programme. The pharmaceutical division of Yakult Honsha Co. Ltd., 653-1, Aza Juzaburo, Shimowada, Susono-shi, Shizuoka 410-1105, Japan, are providing the probiotic for the duration of the trial, but will have no role in the conduct of the trial, the analysis of the findings, or the publication of the results.

Queen Mary, University of London Clinical Trials, Professional Negligence and Public Liability Insurances are all arranged through Zurich. The University reserves the right to place other alternative risk transfer mechanisms in place, and which will provide a level of cover in line with those presently arranged through the insurance market.

14. PUBLICATION POLICY

After Research Ethics Committee approval has been obtained this protocol will be submitted for publication and will be posted on the NPEU website.

The Chief Investigator will co-ordinate dissemination of data from this study. All publications using data from this study to undertake original analyses will be submitted to the TSC for review before release.

To safeguard the scientific integrity of the trial, data from this study will not be presented in public before the main results are published without the prior consent of the TSC. The success of the trial depends on a large number of neonatal nurses, neonatologists and parents. For this reason, chief credit for the results will not be given to the committees or central organisers, but to all who have collaborated and participated in the study. Acknowledgement will include all local co-ordinators and collaborators, members of the trial committees, the Trial Co-ordinating Centre and trial staff. Authorship at the head of the primary results paper will take the form “The Probiotics in Neonates Study Collaborative Group”, this avoids giving undue prominence to any individual; the writing will be the responsibility of a writing committee including all of the investigators. All contributors to the study
will be listed at the end of the report, with their contribution to the study identified.

Those responsible for other publications reporting specific aspects of the study, such as detailed microbiological outcomes, may wish to utilise a different authorship model, such as “[name], [name] and [name] on behalf of the Probiotics in Neonates Study Collaborative Group”. Decisions about authorship of additional papers will be discussed and agreed by the trial investigators and the TSC.

Parents will be sent a summary of the final results of the study, which will contain a reference to the full paper. A copy of the journal article will be available on request from the Chief Investigator.

**15. ORGANISATION OF THE TRIAL**

The trial will be overseen by a Trial Steering Committee (TSC) consisting of the investigators, research nurse, project team at the NPEU plus independent members. This group will meet regularly throughout the trial to review progress and resolve problems, receive reports from the DMC and take decisions about the trial’s conduct.

The trial co-ordinating centre will be at the NPEU where the Trial Manager will be based. The NPEU will be responsible for programming, data management and statistical analyses and, in collaboration with the Chief Investigator and Study Research Nurse(s) at Queen Mary, University of London, for day to day running of the study including recruitment of centres and training of staff.

**16. LOCAL CO-ORDINATION**

Each participating centre will identify a site specific Principal Investigator who will nominate a local co-ordinator for that centre (this may be him/herself) whose responsibilities will be to:

- be familiar with the Trial
- liaise with the Trial co-ordinating centre at the NPEU
- ensure that all staff involved in the care of eligible babies are informed about the Trial and have received requisite training
- ensure that mechanisms for recruitment of eligible babies, including the ready availability of parent information, are in place; monitor their effectiveness and discuss the reasons for non-recruitment with relevant staff
- ensure that packages of active intervention and placebo are available, clearly identified and suitably stored
- ensure that stool samples are collected from babies in the study at the appropriate time and that arrangements are made to send samples as per protocol to the microbiological laboratory at Barts and the London
- ensure that supplies of data collection forms are available, that they are completed and returned to the Trial Co-ordinating Centre promptly, and to deal with any queries arising
- notify the Trial Co-ordinating Centre of any serious adverse events
• make data available for verification, audit and inspection processes as necessary
• ensure that the confidentiality of all information about Trial participants is respected by all persons

16.1 NHS R&D Research Governance Arrangements

Babies will be recruited into the PIPS study and begin the intervention in one of around 20 participating neonatal intensive care units. The intervention is continued until 36 weeks post-menstrual age (36w +0d). During their stay in hospital, approximately 50% of these babies will be well enough to be transferred from the Neonatal Intensive Care Unit of recruitment to another unit (the continuing care site) nearer to the baby’s family home; sometimes there may be multiple transfers between sites. If such a transfer occurs before 36 weeks the intervention will need to be continued and any outstanding stool samples will need to be collected; after 36 weeks all that is necessary is the completion of a short data collection form at discharge or death. Some, but not all of the ‘continuing care’ sites will also be recruiting sites.

There are therefore two levels of involvement in the PIPS sites:
1. Recruiting sites which will sometimes also be continuing care sites receiving babies who have had intensive care elsewhere.
2. Continuing care sites that are not recruiting sites but if the baby is below 36 weeks will need to continue the intervention and collect any outstanding stool samples; if the baby is over 36 weeks they will need only to collect a small dataset at discharge or death.

The responsibilities for these two levels vary; requirements are as follows:

16.2 Recruiting sites

Each recruiting site will have a nominated Principal Investigator who will delegate responsibility for the recruitment of eligible babies to members of their team once they are satisfied that the relevant member(s) of staff is/are both competent and confident in:
1. Their knowledge of the study and their ability to answer questions raised
2. Their competence in obtaining informed consent from the families
3. Making up the study intervention following training provided by a member of the PiPS study team or another member of the local research team who has already received this training
4. Collection and reporting of trial data following training provided by a member of the PiPS study team or by another member of the local research team who has already received this training
5. They are adequately trained by experience or have received training in GCP relative to their role in the trial
Standard operating procedures will be provided to all recruiting sites which explain the process for reporting SUSARs and a log of delegated responsibilities will be maintained in the Study Site File.

Each site will have a full Site Specific Assessment, Trust Research & Development Approval and a contract between the Trust and the study Sponsor, Queen Mary, University of London. The setting up of each contract will be negotiated by the staff of the National Perinatal Epidemiological Unit (NPEU) as part of the agreed delegated responsibilities between the University of Oxford and Queen Mary.

16.3 Continuing Care sites

Some of these will also be recruiting sites in which case they will have full Site Specific Assessment etc. as detailed above.

If a baby is transferred from a recruiting site to a continuing care site before the baby is 36 weeks’ pma the remaining sachets containing the final doses of the intervention will be transferred with the baby together with requisite syringes etc.

16.4 Continuing care sites identified as likely to receive study babies that are not recruiting sites

For those continuing care sites that are not recruiting sites the level of responsibility taken is lower than at a recruiting site however babies being admitted below 36 weeks will still need to receive the intervention.

During the setting up phase of the study non-recruiting centres who are likely to receive study babies will be approached and a Principal Investigator identified. The approvals required will be as for a recruiting centre with full Site Specific Assessment, Trust Research & Development Approval and a contract between the Trust and the study Sponsor, Queen Mary, University of London. The setting up of each contract will be negotiated by the staff of the National Perinatal Epidemiological Unit as part of the agreed delegated responsibilities between the University of Oxford and Queen Mary.

The Principal Investigator will delegate responsibility to members of their team once they are satisfied that the relevant members of staff are both competent and confident in:

1. Their knowledge of the study and their ability to answer questions raised
2. Making up the study intervention following training provided by a member of the PiPS study team or another member of the local research team who has already received this training
3. Collection and reporting of trial data following training provided by a member of the PiPS study team or by another member of the local research team who has already received this training
4. They are adequately trained by experience or have received training in GCP relative to their role in the trial
Standard operating procedures will be provided to all continuing care sites which explain the process for reporting SUSARs and a log of delegated responsibilities will be maintained in the Study Site File.

16.5 Babies transferred outside recognised clinical pathways and to centres where the study is not registered

The majority of recruiting sites will be in and around London with largely predictable pathways of care. However unpredictable referrals will occur because women unexpectedly go into preterm labour and because long distance transfers are sometimes necessary because of lack of neonatal unit capacity.

Inevitably babies in the study will be transferred to hospitals with whom the study has not been registered in advance. It is essential that we maximise the ease with which these babies can complete the study protocol – not only because we need to minimise protocol violations to ensure the scientific integrity of the trial, but also because ethical issues are raised if we are unable to complete the intervention for babies whose parents have given consent simply because of transfer to another hospital within the NHS.

To ensure babies recruited to the study are able to complete the trial Protocol non-recruiting centres who are outside recognised clinical pathways but who may still receive study babies will be approached. We will seek NHS permissions from all Trusts with neonatal units (that have not already been identified) within England and Wales as continuing care sites. Applications will be sent via the generic SSI system in IRAS and all documentation required for approval will be available on CSP.

The approvals required will be a full Site Specific Assessment, Trust Research & Development Approval and a statement setting out responsibilities between the Trust and the study Sponsor, Queen Mary, University of London. The Statement of Responsibilities will set out in detail the process for obtaining approvals via the generic SSI system.

The Principal Investigator will delegate responsibility to members of their team once they are satisfied that the relevant members of staff are both competent and confident in:

1. Their knowledge of the study and their ability to answer questions raised
2. Making up the study intervention following training provided by a member of the PiPS study team, another member of the local research team who has already received this training or by completing the relevant training material contained on the PiPS website
3. Collection and reporting of trial data following training provided by a member of the PiPS study team, another member of the local research team who has already received this training or by completing the relevant training material contained on the PiPS website
4. They are adequately trained by experience or have received training in GCP relative to their role in the trial
Standard operating procedures will be provided to all continuing care sites which explain the process for reporting SUSARs and a log of delegated responsibilities will be maintained in the Study Site File.

All recruiting centres will be provided with regularly updated lists of those centres with whom the study is registered. At recruitment the local investigator will complete a short data form that will include the family postcode, a note of where the mother booked for delivery and a question about where any baby not booked in the recruiting hospital is likely to be transferred when intensive care is no longer needed. If that hospital is not already involved they will immediately be contacted by the study manager based at the NPEU and steps taken urgently to register the study; at the same time the study team will arrange for training about the study and the intervention to be provided.

The R&D department of all hospitals in England with a neonatal unit (n=178), the lead clinician in each (names obtained via neonatal network clinical leads) and the administrators of Research Ethics Committees will be contacted during the set-up phase of the study to make them aware of the study and of the remote likelihood that we might need to request to involve them during its progress.

After 36 weeks post-menstrual age we will need only a short discharge form to be completed either when the baby is transferred from that site, is discharged home or dies. These units will not require full SSA. If a baby is transferred to such a site for which we have no approval already in place after 36 weeks we will contact the R&D department to request formal permission to collect the routine discharge data.
17. REFERENCES


24. Costeloe KL, Jesudass R, Al Nakib L, Whiley A, Wilks M, Millar MR. The early administration of Bifidobacterium breve strain BBG to very low birthweight infants:


34. Mario A. Rojas, Juan M. Lozano. Prophylactic Probiotics To Prevent Death or Nosocomial Infection in Preterm Infants in Colombia. Published on-line at www.pas-meeting.org/2012Boston


**Investigator Agreement**

"I have read this protocol and agree to abide by all provisions set forth therein.

I agree to comply with the International Conference on Harmonisation Tripartite Guideline on Good Clinical Practice."

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Principal Investigator | PI Signature | Date
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[personalised to PI at each site to sign]

Kate Costello | K. L. Costello | 31/07/2012

Chief Investigator | CI Signature | Date
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Appendix 1: Definitions of episodes of infection

Appendix 2: NEC, Bell staging

Appendix 3: Definitions of BPD

Appendix 4: BAPM 2001 definitions of intensive, high-dependency and special care

Appendix 5: Product details, stability and storage

Appendix 6: Microbiological protocols
Appendix 1

Rationale and definitions for microbiological endpoints

The primary outcome: ‘An episode of blood stream infection, with any organism other than a skin commensal, diagnosed on a sample of blood drawn after 72 hours and before 46 weeks post-menstrual age, death or discharge from hospital whichever is soonest. Skin commensals include coagulate negative staphylococci (CoNS) and Corynebacteria.’

Late onset blood stream infection in the preterm baby carries high mortality and morbidity; this is particularly true for infections with Staphylococcus Aureus and Gram negative bacilli (GNB) which make up around 15% of positive blood cultures. The main reservoir of these organisms in the baby is in the gut from which it is believed that they invade the blood stream by translocation of the intestinal wall. If probiotic administration is to be effective in reducing infection in the newborn it is most likely that it will be through a combination of reducing colonisation of the gut by these organisms and promoting intestinal epithelial health.

The majority of positive blood cultures are with Coagulate negative Staphylococci (CoNS). These organisms likewise colonise the gut of infants but they are also important skin commensals of healthcare workers; this reservoir of colonisation will not be affected by probiotic administration to infants. CoNS bloodstream infection is thought usually to arise as a result of colonisation of an intravascular device, most importantly an intravenous central feeding line, during handling and manipulation by healthcare workers, rather than from bacterial translocation through the intestinal wall. Thus while probiotic use, if it is associated with better nutrition and better general health, might reduce need for intravascular devices and might be related to less CoNS sepsis it seems probable that the greater benefit of probiotics in neonatal infection will be through reduction of the more serious infections with organisms that have colonised the intestine such as GNB, Staph Aureus and fungi such as Candida.

Furthermore there is a difficulty in accurate diagnosis of CoNS infection. While a positive blood culture with the clearly pathogenic Staphylococcus aureus or GNB is taken as definite evidence of infection it is widely acknowledged that many CoNS positive blood cultures are contaminants, arising largely through deficient blood culture technique with inadequate skin cleansing. Many schemes have been presented, to explore whether the presence of an organism in a culture sample represents real infection rather than a contaminant. These involve different combinations of clinical signs (lethargy, temperature instability, etc.) and laboratory markers of sepsis (WBC counts, CRP etc.). None of these is accepted as a gold standard and if used in a clinical trial such as PiPS would result in a significant increase in the burden of data collection for participating centres without clear evidence of benefit in the specific circumstances of this randomised trial.

In summary: because of the greater clinical importance of blood stream infection with non skin commensals, the possibility that they are more likely to be reduced by probiotic administration and in the cause of simple clearly defined items for data collection it has been agreed that the microbiological primary endpoint for this study should be blood stream infection with non skin commensals, i.e. positive cultures with bacteria such as E. coli, Klebsiella, S. aureus and with fungi such as Candida.

Secondary outcomes:

While the single most important microbiological clinical outcome is reduction of blood stream infection with non skin commensals there are other possible effects of probiotic use that are important to study:
Infection with skin commensals, secondary outcomes #2-4:

Because details of clinical events and markers of sepsis are not being collected around episodes of suspected infection, the total number of positive blood cultures with skin commensals (the majority of which will be CoNs) will include contaminants; it will however give a guide as to whether or not probiotic use is impacting on skin commensal sepsis as the contaminants should be balanced between the two arms of the study. This information will be augmented by studying whether or not there is a difference in the extent of sampling (secondary outcomes 3&4) in the two arms.

Infections with pathogens: GNB, S. aureus etc. by organism and antibiotic resistance, secondary outcome #5:

The bowel provides a major reservoir for antibiotic resistant bacteria and is also an important site for the transfer of antibiotic resistance genes. If probiotics are not associated with the hoped for reduction in serious blood stream infection they may nonetheless impact upon the type of organisms causing infection and be associated with less antibiotic resistance. To explore this, the types of organisms causing blood stream infection and their patterns of antibiotic resistance will be studied in the two arms of the study.

Blood culture negative episodes of infection:

A further complication in the accurate assessment of the burden of infection is the difficulty of reliably identifying clinical episodes that are considered by the attending staff to be infections but are associated with a negative blood culture; this may arise because the sample of blood is too small but is more often because the baby, at the time of sampling, is already on antibiotics which inhibit bacterial growth. The total number of samples taken, secondary outcome #4, will to some extent provide a surrogate for this.

Data collection to support these endpoints:

Investigators will provide the study centre with details of admission and discharge dates; this might involve multiple hospitals per baby. Microbiological data will be obtained directly from hospital microbiological laboratories who will be asked to provide a download with details of all microbiological investigations from admission, including time and site of sampling and details of any positive cultures with information about antibiotic resistance.

The total days of antibiotic use, for treatment of suspected or proven sepsis, and excluding prophylactic use, will be collected using the study data collection forms.

Stool samples will be collected for the study as close as possible to 2 weeks post-natal age and 36 weeks postmenstrual age and sent to the study centre where they will be examined for colonisation with Bifidobacterium breve BBG and subjected to quantitative microbiology to study patterns of microbiological colonisation and antibiotic resistance.
**Appendix 2: Definitions of Necrotising Enterocolitis**

NEC will be classified using Modified Bell’s criteria with further minor modification excluding recording of positive occult blood in stools and noting of bowel sounds:

<table>
<thead>
<tr>
<th>Bell stage</th>
<th>Systemic signs</th>
<th>Gastro-intestinal signs</th>
<th>Radiographic signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage IIA</td>
<td>Increased desaturations and/or bradycardia</td>
<td>Increased pre-feed gastric aspirate</td>
<td>Definite abdominal dilatation</td>
</tr>
<tr>
<td>(Definite NEC: mildly ill)</td>
<td>Temperature instability</td>
<td>Definite abdominal distension</td>
<td>Pneumotosis intestinalis</td>
</tr>
<tr>
<td></td>
<td>Lethargy</td>
<td>Possible abdominal tenderness</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Possibly bloody stools</td>
<td></td>
</tr>
<tr>
<td>Stage IIB</td>
<td>As Stage IIA with platelets (&lt;100 \times 10^{12}) and/or metabolic acidosis: base excess (&lt;-8\text{meq/l})</td>
<td>Abdominal distension with definite tenderness</td>
<td>As IIA with portal vein gas</td>
</tr>
<tr>
<td>(Definite NEC: moderately ill)</td>
<td>As Stage IIB with definite acidosis: pH (&lt;7.2)</td>
<td>Possible abdominal wall oedema and/or erythema</td>
<td>Possible ascites</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>As IIB plus mixed acidosis: pH (&lt;7.2) DIC</td>
<td>Generalised peritonitis with severe tenderness with abdominal wall induration</td>
<td>As IIA with definite ascites</td>
</tr>
<tr>
<td>(Advanced NEC: bowel intact)</td>
<td>Neutropaenia (&lt;1\times10^{9}/\text{l})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe apnoea</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypotension requiring inotropes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>As IIIA</td>
<td>As IIIB</td>
<td>As IIIA with pneumoperitoneum</td>
</tr>
<tr>
<td>(Advanced NEC: bowel perforated)</td>
<td>As IIIB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**References**

Appendix 3: Bronchopulmonary dysplasia (BPD)

Principal definition for secondary outcome: A baby who is still receiving supplementary oxygen at 36 weeks postmenstrual age.

BPD is one of the most important complications of preterm birth, lengthening hospital stay, increasing the burden on parents particularly through the frequent need for home oxygen and being associated with longer term morbidity. The definition above, which is the standard version that has been used in many clinical trials and clinical studies of neonatal outcomes is imprecise in that whether or not a baby receives oxygen is to a considerable extent dependent upon local practice and the whim of the clinical staff looking after the baby on that particular day. There has been considerable interest in making the definition more objective either by relating it more precisely to physiological measures of gas exchange\(^1,2\) (Ref Walsh, Quine) or by strengthening the underpinning clinical information\(^3\) (Ref Jobe & Bancalari). This outcome is of particular importance and interest for this study since in the pilot study undertaken by the investigators there was a significant reduction of BPD in association with \textit{B. breve BBG} colonisation. The ‘physiological’ assessment of BPD severity is not yet adequately evaluated in terms of its reliability and reproducibility to use it as an outcome measure in a clinical trial such as this. Clinical data to support the categorisation of babies using the system proposed by the NICHD\(^3\) has been collected for babies in EPICure 2 (all births <27w in 2006). Preliminary analysis of data from 869 of 870 possible surviving infants show a significant relationship between the severity of BPD using this classification at 36w pma and the likelihood of going home in oxygen. It is proposed that babies in PIPS are likewise classified at 37w using this scheme and the numbers of babies with different degrees of severity of BPD compared between the probiotic and placebo groups:

No BPD: in air by 28d post natal age.

Mild BPD: in oxygen at 28d pna but in air and not receiving mechanical ventilatory support at 36w post menstrual age.

Moderate BPD: in oxygen but <30% or ≤0.1l/min or on mechanical support in air at 36w post menstrual age.

Severe BPD in oxygen ≥30% or >0.1l/min at 36w post menstrual age

References


Appendix 4: BAPM definitions of intensive, high-dependency and special care

Standards for hospitals providing intensive and high-dependency care: British Association of Perinatal Medicine, 2001

Intensive Care

Any baby who is:

1. receiving any respiratory support via a tracheal tube and in the first 24 hours after its withdrawal
2. receiving NCPAP for any part of the day and less than five days old
3. below 1000g current weight and receiving NCPAP for any part of the day and for 24 hours after withdrawal
4. less than 29 weeks gestational age and less than 48 hours old
5. requiring major emergency surgery, for the pre-operative period and post-operatively for 48 hours
6. requiring complex clinical procedures:
   - full exchange transfusion
   - peritoneal dialysis
   - infusion of an inotrope, pulmonary vasodilator or prostaglandin and for 24 hours afterwards
7. a baby on the day of death.

High Dependency Care

Any baby who is:

1. receiving NCPAP for any part of the day and not fulfilling any of the criteria for intensive care
2. below 1000g current weight and not fulfilling any of the criteria for intensive care
3. receiving parenteral nutrition
4. having convulsions
5. receiving oxygen therapy and below 1500g current weight
6. requiring treatment for neonatal abstinence syndrome
7. requiring specified procedures that do not fulfil any criteria for intensive care:
   - Care of an intra-arterial catheter or chest drain
   - Partial exchange transfusion
   - Tracheostomy care until supervised by a parent
8. requiring frequent stimulation for severe apnoea.

Special Care

Special care is provided for all other babies who could not reasonably be expected to be looked after at home by their mother.
Appendix 5: Product details, stability, storage, temperature tolerance etc

The product to be tested is *Bifidobacterium breve* strain BBG-01. The product is supplied freeze dried with corn starch; the placebo is corn starch alone. Both products are manufactured in Japan by the pharmaceutical division of Yakult Honsha Co. Ltd. Batches of both products have been manufactured specifically for this study. There has been an independent on-site audit of the manufacturing process against EU Good Manufacturing Practices for Investigational Medicinal Products (Directive 2003/94/EC) undertaken to support the application in the UK to the MHRA for a Clinical Trials Certificate for this study. The company’s systems were considered to be in compliance with relevant standards, and acceptable for the manufacture of *B. breve* strain BBG-01 for clinical trials in the UK.

The *B. breve* is cultured on-site. All other starting materials are obtained from approved suppliers. Lactose is obtained from DMV (The Netherlands) and is TSE-free. Process water is distilled and sterilised in the production vessels before use.

*B. breve* is regularly produced for use in many products. The culture is produced in closed production vessels, which are sterilised before use. The cultivation media are checked for absence of bacteria including *E. coli* at the start of the process and after completion of incubation. *Bifidobacterium* content is checked at key stages of the process. The starting material consists of the *Bifidobacterium breve* blended with corn starch.

The Investigational Medicinal Product is manufactured by ethanolic granulation of the *B. breve* and excipients, fluid bed drying at 60ºC and packing into sealed 1g foil sachets.

**Product stability**

The freeze dried products in sealed foil sachets are stable at room temperature with a shelf life of 3 years from manufacture.

**Preparation of products for administration**

When used routinely in Japanese hospitals *B. breve* BBG-01 is resuspended in water, the starch allowed to settle and the supernatant containing the bacteria used. If the active product and the placebo are resuspended like this in water it is easy to distinguish between them as the supernatant of the active product is cloudy and the placebo clear. For this study both products will be resuspended in dilute (1/8th strength: 1 scoop to 240ml of sterile water) Neocate which renders them indistinguishable. Neocate is a cow’s milk free elemental (amino acid based), lactose free, ‘hypo-allergenic’ infant formula specifically manufactured by SHS Laboratories for use in babies with intestinal complications. It is widely used for establishing feeding in babies recovering from NEC and from gastro-intestinal surgery.

3 ml of freshly prepared 1/8th Neocate is measured into a sterile plastic screw cap ‘bijou’ bottle and the entire contents of one sachet are carefully poured in and the lid screwed down firmly. The contents are dispersed either by shaking by hand or by mixing on a vortex mixer for 10 seconds; this produces a dense turbid suspension. The starch is then allowed to settle for 30 minutes and 1 ml of supernatant is withdrawn for administration to the baby.

The products are administered via a naso-gastric or oro-gastric tube or, for babies no longer tube fed, directly into the mouth using a syringe.

*Bifidobacterium breve* BBG-01 content of supernatant and stability after resuspension

When prepared under these conditions 1ml of supernatant contains $6.7 \times 10^7 - 6.7 \times 10^9$ colony forming organisms. There is no significant change in this bacterial count for 5 hours after resuspension with the product kept at room temperature.
The mean starch level when the product is prepared in this way is low at 10.4 mg/ml of supernatant.

For the trial it is recommended that until administration the product remains in the sterile bijou bottle with its lid screwed on and that it is used within 3 hours of preparation. Any product not used by this time should be discarded.
Appendix 6:
Microbiology Protocols

Specimen Collection The study involves the collection of stool samples to perform quantitative microbiology to determine colonisation by *B. breve* BBG and to study other components of the bowel flora. Stool samples will be collected from all infants at 2 weeks postnatal age and 36 weeks post-menstrual age. Staff will be encouraged to collect samples early in the week.

Transport, receipt and storage of specimens

Samples will be transported to the Microbiology Laboratories, 3rd Floor, Pathology and Pharmacy Block, 80, Newark Street, London E1 2ES. A predefined delivery system will be used to allow for overnight transport of specimens. Upon receipt in the laboratory, specimens will be weighed and divided into two equal parts. One half will be frozen and stored at -80°C without any treatment. The other half will be diluted 1:10 in a cryopreservative (Brain heart Infusion Medium (Oxoid) containing 10% glycerol (w/v)), mixed by vortexing for 10 seconds, and then placed in 1 ml aliquots into sterile microtubes (Eppendorf 1.5ml) before freezing at -80°C.

The frozen sample without cryopreservative will be available for additional chemical, immunological and molecular analyses including in situ hybridisation to identify unculturable components of the bowel flora.

The study number, date of receipt and number of aliquots of each specimen will be recorded

Culture and characterisation of isolates

Samples stored with cryopreservative will be removed from the -80°C freezer and allowed to thaw at room temperature.

After mixing by vortexing, 10 fold serial dilutions will be prepared in prereduced PBS. 50 µl aliquots of the 10⁻², 10⁻⁴ and 10⁻⁶ dilutions will be inoculated on to agar media plates. These will include a selective medium (TOS agar containing carbenicillin (10 µg/ml) and kanamycin (50 µg/ml)) for the growth of *Bifidobacterium* spp (provided by Yakult ltd), Oxoid Chromogenic MRSA Agar, MacConkey (Oxoid ltd.) for the detection of Enterobacteriaceae and Slanetz & Bartley agar with and without vancomycin (Oxoid ltd.) for the detection of enterococci. TOS agar is incubated for 48-72h anaerobically. Other plates will be incubated at 37°C for 24-36 hours in air.

Identification and enumeration of *Bifidobacterium breve*

*B breve* grows on TOS agar producing a characteristic white convex colony. The faecal concentration of *B. breve* will be determined by counting the number of colonies on TOS agar. Representative colonies will be subcultured for identification by 16S rDNA sequencing and by ELISA using a monoclonal antibody (provided by Yakult). RAPD PCR will be used to further confirm the identity of *B breve* BBG1 (*Kado et al J Intestinal Microflora, 15: 9-14, 2001*).

Identification and enumeration of antibiotic resistant bacteria

Bacterial colonies growing on selective agars will be counted – allowing calculation of the bacterial numbers in undiluted samples. Gram negative bacilli which grow on MacConkey agar will be tested for susceptibility to a range of antibiotics (including ESβL testing) using British Society for Antimicrobial Chemotherapy methods and interpretive criteria (www.BSAC.org.uk). Antibiotics tested will include cefuroxime, cefazidime, cefpodoxime, ampicillin, gentamicin,
piperacillin/tazobactam, augmentin, tetracycline, trimethoprim, amikacin, tobramycin, imipenem, ertapenem, tigecycline, colistin, and chloramphenicol. Isolates will be identified using standard laboratory methods including the API system (Biomerieux) and 16S rDNA sequencing. MRSA and antibiotic resistant enterococci (including vancomycin resistant) will be identified using standard laboratory methods.

**Identification of Carbapenem (imipenem, meropenem) Resistant Enterobacteriaceae (CRE)**

If CRE is isolated from a stool sample culture the trial team will notify the site Principal Investigator and the nurse in charge of the neonatal unit caring for the infant and provide details of the infant. It will be recommended that they immediately inform the hospital control of infection staff. This will allow actions to be taken at a local level (in line with local infection control policy). No additional Microbiology information will be provided.