Do probiotics modulate the intestinal microbiome in extremely premature infants?

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Project Title: **Do probiotics modulate the intestinal microbiome in extremely premature infants?**

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Project Summary:
The gut microbiome plays a significant role in balancing the inflammatory system in the immature gut. A breakdown in this balance with altered colonization of the microbiota in very low birth weight (VLBW) preterm infants is associated with increased feeding intolerance, NEC and sepsis. Probiotics are proposed to normalize microbial populations and decrease intestinal disease in preterm infants. There is limited data linking clinical outcomes with the biology of probiotics. We aim to study the colonization of the GI tract with probiotic species contained in a specific probiotic blend – Florababy – in VLBW preterm infants.
Stool microbiome will be analyzed at 4 time points in 2 groups (one given Florababy and the other no) of infants less than 1000g birth weight and < 29 weeks gestation. A comparison of stool microbiome analysis and the incidence of feeding intolerance and time to reach full feeds in the two groups will be made.

1. What you want to do.

Research question
Does Florababy change the gut colonization pattern in a measurable way in a population of very premature neonates?

Hypothesis:
Florababy probiotics can multiply in the gut and change the colonization pattern of the premature intestinal tract in a measurable way.

Primary outcome: difference in stool microbiome after 4 weeks of Florababy or compared to infants who didn't receive Florababy.
Secondary outcomes: i) difference in stool microbiome 1week after the study drug administration is stopped.
ii) incidence of feeding intolerance and time to reach full feeds.

3. Why this is important.
At a glance:
a) Probiotics are proposed as universal supplements in preterm neonates. Results of probiotic administration in neonates are conflicting.
b) There is very little data linking clinical outcomes with the biology of probiotics. Gaps in current knowledge are the ecology of probiotics, their fate after administration of a specific product to a given infant, and the degree of influence on the pre-existing and/or developing microbiome.
c) It is essential that biological and mechanistic data are obtained and correlated with clinical data for a given probiotic.

2.1 Microbiome and the neonate.
There is growing recognition of the importance of the relationship between the human body and the microbial flora that coexist within it. The "human" body contains over 10 times more microbial cells than human cells, carrying 150 times as many genes as the human genome. The "microbiome" describes the collective genomes of these microorganisms. Microbial populations residing in the GI tract are the largest and most complex microbiome in our body. Studies of the intestinal microbiome have revealed a wide and variable microbial population whose composition is affected by host factors such as genetics, the immune system and environmental factors. The microbiome has a myriad of roles locally and systemically. There has been an explosion of information about its effects on local and systemic immunity and inflammation, the nervous system, the endocrine environment and metabolic states, etc. with several reviews that have been published recently (1-5).

The fetal intestine was initially thought to be sterile. However, recent studies suggest that it may be exposed to microbes via the amniotic fluid (6-8). During and after birth, microorganisms from the maternal vagina and intestine, from the maternal skin surface, from maternal breastmilk and oral cavity, and from the environment colonize the neonatal GI tract. Many factors have been shown to alter the gut flora in neonates such as gestational age and feeding patterns. Data on the development of the normal microbiome in well term infants is still limited. It shows a very complex process where waves of microbial species get established in a careful orchestrated order with multiple interactions between them and the host (9-14). Many details of this process remain unknown.
Furthermore, the intestinal microbiomes of infants admitted to NICUs have different patterns compared to infants not admitted to NICUs. This is due to exposure to a different environment in NICUs, and the use of antibiotics and sterile formulas. There is even less data about the establishment of the microbiome in very preterm infants, how that evolves with time, what constitutes a "good" profile and how it correlates with intestinal or systemic pathology. What we know shows that the microbiome in preterm infants demonstrates decreased diversity, altered species proportions and even different behavior of the same species when compared with the term infant (15-17).

2.2 Microbiome and intestinal disease in the neonate.
The microbiome plays an essential role in nutrition, mucosal immunity, and GI epithelial integrity. Current evidence suggests that interactions between the altered microbiome and the intestinal epithelium play an important role in the development of feeding intolerance, allergy, sepsis and necrotizing enterocolitis (NEC) (18-20).
NEC is a multifactorial disease and one of the major pathologies affecting preterm infants. It is associated with significant mortality and morbidity, including long term neurologic disability. Systematic interventions to predict and prevent NEC have generally remained elusive. Feeding breastmilk and colostrum to preterm infants is helpful and use of probiotics has shown good results in a number of studies, though significant concerns remain. Concerns include: the limited safety data for infants born < 1000g and a domestically available probiotic formulation with demonstrated efficacy, safety, and regulatory approval. There remain concerns that the regulatory framework for approval of probiotics does not approach the rigor for drugs (21-22). Systematic reviews of randomized controlled trials concluded that probiotics as a group significantly reduce the incidence of severe NEC (23). The principle and methodology of such generalizations is controversial (24-26). Better tools are needed to aid in the understanding and monitoring of probiotic administration.
The technology available to define the intestinal microbiome has been a sizable obstacle. The vast majority of species cannot be cultured and the genomic technologies have just only very recently reached a level where the ratio of cost and resolution allows for reasonably efficient research use.

2.3 Techniques to study gut microbiota and microbiome.
The composition of the gut microbiota has been classically analyzed using differential media to distinguish between populations of bacteria based on their metabolic requirements. The capability of these culture-based techniques to discriminate between different bacterial phylogenetic groups is limited. It is estimated that 80% of the gut microbiota cannot be cultured using these techniques. Recent reviews emphasized the role of genomic technology in mapping the microbiome, providing an unprecedented amount of detail on the constituent species (27-29). These genomic techniques are capable of identifying commercial probiotic strains. One of the leading techniques employed currently is 16S rDNA amplification and sequencing. It is available in Calgary through the Genomics facility at Alberta Children's Hospital Research Institute (ACHRI).

3.1 Probiotic administration results in preterm infants
The subject of probiotic administration to preterm infants is important. There are at least 20 meta-analyses and systematic reviews identified with a Pubmed search. This is a large number despite only 24 trials in the latest Cochrane review and a meta-analysis from April 2014 (23). It is also important to note the contradictory statements in that review. It begins by stating: "Based on the available evidence for probiotics efficacy and safety in preterm infants, the number of infants enrolled, the narrow confidence intervals, and the probiotics safety profile, a change in practice is warranted at this stage. More studies to address the optimal preparation, dosing, and duration of therapy are still needed in head to head comparative studies rather than placebo controlled trials." Immediately followed by: "Although all included trials evaluated probiotics use in preterm infants, the trials were highly variable with regard to enrolment criteria (that is birth weight and gestational age), baseline risk of NEC in the control groups, timing, dose, formulation of probiotic used, and feeding regimens.(23)The validity of our review's results is potentially compromised as it included trials that utilized different preparations and dosing regimens of the intervention under study; and data on the highest risk population (ELBW infants) could not be retrieved."

Two sizeable recent studies were published or had outcome data released after the Cochrane review. The recent Probiotics in Preterm babies Study (PiPS) was done in Britain and enrolled 1315 infants of 30 weeks gestational age or less. It used a single bacterial strain – *Bifidobacterium breve*. This study did not find any significant clinical impact of that particular probiotic and the findings do not support the use of *Bifidobacterium breve* for the prevention of NEC (30). Another recent RCT studied Lactobacillus Reuteri in 400 VLBW infants of less than 32 weeks gestation and it concluded that there was no effect on NEC

or NEC and mortality though late onset sepsis was significantly reduced (31). The combined size of these 2 studies is large, over 1/3rd of all previous RCTs' population in the 2014 Cochrane review.

3.2 Florababy.
Florababy is a 5 strain proprietary probiotic blend produced by Renew Life Canada. It contains 4 billion total CFU of 4 *Bifidobacterium* species (*Breve, bifidum, infantis and longum*) together with *Lactobacillus rhamnosus*. The non-medicinal ingredients are maltodextrin and ascorbic acid. Florababy has been used for only a few years in neonates in Canada. A recently published retrospective cohort study compared data from 17 months before and after introduction of Florababy in a level III NICU for all infants less than 32 weeks gestational age at birth (32). It showed a positive impact on NEC stage 2 or more from a baseline of 10% in infants less than 32 weeks, double the current rate in Calgary. The amount of breastmilk feeding in that cohort is not specified. There was no impact on health care associated infections.

3.3 Biology of probiotics and relation with effects – gaps in knowledge.
The above underscores the need for specific product trials in this population as well as the pressing need for markers and biological understanding. A lot of the limited data concerning the fate of probiotics in preterm infants may not be applicable to current NICU care and feeding practices or was generated before latest genomics technology was available (33-43). The percentage of infants and interval of time in which specific probiotic strains are established in the gut, any differences between strains, differences within a population fed fresh breastmilk and correlation with clinical effects including tolerance of enteral feeds and time to reach full feeds are very important aspects. They need to be clarified for contemporary practices, with best methods, in order to assess and optimize the use of probiotics in the neonate. Using meta-analysis to advocate for an intervention when the biological pathways were not clear has been a source of error before in medicine (44).

3.4 Definition of Feeding Intolerance.
Feeding intolerance will be defined as the inability to digest enteral feedings associated with: i) gastric residual volumes of more than 50% of the volume of the previous feed, ii) abdominal distention and/or emesis, iii) disruption of the baby's feeding plan (decrease, delay or discontinuation of enteral feedings).

4. How you are going to do it.

4.1 Study design
This study will occur in a randomized controlled trial setup in the Calgary NICUs. Florababy probiotic will be administered to infants in the drug arm and clinical outcomes and gut microbiome data will be compared between the two groups.
The projected starting time is Oct 2017. Study duration will be 18 months.

4.2 Study Population
Premature infants born less than 29 weeks gestation and < 1000g birth weight admitted to the NICU at Foothills Medical Centre in Calgary.
a) Inclusion Criteria: Preterm infants born at less than 29 weeks gestation and < 1000g birth weight.
b) Exclusion Criteria: Infants with major congenital anomalies, hypoxic-ischemic injury and NEC or bowel perforation before 72 hours of life.

4.3 Sample Size and feasibility
A total of 100 infants of birth weight <1000g are born annually and admitted to FMC NICU.
Assuming that 80% of parents' consent to their infant's participation in the study, it will be feasible to complete enrollment of 60 babies in the first year of the study. This study will randomize 30 infants to be given probiotics and 30 infants be in the control group where no probiotic given. The 2 groups will be matched by gender, gestational age +/- one week, birth weight +/- 100g, SGA vs AGA/LGA status and type of delivery.
A sample size of 30 infants **per group** will allow for a power of 0.8 and an alpha of 0.05 for a two-tailed Wilcoxon-Mann-Whitney test to detect a 0.7 effect size when comparing relative abundance of stool probiotic microbial species in the group given Florababy versus the one given placebo, conservatively assuming a background logistic distribution (G-Power 3.1 software).

4.4 Variables collected
a) Demographic variables: Gestational age, birth weight, head circumference at birth, gender, mode of delivery, singleton/multiple and rank, maternal administration of corticosteroids and antibiotics.
b) Clinical variables: Apgar scores, umbilical cord pH and base excess, first patient blood gas pH and lactate, day of life at start of feeds and at start of study, total days of study drug, antibiotic use during NICU, use of inotropes and vasoactive substances, steroids and medical and/or surgical treatment for PDA, type of feeds during NICU and at discharge.

c) Outcome variables: mortality; total days of TPN, total days NPO and number of separate NPO events; age, head circumference and weight at discharge home; diagnosis of: feeding intolerance, time taken to reach full enteral feeds (at least 150ml/kg/day), suspected NEC, NEC, cow milk protein allergy, late onset sepsis, BPD, ROP.

4.5 Study protocol.

Infants will be identified within 24 hours of birth and parents will be approached for informed consent.

Once consent is obtained, subjects will be randomly assigned in blocks of 4 to receive either Florababy probiotics orno. Investigators will conduct the randomization using a computer-generated table of random numbers generated at the University of Calgary.

Preparation and Administration of Study Drug

The study probiotic will be started after the first stool sample is obtained and after informed parental consent. Very small amounts of breast milk/colostrum (0.1ml to 0.2ml every 4 hours) are usually given to all premature babies within the first 48 hours of life. Actual feeds of breast and/or human milk (donor milk) are initiated from 72 hours onwards (starting at 10ml/kg/day and increasing slowly thereafter). The study probiotic (at a dose of 0.5g per day) will be started at or after 72 hours of life. It will be administered in 1 ml of sterile water prior to a feed.. No drug will be given to infants in the control group If the infant is placed NPO, the study drug will be stopped and restarted together with refeeding.

The study probiotic will be given until 34 weeks corrected gestational age.

Sample collection

Nurses will collect the stool samples at 4 time points: prior to, two weeks and four weeks after probiotic administration is commenced, and one week after it is stopped. Stool samples for the control group will be done at a matched feeding and gestational age time points.

Stool will be collected directly from the infant's diaper with a sterile spatula. As soon as a sample is collected, the nurse will contact the investigators. The samples will then be placed in a laboratory freezer (-80°C) within 24 hours of collection. Batched samples will be transported to the ACH ACHRI Genomics laboratory for subsequent microbiome processing.

4.6 Microbiome DNA extraction, amplification and species identification.

DNA extraction

DNA will be extracted from ~200 mg of frozen (-80^{o}c) stool using the Zymo ZR Fecal DNA Stool MiniPrep Kit (Zymo Research Corp, USA).

Real-time quantitative PCR (qPCR)

Quantification of target bacterial DNA will be measured using the SsoFast™ EvaGreen® Supermix (Biorad, Mississauga, Canada). Samples and standards (10 ng of DNA from known bacteria) will be prepared and run in duplicate in a final volume of 20 µl on 96 well plates using the CFX96 detection system (Biorad, Mississauga, Canada). All collected fecal samples will be quantified for *Bacteroides*, *Prevotella*, *Clostridium coccoides* and *C. leptum*, *Enterococcus* and *Enterobacteriacae* sp. as indicators of microbiome health. [45]

Library preparation and sequencing

Sequencing of the V3-V4 region of the 16S rRNA gene will be performed on all cases and control samples. We will aim to recruit no more that 50% of our cases from VRE positive patients. Preparation of 16S metagenomics libraries and deep sequencing will be carried out by the University of Calgary's Core DNA Services. DNA libraries be prepared using the 16S Metagenomic sample preparation protocol (Illumina, San Diego, CA). The quality of the prepared library will be checked using Agilent TapeStation D1000 screen tape (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instructions. Indexed DNA libraries are normalized to 4 nM and Illumina Experiment Manager used to build library plates and create sample sheet. Paired-end 300bp sequencing will be performed on the MiSeq instrument using the V3 600 cycle MiSeq cartridge and MiSeq v3 reagents. The completed run will be demultiplexed with Illumina's Casava software and stored in BaseSpace (Illumina) for downstream analysis.

Bioinformatic analysis

Sequences will be processed with the UPARSE pipeline as implemented in USEARCH v 8.0.1623.[46] Details for each processing step are outlined. For quality filtering the forward and reverse 16S PCR primers along with any sequencing adapter will be trimmed off the raw reads using cutadapt v1.8. Following this the forward and reverse reads will be overlapped to reconstruct the full amplicon. This will be done using the fastq_mergepairs option in USEARCH v 8.0.1623. Following this the reads will be quality filtered using the maximum expected error method and then truncated at a length of 173 base pairs. Sequences will be dereplicated, where identical reads are merged and counts of these reads are retained, with vsearch v1.1.3

hosted on GitHub ("https://github.com/torognes/vsearch"). Singleton reads will be filtered out with vsearch prior to clustering. The remaining sequences are then clustered into groups of 97% identity, designated as Operational Taxonomic Units (OTUs) with the cluster_otus command in USEARCH v 8.0.1623. To construct an OTU table the filtered reads, with singletons included, were searched against the clustered OTU sequences with usearch_global in USEARCH v 8.0.1623.
Taxonomy will be assigned to each of the representative sequences using the RDP Naïve Bayesian Classifer v 2.2[47] as implemented in Qiime 1.9.0 [48] using the most recent version of the Greengenes database[49]. The RDP classifier uses a bootstrap procedure to assign confidence values to the taxonomic assignments at each level of the taxonomy. The OTU sequences will be aligned with PyNAST, a flexible tool for aligning sequences to a template alignment[50]. Gaps in the alignment will be removed using Qiime's 'filter_alignment.py', and a phylogeny constructed using FastTree, a tool for inferring phylogenies for alignments with up to hundreds of thousands of sequences[51], using default parameters. All remaining analysis will be done in R, a programming language and software environment for statistical computing and graphics (R Core Team, 2015) using functions from phyloseq[52] DESeq2[53] and vegan.
The diversity within each sample, also known α diversity, will be measured using the Shannon index. This is a measure of both the organismal richness of a sample and the evenness of the organisms' abundance distribution[54]. A two-sided Mann-Whitney test (with False Discovery Rate multiple test correction) will be used to determine if there were significant differences in Shannon diversity between cases and controls.
Diversity between samples (β -diversity) will be evaluated using weighted UniFrac, a rank-based ordination method and visualized with Non-metric Multidimensional Scaling (NMDS). β-diversity represents the explicit comparison of microbial (or other) communities based on their composition or relative abundance. β diversity metrics thus assess the differences between microbial communities. To test whether there are significant differences in community structure between groups the UniFrac distance matrix will be tested with permutational multivariate ANOVA (PERMANOVA) implemented in the adonis function in the vegan R package. Differential abundance of individual OTUs between groups will be assessed using generalized linear models with a negative binomial distribution as is used commonly for RNA-seq[53]. The similarity in data structure between RNA-seq and 16S allow us to adapt already mature methods[55]. This analysis will be performed in DESeq2 v1.8.1 using default parameters.

5. Why you/your group should do it (relevant prior experience and skills, collaborators for technical gaps, preliminary data showing feasibility):
Dr. Amin has a lifelong interest and expertise as well as publications in neonatal GI pathophysiology and NEC.
Dr. Alshaikh has completed a year of supplementary training in the study of the neonatal intestinal tract in Philadelphia. He is also skilled in study design and statistical analysis and has a Master degree in Epidemiology.
The collaborators include Dr. Pon, the director of the ACHRI Genomics facility, Dr. Arrieta is a research scientist at -University of Calgary and an expertise in microbiome, Dr Amuchou Soraisham (neonatologist and epidemiologist), and D. Dersch-Mills – Pediatric Pharmacists.

6. Plans for dissemination or translation of results and plans for next steps in study of topic: The microbiome data will constitute a comprehensive clinical and biological study of probiotic administration in this population. Results will be presented at national and international meetings and submitted for publication.
Findings from this study will also generate insight into the systemic effects of probiotics such that further hypotheses can be generated and tested and the best probiotic species and/or blends can be designed for use in preterm infants.

Budget:

1. Budget and considerations relating to budget

	Per sample cost	Costing
Consumables	-	$1000
DNA extraction (Zymoresearch) Library preparation (Uof C sequencing centre) Sequencing	$85	$85 x 60 x4 = $20,400
Equipment	Pharmacy set up for Florababy for 60 patients in 1st year of study Phase III set up fee Dispensing fee for 60 patients ($30.00 per vial for each patient for 1 week) x 6 weeks = 60x30x6	 $1,500.00 $1,000.00 $10,800.00
Total	-	$33,700.00

REFERENCES

1. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. Physiological reviews. 2010;90(3):859-904. Epub 2010/07/29.
2. Group NHW, Peterson J, Garges S, Giovanni M, McInnes P, Wang L, et al. The NIH Human Microbiome Project. Genome research. 2009;19(12):2317-23. Epub 2009/10/13.
3. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007;449(7164):811-8. Epub 2007/10/19.
4. Spor A, Koren O, Ley R. Unravelling the effects of the environment and host genotype on the gut microbiome. Nature reviews Microbiology. 2011;9(4):279-90.
5. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature 2012, Sep 13;489(7415):220-30.
6. DiGiulio DB. Diversity of microbes in amniotic fluid. Semin Fetal Neonatal Med. 2012;17(1):2-11. Epub 2011/12/06.
7. DiGiulio DB, Romero R, Kusanovic JP, Gomez R, Kim CJ, Seok KS, et al. Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm pre-labor rupture of membranes. Am J Reprod Immunol. 2010;64(1):38-57. Epub 2010/03/25.
8. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. PloS one. 2008;3(8):e3056. Epub 2008/08/30.
9. Adlerberth I, Wold AE. Establishment of the gut microbiota in Western infants. Acta Paediatr. 2009;98(2):229-38. Epub 2009/01/16.
10. Jost T, Lacroix C, Braegger CP, Chassard C. New insights in gut microbiota establishment in healthy breast fed neonates. PloS one. 2012;7(8):e44595. Epub 2012/09/08.
11. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics. 2006;118(2):511-21. Epub 2006/08/03.
12. Putignani L, Del Chierico F, Petrucca A, Vernocchi P, Dallapiccola B. The human gut microbiota: A dynamic interplay with the host from birth to senescence settled during childhood. Pediatr Res 2014, Jul;76(1):2-10.
13. Neu J. The developing intestinal microbiome: probiotics and prebiotics. World review of nutrition and dietetics. 2014;110:167-76. Epub 2014/04/23.
14. Walker WA, ShubaIyengar R. Breastmilk, microbiota and intestinal immune homeostasis. Pediatr Res 2014,Oct 13.
15. Barrett E, Kerr C, Murphy K, O'Sullivan O, Ryan CA, Dempsey EM, et al. The individual-specific and diverse nature of the preterm infant microbiota. Arch Dis Child Fetal Neonatal Ed 2013, Jul;98(4):F334-40.
16. Unger S, Stintzi A, Shah P, Mack D, O'Connor DL. Gut microbiota of the very low birth weight infant. Pediatr Res 2014, Oct 13.
17. Andriantsoanirina V, Teolis AC, Xin LX, Butel MJ, Aires J. Bifidobacterium longum and bifidobacterium breve isolates from preterm and full term neonates: Comparison of cell surface properties. Anae
18. Canani RB, Di Costanzo M. Gut microbiota as potential therapeutic target for the treatment of cow's milk allergy. Nutrients. 2013;5(3):651-62. Epub 2013/03/05.
19. Torrazza RM, Neu J. The altered gut microbiome and necrotizing enterocolitis. Clinics in perinatology. 2013;40(1):93-108. Epub 2013/02/19.
20. Robinson DT, Caplan MS. Linking fat intake, the intestinal microbiome and necrotizing enterocolitis in premature infants. Pediatr Res 2014, Oct 10.
21. Neu J, Walker WA. Necrotizing enterocolitis. N Engl J Med 2011, Jan 20;364(3):255-64.
22. Neu J. Necrotizing enterocolitis: The mystery goes on. Neonatology 2014;106(4):289-95.
23. AlFaleh K, Anabrees J. Probiotics for prevention of necrotizing enterocolitis in preterm infants. Cochrane database of systematic reviews. 2014;4:CD005496. Epub 2014/04/12.
24. Neu J. Probiotics and necrotizing enterocolitis. Clin Perinatol 2014, Dec;41(4):967-78.
25. Millar M, Wilks M, Fleming P, Costeloe K. Should the use of probiotics in the preterm be routine? Arch Dis Child Fetal Neonatal Ed 2012, Jan;97(1):F70-4.
26. Tarnow-Mordi WO, Wilkinson D, Trivedi A, Brok J. Probiotics reduce all-cause mortality and necrotizing enterocolitis: It is time to change practice. Pediatrics 2010, May;125(5):1068-70.
27. Morgan XC, Huttenhower C. Meta'omic analytic techniques for studying the intestinal microbiome. Gastroenterology 2014, May;146(6):1437-1448.e1.
28. Norman JM, Handley SA, Virgin HW. Kingdom-agnostic metagenomics and the importance of complete characterization of enteric microbial communities. Gastroenterology 2014, May;146(6):1459-69.
29. Davis C. Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. J Microbiol Methods 2014, Aug;103:9-17.
30. Costeloe K, Hardy P, Juszczak E, Wilks M, Millar MR, on behalf of The Probiotics in Preterm Infants Study Collaborative Group. The Lancet November 2015. http://dx.doi.org/10.1016/S0140-6736(15)01027-2.

31. Oncel MY, Sari FN, Arayici S, Guzoglu N, Erdeve O, Uras N, et al. Lactobacillus reuteri for the prevention of necrotising enterocolitis in very low birthweight infants: A randomised controlled trial. Arch Dis Child Fetal Neonatal Ed 2014, Mar; 99(2): F110-5.
32. Janvier A, Malo J, Barrington KJ. Cohort study of probiotics in a north american neonatal intensive care unit. The Journal of pediatrics. 2014;164(5):980-5. Epub 2014/01/15Bezirtzoglou E, Stavropoulou E. Immunology and probiotic impact of the newborn and young children intestinal microflora. Anaerobe 2011, Dec;17(6):369-74.
33. Akiyama K, Shimada M, Ishizeki S, Takigawa I, Imura S, Yamauchi K, *et al.* Effects of administration of Bifidobacterium in extremely premature infants; development of intestinal microflora by orally administered Bifidobacterium longum in comparison with Bifidobacterium breve. Acta Neonatol Japonica 1993;30:257-6
34. Millar MR, Bacon C, Smith SL, Walker V, Hall MA. Enteral feeding of premature infants with lactobacillus GG. Arch Dis Child 1993, Nov;69(5 Spec No):483-7.
35. Kitajima H, Sumida Y, Tanaka R, Yuki N, Takayama H, Fujimura M. Early administration of bifidobacterium breve to preterm infants: Randomised controlled trial. Arch Dis Child Fetal Neonatal Ed 1997, Mar 1;76(2):F101-7.
36. Piano MD, Carmagnola S, Ballarè M, Balzarini M, Montino F, Pagliarulo M, et al. Comparison of the kinetics of intestinal colonization by associating 5 probiotic bacteria assumed either in a microencapsulated or in a traditional, uncoated form. J Clin Gastroenterol 2012, Oct;46 Suppl:S85-92.
37. Underwood MA, Kalanetra KM, Bokulich NA, Lewis ZT, Mirmiran M, Tancredi DJ, Mills DA. A comparison of two probiotic strains of bifidobacteria in premature infants. J Pediatr 2013, Dec;163(6):1585-1591.e9.
38. Tobin JM, Garland SM, Jacobs SE, Pirotta M, Tabrizi SN. Rapid assay to assess colonization patterns following in-vivo probiotic ingestion. BMC Res Notes 2013; 6:252.
39. Li Y, Shimizu T, Hosaka A, Kaneko N, Ohtsuka Y, Yamashiro Y. Effects of bifidobacterium breve supplementation on intestinal flora of low birth weight infants. Pediatr Int 2004, Oct;46(5):509-15.
40. Lee SJ, Cho SJ, Park EA. Effects of probiotics on enteric flora and feeding tolerance in preterm infants. Neonatology 2007;91(3):174-9.
41. Wang C, Shoji H, Sato H, Nagata S, Ohtsuka Y, Shimizu T, Yamashiro Y. Effects of oral administration of bifidobacterium breve on fecal lactic acid and short-chain fatty acids in low birth weight infants. J Pediatr Gastroenterol Nutr 2007, Feb;44(2):252-7.
42. Arboleya S, Salazar N, Solís G, Fernández N, Hernández-Barranco AM, Cuesta I, et al. Assessment of intestinal microbiota modulation ability of bifidobacterium strains in in vitro fecal batch cultures from preterm neonates. Anaerobe 2013, Feb;19:9-16.
43. Patole S, Keil AD, Chang A, Nathan E, Doherty D, Simmer K, et al. Effect of bifidobacterium breve M-16V supplementation on fecal bifidobacteria in preterm neonates--a randomised double blind placebo controlled trial. PLoS ONE 2014;9(3):e89511.
44. Borzak S, Ridker PM. Discordance between meta-analyses and large-scale randomized, controlled trials. Examples from the management of acute myocardial infarction. Ann Intern Med 1995, Dec 1;123(11):873-7.
45. Louie TJ, Cannon K, Byrne B, et al. Fidaxomicin preserves the intestinal microbiome during and after treatment of Clostridium difficile infection (CDI) and reduces both toxin reexpression and recurrence of CDI. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* Aug 2012;55 Suppl 2:S132-142.
46. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods.* Oct 2013;10(10):996-998.
47. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology.* Aug 2007;73(16):5261-5267.
48. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nature methods.* May 2010;7(5):335-336.
49. DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology.* Jul 2006;72(7):5069-5072.
50. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics.* Jan 15 2010;26(2):266-267.
51. Price MN, Dehal PS, Arkin AP. FastTree 2--approximately maximum-likelihood trees for large alignments. *PloS one.* 2010;5(3):e9490.
52. McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS computational biology.* Apr 2014;10(4):e1003531.
53. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology.* 2014;15(12):550.
54. Morgan XC, Huttenhower C. Ch12: Human microbiome analysis. *PLoS computational biology.* 2012;8(12):e1002808.
55. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one.* 2013;8(4):e61217.