

## **Appendix (1 and 2)**

### **Appendix 1: Faecal sample collection, DNA extraction, microbiota analysis and shotgun sequencing and Study protocol for PANTS trial**

#### **SiMPro trial**

##### **Faecal sample collection**

Two stool samples were collected in sterile containers from each participating infant; first sample (T1) was collected as soon as possible after birth/admission (week 1). The second sample (T2) was collected between 21-28 days of life (week 3-4). All samples were labelled, weighed and stored at  $-80^{\circ}\text{C}$ . After completing recruitment, samples were shipped on dry ice (carbon dioxide) to the University of New South Wales (Sydney, Australia), for microbial analysis. Acidified samples were shipped on dry ice to the School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore for SCFA analyses.

##### **DNA extraction**

DNA was extracted from stool samples (0.3g) using the Qiagen Powersoil kit (cat# 1288-100). However, instead of vortexing, samples were subjected to physical lysis in a bead-beater (Precellys 24, Bertin Instruments) for 5min at 6500rpm. DNA was eluted in molecular grade water and stored at  $-80^{\circ}\text{C}$ .

##### **Quantification of SCFA**

SCFA from faecal samples were extracted and analysed using modified GC-MS based method. Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, 4-methyl valeric acid (internal standard, IS), ethyl acetate and meta-phosphoric acid were purchased from Merck (Singapore). For sample preparation, 0.2 g of the faecal sample was first diluted 5-fold in 1ml of 1% phosphoric acid and frozen at  $-20^{\circ}\text{C}$  immediately after collection. Before SCFA extraction, the frozen samples were thawed and added 100  $\mu\text{L}$  of 10% meta-phosphoric acid solution to adjust the pH to around 2. Samples were vortexed for about 10

min and centrifuged for 30 min at  $20817 \times g$ . After that, 1 ml of aqueous supernatant was transferred into a new tube and added 4-methyl valeric acid to a final concentration of 500  $\mu\text{M}$ . Then, 500  $\mu\text{L}$  of ethyl acetate was added to and the mixture was vortex for 30 min and centrifuged for 10 min at  $20817 \times g$ . Then, 2  $\mu\text{L}$  of the organic extracts were injected in split less mode into an Agilent GC-MS system (Agilent Technologies 7890B-5977B Bundle with Stainless Steel Source) and separated by a HP-FFAP capillary column (30 m $\times$ 0.250 mm  $\times$ 0.25  $\mu\text{m}$ ; Agilent). Helium was used as the carrier gas at 1 mL/min. The column temperature was initially 80°C (1min), then increased to 120 °C at 20 °C/min, and finally to 210 °C at 6.13 °C/min and kept at this temperature for 2 min. Solvent delay was 3.5 min. The detector was operated in SIM acquisition mode with 30–250 m/z range. The injector, ion source, quadrupole, and interface were set at 250 °C, 230°C, 150°C and 280 °C, respectively. SCFAs was identified by comparing with standards and double confirmed with the NIST 17 library. Quantifications were performed in Mass Hunter Quantitative software (version B.09.00) with base peak ion selected as quantifier for each SCFA. The calibration graphs were constructed by plotting the relative response (ratio of peak area of SCFA/peak area of IS) versus relative concentration for each SCFA. The final concentrations were expressed as microgram of SCFA per gram wet weight faecal sample. All the analyses were performed in duplicate. Wilcoxon rank analysis was performed to compare SCFA concentrations between different groups.

### **Linearity and sensitivity**

A stock solution containing the mixture of standards (20mM final concentration each) in ethyl acetate was diluted to obtain a calibration curve ranging from 2 to 15 000  $\mu\text{M}$ . IS was added to each diluted standards mixture (500  $\mu\text{M}$  final concentration). The calibration graphs were constructed by plotting the ratio of peak area SCFA/peak area IS vs. concentration for each individual SCFA. By normalizing the peak area to that of the IS, the variability in the

extraction step and the instrument response was corrected (in particular, the injection volume variability and the MS response). Each point of the calibration graph corresponds to the mean value from independent replicate injections. The limits of detection (LOD) and quantification (LOQ) of the individual analytes were obtained by injecting successively more diluted standard solutions and were calculated according to the International Union of Pure and Applied Chemistry<sup>16</sup> (ref) method based on a signal-to-noise ratio (S/N) of 3 for the LOD and of 10 for the LOQ.

### **Microbiota analysis**

#### **PCR amplification and 16S rRNA gene sequencing**

Genomic DNA were submitted to the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) for library preparation and sequencing on the Illumina MiSeq platform using the MiSeq Kit v3 (2x300 cycles) using V3-V4 primers.<sup>1</sup>

#### **16S rRNA gene sequence analysis**

16S rRNA sequence data were quality filtered and trimmed using TRIMMOMATIC VERSION 0.36 truncating reads if the quality was below 12 in a sliding window of 4bp. USEARCH version 10.0.240 was used to merge, and quality filter sequencing reads between 350 and 500 nucleotides.<sup>2</sup> Unique sequences that appeared less than 8 times were removed. Processed reads were then concatenated and clustered into operational taxonomic units (OUT) at 97% sequence similarity using UPARSE. Chimeras were removed de novo in reference mode using UCHIME together with the SILVA SSURef NR99 database (version 132) and OTU sequences were identified taxonomically using BLASTN alignments against the SILVA database.<sup>3</sup>

For alpha diversity measures, each sample was subsampled 100 times to a count of 20000 counts per sample and the average was taken. OTU richness and diversity indices; Simpson, Shannon, ACE and Chao1 were calculated in R (version 3.6.0) using the vegan package.

Relative abundance analysis at Phylum, Family, Genus and Species levels were carried out using phyloseq package in R.<sup>4</sup> Data were visualized using ggplot2<sup>5</sup> and ggpubr packages.<sup>6</sup> For beta diversity both weighted UniFrac and Bray Curtis calculations were used. For weighted Unifrac, the data were transformed to relative abundance. To generate a phylogenetic tree for diversity computations, zOTUs were aligned with MAFFT,<sup>7</sup> and the tree was calculated with FastTree.<sup>8</sup> Weighted unifrac distances were calculated and visualized on a principal coordinate analysis (PCoA) plot. For Bray Curtis, the data were square root transformed and data were visualized on a non-metric multi-dimensional (NMD) scaling plot.

### Shotgun metagenomic analysis

Quality of the sequencing reads were checked using FastQC (version 0.11.9). Cutadapt (version 1.8.1) was used for quality filtering of the raw reads to remove adapters and low-quality bases using `-e 0.1, -q 20, --minimum-length 30` as parameters. Bowtie2 (version 2.3.5) was used for mapping the trimmed reads with the reference database, GRCH38 to remove human host reads using `--un-conc`, `--very-sensitive-local` as parameters.

### Taxonomic profiling

Kaiju classifier (version 1.7.0) was used for taxonomic classification of metagenomic reads. The database index is created from the reference database, nr\_euk (2019/02/05) downloaded from the source. MEGAN6 was used for calculating the normalized read counts of bacteria at species level. To obtain sub-species information for *Bifidobacterium longum*, Kraken2 with default database with 'report option' was used. The read counts for each taxon are the number of reads covered by the clade rooted to this taxon.

### References

1. Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* 2013;41(1):e1.
2. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26(19):2460-1.

3. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;41(Database issue):D590-6.
4. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. Paul J. McMurdie and Susan Holmes (2013) PLoS ONE 8(4):e61217.
5. Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.
6. Alboukadel Kassambara (2020). ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.4.0. <https://CRAN.R-project.org/package=ggpubr>
7. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013;30(4):772-80.
8. Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 2009;26(7):1641-50.

### Study protocol- use of PANTS REF group in SiMPro trial

The study protocols for both PANTS and SiMPro were similar except for the eligible gestational age (PANTS: <33 weeks and SiMPro: <28 weeks. PANTS trial protocol details:

**Eligibility criteria:** (1) Gestation up to 32 weeks and 6 days (2) VLBW: Birth weight under 1500 grams (3) Ready to commence or on enteral feeds for <12 hours.

**Exclusion criteria:** (1) Major congenital malformation (2) Chromosomal aberration (3) Lack of informed parental consent (4) On enteral feeds for  $\geq$ 12 hours (5) Contraindications for enteral feeds (6) Life threatening illness.

**Outcomes:** The primary outcome was the effect of *B. breve* M-16V supplementation on levels of *B. breve* in the stools of preterm neonates as detected by quantitative PCR. Secondary outcomes included evidence of a bifidogenic effect (elevation of total bifidobacteria in stools); incidence of NEC ( $\geq$ Stage II), and all cause death; time to reach full enteral feeds (150 ml/kg/day) and blood culture positive late onset sepsis (LOS) beyond 72 hours of life.

**Safety:** This was assessed by monitoring for (1) blood culture positive sepsis by *B. breve* M-16V and (2) adverse effects such as abdominal distension, vomiting, and diarrhea leading to cessation of the supplementation.

All outcomes and safety parameters were monitored from enrolment till death or reaching the corrected age of 37 weeks.

**Pre-planned subgroup:** A subgroup analyses was planned for extremely preterm neonates (Gestation <27 weeks) who are at the highest risk for mortality and morbidities, such as NEC, infections, and feed intolerance

**Randomisation, allocation concealment, and blinding** Group assignment was allocated by a computer generated randomisation sequence in randomly ordered block sizes of 2 and 4, and stratified by gestational age at birth (up to 27+6 weeks and  $\geq 28$  weeks) to ensure that extremely preterm neonates were equally distributed between the two arms of the trial. Opaque, sealed, coded envelopes were used for randomisation. Neonates of multiple pregnancies were considered as separate individuals. Allocation concealment was optimised by prescribing allocation only after informed parental consent and recording the basic neonatal data. The Clinical Trial Pharmacist (CTP) supplied the randomisation sequence and the sachets (identical design, weight, smell, and taste) containing either the probiotic (B. breve M-16V;  $5 \times 10^9$  cfu per sachet with dextrin as carrier) or placebo (equal volume of dextrin) manufactured by Morinaga Milk Industry Co., Ltd, Japan, to the nursing staff. This assured masking of all investigators, clinical and non-clinical outcome assessors, nursing staff and parents with regards to the allocation status of enrolled neonates.

**Probiotic protocol:** When ready for enteral feeds, enrolled neonates were supplemented with the freshly reconstituted contents of the allocated sachets every day, and continued until the corrected age 37 weeks. Reconstitution of the dry powder in the sachets was done using sterile water for injection or breast milk when available. Care was taken during reconstitution to reduce the risk of cross contamination by adhering to strict hand hygiene, preparing doses for individual neonates separately, and avoiding contact with indwelling lines, tubes, and catheters. The dose was  $3 \times 10^9$  cfu/day (1.5 mls of the reconstituted solution), given as a single dose via the orogastric feeding tube. The dose and duration of supplement was based on the previous clinical, and experimental (oral toxicity) studies of this strain in preterm

neonates. For neonates  $\leq 27$  weeks the daily dose was  $1.5 \times 10^9$  cfu per day until reaching milk feeds of 50 ml per kg per day. It was then increased to  $3 \times 10^9$  cfu per day. Considering the risk of probiotic sepsis, supplementation was stopped when enteral feeds were stopped by the attending neonatologist for indications such as sepsis and NEC.

The manufacturer Morinaga Milk Industry Co., Ltd, Japan was not the sponsor but only supplied the product free for the trial.

**Stool samples:** Two stool samples were collected for quantitative cultures from each neonate: One before and one 3 weeks after starting the probiotic supplementation. Samples were frozen after collection and stored at  $-80$  degree Centigrade prior to analysis. The investigators involved in stool culture studies were masked to the allocation status of the enrolled neonate, assuring masking of the primary outcome assessor.

**Stool cultures:** The stool samples were thawed on ice prior to analysis. Stool samples with very inadequate volume were not analysed. The total viable bifidobacteria were enumerated in triplicate by 10-fold serially diluting samples in Wilkins Chalgren broth and plating aliquots on Reinforced Clostridial Agar supplemented with aniline blue (0.03%) as previously described. Plates were incubated at 37 C for 48 hours. The aniline blue and propionic acid in this medium were selective for the bifidobacteria. Pale blue colonies were presumptively identified as bifidobacteria. Results of the total viable bifidobacteria were expressed as cfu per gram (cfu.g<sup>-1</sup>). The *B. breve* was enumerated by quantitative PCR of DNA extracted from the stool samples according to the method of Matsuki et al (2003). Briefly, the DNA was released from washed cell suspensions using lysate buffer (100 mM Tris-HCl, 40 mM EDTA, 1% SDS, pH 9.0), 0.1 mm glass beads and a bead beater and then treated with phenol-chloroform-isoamyl alcohol (25:24:1) prior to precipitation with 3M sodium acetate in 95% ethanol. The *B. breve* specific primer set (BiBRE-1 CCGGATGCTCCATCACAC and BiBRE-2 ACAAAGTGCCTTGCTCCCT) was used, and

in order to enhance specificity, real time PCR conditions were optimised using SsoFast Evagreen (BioRad) as the DNA binding dye instead of SYBR green as used by Matsuki et al. A *B. breve* M-16V strain-specific-primer reported by Schouten et al (2009) was not used due to the potential for cross amplification of *B. breve* other than the M-16V strain. The amplification consisted of a cycle at 98°C for 2 min, 40 cycles of 20 secs at 95°C then 63°C, 72°C for 30 secs, 83°C for 20 secs followed by analysis of melt curves from 65 to 95°C.

### Reference

Patole S, Keil AD, Chang A, Nathan E, Doherty D, Simmer K, Esvaran M, Conway P. Effect of *Bifidobacterium breve* M-16V supplementation on fecal bifidobacteria in preterm neonates--a randomised double blind placebo controlled trial. *PLoS One*. 2014 Mar 3;9(3):e89511.

## **Appendix 2: Approach to statistical analysis of clinical, SCFA and microbiome data**

### **(1) Clinical data**

The analysis was based on the intention to treat principle. Continuous data were summarised using median, interquartile range and range. Categorical data were summarised using frequency distributions. Univariate comparisons for continuous data were made using the Mann Whitney test. For categorical data the Chi-square or Fisher exact test was used. TFF, duration of PN and length of hospital stay were analysed [using survival analysis](#) with Kaplan Meier survival estimates and Cox proportional hazards regression models. [Deaths were censored for time to event analyses to enable the inclusion of their data also in the analysis until the time of death.](#) Data on recruited infants transferred to a peripheral hospital were censored for the length of hospital stay comparison. Hazard ratios (HR) and their 95% confidence intervals (CI) were reported. [Post-hoc sensitivity analysis was performed to assess the consistency of treatment effects when an adjustment for siblings was included.](#) [Results did not change significantly when analyses were adjusted for multiple births \(siblings\).](#) Data analysis was performed using SPSS version 22.0 statistical software (Armonk, NY: IBM Corp).

### **(2) SCFA data**

SCFA measurements were analysed using linear mixed model effects (LME) test to examine statistical differences between the groups over time as well as between the groups at the two time points. In this analysis, subjects were modelled as a random factor and time and treatment as fixed factors. Post-hoc pairwise comparisons between the groups were performed using Tukey's Honestly Significant Difference (HSD) method to adjust for multiple comparisons. A p value <0.05 was considered statistically significant for both analyses.

### **(3) Microbiome data**

All data analyses were conducted with R version 3.6.0. For microbial richness, linear mixed model effects (LME) test (MASS, lme4 and lmerTest packages) was used to identify if there were statistical differences between the groups over time as well as between the groups at the two time points. In our model, subject ID was a random factor, whilst time and treatment were used as fixed factors. Post-hoc pairwise comparisons between the groups were performed using Tukey's HSD method to adjust for multiple comparisons.

Taxa enriched between the groups over time were identified at phylum, family, genus and species levels using the Analysis of Composition of Microbiomes (ANCOM; v2.1)<sup>1</sup>. ANCOM evaluates statistical significance of the taxa or predicted functions using log-ratio transformed data. For this study ANCOM v2.1 code was used which allows for analysis of longitudinal samples and can be adjusted for confounding variable (duration of antibiotic). Cut-off level of 'detected 0.7' was used to accept taxa as significantly enriched across groups and time. To identify differences between the groups at timepoints, Wilcoxon Rank Sum Test with Benjamini-Hochberg correction to adjust for multiple testing was used.

For beta diversity, PERMANOVA was used to check if community structures differed between the groups at the two time points followed by pairwise Adonis test for comparisons between the groups (<https://github.com/bwemheu/pairwise.adonis>). P values were adjusted for multiple testing using the Benjamini-Hochberg correction.

For all analyses, an adjusted P value <0.05 was considered statistically significant.

## References

1. Mandal S, Van Treuren W, White RA, et al. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis* 2015;26:27663.