BMJ Open Gastroenterology

Effect of single versus multistrain probiotic in extremely preterm infants: a randomised trial

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To cite: Athalye-Jape G, Esvaran M, Patole S, *et al*. Effect of single versus multistrain probiotic in extremely preterm infants: a randomised trial. *BMJ Open Gastro* 2022;**9**:e000811. doi:10.1136/bmjgast-2021-000811

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/bmjgast-2021-000811).

Received 18 October 2021
Accepted 12 January 2022

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ABSTRACT

Objective Evidence indicates that multistrain probiotics benefit preterm infants more than single-strain (SS) probiotics. We assessed the effects of SS versus triple-strain (TS) probiotic supplementation (PS) in extremely preterm (EP) infants.

Design EP infants (gestational age (GA) <28 weeks) were randomly allocated to TS or SS probiotic, assuring blinding. Reference (REF) group was EP infants in the placebo arm of our previous probiotic trial. PS was commenced with feeds and continued until 37 weeks' corrected GA. Primary outcome was time to full feed (TFF: 150 mL/kg/day). Secondary outcomes included short-chain fatty acids and faecal microbiota collected at T1 (first week) and T2 (after 3 weeks of PS) using 16S ribosomal RNA gene sequencing.

Results 173 EP (SS: 86, TS: 87) neonates with similar GA and birth weight (BW) were randomised. Median TFF was comparable (11 (IQR 8–16) vs 10 (IQR 8–16) days, p=0.92). Faecal propionate (SS, p<0.001, and TS, p=0.0009) and butyrate levels (TS, p=0.029) were significantly raised in T2 versus T1 samples. Secondary clinical outcomes were comparable. At T2, alpha diversity was comparable (p>0.05) between groups, whereas beta-diversity analysis revealed significant differences between PS and REF groups (both p=0.001). Actinobacteria were higher (both p<0.01), and Proteobacteria, Firmicutes and Bacteroidetes were lower in PS versus REF. Gammaproteobacteria, Clostridia and Negativicutes were lower in both PS versus REF.

Conclusion TFF in EP infants was similar between SS and TS probiotics. Both probiotics were effective in reducing dysbiosis (higher bifidobacteria and lower Gammaproteobacteria). Long-term significance of increased propionate and butyrate needs further studies.

Trial registration number ACTRN 12615000940572.

Summary box

What is already known about this subject?

► Evidence from systematic reviews suggests that multistrain probiotics may benefit enteral nutrition more than single-strain (SS) probiotics in preterm infants. There are limited data on the effect of multistrain probiotic on faecal short-chain fatty acids and microbiome, especially in extremely preterm (EP) infants.

What are the new findings?

► This randomised trial compared the effect of SS versus triple-strain (TS) probiotic on the time to full feeds (TFF) in EP infants. TFF was comparable between the SS and TS probiotic groups. Both probiotics were effective in reducing dysbiosis (higher bifidobacteria and lower Gammaproteobacteria). Long-term significance of increased propionate and butyrate needs further evaluation.

How might it impact on clinical practice in the foreseeable future?

► These results will help in designing future trials comparing SS versus multistrain probiotics in EP infants and assessing the long-term significance of increased propionate and butyrate in early life.

INTRODUCTION

Late-onset sepsis (LOS) and necrotising enterocolitis (NEC ≥stage II) contribute to significant mortality and morbidity, including long-term growth and neurodevelopment in preterm infants, especially those born before 28 weeks' gestation.[1–3] Recently, NEC and LOS have been shown to be preceded by gut dysbiosis.[4–6] Preterm infants are at a high risk of gut dysbiosis due to gut immaturity further complicated by environmental exposures (eg, mode of delivery, chorioamnionitis and neonatal intensive care), feeding intolerance and antibiotic exposure.[7] Probiotic supplementation (PS) has been proposed to reduce the risk of dysbiosis.[5 8]

Probiotics are live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host.[9] Systematic reviews of randomised controlled trials (RCTs) and non-RCTs have shown that probiotics significantly reduce the risk of all-cause mortality ((relative risk (RR) 0.76, 95% CI 0.65 to 0.89; 51 trials, n=10 170 infants; I^2=0%; level of evidence (LoE): moderate),[10] NEC (RR: 0.54, 95% /CI 0.45 to 0.65; 54 RCTs,

n=10 604 infants; I^2=17%; LoE: low),[10] LOS (RR 0.89, 95% CI 0.82 to 0.97; 47 trials, n=9762 infants; I^2=19%; LoE: moderate)[10] and duration of hospitalisation while facilitating enteral nutrition in preterm infants.[10–12] The mechanisms of benefits of probiotics include colonisation and normalisation of perturbed intestinal microbial communities, competitive exclusion of pathogens, bacteriocin production, increasing mucin production, modulating intestinal innate immunity and production of short-chain fatty acids (SCFAs), which strengthen the gut epithelial barrier and mediate anti-inflammatory, antimicrobial and immunomodulatory effects.[13 14] Probiotics, particularly bifidobacteria, specifically use human milk oligosaccharides and facilitate establishment of a bifidobacteria dominant gut ecosystem while inhibiting pathogenic micro-organisms.[15]

Evidence suggests that a mixture of probiotic strains may confer more benefits compared with single-strain (SS) probiotics.[16–18] Multistrain probiotics containing bifidobacteria have been shown to be effective in preventing NEC, LOS and other morbidities in preterm infants.[19] Ishizeki *et al* have reported that in preterm very low birthweight (VLBW) infants, supplementation with a mixture of three strains (5×10^8 colony-forming unit (CFU) of each strain, three strains/intervention group) for 6 weeks significantly increased and prolonged detection rates and colony counts of faecal bifidobacteria compared with the SS *Bifidobacterium breve* M-16V group.[20]

Based on the evidence in totality and the results of our clinical trial of SS (*B. breve* M-16V) supplementation, we have been providing routine probiotic prophylaxis using this strain for all preterm infants born <34 weeks' gestation since 2011.[21 22] Considering that multistrain probiotics may be better than SS probiotics, we decided to study this issue in our population of preterm infants. Probiotics are known to improve gut motility and feeding intolerance in preterm infants.[23 24] Rapid attainment of full feeds is associated with shorter hospital stay, improved postnatal growth and potentially improved long-term neurodevelopmental outcomes.[16 18 25 26] Given these data, we focused on the time to full feeds (TFFs) in our study comparing SS with multistrain bifidobacteria probiotic. We also aimed to assess gut microbiota and faecal SCFA as potential pathways of benefits of probiotics.

METHODS

Hypothesis and aim

Our primary aim was to assess the TFF in extremely preterm (EP, gestation <28 weeks) infants supplemented with either an SS or triple-strain (TS) probiotic. Secondary aims included faecal SCFA and microbiota in the SS and TS probiotic groups. We hypothesised that (1) compared with SS, the TS probiotic will reduce TFF by improving gut motility; (2) infants supplemented with SS or TS probiotic will significantly reduce dysbiosis compared with EP infants who received placebo in our previous probiotic trial serving as the reference (REF) group (online supplemental appendix 1).[22]

Participant recruitment

Design and setting: A double-blind RCT in EP infants in our tertiary neonatal intensive care unit.

Eligibility criteria: (1) gestation of <28 weeks, (2) readiness to commence on feeds/on feeds for <12 hours and (3) informed parental consent.

Exclusion criteria: (1) congenital malformations, (2) chromosomal aberrations, (3) not being ready for feeds/on feeds for ≥12 hours.

SS probiotic group: *B. breve* M-16V (3×10^9 CFU/day).

TS probiotic group: mixture of *B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536 (3×10^9 CFU/day).

REF (ie, no probiotic) group: EP infants from the placebo arm of a previous RCT were used as a REF group only for the microbiome analysis in this study.[22]

Valid comparison: The comparison of SS versus TS probiotic was robust as (1) the SS (*B. breve* M-16V) was one of the components of the TS product, and (2) the total probiotic dose (3×10^9 CFU/day) was identical in both groups.

Rationale for selecting the three *Bifidobacterium* strain products: This was based on clinical and preclinical studies reporting benefits of multistrain probiotic, particularly of *Bifidobacterium* species in preterm infants.[18 19 27 28]

Sample size: The mean (±SD) TFF in EP infants was 24 (±14.8) days in our unit.[22] Sample size of 75 neonates per group was estimated to achieve 80% power (alpha 0.05) to detect a 30% reduction (clinically significant) in TFF in the SS versus TS probiotic group. To allow for attrition (15%), the sample size was increased to 172.

Primary outcome: TFF measured as the time to reach 150 mL/kg/day feeds from the time feeding was commenced.

Secondary outcomes:

Clinical: NEC ≥stage II, all-cause mortality, duration of parenteral nutrition (PN), length of hospital stay, LOS, intestinal transit time (ITT) using carmine red dye[29] and growth at discharge.

Laboratory based: (1) faecal SCFA levels assessed by modified gas chromatography–mass spectrometry[30]; (2) faecal microbiota assessed using 16S ribosomal RNA gene sequencing. A subset of samples was assessed using next-generation sequencing.

Safety: (1) sepsis due to administered bifidobacteria; (2) abdominal distension, diarrhoea and vomiting leading to cessation of PS. An independent data safety committee monitored all outcomes from enrolment until death or corrected gestational age (CGA) of 37 weeks.

Preplanned subgroup: infants small for gestational age (SGA: birth weight (BW) <10th centile for gestational age (GA)) due to intrauterine growth restriction, considering they are at high risk of mortality and morbidities (eg, NEC, LOS and feed intolerance).[31 32]

Randomisation, allocation concealment and blinding

Group allocation was based on computer-generated randomisation sequence in random block sizes of 2 and 4. Opaque, sealed and coded envelopes were used for randomisation. Allocation concealment was optimised by prescribing allocation only after obtaining informed parental consent and recording basic neonatal data. The clinical trial pharmacist supplied the randomisation sequence and the sachets (identical design, weight, smell and taste) containing either the SS (*B. breve* M-16V, 6×10^9 /g sachet) or the TS (*B. breve* M-16V, *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *longum* BB536; 2×10^9 of each strain/g sachet) probiotic manufactured by Morinaga Milk Industry Co., Japan, to the nursing staff. This assured masking of all investigators, including outcome assessors, nursing staff and parents.

Probiotic protocol

When ready for feeds, enrolled infants were supplemented with freshly reconstituted contents of the allocated sachets every day and continued until CGA of 37 weeks. The dry lyophilised powder in the sachets was reconstituted using mum's own milk (first choice) or sterile water for injection. During reconstitution, care was taken to reduce the risk of cross-contamination by adhering to strict hand hygiene, separate preparation of individual doses and avoiding contact with indwelling central lines, tubes and catheters. The single dose (1.5×10^9 CFU/day as 1 mL of the reconstituted solution) was given via the feeding tube until reaching feeds of 50 mL/kg/day.[21 22] It was increased thereafter to 3×10^9 CFU/day (1 mL reconstituted solution two times per day) once feeds exceeded 50 mL/kg/day. Considering the risk of probiotic sepsis, supplementation was discontinued when feeds were stopped for suspected or proven sepsis and NEC.

Data handling, storage and confidentiality

The National Health and Medical Research Council (NHMRC) Australian guidelines were followed for confidentiality and data storage.[33]

Reporting

The revised Consolidated Standards of Reporting Trials (CONSORT) guidelines[34] were used for reporting the results as highlighted on the EQUATOR network (https://www.equator-network.org/reporting-guidelines/, accessed October 2021).

Faecal sample collection, DNA extraction, SCFA assessment and microbiota analysis details are included in online supplemental appendix 1.

Approach to statistical analysis of clinical, SCFA and microbiome data are included in online supplemental appendix 2.

RESULTS

A total of 173 neonates were randomised (SS: 87, TS: 86) between September 2015 and May 2017. Figure 1 outlines the CONSORT flow diagram. Maternal and neonatal demographic characteristics were comparable between the two probiotic groups (table 1).

Primary outcome (TFF: 150 mL/kg/day)

The median TFF was comparable between the SS and TS groups (11 (IQR 8–16) vs 10 (IQR 8–16) days; HR 1.02, 95% CI 0.74 to 1.40; p=0.920).

Secondary outcomes

Clinical

There were no significant differences between the SS and TS groups in all-cause mortality (12/86 (14%) vs 8/87 (9.2%), p=0.328); NEC ≥stage II (3/86 (3.5%) vs 3/87 (3.4%), p=1.000); time until NEC or death (HR 0.59, 95% CI 0.24 to 1.46; p=0.253); median duration of PN (10 days in both groups, p=0.265); hospital stay (114 vs 116 days, p=0.750); suspected LOS (44/85 (51.8%) vs 41/86 (47.7%); p=0.593); and blood culture positive LOS (21/85 (24.7%) vs 15/86 (17.4%), p=0.244). Median ITT was comparable (17 hours vs 18 hours, p=0.826). Two infants in the SS group needed surgery for NEC compared with none in the TS group.

Of the 20 deaths in the enrolled infants, five occurred before commencing trial supplementation (SS: 3, TS: 2). Median age at death was similar between groups (10 (IQR 6–24) vs 10 (IQR 4–20) days, p=0.740).

There were no significant differences between the SS and TS groups for other neonatal outcomes such as anthropometry at discharge or incidence of postnatal growth restriction (table 2). Results did not change significantly when analyses were adjusted for multiple births. A 'per protocol' analysis including only infants who had at least one treatment dose showed no change in results.

Safety

There were no cases of probiotic sepsis or related adverse effects during the trial period.

Laboratory based

Faecal SCFA

Total fatty acid levels were comparable between the groups at both time points (T1, p=0.92, and T2, p=0.151). Levels were higher in the SS (T2: median 885 (IQR 501–1643) vs T1: median 557 (IQR 290–919) µg/g of wet faeces, p=0.059) and the TS (T2: median 1189 (IQR 674–1825) vs T1: median 422 (IQR 245–1291) µg/g of wet faeces, p=0.014) groups, reaching significance only in the TS group (p=0.014) but not in the SS group (p=0.058). Propionic acid levels were significantly higher at T2 compared with T1 in both SS (T2: median 112 (IQR 34–249) vs T1: median 2 (IQR 1–2), p<0.001) and TS (T2: median 123 (IQR 26–241) vs T1: median 2 (IQR 1–12), p<0.001) groups, whereas butyric acid levels were significantly higher at T2 compared with T1 only in the TS group (T2: median 8 (IQR 2–263) vs T1: median 1 (IQR 1–3), p=0.029) but not in the SS group

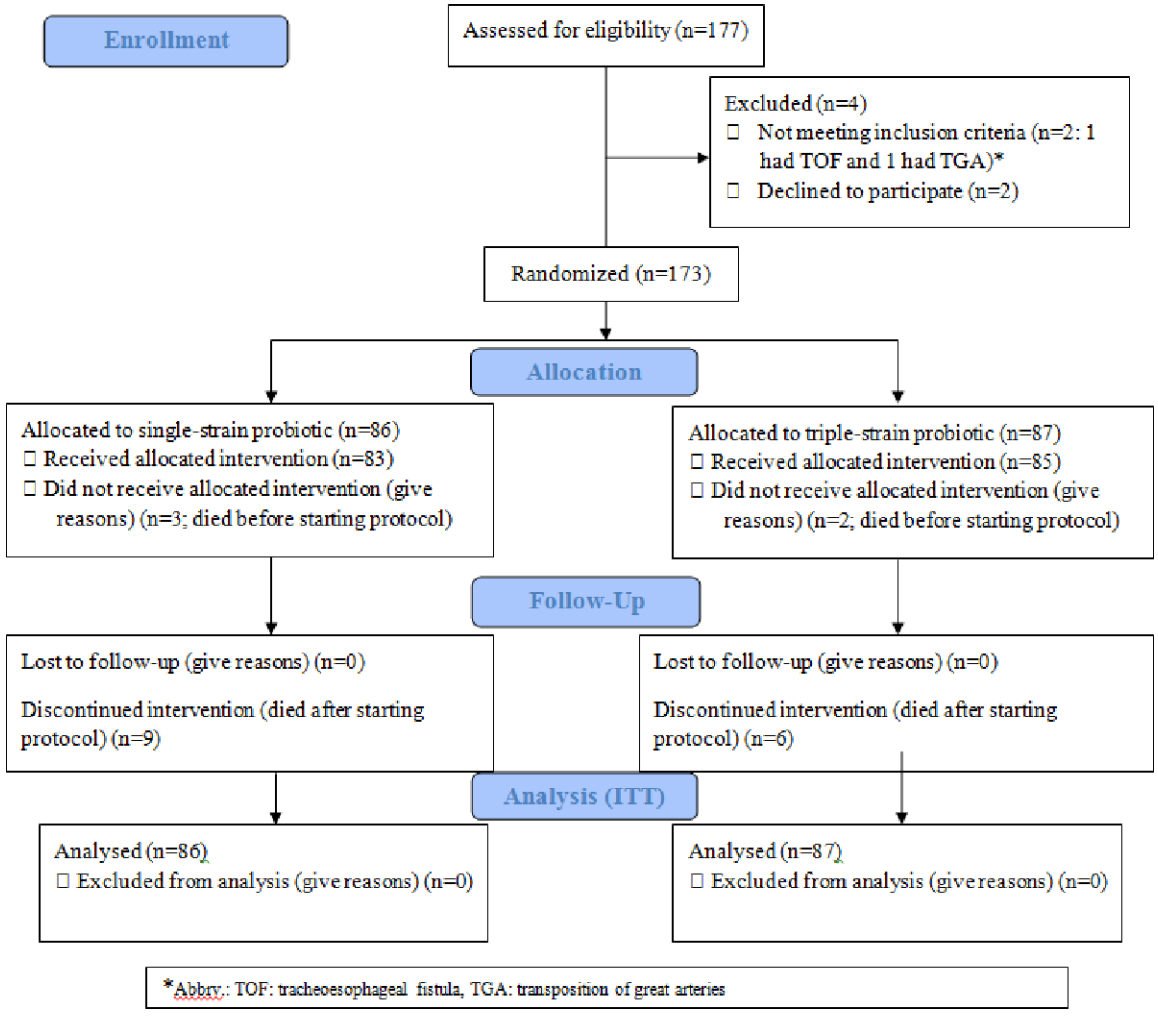

Figure 1 Consolidated Standards of Reporting Trials flow diagram showing details of the study recruitment. TGA, Transposition of Great Arteries; TOF, Tracheo-oesophageal fistula; ITT: Intention To Treat.

(T2: median 8 (IQR 3–111) vs T1: median 2 (IQR 1–3), p=0.188) (figure 2A–F).

Subgroup analysis

Clinical outcomes were comparable in the subgroup of 12 SGA infants (six per group) (online supplemental table 1). Highly variable SCFA levels and small numbers in each group made it difficult to reach any conclusion. Linear mixed effects model test showed non-significance for all cross-sectional and longitudinal analyses.

Faecal microbiome analysis

Prior to using EP infants in the placebo arm from our previous clinical trial[35] as the REF group, diversity analyses were conducted to ensure that faecal community structures of infants at T1 from both studies were similar (online supplemental figures 1 and 2). Alpha-diversity measures: Simpson (REF vs SS, p=0.84; REF vs TS, p=0.84), Shannon (REF vs SS, p=0.74; REF vs TS, p=0.63), ACE (REF vs SS, p=0.27; REF vs TS, p=0.29) and Chao (REF vs SS, p=0.20; REF vs TS, p=0.20) showed no differences (online supplemental figure 1A–D). Beta-diversity measures: weighted Unifrac (REF vs SS, p=0.114; REF vs TS, p=0.429) and Bray Curtis (REF vs SS, p=0.204; REF vs TS, p=0.325) analyses demonstrated that infants from both studies had similar community structures at T1 (online supplemental figure 2A,B).

Richness and diversity

Alpha-diversity analysis showed all groups (REF, SS and TS: SiMPro) had significantly increased bacterial richness at T2 versus T1 (all p<0.001, online supplemental figure 3A,B). However, increased bacterial evenness was observed in infants only in the SS group (p<0.001; online supplemental figure 3C,D). At T2, bacterial richness indices were comparable between all groups (ACE (REF vs SS, p=0.38; REF vs TS, p=0.93; SS vs TS; p=0.38) and Chao1 (REF vs SS, p=0.54; REF vs TS, p=0.58; SS vs TS, p=0.58); figure 3A–D). Alpha-diversity measures showed no difference in within-group variability between REF and SiMPro groups (Shannon (REF vs SS, p=0.79; REF vs TS, p=0.17) and Simpson (REF vs SS, p=0.70; REF vs TS, p=0.16); figure 3C,D). However, TS demonstrated reduced within-group variability compared with SS (Shannon, p=0.02; Simpson, p=0.005; figure 3C,D).

Table 1 Baseline characteristics of probiotic supplemented (SS and TS) and REF groups

	TS group N=87	SS group N=86	REF group (Probiotics and NeonaTes Study (PANTS) placebo N=29	P value (TS/SS vs REF)
Gestation (weeks)	26.3 (24.7–27.1)	26.2 (24.4–27.2)	26.1 (25.2–26.9)	0.990
Birth weight	870 (700–1050)	828 (679–971)	810 (685–970)	0.412
Male	46 (52.9)	48 (55.8)	16 (55.2)	0.933
Caesarean delivery	47 (54.0)	52 (60.5)	13 (44.8)	0.214
Maternal antibiotics*	30 (34.5)	29 (33.7)	14 (48.3)	0.142
Early-onset sepsis				
Suspected	84 (96.6)	82 (95.3)	29 (100)	0.596
Proven	7 (8.0)	4 (4.7)	2 (6.9)	1.000
Courses	2 (2–2)	2 (2–2)	2 (2–2)	0.878
Days of exposure	3 (3–4)	4 (3–5)	4 (3–6)	0.388
Late-onset sepsis	N=86	N=85		
Suspected	41 (47.7)	44 (51.8)	19 (65.5)	0.115
Proven	15 (17.4)	21 (24.7)	7 (24.1)	0.708
None	71 (82.6)	64 (75.3)	22 (75.9)	0.932
1 episode	14 (16.3)	17 (20.0)	6 (20.7)	
2+ episodes	1 (1.2)	4 (4.7)	1 (3.4)	
Antibiotic courses	3 (2–4)	2 (2–4)	2 (2–3)	0.425
Days of exposure†	8 (5–17)	9 (5–10)	8 (5–14)	0.702
Mortality	8 (9.2)	12 (14.0)	0 (–)	0.085
NEC ≥stage II	3 (3.4)	3 (3.5)	0 (–)	0.597
Age commenced probiotic supplementation (days)	3 (2–4) N=85	3 (2–4) N=83	7 (5–10)	<0.001
Faecal specimen collection				
First sample taken	84/87 (96.6)	82/86 (95.3)	29 (100)	0.395
Postnatal (PN) age (days)	5 (3–7)	5 (3–6)		
Second sample taken	79/87 (90.8)	75/86 (87.2)	29 (100)	0.081
PN age (days)	24 (22–27)	24 (22–27)		

P values represent the comparison between the combined SS/TS groups and the REF group.
*Maternal antibiotic exposure: based on chorioamnionitis and PPROM.
†Data represent median and 25th–75th percentile Kaplan-Meier survival estimates.
‡Data represent number (%).
§Median and IQR.
NEC, necrotiing enterocolitis; PN, parenteral nutrition; PPROM, Preterm prolonged rupture of membranes; REF, reference; SS, single-strain; TS, triple-strain.

Beta-diversity analysis showed significant difference in bacterial community structure of all groups at T2 versus T1 (all p<0.05, online supplemental figure 3E–I). At T2, community structures were significantly different in REF versus both SiMPro groups (SS vs REF: R^2=0.135, p=0.001; TS vs REF: R^2=0.194, p=0.001; figure 3E) but comparable between SiMPro groups (p=0.149, figure 3E). Community structure in groups was not affected by factors such as ethnicity, gender, mode of delivery and GA but was influenced by duration of antibiotic exposure (PERMANOVA: ethnicity, p=0.93; gender, p=0.50; delivery, p=0.677; GA, p=0.109; duration of antibiotic, p=0.001; online supplemental figure 4).

Relative abundance (RA) of bacterial taxa

Actinobacteria, Proteobacteria, Bacteroides and Firmicutes were the most prevalent phyla in the faecal samples (figure 4B). Analysis of Composition of Microbiomes with bias correction (ANCOM) analyses revealed Actinobacteria to be significantly enriched in the SiMPro groups compared with the REF group at T2 (online supplemental table 2). At T2, SiMPro groups had increased RA

Table 2 Primary and secondary outcomes

	TS group (N=87)	SS group (N=86)	P value
Primary outcome			
Time to full enteral feeds of 150 mL/kg/day (days)*	10 (8–16)	11 (8–16)	0.920
Secondary outcomes	N (%)	N (%)	
All-cause mortality†	8 (9.2)	12 (14.0)	0.328
Definite NEC†	3 (3.4)	3 (3.5)	1.000
Total Parenteral Nutrition (TPN) duration (days)*	10 (8–16)	8–15	0.693
Intestinal transit time (hours)‡ (N_A=77, N_B=75)	18 (12–24)	17 (13–24)	0.826
Length of hospital stay (days)*	116 (91–136)	114 (105–137)	0.750
Early-onset sepsis†			
Suspected	84 (96.6)	82 (95.3)	0.720
Proven	7 (8)	4 (4.7)	0.360
Duration of antibiotics (days)‡	3 (3–4)	4 (3–5)	0.757
Late-onset sepsis (SS: 85, TS: 86)			
Suspected†	41 (47.7)	44 (51.8)	0.593
Age at first episode (days)‡	12 (8–27)	11 (6–26)	0.606
Total episodes‡	1 (1–3)	1 (1–2)	0.449
Duration antibiotics (days)‡	8 (4–14)	5 (3–12)	0.144
Proven†	15 (17.4)	21 (24.7)	0.244
Age at first episode (days)‡	17 (10–27)	17 (13–31)	0.910
Total episodes‡	1 (1–1)	1 (1–1)	0.285
Duration antibiotics (days)‡	7 (5–16)	8 (4–10)	0.478
Human milk fed†	87 (100)	86 (100)	–
Antibiotic courses	4 (2–7)	4 (2–6)	0.696
Exposure to antibiotics (days)*	8 (5–17)	9 (5–10)	0.820
Age probiotic commenced (days)	3 (2–4)	3 (2–4)	0.772
Duration of probiotic supplementation (days)	71 (63–80)	70 (63–87)	0.545
Discharge anthropometry			
Weight z-score§	−0.70 (0.98)	−0.72 (1.06)	0.922
Length z-score§	−0.88 (1.35)	−1.28 (1.76)	0.144
Head Circumference (HC) z-score§	−0.09 (1.9)	−0.17 (1.2)	0.745
PN growth restriction†	17/85 (20)	15/83 (18.1)	0.750

*Data represent median and 25th–75th percentile Kaplan-Meier survival estimates,.
†Number and percentages.
‡Median and IQR.
§Represents data as mean and SD.
NEC, necrotising enterocolitis; PN, parenteral nutrition; REF, reference; SS, single strain; TS, triple strain.

of Actinobacteria compared with the REF group (both p<0.001, online supplemental table 2). Although not significant, SiMPro groups also exhibited decreased RA of Proteobacteria (SS (median 51.7, IQR 38.3–69.8) and TS (median 50.2, IQR 37.2–66.6) compared with the REF group (median 73.2, IQR 60.8–86.5). RA of Firmicutes (SS (median 11.4, IQR 3.9–27.9) and TS (median 10.4, IQR 6.5–21.9) was also reduced in the SiMPro group versus the REF group (median 20.3, IQR 12.4–33.6). At class level, ANCOM analyses revealed Actinobacteria to be significantly different between the groups (online supplemental table 3). At T2, ANCOM analysis showed both SiMPro groups to have significantly increased RA of Actinobacteria (both p<0.0001, figure 4A). Although not significant, Clostridia levels were reduced in both SS (median 0.01, IQR 0–3.04) and TS (median 0, IQR 0–0.01) compared with REF (median 2.95, IQR 0.14–9.41). In addition, Gammaproteobacteria levels were reduced in SS (median 51.7, IQR 38.3–69.8) and TS (median 50.2, IQR 37.7–66.6) compared with REF (median 73.1, IQR 60.8–86.5) (online supplemental table 3). Potentially pathogenic families of Clostridiaceae and Streptococcaceae

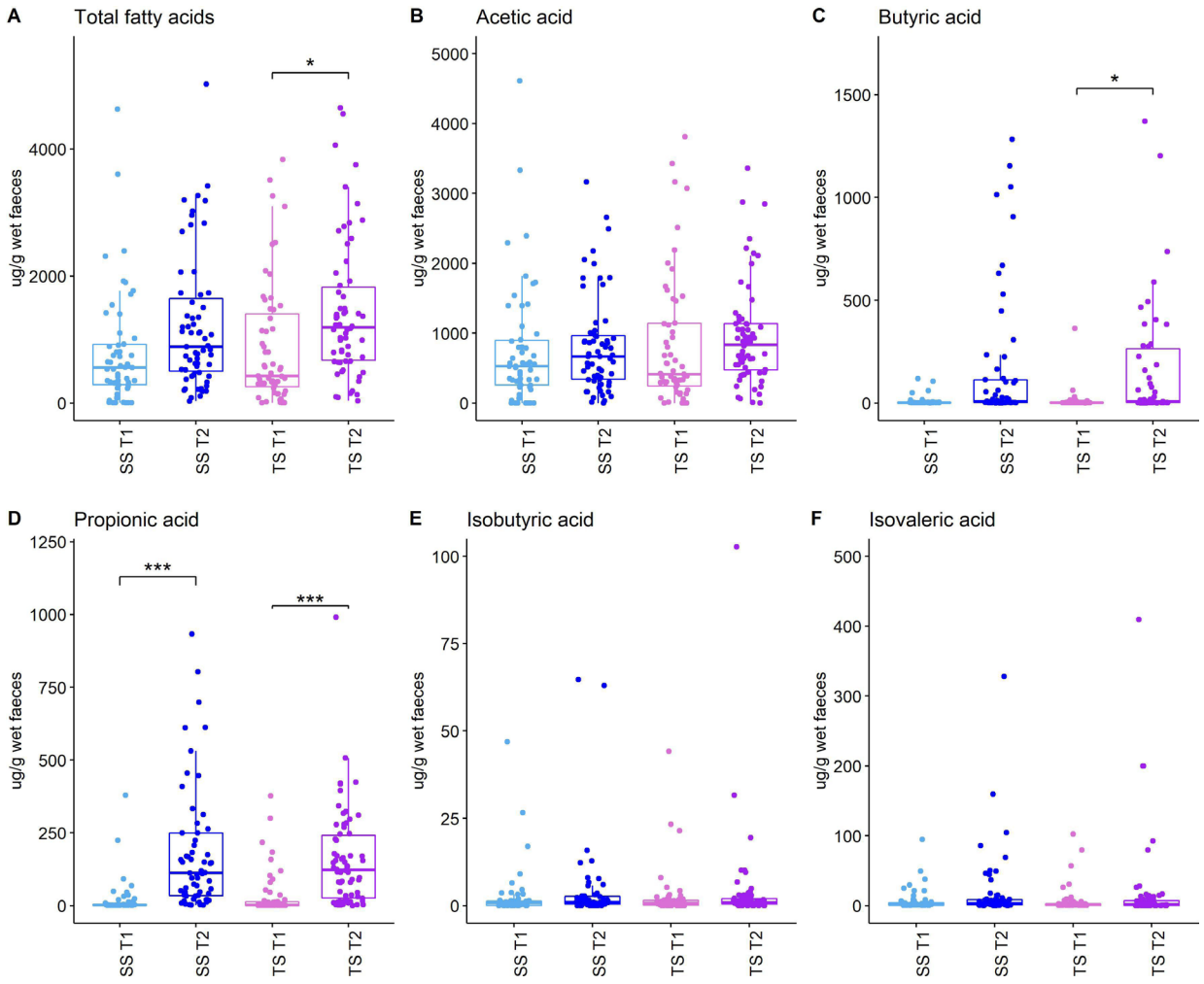

Figure 2 Combined SCFA (butyric acid, propionic acid and acetic acid) and BCFA (isobutyric and isovaleric acid) for all groups. SCFA and BCFA levels. SCFA (acetic acid (B), butyric acid (C) and propionic acid (D)) and BCFA (isobutyric (E) and isovaleric acid (F)) levels in SimPro groups (SS and TS). Box shows IQR; the line, median and the dots represent an individual sample. Differences between groups and time were calculated using linear mixed effects test with Tukey correction to adjust for multiple testing. Significant differences are indicated by *p<0.05, ***p<0.001. BCFA, branched-chain fatty acid; SCFA, short-chain fatty acid; SS, single strain; TS, triple strain.

were significantly lower in both SiMPro groups and REF group at T2 (all p<0.05, online supplemental table 4), while Bifidobacteriaceae (both p=0.00062) was increased (online supplemental table 4). At the genus level, the SiMPro groups had significantly increased RA of *Bifidobacterium* (all p<0.0001, figure 4C) and decreased RA of *Streptococcus* (both p<0 .0001, figure 4C) and *Clostridium sensu stricto 1* (both p<0 .0001, figure 4C) compared with the REF group (online supplemental table 5). At the species level (using metagenomics data), 28 species were found to be differentially distributed; most of them were of *Bifidobacterium* species (online supplemental table 6). Of these, 14 species had a mean RA of >0.01% (figure 4D). The REF group was enriched in *Clostridium butyricum*, *Streptococcus salivarius* and *S. thermophilus* (all p<0.01) compared with the SiMPro groups. At this level, differences were found in the SiMPro groups. At T2, the SS group had significantly higher *B. breve* (p<0.01) and *B. bifidum* (p<0.05) compared with the TS group, whereas the TS group had significantly increased *B. longum* (p=0.005), *B. longum* CAG:69 (p=0.012), *B. reuteri* (p=0.024), *B. pseudocatenulatum* CAG:263 (p=0.043), *B. pseudocatenulatum* (p=0.047), *S. pyogenes* (p=0.005) and *Gardnerella vaginalis* (p=0.048) compared with SS group (figure 4D). Subspecies analysis revealed that the SiMPro groups had significantly increased *B. longum* subsp. *infantis* and *B. longum* subsp. *longum* (all p<0.0001) versus the REF group. However, the TS group had significantly increased *B. longum* subsp. *infantis* (p=0.0024) and *B. longum* subsp. *longum* (p=0.0001) compared with the SS group (figure 4E).

DISCUSSION

The results of our double-blind RCT conducted exclusively in EP infants showed that TFF was comparable in SS and TS bifidobacteria-supplemented groups. Furthermore, there were no significant differences in NEC ≥stage II, LOS, all-cause mortality, duration of hospitalisation and ITT. Both groups showed comparable SCFA levels. At T2, propionic

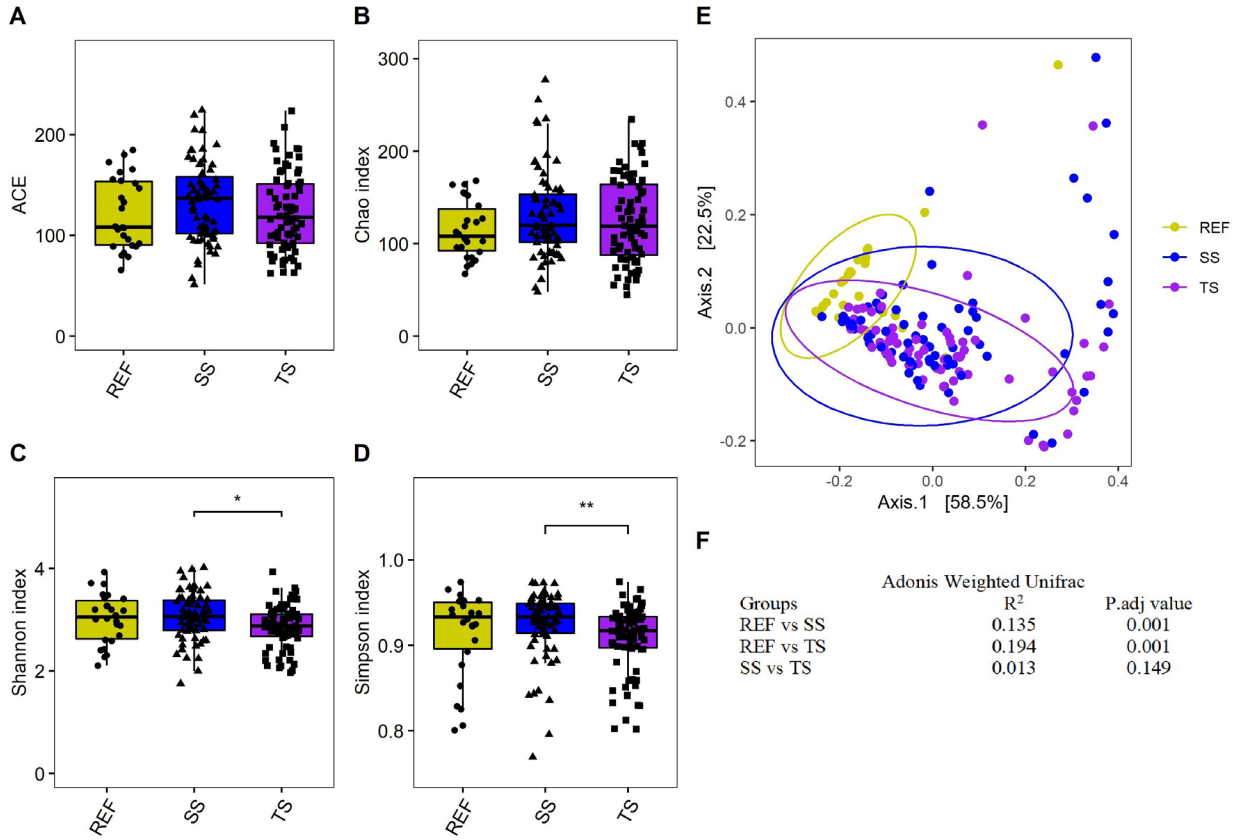

Figure 3 (A–I) Alpha and beta diversity at T2. Box plots of bacterial richness measures; Ace (A) and Chao1 (B) and alpha evenness measures; Shannon (C) and Simpson index (D). Box shows IQR; the line, median and the error bars; the range and the dots are outliers. Differences between groups were calculated using Wilcoxon rank-sum test with Benjamini-Hochberg correction to adjust for multiple testing. Significant differences are indicated by *p<0.05, ** <0.01. (E) Principal coordinate analysis based on weighted Unifrac distances. Each sample is depicted as a dot. (F) Permutational Analysis of Variance (PERMANOVA) was used to identify if there were differences in the community structures by group and time followed by pairwise Adonis test for comparisons between the groups. REF, reference; SS, single strain; TS, triple strain.

acid levels were significantly higher in both SiMPro groups, whereas butyric acid levels were significantly higher only in the TS group. When compared with the REF group (placebo arm of the PANTS trial), both SiMPro groups showed significantly higher bifidobacteria and lower Gammaproteobacteria. Microbial profiles were different at species levels between the SS and TS groups. SiMPro supplements were well tolerated without any adverse effects, including sepsis, due to the administered probiotic strains.

Our study showed no significant difference in the median (IQR) TFF between the infants in the SS and TS groups. It is important to note that sample size for this study was based on the mean (±SD) TFF (24±14.8 days) from our previous placebo controlled RCT assessing product quality and effect of *B. breve* M-16V supplementation on faecal bifidobacteria counts before introducing routine PS from June 2012.[22] Updated data showed significant reduction in mean (±SD) TFF (12±6.5 days) following routine probiotic supplementation (RPS) with *B. breve* 3 M-16V in infants of <29 weeks' gestation.[21] Using the new estimate of TFF, the SiMPro trial has 95% power to detect a 30% reduction in TFF as desired originally during planning.

It is possible that the median duration of 10–11 days represents the shortest possible TFF considering the strategies for optimising enteral nutrition of EP infants in our unit.[35–37] However, it is equally possible that TS probiotic was not superior in reducing TFF compared with SS, or its effect size was smaller than expected. It is important to note that other investigators have reported TFF of 11±3.6,[38] 12 (9–16)[39] and 14 (10–22)[40] days in very preterm infants. Boscarino *et al* concluded that high-energy intake administered through the enteral route was positively correlated to cerebral growth, whereas energy intake via the parenteral route resulted in poorer cerebral growth.[41]

Gómez-Rodriguez *et al* conducted an RCT assessing the effect of SS versus multistrain probiotic in 90 very preterm infants.[42] Median TFF was 18 (0–56) days vs 15 (0–39) days for the SS group versus multistrain group, respectively. NEC incidence and faecal sIgA levels were comparable between groups.[42] Compared with their study,[42] exposure to antenatal steroids was higher, and incidence of caesarean delivery, median gestation and BW of study participants and median duration of antibiotics for LOS was lower in our trial. Additionally, median

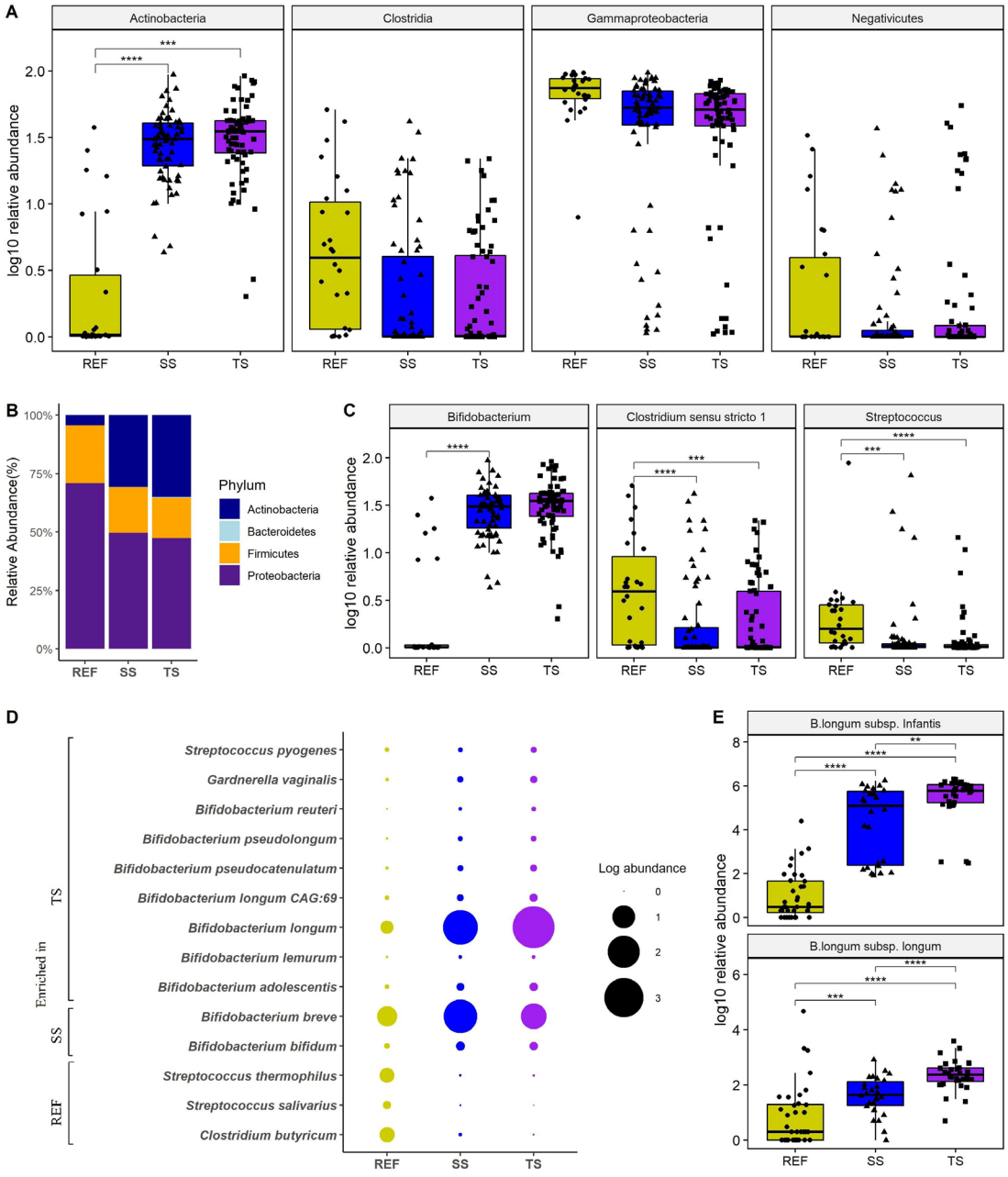

Figure 4 (A–E) Infant gut microbiota by taxa at T2. Box plots showing RA of class (A), genus (C) and subspecies (E). Box shows IQR; the line, median and the dots represent an individual sample. (B) Shows phylum level composition. (D) Bubble plot of significantly different Species with RA >0.01%. Differences between groups were calculated using Wilcoxon rank-sum test with Benjamini-Hochberg correction to adjust for multiple testing. Significant differences are indicated by **p<0.01, ***p<0.001, ****p<0.0001. RA, relative abundance; REF, reference; SS, single-strain; TS, triple-strain.

TFF was significantly shorter (15–18 days vs 10–11 days); postnatal age at starting probiotics was earlier (5 days vs 3 days); and exclusive human milk feeding was higher. Priyadarshi *et al* reported no difference in NEC, TFF and LOS in their retrospective observational study comparing 180 very preterm (gestation <32 weeks) infants (two-strain probiotic group: *B. bifidum*+*Lactobacillus acidophilus*, 1×10^9 CFU each) vs 196 very preterm infants (SS: *B. breve* M-16V, 2.5×10^9 CFU/day). TFF was 17.4±11 vs 15±9 days for the two-strain and SS groups, respectively.[43]

A Cochrane review in 2014 had reported that probiotics reduce the risk of NEC in preterm infants.[12] The updated (year 2020) Cochrane review (56 RCTs, n=10 812) also found that probiotics reduce the risk of NEC in very preterm (VP) VLBW infants (evidence grade: 'low certainty'; RR: 0.54, 95% CI 0.45 to 0.65). Trials using multistrain probiotics showed larger effect size for NEC.[10] Evidence for LOS and mortality was graded as 'moderate certainty'.[10] Furthermore, in their systematic review and network meta-analysis, Morgan *et al* reported moderate to high evidence for superiority of combinations of probiotics containing one or more *Lactobacillus* or *Bifidobacterium* compared with other multistrain probiotics.[18] It is important to note that probiotic effects are

strain-specific, and different strains in a mixture can have synergistic, compatible or antagonistic effects.[12 27 44] The reported improved efficacy of multistrain probiotics could simply be due to the higher cumulative dose. Hence, ideal comparison involves identical total dose in SS versus multistrain arms of the trial where SS is also a component of the multistrain probiotic. Our trial involves such a comparison.

Comparing our results with previous studies of SCFA in preterm infants exposed to probiotics is important.[45 46] Wang *et al* randomised 66 preterm infants (extremely low birth weight (ELBW), VLBW, low birth weight: 22 per group) to receive probiotic (*B. breve* M-16V, 1.6×10^8 CFU two times per day) or no probiotic (control).[45] Compared with birth, faecal acetate and total SCFA were significantly higher at 2 and 4 weeks, but butyric acid levels were significantly lower.[45] Considering the differences in eligibility criteria (ELBW vs EP), method of SCFA assay and units of measurement, comparing results of the 22 ELBW infants in Wang *et al* with 86 infants (SS group) in our study is difficult. Infants in our study received exclusive human milk diet and higher probiotic dose for longer duration.

Long-term follow-up of our cohort is important, considering the clinical significance of increased propionate and butyrate levels (reduced allergy, asthma, obesity, metabolic syndrome and improved neurodevelopment).[47–49] SCFAs benefit through their influence on Treg biology, epithelial integrity, gut homeostasis, dendritic cell biology, gene transcription and IgA antibody responses.[13 14] Gut microbiota may regulate neurodevelopment and neurobehaviour by various mechanisms including SCFA modulation through the gut–brain axis.[49]

To our knowledge, there are no previous RCTs reporting on faecal microbiota of SS versus multistrain probiotics in preterm infants. Previous RCTs of SS[40 50 51] or multistrain probiotic[39] versus placebo have reported variable effects on gut colonisation. Previous studies have reported increased Proteobacteria, Firmicutes and coagulase-negative staphylococci (CONS) in infants with NEC.[4] Both SiMPro groups had decreased Gammaproteobacteria and Clostridia and reduced CONS, especially in the TS group versus REF group.

To our knowledge, SiMPro is the first RCT with robust design for comparing SS and multistrain probiotics with adequate power (95%) for a clinically important primary outcome (TFF) in EP infants.[41] Our comprehensive microbiome analysis was based on 16SrRNA gene and metagenomic sequencing. Our trial was not powered for NEC as primary outcome considering its low incidence in our unit.[21 22] SiMPro groups had significantly increased levels of *Bifidobacterium* at T2 versus T1 compared with REF clearly demonstrating effect of PS on infant gut microbiome. The absence of a placebo arm in our RCT due to ethical difficulties was another limitation. Although we used the placebo arm of the PANTS study as our REF group, we cannot discount the possibility of *batch effect* as a confounder in our study. Furthermore, this also resulted in being unable to provide REF SCFA levels in EP infants not receiving probiotic. Previous studies are not helpful in this context due to methodological differences.[45 46]

In conclusion, TFF and other clinical outcomes in EP infants were similar between SS and TS strain probiotics. The long-term significance of raised propionate and butyrate needs to be studied.

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Acknowledgements We sincerely thank the following: Professor Fumiaki Abe and Noriyuki Iwabuchi of Morinaga Milk Industry, Japan, for providing trial supplements free of cost; Annie Chang, Melanie McDougall and Chooi Yen Kok: nursing research assistants, data collection, recruitment, sample collection and preparation for storage; Nabeelah Mukadam and Michael Petrovski, KEMH pharmacy department; Dr J Tan, consultant neonatologist at Princess Margaret and Perth Children's Hospital for his role on the data monitoring committee for the study duration; nursing staff at King Edward Memorial and Princess Margaret Hospital for collecting stool samples and administering the trial supplements to study infants; parents for providing informed consent for participation of their infants in the randomised trial; Mr Rikky Purbojati, Nanyang Technological University, Singapore, and Dr Daniela Moses, Deputy Research Director, Meta-'omics and Microbiomes, Nanyang Technological University, Singapore: assistance with bioinformatics analysis and interpretation. Dr J Tan and Dr R Jois, Department of Neonatology, Joondalup Health Campus (JHC), Perth, Western Australia: assistance with trial protocol continuation at JHC in trial participants who were transferred before 37 weeks completed GA and for provision of relevant data at discharge. Dr M Deshmukh, Dr. J Du-Plessis and Dr S Mehta, Department of Neonatology, Fiona Stanley Hospital (FSH), Perth, Western Australia: assistance with trial protocol continuation at FSH in trial participants who were transferred before 37 weeks completed GA and for provision of relevant data at discharge.

Contributors GJ contributed to data acquisition, infant recruitment, ethics, governance and TGA application, funding application, setting up the clinical trial including liaising with pharmacy, ordering trial equipment for storage of faecal samples, supervision of project running, data interpretation, writing first and final draft of manuscript and revision of manuscript for critically important intellectual content. ME contributed to analysis and interpretation of data including bioinformatics details, critical revision of the manuscript for important intellectual content. SP and KS contributed to conception and design, data interpretation, revision of manuscript for critical important intellectual content, supervision of project running and manuscript writing. SP was also the guarantor for the work conducted in this study. EN and DD contributed to statistical input into design, analysis and interpretation of clinical and short-chain fatty acid (SCFA) data and revision of the manuscript for critical important intellectual content. AK contributed to conception and design, and revision of the manuscript for critical important intellectual content, assuring independent safety check of trial probiotic products. SR assisted with funding application and revision of the manuscript for critically important intellectual content. LChe contributed to sample analysis for SCFA, SCFA data analysis and interpretation and revision of manuscript for critically important intellectual content. LCha and SS contributed to sample analysis for metagenomic sequencing and data interpretation; CK contributed as research assistant and for data collection, data cleaning, participant recruitment, sample collection and preparation for storage. PC contributed to critical revision of the manuscript

for important intellectual content. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

Funding Telethon-WIRF Channel-Seven Trust Grant and Princess Margaret Hospital Foundation (PMHF) Translational Grant.

Disclaimer The funding organisations played no role in the design and conduct of the study. The manufacturer Morinaga Milk Industry Co, Japan, was not the sponsor but only supplied the probiotic products free of cost for the trial and was not involved in the design, conduct, analysis and reporting of the trial.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Women and Newborn Health Ethics Committee. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. Our clinical trial was commenced in 2015 and completed in 2017.

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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^{0}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^{0}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℃ immediately after collection. Before SCFA extraction, the frozen samples were thawed and added 100 μL 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 × 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 µM. Then, 500 µL of ethyl acetate was added to and the mixture was vortex for 30 min and centrifuged for 10 min at 20817 ×g. Then, 2 µL 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×0.250 mm ×0.25 µm; Agilent). Helium was used as the carrier gas at 1 mL/min. The column temperature was initially 80℃ (1min), then increased to 120 ℃ at 20 ℃/min, and finally to 210 ℃ at 6.13 ℃/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 ℃, 230℃, 150℃ and 280 ℃, 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 µM. IS was added to each diluted standards mixture (500 µM 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 Chemistry16 (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.[1]

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.[2] 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.[3]

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.[4] Data were visualized using ggplot2 [5] and ggpubr packages.[6]

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,[7] and the tree was calculated with FastTree.[8] 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

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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 ≥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 (≥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 ≥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; 5×10^9 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 3×10^9 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 ≤27 weeks the daily dose was 1.5×10^9 cfu per day until reaching milk feeds of 50 ml per kg per day. It was then increased to 3×10^9 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−1). 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)[1]. 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

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Supplemental Tables and Figures

Supplemental Table 1: Subgroup analysis of IUGR infants

Primary outcome	**SS (N=6)**	**TS (N=6)**
Time to full enteral feeds (150ml/kg/day)[#] (days) (N_{SS}=5, N_{TS}=4)	11 (10-14)	12.5 (10-27)
Secondary outcomes		
All-cause mortality	1 (16.7%)	2 (33.3%)
Definite NEC	1 (16.7%)	1 (16.7%)
Intestinal transit time (h)[#] (N_{SS}=5, N_{TS}=4)	17 (10-23)	13 (5-22)
Length of hospital stay (days) (N_{SS}=5, N_{TS}=4)	112 (86-161)	146 (73-200)
Late onset sepsis		
Suspected	4 (66.7%)	4 (66.7%)
Proven	3 (50%)	3 (50%)

Supplemental Table 2: Detection of differentially abundant Phyla between groups

Phyla	W[α]	Groups	T1 [β]	T2 [β]	T1 vs T2 [β]
			Median (IQR)		
Actinobacteria	3	REF	**0.16 (0-3.25)**	**0.04 (0.01-1.94)**	**P.adj= 1.00**
		SS	**20.83 (7.84-33.57) [P]**	**29.67 (18.36-39.54) [P]**	**P.adj= 0.224**
		TS	**10.47 (1.23-33.64) [P]**	**34.18 (23.22-41.33) [P]**	**P.adj= < 0.001**
Bacteroidetes	1	REF	0 (0-0.03)	0 (0-0.01)	Padj= ns
		SS	0 (0-0.01)	0 (0-0)	Padj= ns
		TS	0 (0-0.46)	0 (0-0)	Padj= ns
Firmicutes	1	REF	26.48 (4.11-78.31)	20.27 (12.43-33.6)	Padj= ns
		SS	17.26 (5.52-34.04)	11.45 (3.76-27.62)	Padj= ns
		TS	7.94 (0.8-23.67)	10.39 (6.49-21.94)	Padj= ns
Proteobacteria	1	REF	61.04 (0.67-95.86)	73.19 (60.85-86.53)	Padj= ns
		SS	49.42 (0.28-66.04)	51.76 (38.32-69.84)	Padj= ns
		TS	53.08 (22.87-67.38)	50.19 (37.73-66.61)	Padj= ns

[α] W= X for taxon found to be different using repeated measures ANCOM (where Group and Time were fixed factors and SubjectID was a variable factor) relative to 70% of other taxa. Out of the 28 Species that were significantly different only those with relative abundance > 0.01% are shown in this table.

[β] Differences between groups were calculated using linear mixed effects model, where Group and Time were fixed factors and SubjectID was a variable factor. Differences between groups was calculated using post-hoc Tukey test. [P] is P.adj < .05 compared to Reference group. [T] is P.adj < .05 for Triple compared to Single group.

Supplemental Table 3: Detection of differentially abundant Class between groups

Class	W[α]	Groups	T1 [β]	T2 [β]	T1 vs T2 [β]
			Median (IQR)		
Actinobacteria	6	REF	**0.16 (0-3.25)**	**0.04 (0.01-1.94)**	**P.adj= 1.00**
		SS	**20.83 (7.84-33.57)** [P]	**29.67 (18.36-39.54)** [P]	**P.adj= 0.270**
		TS	**10.47 (1.23-33.64)** [P]	**34.18 (23.22-41.33)** [P]	**P.adj= < 0.001**
Alphaproteobacteria	1	REF	0.05 (0.03-5.18)	0.05 (0.04-0.07)	Padj= ns
		SS	0.03 (0.01-0.23)	0.04 (0.03-0.07)	Padj= ns
		TS	0.05 (0.02-0.65)	0.03 (0.02-0.05)	Padj= ns
Bacilli	1	REF	25.51 (3.58-77.85)	9.17 (3.53-14.77)	Padj= ns
		SS	14.48 (2.44-32.45)	7.48 (2.18-14.43)	Padj= ns
		TS	5.7 (0.8-23.02)	7.5 (2.3-11.88)	Padj= ns
Clostridia	1	REF	0 (0-0.25)	2.95 (0.14-9.41)	Padj= ns
		SS	0 (0-0.11)	0.01 (0-3.04)	Padj= ns
		TS	0 (0-0.01)	0 (0-0.01)	Padj= ns
Gammaproteobacteria	1	REF	45.06 (0.64-95.81)	73.14 (60.8-86.49)	Padj= ns
		SS	27.59 (0.27-61.73)	51.72 (38.27-69.78)	Padj= ns
		TS	52.5 (22.28-67.34)	50.17 (37.69-66.55)	Padj= ns
Negativicutes	1	REF	0 (0-0)	0.01 (0-2.98)	Padj= ns
		SS	0 (0-0.01)	0 (0-0.12)	Padj= ns
		TS	0 (0-0.01)	0 (0-0.22)	Padj= ns

[α] W= X for taxon found to be different using repeated measures ANCOM (where Group and Time were fixed factors and SubjectID was a variable factor) relative to 70% of other taxa. Out of the 28 Species that were significantly different only those with relative abundance > 0.01% are shown in this table.

[β] Differences between groups were calculated using linear mixed effects model, where Group and Time were fixed factors and SubjectID was a variable factor. Differences between groups was calculated using post-hoc Tukey test. [P] is P.adj < .05 compared to Reference group. [T] is P.adj < .05 for Triple compared to Single group.

Supplemental Table 4: Detection of differentially abundant Family between groups

Family	W[α]	Groups	T1 [β]	T2 [β]	T1 vs T2 [β]
			Median (IQR)		
Bifidobacteriaceae	21	REF	**0.01 (0-1.51)**	**0.01 (0.01-0.06)**	**Padj= 0.999**
		SS	**14.65 (2.62-29.09)** [P]	**29.67 (17.33-39.38)** [P]	**Padj= 0.016**
		TS	**5.5 (0.92-30.46)** [P]	**34.07 (23.2-41.19)** [P]	**Padj < 0.001**
Clostridiaceae 1	19	REF	**0 (0-0.07)**	**2.94 (0.06-9.41)**	**Padj= 0.002**
		SS	**0 (0-0.01)**	**0 (0-0.87)** [P]	**Padj= 0.512**
		TS	**0 (0-0)**	**0.01 (0-2.95)** [P]	**Padj= 0.910**
Enterobacteriaceae	1	REF	22.4 (0.57-95.8)	72.94 (60.66-84.77)	Padj= ns
		SS	10.31 (0.13-47.47)	50.58 (37.03-69.77)	Padj= ns
		TS	19.39 (10.17-57.74)	50.17 (37.67-66.13)	Padj= ns
Enterococcaceae	1	REF	0.76 (0.01-7.01)	4.76 (1.14-8.73)	Padj= ns
		SS	0.01 (0.0-1.50)	2.22 (0.34-7.65)	Padj= ns
		TS	0.66 (0.06-3.62)	2.07 (0.24-7.08)	Padj= ns
Streptococcaceae	18	REF	**0.02 (0-0.09)**	**0.6 (0.13-1.84)**	**Padj= 1.00**
		SS	**0.01 (0-0.02)**	**0.04 (0.01-0.11)** [P]	**Padj= 0.003**
		TS	**0 (0-0.07)**	**0.02 (0.01-0.08)** [P]	**Padj= 0.898**

[α] W= X for taxon found to be different using repeated measures ANCOM (where Group and Time were fixed factors and SubjectID was a variable factor) relative to 70% of other taxa. Out of the 28 Species that were significantly different only those with relative abundance > 0.01% are shown in this table.

[β] Differences between groups were calculated using linear mixed effects model, where Group and Time were fixed factors and SubjectID was a variable factor. Differences between groups was calculated using post-hoc Tukey test. [P] is P.adj < .05 compared to Reference group. [T] is P.adj < .05 for Triple compared to Single group.

Supplemental Table 5: Detection of differentially abundant Genus between groups

Genus	W[α]	Groups	T1 [β]	T2 [β]	T1 vs T2 [β]
			Median (IQR)		
Bacteroides	5	REF	0 (0-0.01)	0 (0-0.01)	Padj= ns
		SS	0 (0-0)	0 (0-0)	Padj= ns
		TS	0 (0-0)	0 (0-0)	Padj= ns
Bifidobacterium	34	REF	**0 (0-0.02)**	**0.01 (0.01-0.06)**	**Padj= 0.999**
		SS	**11.86 (0.65-23.45)** [P]	**29.67 (17.33-39.38)** [P]	**Padj= 0.004**
		TS	**5.5 (0.92-30.46)** [P]	**34.07 (23.2-41.19)** [P]	**Padj < 0.001**
Clostridium sensu stricto1	30	REF	**0 (0-0.04)**	**2.94 (0.06-8.56)**	**Padj= 0.004**
		SS	**0 (0-0)**	**0 (0-0.63)** [P]	**Padj= 0.580**
		TS	**0 (0-0)**	**0.01 (0-2.95)** [P]	**Padj= 0.919**
Enterobacter	1	REF	0.3 (0.06-7.84)	17.53 (2.67-47.58)	Padj= ns
		SS	0.75 (0.02-3.03)	23.45 (0.64-37.79)	Padj= ns
		TS	0.16 (0.05-4.08)	13.14 (0.36-37.74)	Padj= ns
Enterococcus	1	REF	1.28 (0.01-6.93)	4.77 (1.14-8.73)	Padj= ns
		SS	0.01 (0-1.51)	2.22 (0.35-7.65)	Padj= ns
		TS	0.66 (0.07-3.63)	2.07 (0.25-7.09)	Padj= ns
Escherichia-Shigella	1	REF	0.14 (0.05-1.49)	0.35 (0.09-21.47)	Padj= ns
		SS	0.81 (0.04-4.91)	0.08 (0.03-0.69)	Padj= ns
		TS	3.85 (0.15-12.87)	0.15 (0.03-15.23)	Padj= ns
Klebsiella	4	REF	0.09 (0.04-1.72)	17 (4.32-40.15)	Padj= ns
		SS	0.43 (0.02-17.75)	8.81 (0.24-27.97)	Padj= ns
		TS	0.07 (0.02-0.45)	1.29 (0.1-10.77)	Padj= ns
Pseudomonas	14	REF	0 (0-0)	0 (0-0)	Padj= ns
		SS	0.01 (0-0.11)	0 (0-0)	Padj= ns
		TS	0 (0-0.06)	0 (0-0)	Padj= ns
Staphylococcus	3	REF	1.58 (0.01-22.71)	0.45 (0.15-3.12)	Padj= ns
		SS	2.34 (0.38-26.32)	1.54 (0.58-4.45)	Padj= ns
		TS	0.66 (0.06-9.06)	1.74 (0.33-3.8)	Padj= ns
Streptococcus	26	REF	**0 (0-0.08)**	**0.59 (0.13-1.82)**	**Padj= 1.000**
		SS	**0.01 (0-0.02)**	**0.04 (0.01-0.1)** [P]	**Padj= 0.003**
		TS	**0 (0-0.07)**	**0.02 (0.01-0.07)** [P]	**Padj= 0.897**

[α] W= X for taxon found to be different using repeated measures ANCOM (where Group and Time were fixed factors and SubjectID was a variable factor) relative to 70% of other taxa. Out of the 28 Species that were significantly different only those with relative abundance > 0.01% are shown in this table.

[β] Differences between groups were calculated using linear mixed effects model, where Group and Time were fixed factors and SubjectID was a variable factor. Differences between groups was calculated using post-hoc Tukey test. [P] is P.adj < .05 compared to Reference group. [T] is P.adj < .05 for Triple compared to Single group.

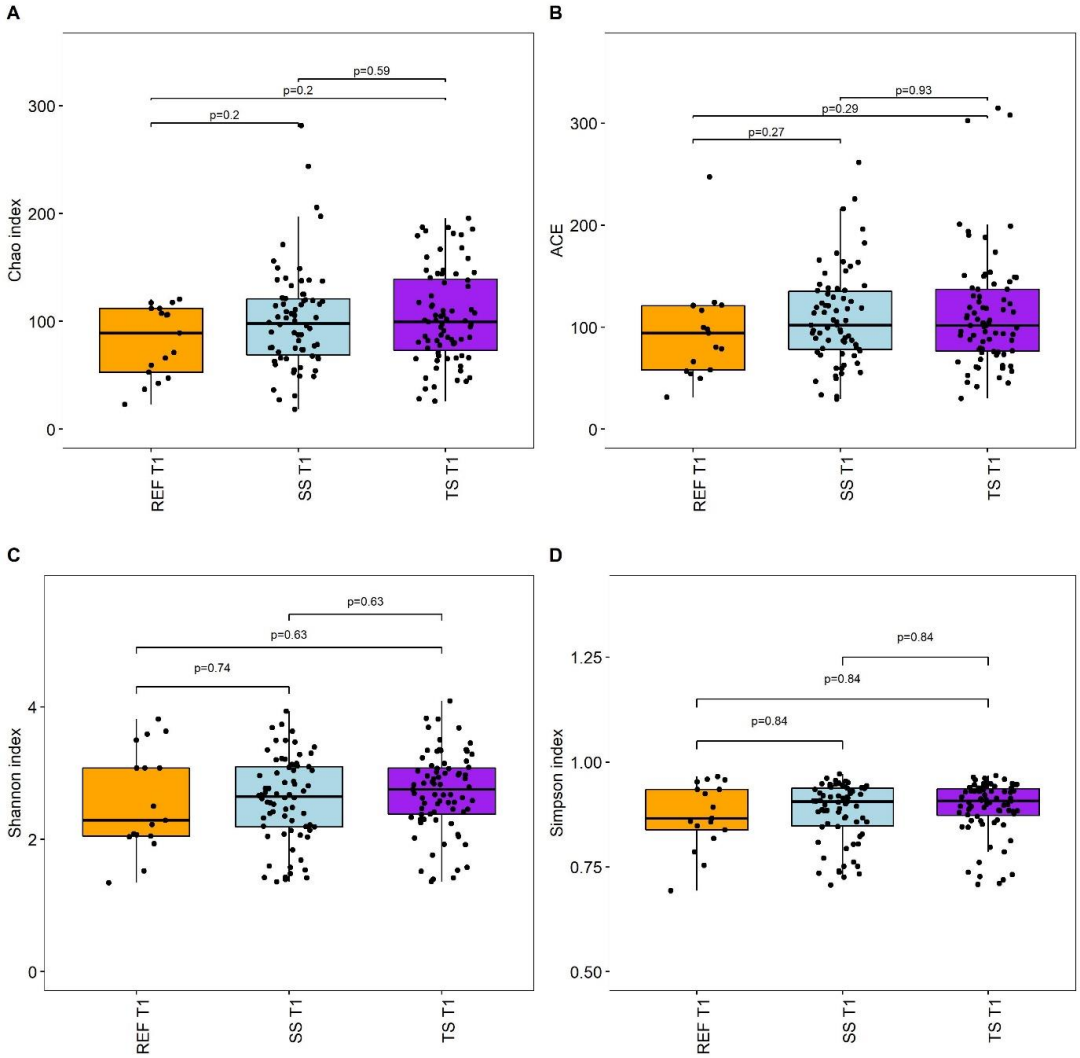
Supplemental Table 6: Detection of differentially abundant Species between groups

Species	W[α]	Groups	T1 [β]	T2 [β]	T1 vs T2 [β]
			Median (IQR)		
Bifidobacterium adolescentis	268	REF	0 (0-0)	0 (0-0.02)	Padj= 0.909
		SS	0.04 (0-0.12) [P]	0.1 (0.04-0.16) [P]	Padj= 0.016
		TS	0.06 (0.03-0.09)	0.13 (0.07-0.19) [P]	Padj= 1.000
Bifidobacterium bifidum	268	REF	0 (0-0)	0 (0-0.04)	Padj= 0.806
		SS	0.07 (0.01-0.11)	0.13 (0.07-0.2) [P]	Padj= 0.001
		TS	0.05 (0.03-0.08)	0.12 (0.05-0.15) [P]	Padj= 0.999
Bifidobacterium breve	268	REF	0 (0-0.63)	0.04 (0-1.29)	Padj= 0.974
		SS	3 (0.72-6.77)	7.9 (3.8-13.99) [P]	Padj= 0.017
		TS	1.35 (0.67-2.02)	1.78 (1.07-4.39) [P T]	Padj= 0.999
Bifidobacterium lemurum	268	REF	0 (0-0)	0 (0-0)	Padj= 0.887
		SS	0.01 (0-0.02)	0.02 (0-0.02) [P]	Padj= 0.015
		TS	0.01 (0-0.01)	0.02 (0.01-0.02) [P]	Padj= 1.000
Bifidobacterium longum	268	REF	0 (0-0)	0 (0-0.44)	Padj= 1.000
		SS	7.51 (0.11-45.25) [P]	26.27 (0.6-46.04) [P]	Padj= 1.000
		TS	27.14 (13.58-40.71) [P]	49.82 (17.44-74.75) [P T]	Padj= 0.998
Bifidobacterium longum CAG:69	268	REF	0 (0-0)	0 (0-0)	Padj= 0.974
		SS	0.02 (0-0.11) [P]	0.06 (0-0.13) [P]	Padj= 0.981
		TS	0.06 (0.03-0.09)	0.13 (0.04-0.17) [P T]	Padj= 0.982
Bifidobacterium pseudocatenulatum	267	REF	0 (0-0)	0 (0-0)	Padj= 0.968
		SS	0.01 (0-0.08) [P]	0.05 (0.02-0.1) [P]	Padj= 0.157
		TS	0.04 (0.02-0.06)	0.08 (0.04-0.11) [P T]	Padj= 1.000
Bifidobacterium pseudolongum	268	REF	0 (0-0)	0 (0-0)	Padj= 0.876
		SS	0.01 (0-0.04) [P]	0.04 (0.01-0.06) [P]	Padj= 0.128
		TS	0.02 (0.01-0.04)	0.05 (0.02-0.07) [P T]	Padj= 0.996
Bifidobacterium reuteri	268	REF	0 (0-0)	0 (0-0)	Padj= 0.995
		SS	0.01 (0-0.03) [P]	0.02 (0-0.03) [P]	Padj= 0.276
		TS	0.02 (0.01-0.02) [P]	0.03 (0.01-0.04) [P T]	Padj= 0.992
Clostridium butyricum	241	REF	0 (0-0)	0.01 (0-0.56)	Padj= 1.000
		SS	0 (0-0)	0 (0-0) [P]	Padj= 0.995
		TS	0 (0-0)	0 (0-0) [P]	Padj= 1.000
Gardnerella vaginalis	267	REF	0 (0-0)	0 (0-0)	Padj= 0.872
		SS	0.02 (0-0.08) [P]	0.06 (0.02-0.09) [P]	Padj= 0.080
		TS	0.04 (0.02-0.07)	0.08 (0.04-0.12) [P T]	Padj= 1.000
Streptococcus pyogenes	268	REF	0 (0-0.12)	0 (0-0.02)	Padj= 0.571
		SS	0.05 (0.03-0.12)	0.02 (0-0.06) [P]	Padj= 0.392
		TS	0.1 (0.08-0.13)	0.05 (0.03-0.07) [P T]	Padj= 0.847
Streptococcus salivarius	228	REF	0 (0-0)	0 (0-0.05)	Padj= 0.823
		SS	0 (0-0)	0 (0-0) [P]	Padj= 0.989
		TS	0 (0-0)	0 (0-0) [P]	Padj= 1.000
Streptococcus thermophilus	267	REF	0 (0-0)	0.13 (0.01-0.9)	Padj= 0.001
		SS	0 (0-0)	0 (0-0.01) [P]	Padj= 0.998
		TS	0 (0-0.01)	0 (0-0) [P]	Padj= 1.000

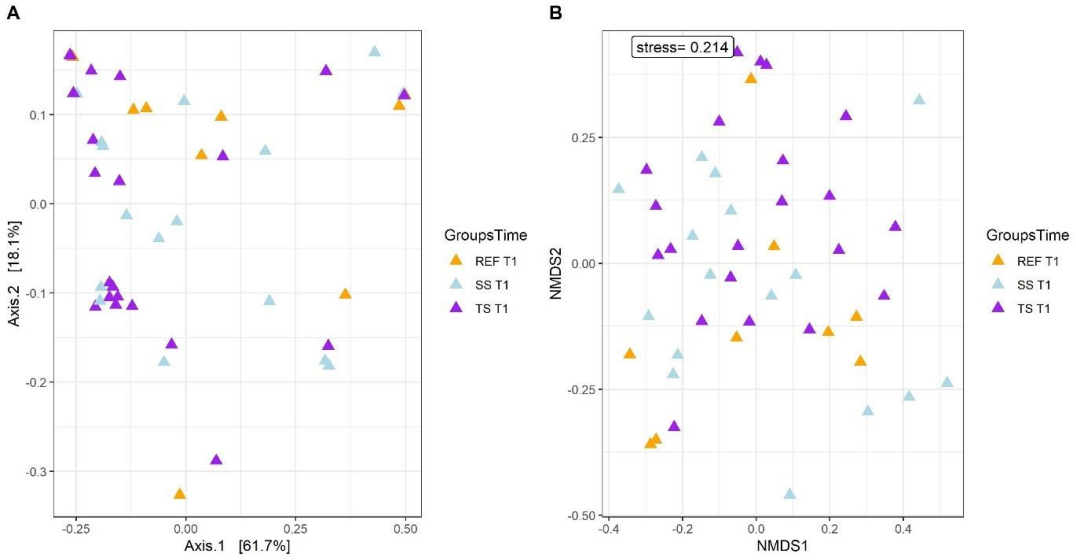
[α] W= X for taxon found to be different using repeated measures ANCOM (where Group and Time were fixed factors and SubjectID was a variable factor) relative to 70% of other taxa. Out of the 28 Species that were significantly different only those with relative abundance > 0.01% are shown in this table.

[β] Differences between groups were calculated using linear mixed effects model, where Group and Time were fixed factors and SubjectID was a variable factor. Differences between groups was calculated using post-hoc Tukey test. [P] is P.adj < .05 compared to Reference group. [T] is P.adj < .05 for Triple compared to Single group.

Supplemental Figure 1: Comparison of alpha diversity between SiMPro and PANTS (REF) at T1



Supplemental Figure 2: Comparison of beta diversity between SiMPro and PANTS (REF) at T1



C

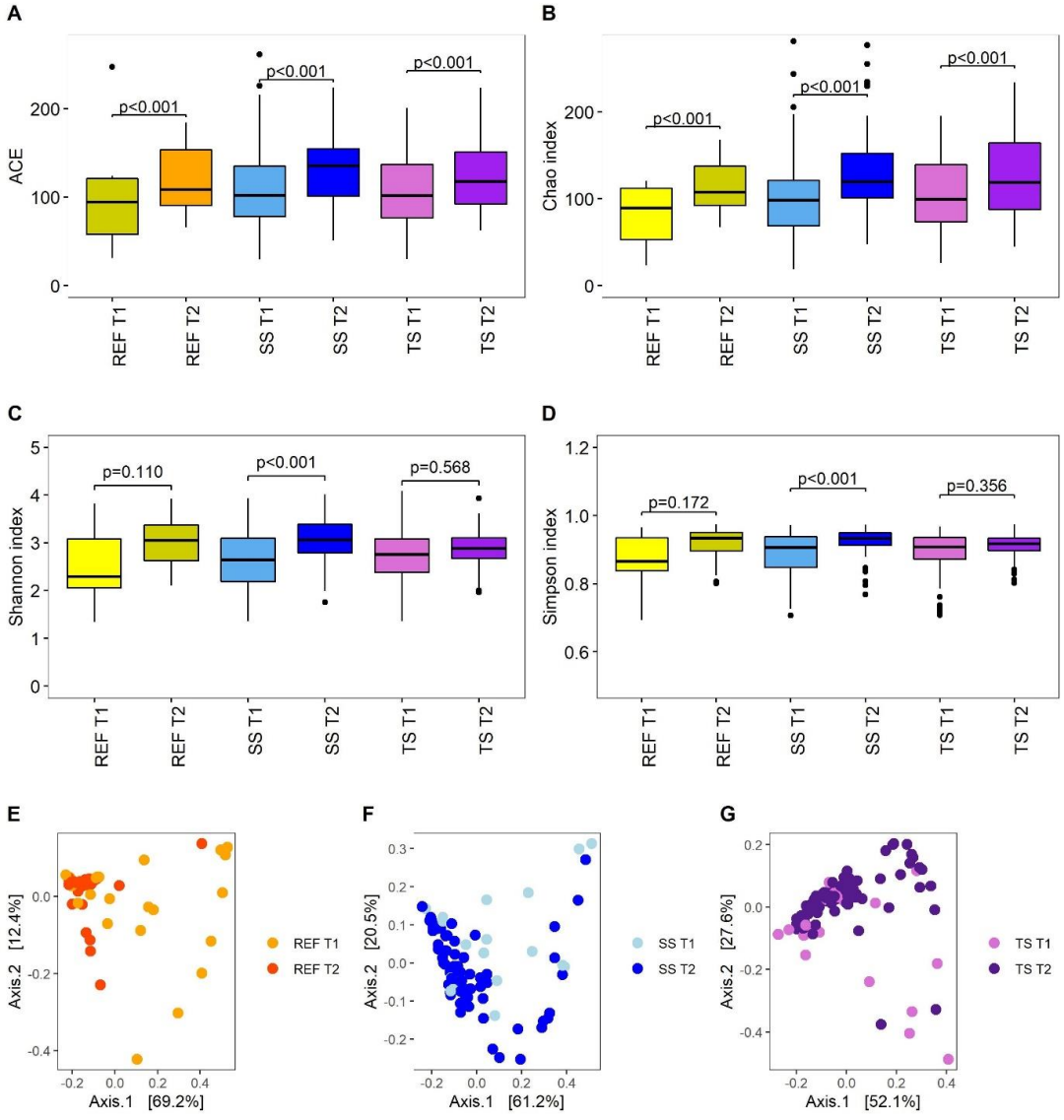
Adonis Weighted Unifrac

Groups at T1	R^2	adjusted P value
REF vs SS	0.09	0.114
REF vs TS	0.03	0.429
SS vs TS	0.04	0.324

Bray Curtis Weighted Unifrac

Groups at T1	R^2	adjusted P value
REF vs SS	0.06	0.204
REF vs TS	0.04	0.325
SS vs TS	0.02	0.450

Supplemental Figure 3: Alpha and beta diversity of all the infants significantly change from T1 to T2.

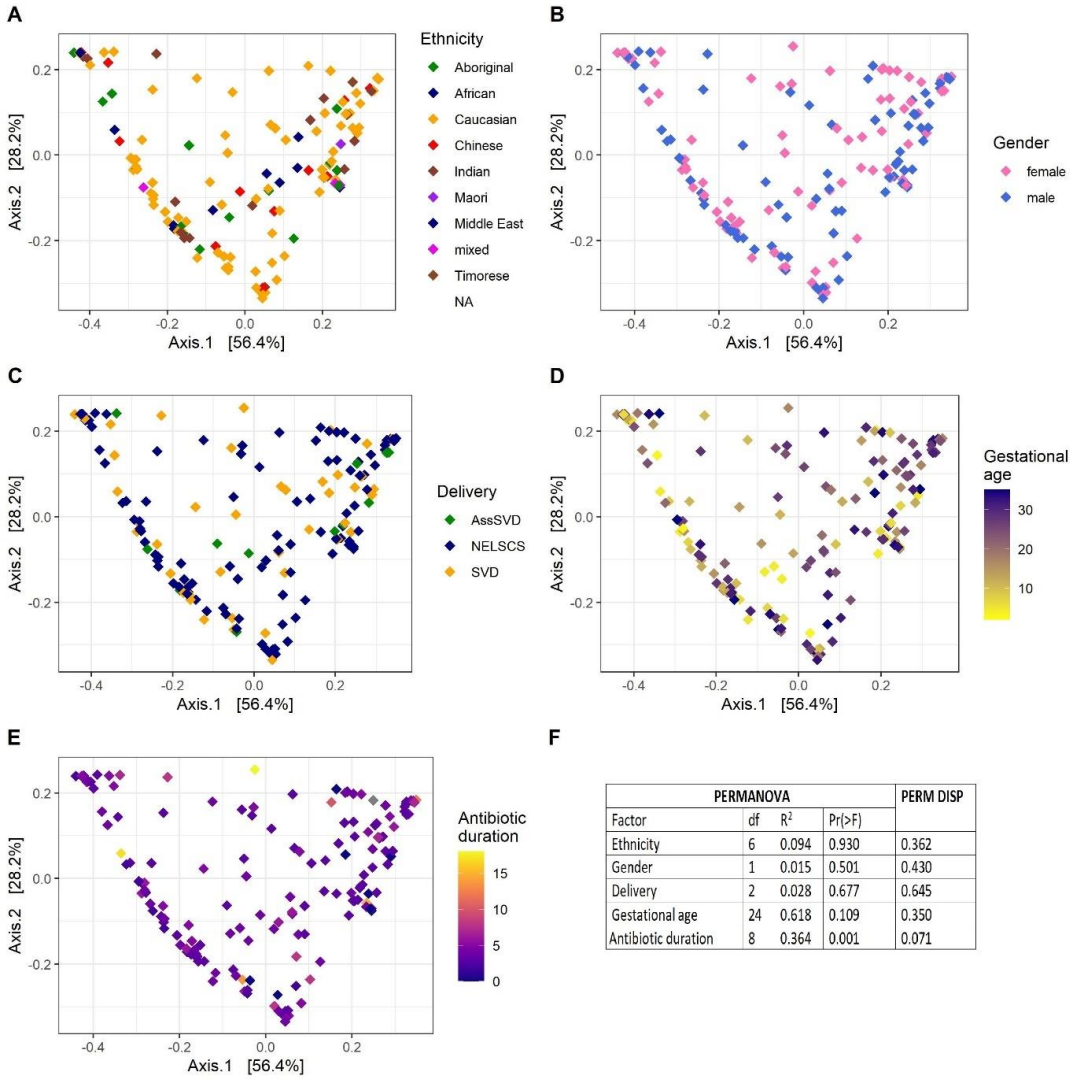


I

Adonis Weighted Unifrac

Groups T1 vs T2	R^2	P.adj value
REF	0.354	0.003
SS	0.071	0.005
TS	0.052	0.009

Supplemental Figure 4: Beta diversity at T1 showing community structures do not depend on ethnicity, gender, mode of delivery and gestational age but on duration of antibiotics. Permutational variance of analysis (PERMANOVA) explored the difference in impact of the factors on community structures. PERMDISP was used to test if there was any difference in dispersion between the groups.



PERMANOVA				PERM DISP
Factor	df	R^2	Pr(>F)	
Ethnicity	6	0.094	0.930	0.362
Gender	1	0.015	0.501	0.430
Delivery	2	0.028	0.677	0.645
Gestational age	24	0.618	0.109	0.350
Antibiotic duration	8	0.364	0.001	0.071