Pediatric RESEARCH

CLINICAL RESEARCH ARTICLE

Gut microbiota in neonates with congenital gastrointestinal surgical conditions: a prospective study

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BACKGROUND: There is limited information on gut microbiota of neonates with congenital gastrointestinal surgical conditions (CGISCs) available.

METHODS: This study compared stool microbiota and short-chain fatty acids (SCFAs) of 37 term infants with CGISCs with 36 term healthy infants (HIs). Two stool samples were collected from each infant: as soon as possible after birth (week 1) and 10–14 days of life (week 2).

RESULTS: Bacterial richness and alpha diversity were comparable between CGISCs and HIs at week 1 and week 2 (all $p > 0.05$). Beta diversity analysis revealed that at week 1, CGISCs had similar community structures to HIs ($p = 0.415$). However, by week 2, community structures of CGISCs were significantly different from HIs ($p = 0.003$). At week 1, there were no significant differences in the relative abundances of genera *Bifidobacterium* and *Bacteroides* between CGISCs and HIs. At week 2, the relative abundance of *Bifidobacterium* was significantly lower in CGISCs (mean percentage 7.21 ± 13.49 vs. 28.96 ± 19.6; $p = 0.002$). *Bacteroides* were also less abundant in the CGISC group (mean percentage 0.12 ± 0.49 vs. 6.59 ± 8.62; $p = 0.039$). Relative abundance of genera *Pseudomonas* and *Escherichia–Shigella* were higher in CGISCs. At week 2, stool concentrations of all SCFAs were lower in CGISCs (all $p < 0.001$).

CONCLUSIONS: During hospitalization, neonates with CGISCs develop gut dysbiosis and deficiency of SCFAs.

Pediatric Research (2020) 88:878–886; https://doi.org/10.1038/s41390-020-0824-7

IMPACT:

- During hospitalisation, neonates with congenital gastrointestinal surgical conditions develop gut dysbiosis with deficiency of Bifidobacteria and Bacteroides and increased abundance of Escherichia-Shigella and Pseudomonas. They also have low levels of short chain fatty acids in their stools compared to healthy infants.
- This is the first study evaluating the gut microbiota using 16S ribosomal RNA sequencing methods and stool short chain fatty acids in neonates with congenital gastrointestinal surgical conditions and comparing them to healthy infants.
- The findings of this study will pave the way for randomised trials of bifidobacterial supplementation in neonates with congenital gastrointestinal surgical conditions.

INTRODUCTION

The major congenital gastrointestinal surgical conditions (CGISCs) are gastroschisis, exomphalos, duodenal atresia, small and large intestinal atresia, oesophageal atresia, congenital short bowel syndrome (SBS), malrotation and volvulus, meconium ileus, hypoplastic left colon, Hirschsprung disease (HD), anorectal malformations and others.

Common morbidities in these conditions are feed intolerance and increased risk of infections. Infants with CGISCs are cared for in intensive care units and do not receive breastmilk in the first few days of life. They undergo invasive procedures and do not receive adequate skin-to-skin contact with their mothers. They get exposed to gastric acid suppressants, parenteral nutrition and multiple courses of antibiotics. All these factors have the potential to increase the risk of gut dysbiosis.[1–3]

While the gut microbiota of extremely preterm non-surgical infants has been well studied using culture-independent genomic approaches,[4] there is very limited information on gut microbiota of term infants with CGISCs. The studies that evaluated gut flora in neonates with surgical conditions in the past were based on the conventional culture-dependent techniques.[5] However, a growing body of evidence in the recent decade has shown the importance of culture-independent, genomic approaches in understanding the role of the human microbiota in health and disease.[6] Hence, we conducted this prospective study to investigate the gut microbiota in term neonates with CGISCs using culture-independent techniques.

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Received: 17 May 2019 Revised: 31 December 2019 Accepted: 1 January 2020
Published online: 16 March 2020

Many of the biological functions of healthy gut microbiota are mediated via short-chain fatty acids (SCFAs), such as acetic acid, butyric acid and propionic acid. SCFAs have important biological functions in humans, such as immune modulation, anti-inflammatory, anti-tumorigenic and antimicrobial effects and enhancement of gut integrity.[7] They are thought to play a key role in microbiota–gut–brain crosstalk.[8] In infants, SCFAs are the by-products of fermentation of human milk oligosaccharides (HMOs) by the anaerobic bacteria in the colon. HMOs are not digested by the small intestine of infants and hence they reach the colon intact, where they are utilized as nutrition by bacteria such as *Bifidobacteria* and *Bacteroides*. Since not all bacteria have the necessary enzymes to utilize HMOs, these milk glycans facilitate the establishment of a highly specialized microbial ecosystem dominated by *Bifidobacteria* and *Bacteroides* among others, while indirectly limiting growth of other bacteria.[9] It is possible that neonates with CGISCs have insufficient amounts of anaerobes such as *Bifidobacteria* and *Bacteroides*, which in turn could result in decreased utilization of HMOs, and hence lower amounts of SCFAs in the colon. Given the importance of SCFAs in human health and the interplay between gut microbiota and SCFAs,[7] we investigated stool SCFA levels in these infants.

METHODS

This was a prospective cohort study in which neonates with CGISCs were recruited from the neonatal intensive care unit of Perth Children's Hospital (PCH) and healthy infants (HIs) were recruited from the postnatal ward of King Edward Memorial Hospital (KEMH), Perth, Western Australia.

The study was approved by the institutional human research ethics committees of both hospitals. Informed parental consent was obtained for all studied infants.

Eligibility criteria

Neonates (≥36 weeks) with gastroschisis, exomphalos, Hirschsprung disease, duodenal atresia, other intestinal atresia, congenital diaphragmatic hernia, oesophageal atresia, congenital SBS and conditions needing enterostomy. Controls were healthy term newborn infants (≥36 weeks). We chose to include infants born at 36 weeks also instead of the conventional definition of 37 weeks because many infants with surgical conditions are born at 36 weeks and would not have been eligible for inclusion, thereby resulting in difficulty in achieving the sample size. Preterm infants <36 weeks were excluded to avoid the confounding effect of prematurity, which itself is a significant risk factor for gut dysbiosis. Even though congenital diaphragmatic hernia is not truly a gastrointestinal (GI) condition, we decided to include it because the intestines are not in the abdominal cavity throughout pregnancy in this condition.

Outcomes

Stool microbiota using 16S ribosomal RNA gene sequencing, and SCFAs using modified gas chromatography-mass spectrometry (GC-MS) were measured on samples collected as soon as possible after birth/admission (week 1) and 10–14 days of life (week 2).

Stool sample collection

Two stool samples were collected in sterile containers from each consented infant. The first sample was collected as soon as possible after birth/admission (week 1) and the second sample was taken between 10 and 14 days of life (week 2). All neonates with CGISCs were inpatients at the time of week 1 as well as week 2 sample collection. The week 1 samples of healthy term infants were collected while in hospital, whereas the week 2 samples were collected in sterile containers using sterile spatula at home by parents and kept in the refrigerator at home. Parents were advised to do thorough hand washing before collecting the samples and to screw the lid tightly immediately after collection to prevent contamination. Accredited couriers retrieved the samples from infants' homes in cooler bags with ice packs within 48 h of collection by parents. Subsequently, all stool samples were initially stored at −20 °C for 3–5 days and subsequently at −80 °C. At the completion of recruitment of all study participants, samples were shipped on dry ice (carbon dioxide) to the University of New South Wales (Sydney, Australia), where microbial analysis was undertaken. Acidified samples were shipped on dry ice to the School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore, where SCFA analysis was carried out.

DNA extraction

DNA was extracted from stool samples using the method of Matsuki et al.[10]. Briefly, stool samples were thawed on ice, diluted 10-fold with sterile water and bacterial cells harvested by centrifugation. DNA was subsequently extracted from the bacterial cells using chemical and physical lysis methods. DNA was stored at −80 °C.

PCR amplification and 16S rRNA sequencing

Polymerase chain reaction (PCR) amplification of the V3–V4 region of the 16S ribosomal RNA (rRNA) gene was conducted using the 341 F′ (5′-CCTACGGGNGGCWGCAG-3′) and 785 R′ (5′-GACTAC HVGGGTATCTAATCC-3′) primers with the Nextera indexes. Purified PCR products were submitted to the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia) for library preparation and sequencing on the Illumina MiSeq platform using the MiSeq Kit v3 (2 × 300 cycles).

rRNA sequence analysis

16S rRNA sequence data were initially quality filtered and trimmed using TRIMMOMATIC VERSION 0.36 truncating reads if the quality was found to be below 12 in a sliding window of 4 bp. Reads shorter than 100 bp were discarded after quality trimming. USEARCH version 11.0.667 was used to merge forward and reverse reads between 350 and 550 nucleotides. Primer sequences were truncated with cutadapt version 2.5.[11] Reads with no detectable primers were removed from the further analysis. Afterwards, reads were quality filtered using USEARCH. All reads with an expected error of more than 2 and more than 1 ambiguous base were removed. All sequences of all samples were concatenated in a single file and subsequently dereplicated to form unique sequences. Unique sequences were clustered into zero-radius operational taxonomic units (zOTUs, also called ASVs) using the UNOISE3 algorithm implemented in USEARCH. Chimeras were removed de novo during clustering and in reference mode using the UCHIME2 algorithm together with the SILVA SSURef NR99 v132. Processed, concatenated sequences were mapped on the final set of zOTUs to determine their occurrence and abundance in each sample using the otu_tab command with an identity cut-off of 97% and termination options disabled, which means that every sequence is searched against every zOTU to find the best hit. Taxonomy was assigned to each zOTU using the SINA aligner version 1.6[12] and the SILVA SSURef NR99 v132 database.

For alpha diversity measures, each sample was subsampled 100 times to a count of 25,400 counts per sample and the average was taken. OTU richness and diversity indices, Shannon, ACE and Chao1, were calculated in R (version 3.5.1) using the *vegan* package. Relative abundance analysis at the Phylum, Family and Genus levels were carried out using *phyloseq* package in R. Data were visualized using *ggplot2* and *ggpubr* packages.

For beta diversity analysis, data were square root transformed. To generate a phylogenetic tree for diversity computations, zOTUs were was aligned with *muscle* (version 3.8.31)[13] and the tree was calculated with RaxML (version 8.2.10)[14] using the GTRGAMMA model. Weighted unifrac distances were calculated and visualized on a principal coordinate analysis plot.

Statistical considerations

At the time of commencing this study, to our knowledge, there were no studies that had evaluated gut microbiota in neonates with CGISCs using culture-independent techniques. In the absence of baseline data, we aimed to study 35 neonates with CGISCs and 35 healthy term infants. Since the gut microbiota changes rapidly in the first few months of life, we attempted to collect the stool samples from cases and respective controls within ±2 days of each other.

Statistical analysis of clinical data

Summary data for continuous variables with normal distribution were reported using mean ± SD. Median and range were used to report data with skewed distribution. Continuous variables were compared using the *t* test for normally distributed data and Wilcoxon' rank-sum test for skewed data. Binary outcomes were compared using the Fisher's exact test. A *p* value of <0.05 was considered statistically significant.

Statistical analysis of microbiota data

All data analyses were conducted with R version 3.5.1. 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 timepoints. In our model, Patient ID was a random factor, while time and treatment were used as fixed factors. Post hoc pairwise comparisons between the groups were performed using Tukey's HSD(honestly significant difference) method to adjust for multiple comparisons.

Differential abundance of phyla and genera were examined using the Wilcoxon's rank-sum test.

For beta diversity, PERMANOVA (permutational multivariate analysis of variance) was used to check if community structures differed between the groups at the two timepoints followed by pairwise.adonis test (https://github.com/bwemheu/pairwise.adonis) for pairwise comparisons between the groups. *P* values were adjusted for multiple testing using the Benjamini–Hochberg correction.

For all analyses, a *p* value of <0.05 was considered statistically significant.

Quantification of SCFAs in faecal samples of study infants

Faecal SCFA analyses were performed using a modified GC-MS method.[15] Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and 4-methyl valeric acid were purchased from Sigma-Aldrich (Merck, Singapore). First, 1 g of the faecal sample was suspended in 5 mL of 1% phosphoric acid and frozen at −20 °C immediately after collection. Once thawed, the faecal suspensions were homogenized with vortex. Then, 100 µL of 10% meta-phosphoric acid solution was added to 0.5 mL baby faecal sample to adjust the pH to about 2.0. Samples were vortexed for about 10 min and centrifuged for 30 min at 20,817 × *g*, 4 °C to solidify the precipitate. After that, 0.5 mL of aqueous supernatant was transferred into a new tube; 4-methyl valeric acid was added as internal standard (IS) to a final concentration of 500 µM. Then, 250 µL of ethyl acetate was added to extract SCFAs with a vortex for about 30 min and centrifuged for 20 min at 20,817 × *g*; lastly, 50 µL volume of organic extracts were transferred into GC glass vial for GC-MS analysis. One microliter of organic extracts was injected into GC-MS system (Agilent Technologies 7890B-5977B) equipped with 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. Split ratio is 30:1. The oven temperature was initially held for 1 min at 80 °C, then increased to 120 °C at 20 °C/min, and finally to 210 °C at 6.13 °C/min and held for 2 min The temperature of the injector, ion source, quadrupole, and interface were 250, 230, 150, and 280 °C respectively. The SCFA data analyses were performed in duplicate. Identification of the SCFAs was based on the retention time of standard compounds and with the assistance of the NIST 17 library. Quantifications were carried out in selected ion monitoring acquisition mode in MassHunter Acquisition software with base peak ion selected as quantifier for each compound. The calibration graphs were constructed in MassHunter Quantitative software (version B.09.00) by plotting the relative response (ratio of peak area SCFAs/peak area IS) vs. relative concentration for each individual SCFAs. The final SCFA concentrations were expressed as microgram per gram wet weight faecal sample. Since the data were not of normal distribution, Wilcoxon's rank analysis was performed to compare SCFA concentrations between the two groups (CGISCs and HIs).

Linearity and sensitivity

A stock solution containing the mixture of standards (20 mM 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 peak area SCFAs/peak area IS vs. concentration for each individual SCFAs. By normalizing the peak area to that of the IS, the variability in 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 limits of 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[16] method based on a signal-to-noise ratio of 3 for the LOD and of 10 for the LOQ.

Reporting

STROBE checklist was followed for reporting the results of this observational study.[17]

RESULTS

In total, 37 CGISCs and 36 HIs were recruited into the study. The surgical conditions in the CGISC group were oesophageal atresia: 4; gastroschisis: 9; malrotation: 4; duodenal atresia: 4; small intestinal atresia: 2; colonic atresia: 1; imperforate anus: 2; HD: 3; meconium ileus needing enterostomy: 2; and congenital diaphragmatic hernia: 6. The relevant clinical details are given in Table 1.

For microbial analysis at week 1, 36 stool samples from CGISCs and 25 samples from HIs were available; at week 2, 32 stool samples from CGISCs and 17 samples from HIs were available.

For SCFA analysis at week 1, 35 stool samples from CGISCs and 23 samples from HIs were available; at week 2, 30 stool samples from CGISCs and 17 samples from HIs were available.

Microbial analysis

For CGISCs, the total number of reads were 2,904,691 (median 76,125; range: 34,601–116,020) at week 1 and 2,313,892 (median 73,228; range: 25,460–126,106) at week 2. For HIs, total reads were 2,424,727 (median 80,631, range: 49,494–131,223) at week 1 and 1,247,024 (median 74,409, range: 54,056–104,347) at week 2.

Richness

Week 1 samples (CGISC vs. HI): There were no statistically significant differences in the number of OTUs between neonates with CGISCs and HIs at week 1 (mean OTU: 121 vs. 100; $p = 0.07$) (Fig. 1a).

Week 2 samples (CGISCs vs. HIs): There were no statistically significant differences in the number of OTUs between neonates with CGISCs and HIs (mean OTU: 83 vs. 73; $p = 0.82$) (Fig. 1a).

Bacterial richness decreased significantly in both CGISC and HI groups from week 1 to week 2 ($p < 0.001$ and $p = 0.048$, respectively) (Fig. 1a).

Table 1. Baseline clinical data of surgical and healthy infants.

	Neonates with CGISCs ($N = 37$)	Healthy term infants ($N = 36$)	P value
Gestational age (weeks)	37.2 ± 1.2	38.9 ± 1.3	<0.0001
Birth weight (weeks)	2946 ± 489.9	3344.9 ± 399.8	0.0002
Female, *N* (%)	13 (35%)	15 (42%)	0.634
Maternal pregnancy-induced hypertension	3 (8%)	0 (0%)	0.240
Chorioamnionitis	2 (5.4%)	0 (0%)	0.493
Antepartum haemorrhage	1 (2.7%)	0 (0%)	1.000
Caesarean section, *N* (%)	16 (43.2%)	12 (33%)	0.472
Apgar at 5 min	9 (IQR: 9–9; range: 5–10)	9 (IQR: 9–9; range: 8–10)	0.026
Age at admission (days)	1 (IQR: 1–2; range:1–9)	1 (IQR: 1–1; range: 1–1)	<0.0001
Age at initial surgery (days)	4 (IQR 2–7; range: 1–15)	NA	NA
Day of life enteral feeds commenced	6 (IQR: 3–9; range: 1–18)	1 (IQR: 1–1; range: 1–1)	<0.0001
Time to full enteral feeds (days)	15 (IQR:9–25, Range: 4–65)	1 (IQR: 1–1; range: 1–1)	<0.0001
Duration of parenteral nutrition (days)	13 (IQR: 7–24; range: 1–62)	0	<0.0001
Duration of antibiotic therapy (days)	10 (IQR: 6–21; range: 2–64)	0	<0.0001
Duration of ventilator support (h)	55 (IQR:38–137; range: 0–616)	0	<0.0001
Duration of hospital stay (days)	22 (IQR: 16–38; range: 6–167)	3 (IQR: 2–4; range: 1–7)	<0.0001
Use of proton pump inhibitors	15 (40.5%)	0	<0.0001
Use of H2 receptor blockers	0	0	NA
Number of surgeries during NICU stay	1 (IQR: 1–2; range: 1–5)	NA	NA
Mortality	0	0	NA
Early-onset sepsis	0	0	NA
Hospital-acquired blood stream infection (HABSI)[a]	8 (21.6%)	0	0.005
Organisms causing HABSI	CONS: 4, *E. cloacae*: 1, *E. coli* and *E. fecalis*: 1; CONS and *E. fecalis*: 1, *E. coli*: 1	NA	NA
Use of breastmilk	32 (89%)	26 (72%)	0.135
Day of life at collection of first stool sample	4 (IQR: 2–6; range: 1–10)	2 (IQR: 2–3; range: 1–6)	<0.0001
Day of life at collection of second stool sample	13 (IQR:12–15; range: 12–19)	13 (IQR:12–15; range: 10–17)	0.621

Data are given as mean ± SD or median (IQR; range) or number (%).
[a]Positive blood culture on a sample collected 48 h after admission to the NICU.

Alpha diversity. Alpha diversity in the study samples was measured using three different measures: Shannon, Chao1 and abundance-based coverage estimators (ACE) (Fig. 1b–d).

Week 1 samples (CGISCs vs. HIs): All alpha diversity measures showed no statistically significant differences between neonates with CGISCs and HIs at week 1 Shannon index (2.33 vs. 1.96; $p = 0.14$).

Week 2 samples (CGISCs vs. HIs): All alpha diversity indices showed no statistically significant differences between neonates with CGISCs and HIs at week 2 (Shannon index: 2.91 vs. 2.00; $p = 0.94$).

Alpha diversity decreased significantly in CGISCs from week 1 to week 2 ($p = 0.014$), but not in HIs ($p = 0.998$).

Beta diversity. Weighted Unifrac and Bray–Curtis distances were used to assess the beta diversity between groups.

Week 1 samples (CGISCs vs. HIs): The microbial community structures of neonates with CGISCs were comparable to HI on both weighted Unifrac and Bray–Curtis measures ($p = 0.415$ and 0.241, respectively) (Fig. 2a, c).

Week 2 samples (CGISCs vs. HIs): The microbial community structures of neonates with CGISCs were significantly different from HI on both weighted Unifrac and Bray–Curtis measures (both $p = 0.003$) (Fig. 2b, d)

Relative abundance of bacteria at the phylum level. In both neonates with CGISCs and HIs, bacteria belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria accounted for more than 99.5% of all bacteria. Cyanobacteria, Deinococcus-Thermus, Fusobacteria, Planctomycetes and Verrucomicrobia accounted for the remaining 0.5%.

Comparison of relative abundance of major phyla on week 1 samples (CGISCs vs. HI): The levels of the four major phyla were comparable between CGISC and HI groups at week 1 (all $p > 0.05$; Fig. 3a).

Comparison of relative abundance of phyla on week 2 samples (CGISCs vs. HIs): CGISC group had significantly less Actinobacteria and Bacteroidetes ($p < 0.0001$ and <0.001, respectively) than HIs. CGISCs and HIs had comparable levels of Firmicutes. The stools of neonates with CGISCs had significantly more Proteobacteria compared to HIs ($p = 0.003$) (Fig. 3b).

Relative abundance at the genus level
Comparison of relative abundance of major genera on week 1 samples (CGISCs vs. HIs): CGISC infants had significantly more

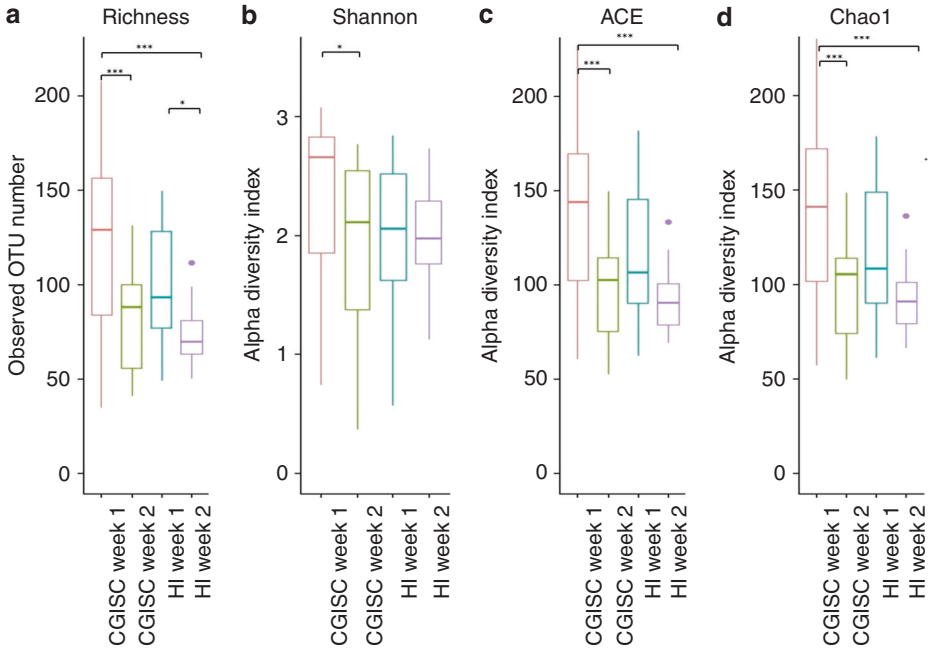

Fig. 1 Richness and alpha diversity measures of faecal microbiota in the study infants. The faecal microbiota of CGISC infants demonstrated significant decrease in bacterial richness and alpha diversity shown by Shannon index, ACE and Chao1 from week 1 to week 2 ($p < 0.05$), while the HI infants exhibited significant decrease in only bacterial richness ($p < 0.05$) (*$p < 0.05$; ***$p < 0.001$).

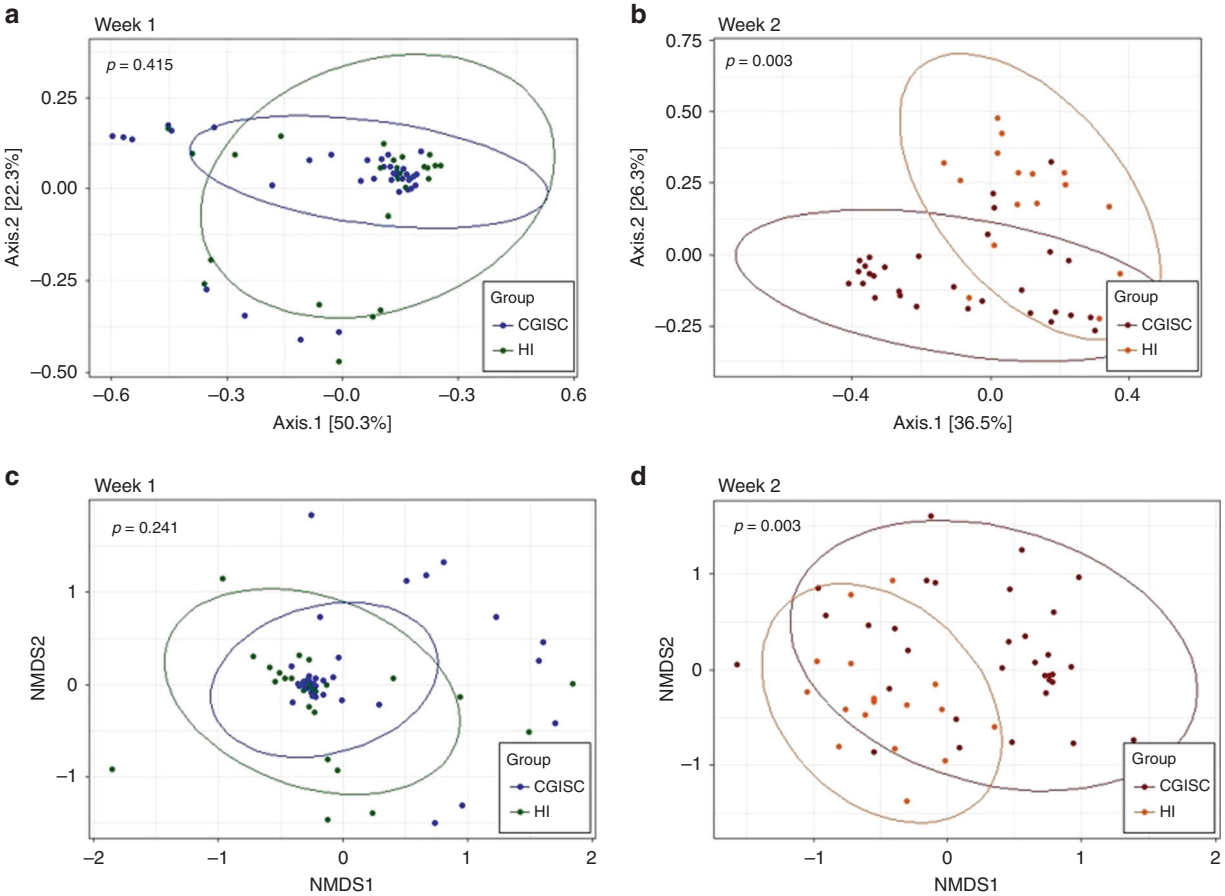

Fig. 2 Beta diversity measures in the study infants. Principal coordinate analysis plots of weighted Unifrac distance of the infants at week 1 (**a**) and week 2 (**b**). NMDS plots on Bray–Curtis dissimilarity at week 1 (**c**) and week 2 (**d**) of the infants. At week 1, HI and CGISC infants had similar community structures (**a**, **c**). However, at week 2, HI had significantly different community structure compared to CGISC infants (**b**, **d**).

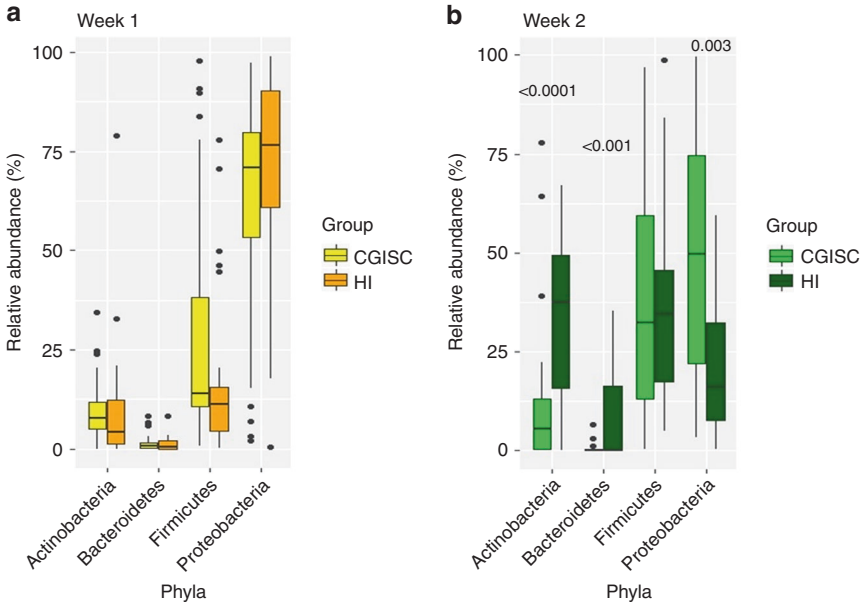

Fig. 3 Relative abundance of the top four phyla in the study infants. Both CGISC and HI infants have similar levels of the four phyla at week 1. However, at week 2, CGISC infants are significantly enriched for Proteobacteria and lower in abundance for Actinobacteria and Bacteroides.

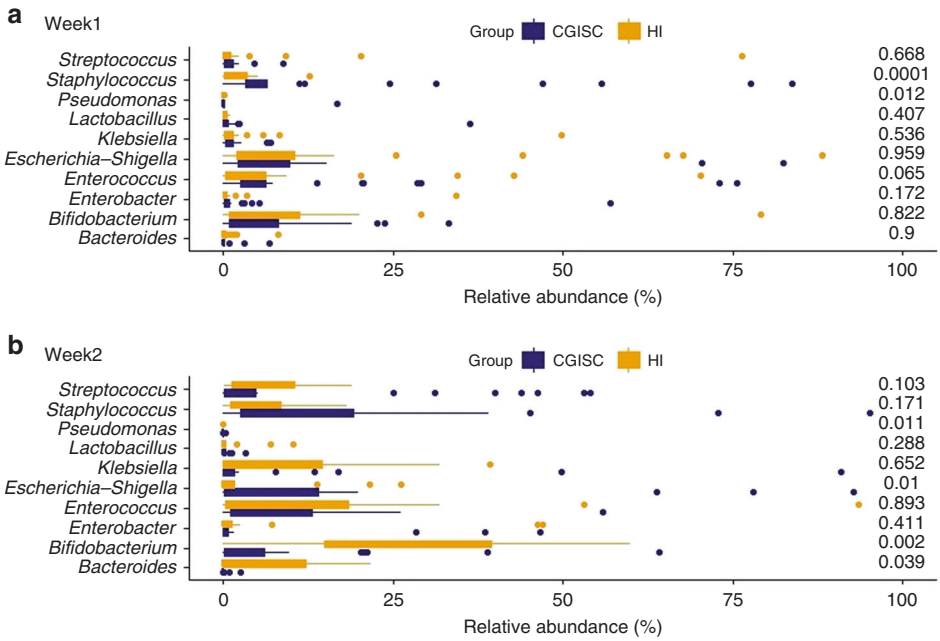

Fig. 4 Comparison of various genera in study infants. CGISC infants have signifncantly increased levels of *Staphylococcus* and *Pseudomonas* in week 1 compared to HI infants. At week 2, CGISC infants have significantly increased *Pseudomonas* and *Escherichia–Shigella*, while HI infants are significantly enriched for *Bifidobacterium* and *Bacteroides*.

Staphylococcus ($p = 0.001$) and *Pseudomonas* ($p = 0.012$) than HI infants at week 1 (Fig. 4 and Supplementary Table S1). There were no significant differences between the groups for the other important bacterial genera (Fig. 4 and Supplementary Table S1).

Comparison of relative abundance of major genera on week 2 samples (CGISCs vs. HI): CGISC infants had significantly lower abundance of *Bifidobacterium* ($p = 0.002$) and *Bacteroides* ($p = 0.039$) and significantly higher abundance of *Escherichia–Shigella* ($p = 0.01$) and *Pseudomonas* ($p = 0.011$) than HIs (Fig. 4 and Supplementary Table S1). There were no significant differences between the two groups for other genera such as *Staphylococcus*, *Enterococcus*, *Enterobacter*, *Klebsiella* and *Streptococcus* (Fig. 4 and Supplementary Table S1).

Results of SCFA analysis

The total SCFA levels were significantly lower in the CGISC group at week 1 (median 407.7, range: 302.2–696.1 µg/g of wet faeces vs. 1208.2, range: 1036.5–6846.9, $p < 0.0001$) as well as at week 2 (median 410.2, range: 300.1–664.1 vs. 1750.6, range: 1046.8–7781.7, $p < 0.0001$) (Fig. 5). Analysis of individual SCFAs found that there were lower levels of acetic acid, isobutyric acid,

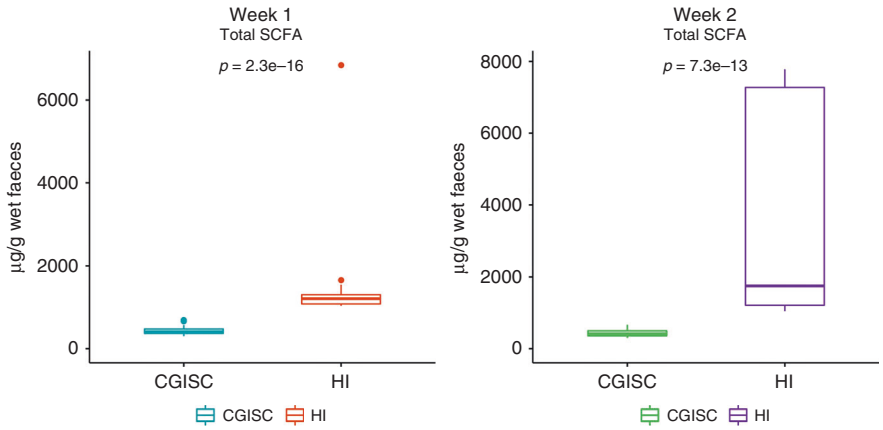

Fig. 5 Stool SCFA levels in study infants. At week 1, CGISC infants have significantly lower amounts of total short-chain fatty acid levels and remain so at week 2.

isovaleric acid, valeric acid, hexanoic acid, and heptanoic acid in the CGISC group as compared to HI at week 1 (Supplementary Table S2). There were no significant differences in concentrations of propionic acid and butyric acid between the groups at week 1 (Supplementary Table S2). At week 2, the concentrations of acetic acid, butyric acid, propionic acid and all other SCFAs were lower in the CGISC group (Supplementary Table S2). Among the CGISC group, there were no significant differences in SCFA levels between week 1 and week 2 samples (all $p > 0.05$). On the other hand, HI group showed significant increases in SCFA levels at week 2 compared to week 1 (all $p < 0.01$ except valeric acid and heptanoic acid where $p > 0.05$).

DISCUSSION

This prospective observational study found that by 10–14 days of life, term neonates with CGISCs developed significant dysbiosis with deficiency of the genera *Bacteroides* and *Bifidobacterium* and increased abundance of the genus *Escherichia–Shigella* and *Pseudomonas*. It also found significant deficiency of SCFAs in the stools of neonates with CGISCs.

Bifidobacterium was the predominant genus during the second week in HIs because they were predominantly breastfed and at home compared to CGISCs, who were still in hospital and had issues, such as feed intolerance, parenteral nutrition and intravenous antibiotics, all of which would have interfered with colonization by *Bifidobacteria*.[18,19] Normally, HMOs are utilized by *Bifidobacteria*, which enables them to grow and enrich the gut.[18] In the absence of adequate intake of breastmilk, there can be delay in the gut colonization by *Bifidobacteria*. In our study, the median age at the collection of second sample was 13 days, whereas the median age at full feeds was 15.5 days in infants with CGISCs. Many infants with CGISCs were still on parenteral nutrition and were being graded up on milk feeds when the second stool sample was collected.

Bifidobacteria are among the first microbial colonizers of the intestines of newborn infants and play key roles in the development of their physiology, including maturation of the immune system.[20,21] Hence, their deficiency in surgical infants is of concern and may contribute to the morbidities faced by these vulnerable infants. A vicious cycle may arise wherein clinical morbidities lead to gut dysbiosis, which in turn worsens clinical morbidities.

In our cohort of HIs, richness was significantly lower at week 2 compared to week 1 in HIs. While the drop in alpha diversity was not discernible on Shannon index ($p = 0.998$), ACE and Chao1 indices suggested a trend towards lower alpha diversity at week 2 compared to week 1 even in HIs (ACE, $p = 0.075$; Chao1, $p = 0.084$, Fig. 1). These findings are similar to that of Chi et al.,[22] who found that alpha diversity drops significantly from week 1 to week 2 of birth in low birth weight as well as healthy term newborn infants, before stabilizing.

The richness and alpha diversity decreased markedly from week 1 to week 2 in surgical infants; in addition to the normal drop that occurs in HIs as observed in our study and the study by Chi et al.,[22] the other contributing factor could be the use of antibiotics during that period. Bokulich et al.[23] reported that antibiotic use significantly diminishes the phylogenetic diversity and richness in the early newborn period.

The genera *Pseudomonas* and *Escherichia–Shigella* were significantly higher in CGISCs at week 2, compared to HIs. There were no significant differences in the relative abundances of other clinically important genera such as *Enterococcus*, *Enterobacter*, *Klebsiella*, *Staphylococcus* and *Streptococcus* between the surgical and HIs. Probable reason could be the extensive use of antibiotics in surgical infants, which might have suppressed these bacteria, and the short duration of follow-up; it takes some weeks before *Bifidobacteria* become well established at higher levels in healthy breastfed infants, replacing the facultative anaerobes.[24,25] Newborn infants have an aerobic intestine at birth.[26] The high level of oxygen in the newborn GI tract favours the appearance of facultative anaerobes (e.g. *Enterobacter*, *Enterococcus*, *Streptococcus*, *Staphylococci*, *Escherichia coli* and *Klebsiella*). These early colonizers gradually create a reduced, anaerobic environment within the GI tract by consuming the available oxygen, consequently facilitating the establishment of obligate anaerobes such as *Bifidobacterium* and *Bacteroides*.

Evidence is mounting that gut microbial metabolites have a major influence on host physiology. SCFAs are volatile fatty acids produced by the gut microbiota in the large bowel as fermentation products from food components that are unabsorbed/undigested in the small intestine.[27] Acetic acid, propionic acid and butyric acid are the most abundant, representing 90–95% of the SCFAs present in the colon. SCFAs result in reduction in the luminal pH in the gut, which inhibits pathogenic microorganisms.[28] Acetate produced by *Bifidobacteria* and other commensals improves intestinal defence mediated by epithelial cells and thereby protects the host against lethal infections by enteropathogens. Butyrate is an important fuel of intestinal epithelial cells and stimulates the mitogen-activated protein kinase signalling pathway in intestinal cells, which is positively correlated with gut defences.[29] Butyrate also enhances the intestinal barrier by regulating the assembly of tight junctions. In addition, SCFAs are known to have anti-inflammatory properties.[7]

Bacteroides are known to increase the intestinal concentrations of acetate as well as propionate,[30] whereas Firmicutes are predominant contributors of butyrate. While *Bifidobacteria* are not butyrogenic by themselves, acetate and other organic acids

produced by them are converted to butyrate by other colonic bacteria via cross-feeding interactions.[31]

It is concerning that SCFAs were lower in CGISCs compared to HIs. The deficiency of SCFAs in neonates with CGISCs has the potential to increase the risk of sepsis due to weakened gut barrier function and other adverse outcomes.

Given that neonates with CGISCs suffer from gut dysbiosis, probiotic supplementation may improve the dysbiosis, SCFA levels and clinical outcomes of these infants. Probiotics are known to inhibit gut colonization with pathogenic bacteria enhance gut barrier function, facilitate colonization with healthy commensals, protect from enteropathogenic infection through production of acetate, reduce antimicrobial resistance, enhance innate immunity and increase maturation of the enteric nervous system and promote gut peristalsis.[32] Through these mechanisms, probiotics have the potential to decrease the risk of sepsis, improve feed tolerance and minimize parenteral nutrition-associated cholestasis in infants with CGISCs.[32]

Meta-analyses of RCTs in preterm infants (non-surgical) have shown probiotic supplementation to be beneficial in decreasing mortality, necrotizing enterocolitis (NEC), late-onset sepsis and improving feed tolerance.[33] Majority of the RCTs included in those meta-analyses used *Bifidobacteria* as the sole or one of the components of probiotic supplements. Recent meta-analyses that focussed on bifidobacterial supplementation found a significant reduction in the incidence of mortality and NEC in preterm infants.[34] In a RCT that included 24 neonates with gastroschisis (probiotics: 12; placebo: 12),[35] significant dysbiosis was noted, and it was partially attenuated by the administration of *Bifidobacterium longum* subsp. *infantis*.[35] The authors stated that their pilot study was not powered to look at clinical outcomes and that further studies are indicated.

In a small RCT by Murakami et al.[36] eight surgical infants were included (*Bifidobacterium*: 4; no *Bifidobacterium*: 4); they reported that unexpectedly there were significantly more Bifidobacteriaceae in the samples from those who did not receive probiotics ($p < 0.05$). Since the sample size was very small, the results may need to be interpreted with caution. The authors concluded that surgical stress appears to affect intestinal microbiota and that probiotic administration requires further clarification.

A meta-analysis that included 198 infants with HD (two RCTs, three observational studies) reported that the incidence of Hirschsprung-associated enterocolitis was 22.6% in the probiotic group vs. 30.5% in the controls, but the difference was not statistically significant (odds ratio 0.72; 95% confidence interval: 0.37–1.39; $p = 0.33$).[37] In an RCT of 30 children (<15 years) undergoing various surgeries, Okazaki et al.[38] reported that supplementation with *Bifidobacterium breve* BBG-001 was well tolerated without adverse effects, and postoperative infectious complications were significantly decreased. Faecal analysis showed increased levels of *Bifidobacterium* and decreased abundances of Enterobacteriaceae, *Clostridium difficile* and *Pseudomonas*.[38]

Evidence is emerging from adult studies regarding the beneficial effects of probiotics in GI surgery[39,40] The meta-analysis by Lytvyn et al.,[39] which included 20 RCTs ($N = 1374$), concluded that probiotic/symbiotic supplementation decreases the risk of surgical site and urinary tract infections in patients undergoing abdominal surgery. Another meta-analysis by Yang et al.[40] that included 28 RCTs ($n = 2511$) involving adult patients undergoing GI surgery came to similar conclusions. The durations of hospital stay and antibiotic therapy were shorter in the probiotics/symbiotic group vs. controls.

Hence, there seems to be adequate rationale for conducting RCTs of probiotic supplementation, especially the one that contains *Bifidobacteria* in neonates with CGISCs.

Currently, two RCTs of probiotic supplementation in neonates undergoing GI surgery are underway (Howlette et al., Canada, https://ichgcp.net/clinical-trials-registry/NCT03266315); Rao et al., Australia, ACTRN12617001401347).

While our study found dysbiosis in infants with CGISCs, it does not address if the dysbiosis is related to the underlying surgical condition or factors, such as surgical stress, delayed introduction of breastmilk, usage of antibiotics and being in the NICU ecosystem. Infants with CGISCs are probably at a higher risk of dysbiosis because they have more prolonged feed intolerance due to the underlying GI pathology. Additionally, gut is the place where these organisms are predominantly located and hence CGISCs are probably more prone to dysbiosis than other types of surgical conditions. Future studies need to compare the gut microbiota of neonates with CGISCs vs. other surgical conditions to address this issue.

The main strength of our study is that it is probably the first study comparing gut microbiota in neonates with CGISCs vs. healthy term infants using culture independent techniques. The only other study was the RCT by Powell et al.,[35] which demonstrated dysbiosis in neonates with gastroschisis; however, the gut microbiota of HIs was not investigated in that study. A limitation of our study is the short duration of follow-up (14 days) and that only two samples were collected from study infants (weeks 1 and 2). The reasons for this approach were logistic feasibility and funding.

We were concerned that there would be significant drop-out rates if a later postnatal age for the second sample collection was chosen (e.g. day 21). As experienced in the present study, even at week 2, there were significant drop-out rates (50%) from HIs. Drop outs would also have occurred in surgical infants because nearly 25% of our surgical infants were discharged home by day 17 and 50% by day 22. It is difficult for busy parents at home to collect samples on a weekly basis. Future studies should allocate adequate resources to have longer duration of follow-up and test multiple stool samples (e.g. once a week for 4–6 weeks).

The other limitation was the fact that HIs were more mature by 1 week compared to the CGISC group. Since the gut microbiota evolves rapidly in the neonatal period, the influence of this 1-week difference cannot be ruled out. Another limitation was that the week 1 samples were collected at an earlier postnatal age (median 2 day) compared to surgical infants (median 4 days). Surgical infants usually have delayed passage of meconium and infrequent passage of subsequent stools because of underlying gut anomaly, administration of narcotic analgesics, delayed commencement of enteral feeds and postoperative ileus.

In summary, during hospitalization, neonates with CGISC develop gut dysbiosis with depletion of the genera *Bacteroidetes* and *Bifidobacterium* and increased abundance of *Pseudomonas* and *Escherichia–Shigella*. They also have deficiency of biologically important SCFAs in their stools. Similar studies with larger sample size and longer duration of follow-up are essential.

ACKNOWLEDGEMENTS

We sincerely thank the midwives at KEMH for facilitating stool collections of healthy term infants from postnatal wards, nursing staff at PCH for collecting stool samples from surgical infants, Mr. Damber Shrestha for providing data from the neonatal database, Ms. Yen Kok, Ms. Annie Chang, and Ms. Julie Hibbert for processing the stool samples and storing them in deep freezer and Dr. Gayatri Jape and Dr. Abhijeet Rakshasbhuvankar for helping with patient recruitment at KEMH. This study was funded by the PCH Grant and grants from the Centre for Neonatal Research and Education, Neonatal Directorate, KEMH for Women, Western Australia.

AUTHOR CONTRIBUTIONS

S.C.R.: Conception and design, acquisition of data, analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. M.E.: Analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. S.K.P.: Conception and design, interpretation of data; revising the article critically for important intellectual content;

and final approval of the version to be published. K.N.S.: Conception and design, interpretation of data; revising the draft of the article critically for important intellectual content; and final approval of the version to be published. I.G.: Conception and design, interpretation of data; revising the draft critically for important intellectual content; and final approval of the version to be published. A.K.: Conception and design, interpretation of data; revising the draft critically for important intellectual content; and final approval of the version to be published. B.W.: Analysis and interpretation of data; revising the draft critically for important intellectual content; and final approval of the version to be published. L.C.: Analysis and interpretation of data, revising the draft for important intellectual content; and final approval of the version to be published. P.L.C.: Conception and design, analysis and interpretation of data; revising the draft critically for important intellectual content; and final approval of the version to be published.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41390-020-0824-7) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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