THE MICROBIOME AND IMMUNITY IN PEDIATRIC SHORT BOWEL SYNDROME

INFLAMMATION, INFECTIONS AND THE MICROBIOME OF CHILDREN WITH SHORT BOWEL SYNDROME

By

JENNA DOWHANIUK, MD, FRCPC

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements for the

Degree Master of Science

McMaster University

MASTER OF SCIENCE 2017 McMaster University (Medical Science)
Hamilton, Ontario

TITLE: Inflammation, infections and the microbiome of children with short bowel syndrome

AUTHOR: Jenna Dowhaniuk, MD, FRCPC (McMaster University)

SUPERVISOR: Dr. Elyanne Ratcliffe, MD, FRCPC

NUMBER OF PAGES: xv, 111

LAY ABSTRACT

Short bowel syndrome is a condition caused by surgical removal of a large portion of the intestine. This research identified the most common bacteria which cause serious bloodstream infections and characterized the bacterial composition, known as the microbiome, in their intestine. The microbiome, in this population, exhibited a lower number and variety of bacteria, fewer 'good' bacteria and an overgrowth of specific bacteria usually in very low abundance in the intestine of healthy children. Children with short bowel syndrome had less bacteria by-products, which help maintain a healthy digestive tract. We did not identify differences in the permeability of the intestine or evidence of inflammation. These results are important to understand the interactions between bacteria of the intestine, the digestive complications of short bowel syndrome, and to establish a link between the microbiome, bloodstream infections and inflammation, which in the future can help advance future treatments for this serious disease.

ABSTRACT

Children with short bowel syndrome have reduced gastrointestinal mass due to surgical resection in the neonatal period. This multi-pronged health translational study investigated the previous infectious history, microbiome, and inflammation of twelve children with this disease. We sought to further understand how dysbiosis, bacterial products such as butyrate, and intestinal permeability may link with the type and frequency of bloodstream infections and systemic inflammation.

The most frequent pathogens identified in bloodstream infections were coagulase-negative *Staphylococcus*, *Enterococcus* species, *Klebsiella* species and *Candida* species. Sixty-one percent of cultured microbes could be considered enteric with a gastrointestinal origin.

With high-thoroughput 16S rRNA fecal analysis, children with intestinal failure demonstrated the most significant dysbiosis with the lowest Shannon diversity and an abundance of the *Escherichia* genus seemingly attributable to the pro-inflammatory species *E. coli.* Commensal anaerobes known to produce SCFA including *Ruminococcaceae* and *Lachnospiraceae* were significantly reduced in those with IF. Similarly, measured butyric acid was significantly reduced in children with IF compared to controls (median 0.37nmol/mg vs 10.92nmol/mg; $p<0.0001$).

Serum analysis of intestinal permeability markers including circulating bacterial products lipopolysaccharide and muramyl dipeptide (MDP) were similar between children with SBS and controls. Furthermore, no differences were detected in gene expression of pro- or anti-inflammatory transcription factors, cytokine concentration, or fecal calprotectin.

Children with SBS exhibit dysbiosis, a reduction in SCFA-producing microbes and reduction in butyrate concentration. Fecal analysis has identified a significant abundance of likely *E. coli*

however it remains the sixth most common microbe cultured in bloodstream infections. The microbial community is significantly altered in children with intestinal failure with implications on complications of this disease including feed advancement, bacterial overgrowth, gastrointestinal motility and infection. Further research will be needed to investigate if manipulation of the microbiome can influence important clinical outcomes and alter bacterial translocation and infection.

ACKNOWLEDGEMENTS

I want to kindly thank my supervisor Dr. Elyanne Ratcliffe for her incredible guidance and support throughout the years. Her intellectual guidance during my graduate studies has been invaluable. She has many years of clinical insight to the complications and struggles families with short bowel syndrome have as well as instrumental knowledge as a Clinician Scientist in Pediatric Gastroenterology and as a member of the Farncombe Family Digestive Health Research Institute. I would also like to express my gratitude to the expertise and guidance of Dr. Dawn Bowdish and Dr. Mike Surette as members of my graduate committee. Additional acknowledgements to the support and assistance of Dr. Matt Miller, Dr. Chris Verschoor, Dessi Loukov, Grace Tesky, Kyle Novakowski, Laura Rossi, Sam Chorlton, Joseph Chon, Michelle Shah, Rose-Frances Clause, Jillian Owens, Heather Mileski, Jake Szamosi and the McMaster Pediatric Gastroenterology Division. I would like to thank the Regional Medical Association of Hamilton, the Canadian Association of Gastroenterology and the Farncombe Family Digestive Health Research Institute for supporting my research as well as the families and children with short bowel syndrome who participated in this research. Enormous thanks to my incredible husband and family for their endless love and support.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

PRO-INFLAMMATORY, ANTI-INFLAMAMTORY AND INTESTINAL PERMEABILITY MARKERS IN CHILDREN WITH SHORT BOWEL SYNDROME

LIST OF ABBREVIATIONS

ANC	Absolute neutrophil count
CLABSI	Central line associated bloodstream infection
CoNS	Coagulase-negative *Staphlococcus*
CI	Confidence interval
CRP	C-reactive protein
CVC	Central venous catheter
CT	Cycle threshold
Foxp3+	Forkhead box P3
GI	Gastrointestinal
IF	Intestinal failure
Ig	Immunoglobulin
IL	Interleukin
IFNγ	Interferon Gamma
iNOS	Inducible nitric oxide synthase
IV	Intravenous
IQR	Interquartile range
LPS	Lipopolysaccharide
MDP	Muramyl dipeptide
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NF-κB	Nuclear factor kappa-light-chain of B cells
OTU	Operational taxonomic units
PN	Parenteral nutrition
qPCR	Quantitative real-time polymerase chain reaction
RORγT	RAT-related orphan receptor gamma
SBS	Short bowel syndrome
SCFA	Short chain fatty acids
SIBO	Small intestine bacterial overgrowth
TLR	Toll like receptor
Treg	T-regulatory cells
TNF	Tumour necrosis factor
WBC	White blood cell

DECLARATION OF ACADEMIC ACHIEVEMENT:

MICROORGANISM PROFILE AND LABORATORY RESULTS OF CONFIRMED BLOODSTREAM INFECTION IN CHILDREN WITH INTESTINAL FAILURE: A RETROSPECTIVE COHORT STUDY

Dr. J. Dowhaniuk: Involved in all aspects of study. Study concept, completion of study design, completed research ethics submission and revisions, data abstraction, statistical analysis and first author of the manuscript.

Dr. E. Ratcliffe, Dr. D. Bowdish and Dr. J. Pernica were involved in the study concept, revisions and edits of the manuscript.

Joseph Chon and Sam Chorlton were involved in data abstraction, statistical analysis and editing the manuscript.

ESCHERICHIA ABUNDANCE AND LOW FECAL BUTYRATE IN CHILDREN WITH INTESTINAL FAILURE

Dr. J. Dowhaniuk: Involved in the following aspects of study: Study concept, completion of study design, research ethics submission and revisions, data abstraction, short chain fatty acid analysis preparation, bioinformatics and epidemiologic statistical analysis and first author of the manuscript.

Dr. E. Ratcliffe, Dr. D. Bowdish and Dr. M. Surette were involved in study concept, statistical analysis and revisions and edits of the manuscript.

Laura Rossi and Michelle Shah in the laboratory of Dr. M. Surette completed 16s rRNA gene sequencing on the Illumina MiSeq platform.

Dr. K Green of McMaster Regional Centre for Mass Spectrometry completed the short chain fatty acid extraction of stool samples.

Sam Chorlton was involved as a research assistant collecting samples and demographics and the editing of the manuscript.

Jake Szamosi completed bioinformatics analysis.

PRO-INFLAMMATORY, ANTI-INFLAMAMTORY AND INTESTINAL PERMEABILITY MARKERS IN CHILDREN WITH SHORT BOWEL SYNDROME

Dr. J. Dowhaniuk: Involved in the following aspects of study: Study concept, completion of study design, research ethics submission and revisions, data abstraction, RNA primer optimization, RNA extraction and qPCR, statistical analysis and first author of the manuscript.

Dr. E Ratcliffe, Dr. D. Bowdish and Dr. M. Surette were involved in study concept and design, statistical analysis and revisions and edits of the manuscript.

Grace Teskey completed analysis of circulating bacterial products.

Cytokine analysis was completed at Eve Technologies in Calgary, AB Canada.

INTRODUCTION

1.1 SHORT BOWEL SYNDROME AND INTESTINAL FAILURE

Pediatric short bowel syndrome (SBS) is a rare and potentially debilitating condition following massive surgical intestinal resection in infancy (O'Keefe et al., 2006). The loss of functional small intestinal mass leads to chronic gastrointestinal complications including persistent diarrhea, dysmotility, poor growth and nutrient deficiencies (O'Keefe et al., 2006). While SBS is a rare disease with an estimated incidence of 24.5 per 100,000 live births, the condition poses a significant impact on a child and families' quality of life with frequent lengthy hospitalizations, repeated surgical procedures and surgical devices for enteral and parenteral nutrition (Duggan & Jaksic, 2017; Wales et al., 2004). The severity can range from mild to severe with those with the shortest remaining small intestine requiring long-term parenteral nutrition (PN) through central venous lines to provide life-sustaining fluids, electrolytes, and nutrition (O'Keefe et al., 2006; Squires et al., 2012). This sub-group of patients requiring PN is classified as having intestinal failure (IF) (O'Keefe et al., 2006). With an all-cause mortality of 25%, children with IF also experience high morbidity due to complications such as central line associated bloodstream infections (CLABSI), sepsis, loss of venous access, and liver failure (Duggan & Jaksic, 2017). They often require treatment for small bowel bacterial overgrowth with cycled oral antibiotics as well as treatment with motility agents, and require additional caloric support (Amin, Pappas, Iyengar, & Maheshwari, 2013; Duggan & Jaksic, 2017). Some require repeated surgical procedures to improve motility, to place gastric and jejunal feeding tubes and to obtain venous access for PN. A minority of patients will require intestinal and/or liver transplant with resulting complications including rejection, immune suppression and graft failure (Squires et al., 2012).

1.2 ETIOLOGY OF SHORT BOWEL SYNDROME

Surgical resections during infancy which result in SBS can occur in the setting of multiples etiologies including necrotizing enterocolitis, gastroschisis, midgut volvulus with malrotation and intestinal atresias (Duggan & Jaksic, 2017; Squires et al., 2012). While the average small intestine at term gestation is 157.4cm, children with SBS and IF can have 10-25% of remaining normal length following surgical resection of their diseases segments (Struijs, Diamond, de Silva, & Wales, 2009). Variations in the surgical procedures required depend on the individual's disease state and may include the loss of differing segments of small intestine, as well as loss of the ileocecal valve and colon (Amin et al., 2013). Furthermore, differences in disease and age at gestation predispose some children to surgical resection as a premature infant. Some conditions are associated with improved long term outcome and cessation of parenteral nutrition such as necrotizing enterocolitis, while other conditions are known to be associated with dysmotility and prolonged use of parenteral nutrition (Fallon et al., 2014). Gastroschisis is associated with more pronounced dysmotility, which could contribute to the challenge when attempting to advance enteral feeds in order to wean PN (Fallon et al., 2014). Children with IF secondary to SBS, therefore, represent a heterogeneous group with varied conditions and complications while all requiring parenteral nutrition.

1.3 FUNCTIONAL ADAPTATION AND CLINICAL MANAGEMENT OF SHORT BOWEL SYNDROME

An inter-disciplinary team is required to care for children with SBS and is associated with improved clinical outcomes (Sant'Anna et al., 2012; Stanger, Oliveira, Blackmore, Avitzur, & Wales, 2013). Registered dietitians, nurse practitioners, nurses, pediatric gastroenterologists,

pediatric surgeons, infectious disease specialists and social workers all have a critical role in the long-term care of a child with IF. Maintaining adequate growth in the setting of minimal oral or enteral intake is paramount and requires frequent adjustments of parenteral nutrition (Squires et al., 2012). Parenteral nutrition is provided through surgically-placed central line catheters and amino acids, glucose, and lipids are infused over many hours per day to maintain electrolyte homeostasis and for adequate growth (Engstrand Lilja, Wefer, Nyström, Finkel, & Engstrand, 2015).

A multi-disciplinary team strives to promote oral or G-tube feeds and minimize the use of parenteral nutrition. By providing enteral nutrition, the remaining shortened small intestine can undergo macroscopic and functional changes over time (Duggan & Jaksic, 2017). This process, termed functional adaptation, increases the capacity per unit of small intestinal digestive and absorptive function (Duggan & Jaksic, 2017; Weale, Edwards, Bailey, & Lear, 2005). Macroscopic changes include crypt differentiation and elongation, villi height and diameter expansion, increased nutrient transport expression, cellular proliferation and the increased absorption of carbohydrate, water and electrolytes (Weale et al., 2005). This process of adaptation can begin immediately following surgery and can continue up to 5 years post-resection. To promote functional adaptation, the health care team must minimize enteral deprivation to the GI tract when possible and provide cycled enteral nutrients, with particular emphasis on feeding human breast milk in infancy, whole protein if tolerated, mixed long and medium-chain fatty acids and complex carbohydrates (Goulet et al., 2013). Furthermore, this process of adaptation may require months to many years depending on the segment and total length resected (Duggan & Jaksic, 2017; Goulet et al., 2013). While encouraging oral feeds is crucial, many barriers to

functional adaptation exist including oral aversion, dysmotility, abdominal pain, excess gas production, as well as diarrhea. A multi-disciplinary team manages and treats each complication and barrier while slowly titrating enteral feeds and weaning parenteral nutrition. The ultimate goal is one of enteral autonomy where a child with SBS no longer requires parental nutrition and no longer is considered to have IF (Duggan & Jaksic, 2017).

1.4 PARENTERAL NUTRITION AND VENOUS ACCESS

While PN is life-sustaining, there are many complications to its long-term use. To maintain infusion rates required to meet the caloric needs of children, a surgically placed central line is required (Diamanti et al., 2007). Children with IF may require their central line to be removed and replaced many times over their life predisposing them to loss of venous access as they age. Loss of venous access remains an indication for small intestinal transplantation which carries a high risk of mortality (Kaufman et al., 2001). Minimizing central line replacements when possible is important to maintain vascular access sites. This remains a challenge in the setting of CLABSI where a central line has been colonized with a fungus or bacteria and the clinician must balance the risk of a contaminated central line leading to recurrent sepsis, with loss of vascular access sites. Further complications of prolonged PN include PN-associated liver disease, bacterial infections, sepsis and thrombosis (Duggan & Jaksic, 2017). For some children, bacterial infection remains the most significant morbidity of IF requiring frequent hospital admission, prolonged IV antibiotics, surgical replacement of venous lines and life-threatening sepsis (Diamanti et al., 2007).

1.5 SBS AND MUCOSAL IMMUNITY

This multi-pronged health translational study sought to investigate how surgical resection and resultant short bowel syndrome impacts a child's microbiome, gastrointestinal inflammation, and infectious outcomes. With abundant Peyer's patches and overlying M cells, the small intestine functions as a link between environment, microbes and host (Rosner & Keren, 1984). This complex interplay allows microbial communities to both prime and regulate mucosal immunity while our immune system can also exert host control over nearby microbiota (Stecher, 2015). Massive small intestinal surgical resection at a young age may impact this cross-talk between microbe and host, influencing antigen-exposure, inflammatory processes and paracellular permeability of intestinal epithelial cells.

1.6 SBS AND THE MICROBIOME

We sought to characterize and compare the microbiome of children with severe SBS and IF from those who achieved enteral autonomy. With culture-independent high-throughput 16s rRNA sequencing of fecal samples of children of SBS, this study will add to the very recent first publications applying metagenomics to understand if dysbiosis plays a role in clinical outcome of pediatric SBS[1-3]. The knowledge already gained from the Human Microbiome Project has demonstrated how host-microbe interactions can impact human health (Turnbaugh et al., 2007). Clinical complications of SBS including diarrhea, motility, nutrient absorption and small bowel bacterial overgrowth could be directly related to microbiome diversity and abundance patterns.

1.7 SBS AND SHORT CHAIN FATTY ACIDS

Beyond the quantitative analysis of microbes, this research will further investigate critical colonic bacterial by-products of fermentation specifically short chain fatty acids (SCFA). Known to improve epithelial barrier function and regulate inflammation, SCFA concentration of stool samples may provide insight into altered intestinal permeability as well as T-regulatory cell expansion (Lührs et al., 2001; Maslowski & Mackay, 2011). We sought to correlate SCFA concentration to known SCFA-producers of the *Clostridia* class specifically *Lachnospiraceae* and *Ruminococcaceae*.

Short chain fatty acids are a link between the commensal obligate aneorobes of the gastrointestinal tract and our mucosal immunity. They are known to expand Foxp3+ T-regulatory cells and anti-inflammatory cytokine IL-10 (Maslowski & Mackay, 2011; Segain et al., 2000). Butryate production maintains a healthy intestinal epithelium, influences cytokine production and paracellular permeability (Kinoshita, Suzuki, & Saito, 2002). Therefore, we sought to complete analysis of cytokine IL-10 as well as mRNA expression of transcription factor FoxP3+ to investigate for a reduction in anti-inflammatory cell signalling.

1.8 MUCOSAL INFLAMMATION

Through toll-like receptors (TLR), pathogenic gastrointestinal microbes induce intestinal epithelial cell-signals to induce inflammation (Lucas & Maes, 2013). Endotoxin lipopolysaccharide (LPS) of gram negative bacteria bind to basolateral membrane bound TLR-4 to ultimately increase transcription of NF-κB and the production of inflammatory cytokines

TNFα, IFN-γ, and IL-6 (Round & Mazmanian, 2009). Inflammation in the gastrointestinal tract can be characterized by an increase of T_H17 cells and their cytokine expression of IL-17, IL-23 and TNFα and infiltration of lymphocytes, macrophages and neutrophils into the lamina propria of the mucosa (Round & Mazmanian, 2009). Neutrophil activation leads to calprotectin release into stool and can be detected in fecal samples (Wang et al., 2015). We investigated inflammatory processes within the gastrointestinal tract by stool measurement of calprotectin as well as pro-inflammatory cytokine concentration, gene expression of TLR4 and transcription factor RORγT.

1.9 BLOODSTREAM INFECTIONS AND INTESTINAL PERMEABILITY

One of the most common and severe complications of SBS and IF is the frequency of bloodstream infections which can result in sepsis and death. With central line infection rates exceeding those of children on chemotherapy, little is understood of the underlying physiology behind frequent blood stream infections in SBS (Alexander et al., 2016). For those children who do not require intestinal transplant, sepsis is now the leading cause of death in SBS (Squires et al., 2012). We completed a retrospective review of previous bloodstream infections of this cohort to identify the common organisms isolated. In addition, total leukocyte counts, neutrophil counts and inflammatory marker C-reactive protein were evaluated to investigate if typical immune response occur at the onset of infection.

We hypothesize that bacterial translocation from the gastrointestinal tract has a central role in the predisposition to bloodstream infections. Intestinal permeability is difficult to reliably evaluate

and many methods exist include serum analysis of circulating bacterial products, lactulose-mannitol ratio testing, antibody concentrations to bacterial products, and transmembrane protein concentrations (D'Antiga, Dhawan, Davenport, Mieli-Vergani, & Bjarnason, 1999; Wang et al., 2015). This study will further analyze if intestinal permeability is abnormal in children with IF via detectable circulating bacterial products lipopolysaccharide (LPS) and muramyl dipeptide (MDP) through binding of their receptors toll-like receptor-4 and nucleotide oligomerization domain 2, respectively as well as gene expression analysis of TLR2 and TLR4 via real-time qPCR.

1.10 CENTRAL HYPOTHESIS

We hypothesize that the natural history of IF including early surgical resection, enteral deprivation, and frequent antibiotics can significantly impact the development and maintenance of a diverse commensal microbiome. An imbalance of tolerogenic commensals and pathogenic bacteria may disrupt intestinal permeability, promoting mucosal inflammation and bacterial translocation leading to clinical infectious complications.

1.11 STUDY OVERVIEW

This research will provide important documentation as to which bacteria are cultured in bloodstream infections for children with SBS, the frequency of such infections and the preliminary leukocyte response at the time of infection. In addition; we sought to complete one of the first high-thoroughput 16S rRNA fecal analysis of their microbiome. Microbiome diversity and composition in this cohort can have clinical implications by influencing feed

advancement, growth, diarrhea and bloodstream infections. Furthermore, our prospective analysis of inflammation and intestinal permeability may advance our understanding of frequent CLABSI and septic events.

MICROORGANISM PROFILE AND LABORATORY RESULTS OF CONFIRMED BLOODSTREAM INFECTION IN CHILDREN WITH INTESTINAL FAILURE: A RETROSPECTIVE COHORT STUDY

[1]Jenna Katherine **Dowhaniuk** (MD, FRCPC), [2]Sam **Chorlton** (MD), [3]Dawn ME **Bowdish** (MSc, PhD), [4]Joseph **Chon**, [1]Jeffrey M. **Pernica** (MD, FRCPC), [1]Elyanne M. **Ratcliffe** (MD, FRCPC)

[1]Department of Pediatrics, McMaster University, Hamilton, Ontario

[2]Department of Undergraduate Medicine, McMaster University, Hamilton, Ontario

[3]Department of Pathology & Molecular Medicine, McMaster University, Hamilton, Ontario

[4]Department of Biochemistry, McMaster University, Hamilton, Ontario

Correspondence to: Dr. Jenna Dowhaniuk, Department of Pediatrics, McMaster University 3A61,1280 Main Street West, Hamilton, Ontario, L8S 4K1, Canada. Tel.: 905-527-2299 x73455; fax: 905-521-2655; e-mail: dowhanjk@mcmaster.ca

Funding and Supports: Source of funding includes Regional Medical Associates of Hamilton and Canadian Association of Gastroenterology

Conflicts of Interest: None.

PREFACE:

MICROORGANISM PROFILE AND LABORATORY RESULTS OF CONFIRMED BLOODSTREAM INFECTION IN CHILDREN WITH INTESTINAL FAILURE: A RETROSPECTIVE COHORT STUDY

J. Dowhaniuk: Involved in all aspects of study. Study concept, completion of study design, completed research ethics submission and revisions, data abstraction, statistical analysis and first author of the manuscript.

Dr. Elyanne Ratcliffe, Dr. Dawn Bowdish and Dr. Pernica were involved in the study concept, revisions and edits of the manuscript.

Joseph Chon and Sam Chorlton were involved in data abstraction, statistical analysis and editing the manuscript.

SUBMISSION: drafted for submission

ABSTRACT

Background: Children with Intestinal Failure (IF) experience frequent bloodstream infections and require prolonged use of central venous catheters (CVC) for parenteral nutrition. We sought to review all confirmed bloodstream infections (BSI) for a cohort of children, including laboratory findings upon presentation. Rates of central line-associated bloodstream infections (CLABSI) were calculated and catheter salvage practices were reviewed.

Methods: We conducted a retrospective review of 12 children with IF secondary to short bowel syndrome. Data was collected for each BSI including microorganism growth, culture growth time and laboratory results at presentation. Only BSI infections that met Infectious Disease Society of America criteria were categorized as CLABSI. For each CLABSI, parameters evaluated included number of organisms detected and catheter salvage rates.

Results: The most frequent pathogens identified were coagulase-negative *Staphylococcus*, *Enterococcus* species, *Klebsiella* species and *Candida* species. Study participants generally presented with normal leukocyte counts, neutrophils and modestly elevated C-reactive protein (median 32.3, IQR 7.5,72.9). There was no significant difference in leukocyte counts based on the microorganism cultured. There were 2.4 CLABSI per 1000 catheter-days. The majority of CVC's (53%) were promptly removed after CLABSI diagnosis; 25% were salvaged and 22% required CVC removal following pathogen regrowth.

Conclusions: In this cohort of IF patients, most bacteremias involved enteric pathogens and 11% of all BSI were Candida infections; consequently, empiric anti-fungal treatment could be considered in the setting of potential CVC infection. Normal leukocyte counts and mild elevation in C-reactive protein levels are commonly observed in the setting of documented BSI in children with IF.

INTRODUCTION

Short bowel syndrome remains the most common cause of intestinal failure (IF) in children[1]. Surgical resections leading to short bowel syndrome (SBS) in the neonatal period are often due to necrotizing enterocolitis, gastroschisis, intestinal atresias and volvulus[1]. Extensive surgical resections can result in an inability to maintain energy and fluid balance on enteral diets requiring long-term life-sustaining parenteral nutrition (PN)[1-3]. Children with IF have substantial morbidity given their reliance on central venous catheters (CVCs) for daily nutrition[1, 3]; they present with frequent central line-associated bloodstream infections (CLABSI) and sepsis represents a significant cause of mortality in this patient population[1, 2, 4]. In addition, recurrent CLABSI can alter a child's clinical outcome as frequent sepsis, loss of central venous access due to thrombosis, and the necessity for repeated catheter replacement after infection remain possible indications for intestinal transplantation[5].

Fever in a child with IF could represent life-threatening sepsis. Pediatric health care providers must consider the possibility of a bloodstream infection (BSI) when evaluating an unwell child with IF, given that fevers in children with CVCs due to a primary gastrointestinal diseases are associated with bacteremia more than half the time[6]. In contrast, children with cancer who had CVCs and presented to the emergency department (ED) with fever were found to have BSI far less frequently (9% of visits) in a previous cohort study[6]. Upon presentation, initial work up for sepsis often includes evaluation for leukocytosis or leukopenia, as well as inflammatory markers such as C-reactive protein (CRP)[7-9], prior to initiating empiric antimicrobial therapy[10]. Antibiotic selection and duration should be further tailored if an organism is identified. Appropriate management of CLABSI often involves removing the catheter, though occasionally it can be

salvaged using antimicrobial therapy alone[10]. Leaving an infected catheter *in situ* increases the risk of infection recrudescence or relapse but reduces surgical procedures to preserve critical CVC access sites.

We completed a single-centered retrospective review of confirmed BSIs in patients with pediatric IF to provide further insight into microbiologic etiology, to delineate leukocyte response and biomarkers in the setting of septicemia, and to review catheter salvage following CLABSI diagnosis.

METHODS:

We performed a retrospective chart review of 12 children followed at McMaster Children's Hospital. Children younger than 18 years of age followed by the specialized Short Bowel Rehabilitation team were eligible for inclusion. These 12 children had been enrolled in ongoing prospective studies and all had required PN for IF delivered through a peripherally or centrally inserted catheter (Broviac, Port-a-Cath). Children were not excluded if they had ultimately achieved enteral autonomy, but only their catheter days with PN were included in the study. For the purpose of this study, intestinal length was obtained from operative reports including the shortest measured length of small intestine, the timing and gestational age corresponding to that measurement. Small intestinal lengths were compared to previously published normal values[11]. Gestational age, total number of catheter days, ileocecal valve and colon preservation were collected from previous records. Ethics approval was obtained from the Hamilton Integrated Research Ethics Board for retrospective analysis.

Microbiology of Bloodstream Infection

We reviewed all blood culture results from included participants while they were receiving PN. Bloodstream infection was defined as 1) a central and/or peripheral culture positive for *Staphylococcus aureus,* gram-negative bacilli, *Candida* species or *Enteroco*ccus species, or 2) a positive central AND peripheral culture OR repeated positive central culture for the following pathogens: coagulase-negative *Staphylococcus* (CoNS), *Bacillus* species., *Leuconostoc* species, or viridans group streptococci. A given 'episode' of BSI referred to the period of time where a patient had blood cultures positive for the same pathogen(s) for the purpose of determining the overall incidence of BSI and the clinical presentation of the patient at the onset of infection. BSI rate per 1000 catheter days was calculated. The time to positivity of all cultures was recorded, as was concurrent antimicrobial therapy.

Laboratory Results

For each BSI episode, the patient chart was reviewed to permit abstraction of the following laboratory tests at the onset of illness: white blood cell count (WBC), absolute neutrophil count (ANC), percentage of total WBC represented as neutrophils, monocytes, and lymphocytes, and CRP. Reference ranges were applied across all age groups as normal total leukocyte count 4.5 to 13.5x10(9)/L, normal ANC 1.5 to 8.0x10(9)/L and lymphocyte range 1.5 to 6.8 x10(9)/L. Distributions of these tests were plotted and stratified by infecting pathogen looking at solely monomicrobial BSIs; non-parametric ANOVA was used to explore potential differences. Pathogens were grouped by genus level or at a species level for clinical relevance.

Central Line Associated Bloodstream Infections

CLABSI was defined as both a positive central and peripheral culture with a differential time to positivity of greater than two hours, as per the Infectious Diseases Society of America (IDSA) definition[10]. We recorded whether CVCs were removed promptly or treated with antimicrobials alone for each episode of CLABSI. We defined 'prompt' CVC removal as a central line immediately removed following positive blood cultures or removed within 7 days due to repeated positive blood cultures. Any regrowth of the same organism was identified if growth was beyond the first seven days. A CVC was considered successfully salvaged if no regrowth of the same organism occurred subsequent to the first week requiring further therapy or line removal. Salvage failure was defined as the re-growth of the initial infecting pathogen in a blood culture later than 7 days from the initial blood culture up to 6 months after the initial infection.

The distribution of results was assessed visually and with a D'Agostino-Pearson omnibus normality test. The groups were not normally distributed and consequently, a non-parametric analysis was performed with Kruskall-Wallis ANOVA and Mann-Whitney U test with Bonferonni correction for multiple testing. Data is presented as means with confidence intervals (CI) or median and interquartile ranges (IQR).

RESULTS:

Demographics

The 12 children with IF enrolled in this study had a cumulative total of 18,166 catheter days with a median of 867 catheter days (IQR 482.8, 2775) per patient. The etiology of IF included necrotizing enterocolitis (41.7%), gastroschisis (33.3%) and malrotation with midgut volvulus

(16.7%). The median length of residual small bowel was 42cm (IQR 26.1-57.5) which is 20.4% of expected small intestine length compared to published norms (IQR 16.1, 48.8%)[11]. Fifty-eight percent of patients had a preserved colon and ileocecal valve. Full demographics are presented in Table 1.

Microbiology of Bloodstream Infection

A total of 186 positive blood culture isolates were considered for analysis between 2006 and October 2016. One hundred and six blood cultures (57%) fulfilled criteria for BSI as determined above. All included patients experienced at least one BSI (range 1-27 per child) and no patients developed BSI subsequent to PN discontinuation or CVC removal. The rate of BSI in our cohort over the study period was 5.84 per 1000 catheter days. There was a total of 27 different organisms isolated from positive cultures (Figure 1). The most prevalent organism was coagulase-negative *Staphylococcus* (n=36, 34%), followed by *Enterococcus faecalis* (n=24, 23%), *Klebsiella oxytoca* (n=11, 10%) and *Klebsiella pneumoniae* (n=11, 10%). As a group, *Enterobacteraceae* were more prevalent than any other bacterial family and accounted for 35% (n=37) of positive cultures. Fungi accounted for 11% (n=12) of all positive blood cultures: *Candida albicans* (n=6, 5.6%) was most prevalent and followed by *Candida tropicalis* (n=2, 1.9%), *Candida parapsilosis* (n=2, 1.9%), *Candida glabrata* (n=1, 1%) and *Candida krusei* (n=1, 1%). No obligate anaerobes were isolated.

Leukocyte and Neutrophil response

Our study participants were found to have median leukocyte and neutrophil counts in the normal reference range at the presentation of a BSI (Figure 2-4). The median total leukocyte count was 6.95 (IQR 4.6-10.2), with a median neutrophil count of 3.7 (IQR 2.0-6.1), and percent ANC

median 58% (IQR 36-69.5%). Leukopenia and leukocytosis were present in 22.2% and 13.3% of our cohort, respectively, leading to a sensitivity of 35.5% for an abnormal WBC count predicting BSI. As a group, 12.4% had neutropenia (ANC<1.5×10^9/L) and 13.5% had neutrophilia (ANC>8×10^9/L) at BSI diagnosis, leading to an overall sensitivity of 25.9% for abnormal ANC. Most patients presented with a mildly elevated CRP (median 32.3 mg/L, IQR 7.5-72.9), though values were only available for 35 BSI episodes (Figure 5).

We next compared the WBC, ANC, and CRP at BSI presentation for the most common isolates including CoNS*, Enterococcus* species, *Klebsiella* species, *Staphylococcus aureus, Candida* species and *Escherichia coli*; there was no statistically significant difference between groups (Figures 2-5).

CLABSI Results

Of the 106 BSI, 43 met the IDSA definition for a CLABSI. The rate of CLABSI in our study cohort was 2.4 per 1000 catheter days. Chart review identified that 47% of children with IF were on oral antibiotic treatment at the time of initial presentation; these consisted of predominantly cycled antibiotics for presumed small bacterial overgrowth with 75% on one or more of metronidazole, gentamicin, trimethoprim-sulfamethoxazole or cephalexin. Of all CLABSI, 28% were polymicrobial. The median time to central positivity was 7.6 hours (IQR 5, 12 hours) and the median time to peripheral positivity was 17.8 hours (IQR 10.5, 27 hours). No central line culture grew after 24 hours, whereas 9 pathogens were isolated after 24 hours in the peripheral cultures. These late-growers included CoNS*, Candida parapsilosis, Candida albicans, Candida*

tropicalis, Acinetobacter species and *Alcaligenes faecalis.* The maximum recorded peripheral culture positivity was 50.4 hours which identified CoNS.

Just over half (22/43, 51%) of these 43 CLABSI episodes were managed in part via prompt removal of the central line. The CVC was successfully salvaged for 25.6% (11/43) of the CLABSI in this cohort; infecting pathogens in this group included CoNS (8/11), *Escherichia coli* (1/11), *Enterococcus* species (2/11) and *Klebsiella pneumoniae* (1/11). The remaining CVC (10/43, 23.3%) that were not removed promptly were later considered salvage failure when they regrew the same organism at an average of 20.8 days (minimum 8 days, maximum 38 days) following therapy. No patient died in this cohort.

DISCUSSION:

Children who have IF represent a unique cohort of children with frequent sepsis and bloodstream infections. Multiple hypothesized factors may contribute to the increased rate of BSI including small bowel bacterial overgrowth, increased intestinal permeability, bacterial translocation, and frequent access to the CVC[4, 6]. This retrospective review of children with IF sought to review microorganism cultures, laboratory results, and CLABSI management.

Our results are similar to previous reports documenting CoNS, *Enterococcus*, *Klebsiella* and *Candida* species as the most common pathogens identified in BSI among children with IF[2, 4, 6, 12]. Other investigators have identified different patterns of BSI in children with primary gastrointestinal disorders as compared to children with other chronic conditions[6]; these include more polymicrobial infections and more infections with common colonizers of the large intestine[6].

We identified that 61% of all BSI in our study population were caused by *Enterobacteriacaeae* (*Escherichia, Klebsiella, Enterobacter, Citrobacter, Proteus, and Serratia species)* or *Enterococcus species.;* this is consistent with previous studies of BSI in children with IF that found up to 61-66% of isolates were these 'enteric' pathogens[2, 6]. The predominance of the genus *Enterococcus,* specifically, is interesting, and potentially speaks to the pathogenesis of BSI in children with IF; previous cohort studies of children with non-peripherally inserted central catheters have generally found many fewer BSIs caused by these pathogens[13]. As anaerobic cultures may have been infrequently ordered, the prevalence of obligate anaerobe infection was likely underestimated. Repeated septicemia with enteric pathogens raises questions about intestinal permeability as a possible source for BSI.

Our study identified a BSI rate of 5.84 per 1000 catheter days and CLABSI rate of 2.4 per 1000 catheter days. This is likely an underestimate given our strict inclusion criteria and application of the IDSA criteria requiring us to exclude a majority of positive cultures. Previous reports have identified higher rates of CLABSI in children with IF (8.9 per 1000 catheter days) which exceed rates in children who are broadly immunocompromised as a result of chemotherapy (2.85 per 1000 catheter days)[1, 14].

We also found that candidemia was a relatively frequent occurrence, comprising 11% of all BSI in our cohort; this is higher than reported in other Canadian pediatric populations[13]. Pediatric IF and receipt of PN or lipids are independent risk factors for *Candida* infections[15]; a previous large study observed that 22% of children with IF experienced candidemia[1, 15]. The majority of fungemias in our cohort were caused by *Candida albicans*, *Candida tropicalis* and *Candida*

parapsilosis, though we did observe infections with the more difficult-to-treat *Candida glabrata* and *Candida krusei*. Multiple candidal infections were documented in only 4 children; these individuals had retained catheters for a median of 3044 days (IQR 2484, 3599), compared to the other 8 children who had lines *in situ* for a median of 492 days (IQR 339, 873). Diagnosis and prompt treatment of invasive *Candida* infections is imperative as candidemia has been associated with mortality rates from 7.7 to 26%[16, 17]. Potential pathogenic mechanisms of fungal BSI in children with IF include gastrointestinal translocation due to altered intestinal epithelial barriers as well as from increased colonization rates resulting from frequent antibiotic use[15, 16]. The IDSA Clinical Practice Guidelines for the Diagnosis and Management of Intravascular Catheter-Related Infections suggest empirical therapy for candidemia in those with risk factors such as total PN or prolonged use of broad-spectrum antibiotics[10]. Given the occurrence of these risk factors in our cohort, further research is required to identify if particular children with IF should also be treated empirically for fungal infections in the setting of presumed sepsis.

Although host response to infection varies among individuals, in this cohort of children, few appeared to mount an increased leukocyte or neutrophil count in the setting of an infectious microorganism in their bloodstream[9]. Normal leukocyte and neutrophil counts were identified in most children on the day they presented with a confirmed-bloodstream infection; this immune response did not seem to vary significantly by infecting pathogen. This finding appears quite different than the typical immune response of healthy children; previous literature identified that up to 65-74% of children with bacterial infection presented with an elevated leukocyte count >15,000cells/mm3[7, 18]. It is unclear if a normal leukocyte count in the setting of bacteremia suggests an alteration in the immune response for children with IF. To date, there are limited

studies examining the immune response in pediatric SBS. In our cohort, children with SBS appeared to have normal quantities of neutrophils; however previous research has observed impaired killing *in vitro* when challenged with CoNS[19].

CRP is a widely available biomarker, well established to correlate with the inflammatory response in pediatric sepsis and line infection[9]. We observed a slight rise in CRP (median 32.3) in children with IF at first presentation of confirmed BSI. Our results indicate that blood tests commonly ordered by front-line physicians evaluating children for invasive bacterial or fungal infections, such as leukocyte counts or CRP, have little to no value in the diagnosis of BSI in children with IF due to their very limited sensitivity; this is in contrast to findings of other cohort studies[12].

We identified that only 25% of children had their CVC salvaged following a CLABSI, lower than other studies reporting salvage rates of 42 to 72% for all monomicrobial infections[12]. However, it is difficult to compare CVC salvage rates between studies, as there is both objective (eg. *Staphylococcus aureus* infection) and subjective (eg. 'septic appearance') criteria governing the appropriateness of catheter removal[10]. Previous publications have reviewed successful CVC salvage in the setting of *Staphylococcus aureus*, enteric organisms and polymicrobial infections[12], however, the benefit of leaving the catheter in place must be carefully balanced against the significant risk of persistent or recrudescent BSI. It should be noted that nearly half of the children who did not have their CVC removed promptly, had an incompletely-sterilized catheter likely cause a second episode of BSI.

Our study presents with several limitations including the within-group variation in type of CVC, age, hospitalization, cycled antibiotics and use of ethanol locks over the ten-year study period that

was not corrected for in the analysis. The majority of patients required multiple types of catheters and antibiotic lock therapy and all data were pooled. As this was a retrospective review, there was no standardization for bloodwork and other ordered investigations leaving some data gaps. A further limitation was the small sample size minimizing the ability to draw on multi-variant analysis. This study provides only descriptive data of laboratory results and was not designed to determine sensitivity or specificity for its use.

Children with IF present with frequent bloodstream infections which are predominantly enteric organisms and CoNS. Fungal bloodstream infections are prevalent in pediatric intestinal failure patients and empiric anti-fungal treatment in sepsis could be considered. For children with IF, there are limitations in applying leukocyte or neutrophil results as a predictor of serious BSI. Identification of severe bacterial infections, CLABSI and sepsis in children with IF requires high clinical concern without reliance on laboratory findings. Further research is required to investigate the inflammatory cascade in the setting of bloodstream infection to further understand any appreciable differences in children with IF.

Table 1. Demographic characteristics of study participants

Variable	Result
Gender, n, *(%)*	
Male	8 (66.7%)
Total catheter days, median *(IQR)*	867 (482.8 - 2775)
Etiology of intestinal Failure, n, *(%)*	
Necrotizing enterocolitis	5 (41.7%)
Gastroschisis	4 (33.3%)
Malrotation with mid-gut volvulus	2 (16.7%)
Isolated jejunal atresias	1 (8.3%)
Gestational age, *weeks,* median *(IQR)*	33.9 (29.1-37.6)
Age at initial intestinal resection, *days,* median *(IQR)*	19 (2-154)
Shortest measured length of small intestine, *cm,* median *(IQR)*	42 (26.1-57.5)
Predicted bowel length at shortest measure, mean *(SD)*	20.4% (16.1-48.8%)
Age of measured shortest length of small intestine, *days*, median *(IQR)*	59 (13.5-180.3)
Preserved ileocecal valve, n *(%)*	7 (58.3%)
Colon Anatomy, n, *(%)*	
Full colon intact	7 (58.3%)
Partial or hemi-colon	4 (33.3%)
Sub-total colectomy	1 (8.3%)

Abbreviations: SD, Standard Deviation; IQR, Interquartile range at 25% and 75%

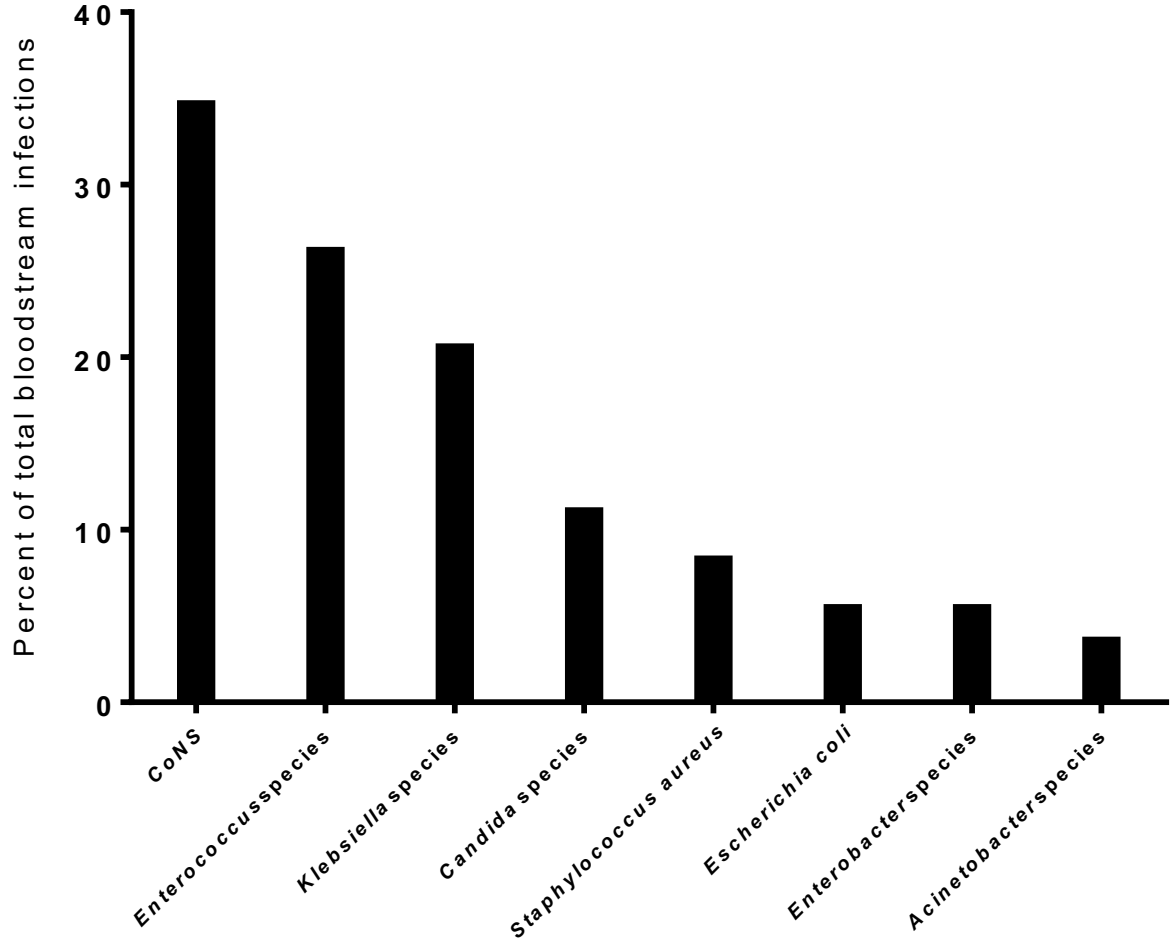

Figure 1. Microbiology of bloodstream infections in children with short bowel syndrome by most prevalent cultured microorganism. Abbreviation: CoNS, coagulase-negative *staphylococci*

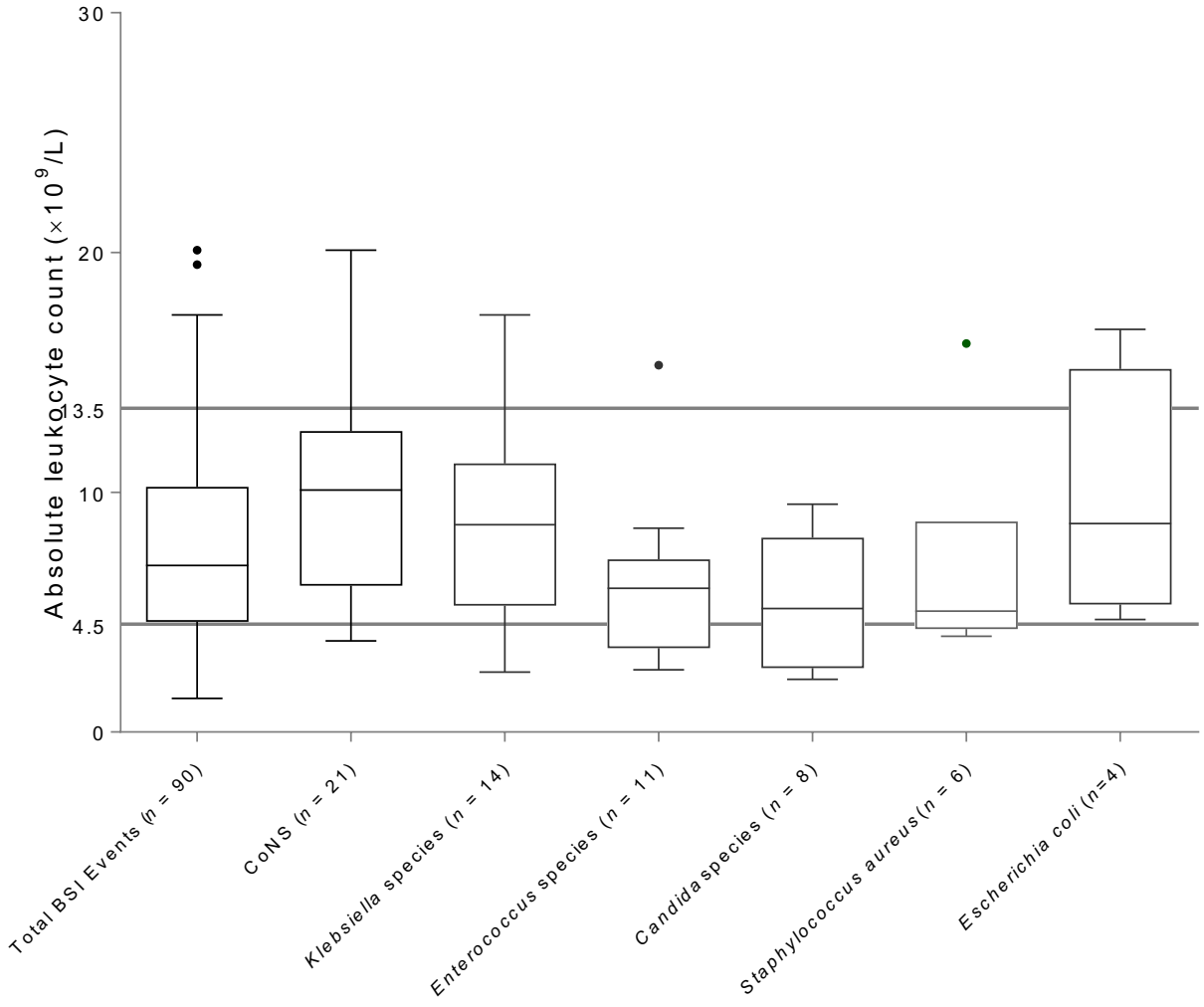

Figure 2. Box and whisker plot of absolute leukocyte count (x10^9/L) at presentation of bloodstream infection in children with short bowel syndrome. Displaying cumulative results of *n*=90 bloodstream infections as well stratified by most frequent cultured microorganism. Reference range applied across all age group depicted by horizon bars as 4.5 to 13.5x10^9/L. Data not complete if bloodwork not available from time of presentation. Abbreviation: CoNS, coagulase-negative *staphylococci;* BSI, bloodstream infections

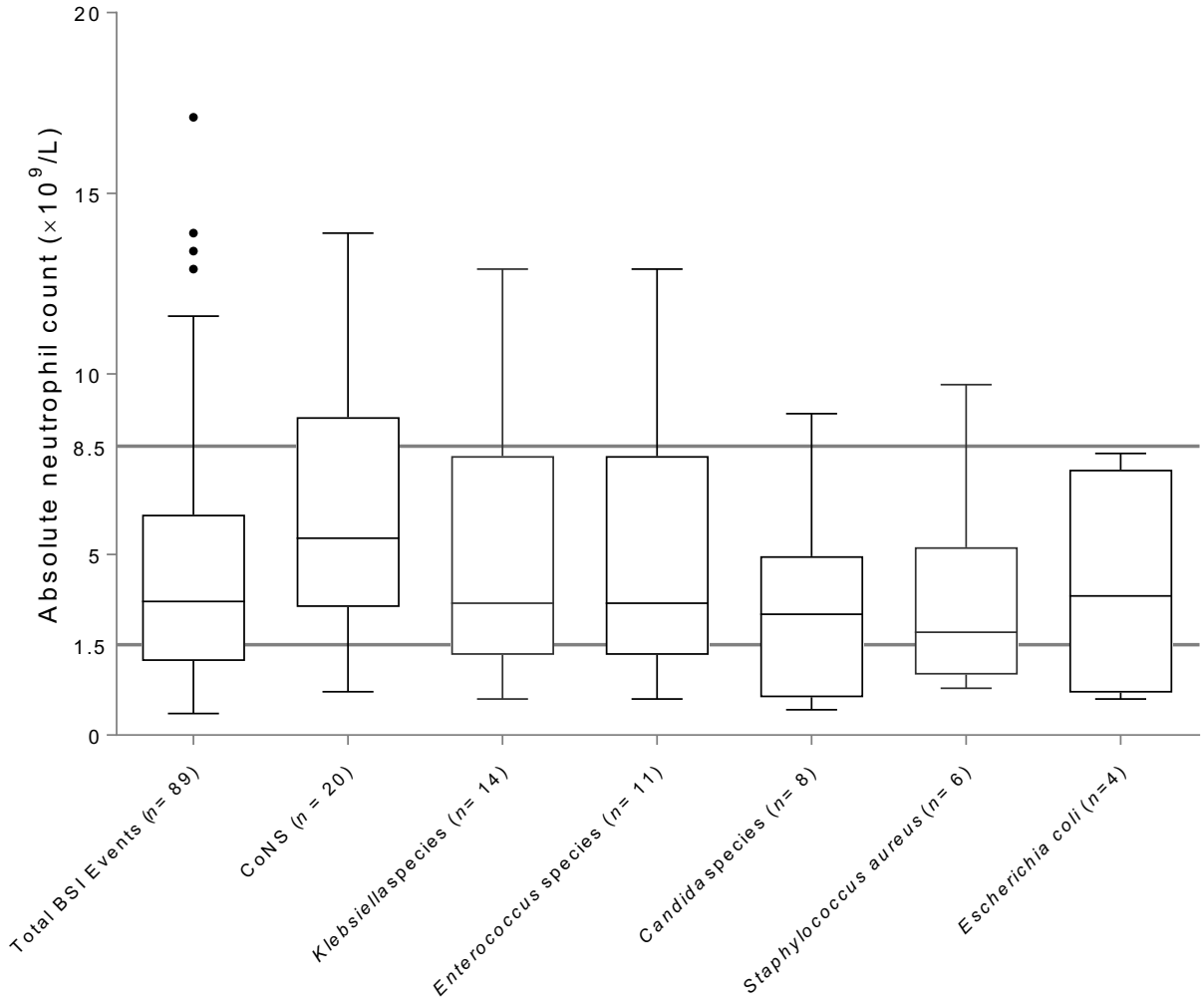

Figure 3. Box and whisker plot of absolute neutrophil count (x10^9/L) at presentation of bloodstream infection in children with short bowel syndrome. Displaying cumulative results of *n*=89 bloodstream infections as well stratified by most frequent cultured microorganism. Reference range applied across all age group depicted by horizon bars as 1.5 to 8.0x10^9/L. Data not complete if bloodwork not available at time of presentation. Abbreviation: CoNS, coagulase-negative *staphylococci;* BSI, bloodstream infections

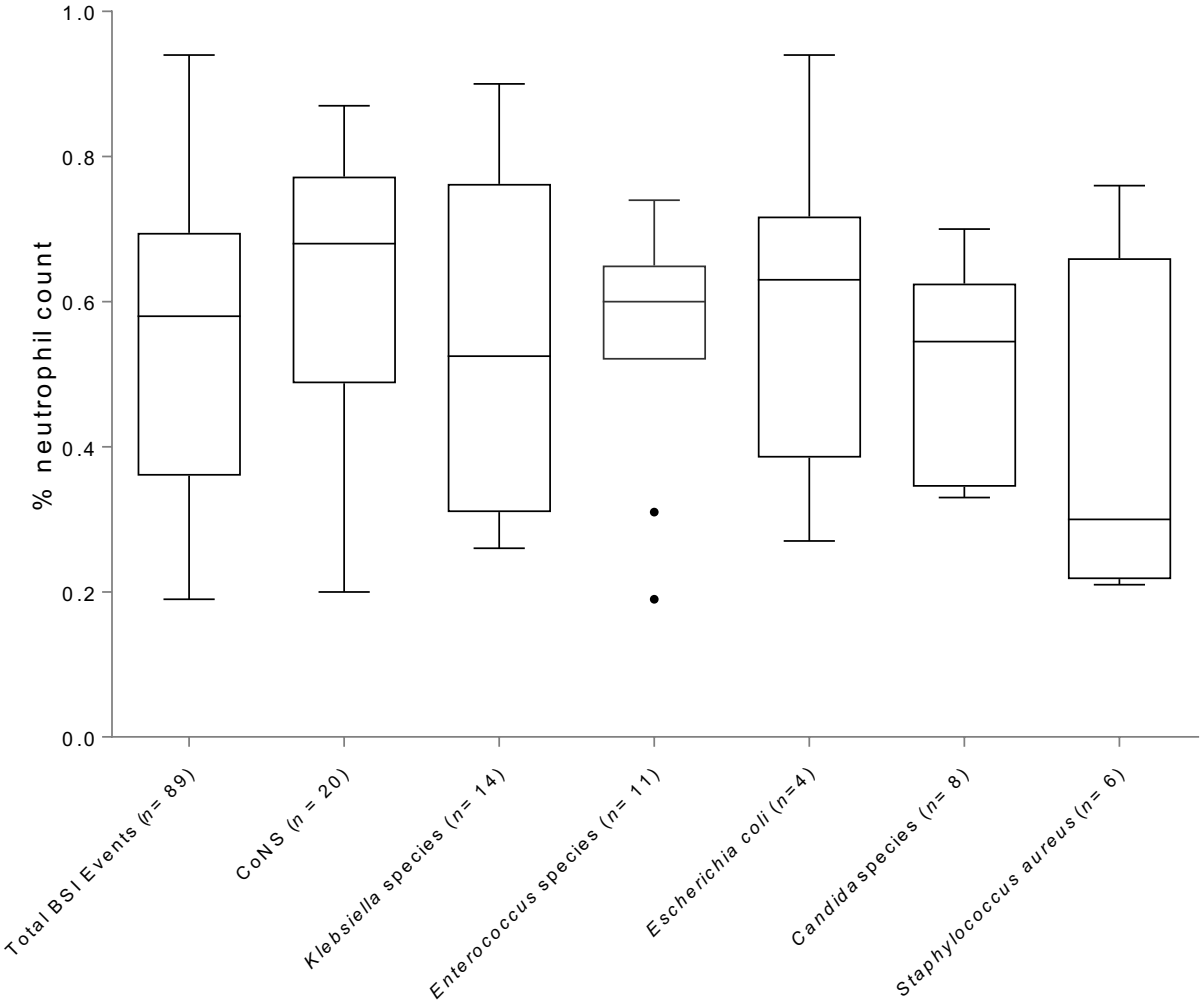

Figure 4. Box and whisker plot of percent neutrophil count at presentation of bloodstream infection in children with short bowel syndrome. Displaying cumulative results of *n*=89 bloodstream infections as well stratified by most frequent cultured microorganism. Data not complete if bloodwork not available at time of presentation. Abbreviation: CoNS, coagulase-negative *staphylococci;* BSI, bloodstream infections

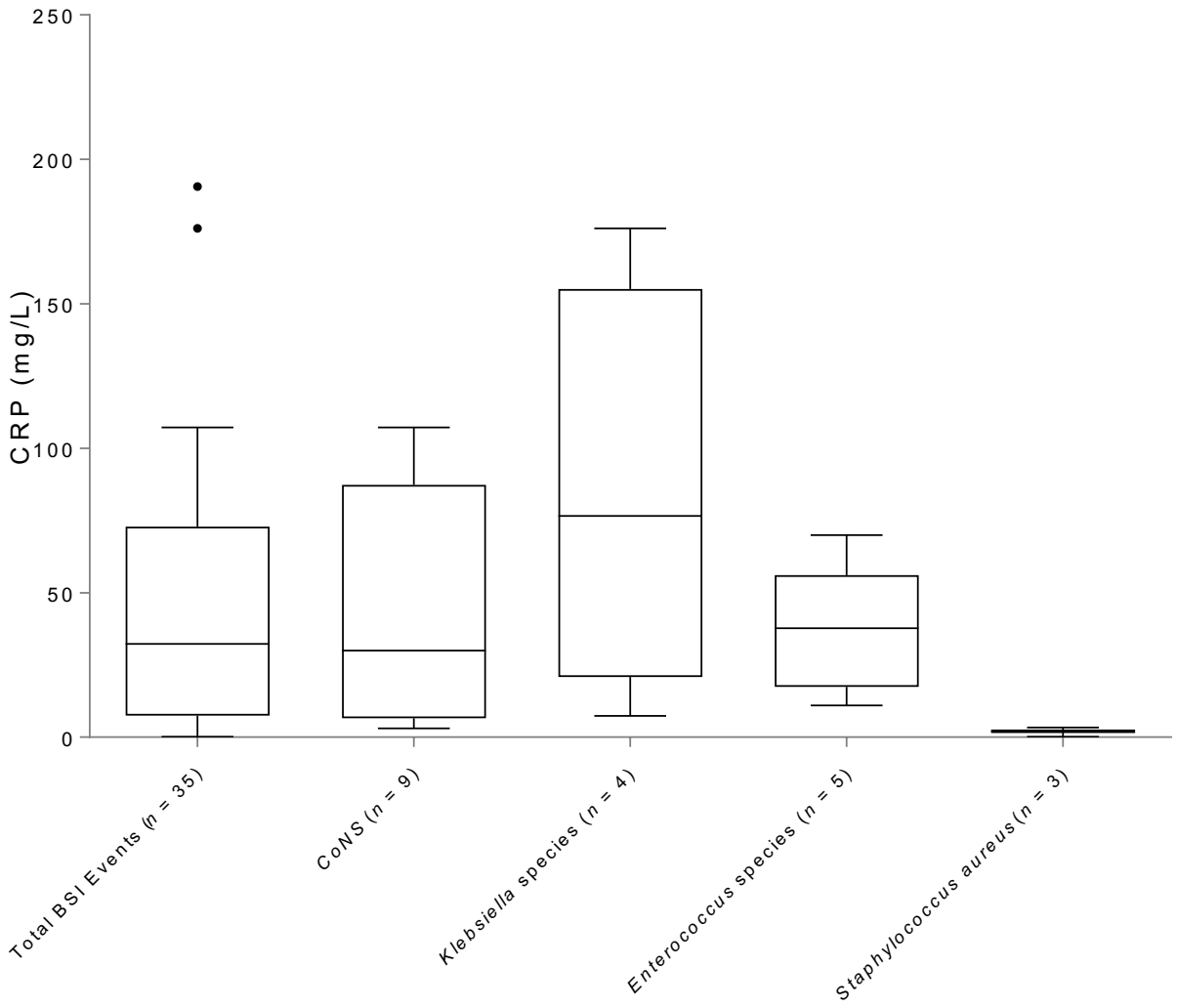

Figure 5. Box and whisker plot of C-reactive protein (mg/L) at presentation of bloodstream infection in children with short bowel syndrome. Displaying cumulative results of *n*=35 bloodstream infections as well stratified by most frequent cultured microorganism. Data not complete if bloodwork not available at time of presentation. Abbreviations: CRP, C-reactive protein; CoNS, coagulase-negative *staphylococci;* BSI, bloodstream infections

REFERENCES:

1. Squires RH, Duggan C, Teitelbaum DH, et al. Natural history of pediatric intestinal failure: initial report from the Pediatric Intestinal Failure Consortium. J Pediatr 2012;161:723-8.e2.
2. Chang MI, Carlson SJ, Nandivada P, et al. Challenging the 48-Hour Rule-Out for Central Line-Associated Bloodstream Infections in the Pediatric Intestinal Failure Population: A Retrospective Pilot Study. JPEN J Parenter Enteral Nutr 2016;40:567-73.
3. O'Keefe SJ, Buchman AL, Fishbein TM, et al. Short bowel syndrome and intestinal failure: consensus definitions and overview. Clin Gastroenterol Hepatol 2006;4:6-10.
4. Cole CR, Frem JC, Schmotzer B, et al. The rate of bloodstream infection is high in infants with short bowel syndrome: relationship with small bowel bacterial overgrowth, enteral feeding, and inflammatory and immune responses. J Pediatr 2010;156:941-7, 947.e1.
5. Burghardt KM, Wales PW, de Silva N, et al. Pediatric intestinal transplant listing criteria - a call for a change in the new era of intestinal failure outcomes. Am J Transplant 2015;15:1674-81.
6. Alexander T, Blatt J, Skinner AC, et al. Outcome of Pediatric Gastroenterology Outpatients With Fever and Central Line. Pediatr Emerg Care 2016;32:746-750.
7. Peltola V, Mertsola J, Ruuskanen O. Comparison of total white blood cell count and serum C-reactive protein levels in confirmed bacterial and viral infections. J Pediatr 2006;149:721-4.

8. Zarkesh M, Sedaghat F, Heidarzadeh A, et al. Diagnostic value of IL-6, CRP, WBC, and absolute neutrophil count to predict serious bacterial infection in febrile infants. Acta Med Iran 2015;53:408-11.

9. Lanziotti VS, Póvoa P, Soares M, et al. Use of biomarkers in pediatric sepsis: literature review. Rev Bras Ter Intensiva 2016;28:472-482.

10. Mermel LA, Allon M, Bouza E, et al. Clinical practice guidelines for the diagnosis and management of intravascular catheter-related infection: 2009 Update by the Infectious Diseases Society of America. Clin Infect Dis 2009;49:1-45.

11. Struijs MC, Diamond IR, de Silva N, et al. Establishing norms for intestinal length in children. J Pediatr Surg 2009;44:933-8.

12. Robinson JL, Casey LM, Huynh HQ, et al. Prospective cohort study of the outcome of and risk factors for intravascular catheter-related bloodstream infections in children with intestinal failure. JPEN J Parenter Enteral Nutr 2014;38:625-30.

13. Carter JH, Langley JM, Kuhle S, et al. Risk Factors for Central Venous Catheter-Associated Bloodstream Infection in Pediatric Patients: A Cohort Study. Infect Control Hosp Epidemiol 2016;37:939-45.

14. Bundy DG, Gaur AH, Billett AL, et al. Preventing CLABSIs among pediatric hematology/oncology inpatients: national collaborative results. Pediatrics 2014;134:e1678-85.

15. Klatte JM, Newland JG, Jackson MA. Incidence, classification, and risk stratification for Candida central line-associated bloodstream infections in pediatric patients at a tertiary care children's hospital, 2000-2010. Infect Control Hosp Epidemiol 2013;34:1266-71.

16. Gokcebay DG, Yarali N, Isik P, et al. Candida Associated Bloodstream Infections in Pediatric Hematology Patients: A Single Center Experience. Mediterr J Hematol Infect Dis 2016;8:e2016018.

17. Tragiannidis A, Tsoulas C, Groll AH. Invasive candidiasis and candidaemia in neonates and children: update on current guidelines. Mycoses 2015;58:10-21.

18. Herz AM, Greenhow TL, Alcantara J, et al. Changing epidemiology of outpatient bacteremia in 3- to 36-month-old children after the introduction of the heptavalent-conjugated pneumococcal vaccine. Pediatr Infect Dis J 2006;25:293-300.

19. Okada Y, Klein NJ, van Saene HK, et al. Bactericidal activity against coagulase-negative staphylococci is impaired in infants receiving long-term parenteral nutrition. Ann Surg 2000;231:276-81.

ESCHERICHIA ABUNDANCE AND LOW FECAL BUTYRATE IN CHILDREN WITH INTESTINAL FAILURE

[1]Jenna Katherine **Dowhaniuk** (MD, FRCPC), [3]Jake **Szamosi** (MSc), [2]Sam **Chorlton** (MD), [5]Jillian **Owens** (RD), [5]Heather **Mileski** (RD), [5]Rose-Frances Clause (NP) [1]Jeffrey **Pernica** (MD, FRCPC), [4]Dawn **Bowdish** (MSc, PhD), [3]Michael G. **Surette** (PhD), [1]Elyanne **Ratcliffe** (MD, FRCPC)

[1]Department of Pediatrics, McMaster University, Hamilton, Ontario

[2]Department of Undergraduate Medicine, McMaster University, Hamilton, Ontario

[3]Department of Medicine and Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario

[4]Department of Pathology & Molecular Medicine, McMaster University, Hamilton, Ontario

[5]Division of Pediatric Gastroenterology and Nutrition, McMaster Children's Hospital

Correspondence to: Dr. Jenna Dowhaniuk, Division of Pediatric Gastroenterology, Department of Pediatrics, McMaster University 3A61,1280 Main Street West, Hamilton, Ontario, L8S 4K1, Canada. Tel.: 905-527-2299 x73455; fax: 905-521-2655; e-mail: jenna.dowhaniuk@medportal.ca

Funding and Supports: Source of funding includes Regional Medical Associates of Hamilton and Canadian Association of Gastroenterology

Conflicts of Interest: None.

PREFACE/ INTRODUCTION:

ESCHERICHIA ABUNDANCE AND LOW FECAL BUTYRATE IN CHILDREN WITH INTESTINAL FAILURE

J. Dowhaniuk: Involved in the following aspects of study: Study concept and, completion of study design, research ethics submission and revisions, data abstraction, short chain fatty acid analysis preparation, bioinformatics analysis, statistical analysis and first author of the manuscript.

Dr. Elyanne Ratcliffe, Dr. Dawn Bowdish and Dr. Surette were involved in study concept and design, statistical analysis and revisions and edits of the manuscript.

Laura Rossi and Michelle Shah in the laboratory of Dr. M. Surette completed 16s rRNA gene sequencing on the Illumina MiSeq platform.

Dr. Kirk Green of McMaster Regional Centre for Mass Spectrometry completed the short chain fatty acid extraction of stool samples.

Sam Chorlton was involved as a research assistant collecting samples and demographics and the editing of the manuscript.

Jake Szamosi completed bioinformatics analysis.

SUBMISSION: Drafted for submission

ABSTRACT

Background: Short Bowel Syndrome (SBS) is the leading cause of intestinal failure (IF) in children. The goal for a child with IF is to undergo sufficient intestinal adaptation to achieve enteral autonomy and thus be able to discontinue parenteral nutrition (PN). The prognosis for each child may be different dependent on anatomic difference such as small intestinal length, surgical history, gestation age and the underlying etiology. The composition of the fecal microbiome may represent an additional independent risk factor for dependence on PN.

Aims: We sought to compare the intestinal microbiome of children who continue to require PN (defined as SBS+IF) to those with SBS who have discontinued PN, and control children using high-throughput sequencing to further understand host-microbe interactions in this population. Furthermore, we quantified the short chain fatty acid (SCFA) production between groups as well as total fecal bacterial load.

Methods: A total of 53 samples were submitted over 6- 15 months. Six children with SBS +IF submitted 34 samples, 6 children with SBS who previously had discontinued PN submitted 15 samples; these were compared to samples from 5 control children. Fecal samples were analyzed by 16S rRNA partial gene sequencing using the MiSeq Illumina sequencer. SCFA levels, including butyric acid, were measured in stool samples by mass spectrometry. Bacterial load was measured by qPCR.

Results: Enteric samples from those with SBS+ IF demonstrated the most substantial dysbiosis with the lowest Shannon diversity and an abundance of the *Escherichia* genus seemingly attributable to the pro-inflammatory species *E. coli.* There was a significant 168-fold increase in the abundance of *Escherichia* compared to control children. Commensal anaerobes known to

produce SCFA including *Ruminococcaceae* and *Lachnospiraceae* were significantly reduced in those with SBS. Similarly, measured butyric acid was significantly reduced in children with IF compared to controls (median 0.37nmol/mg vs 10.92nmol/mg; $p<0.0001$). Children with IF had a significantly reduced bacterial load in stool samples compared to controls (median 124.2nmol/L vs 1225nmol/L; $p=0.006$).

Conclusion: Dysbiosis characterized by a reduction in diversity and over-population of *Escherichia*, as well as a significant reduction in the SCFA butyric acid were identified in children with IF. These findings have potential implications for intestinal epithelium barrier function, intestinal permeability, and host-microbe immune response in this patient population.

INTRODUCTION

Pediatric intestinal failure (IF) is defined as an inability to maintain energy, electrolytes, protein, or fluids without life-sustaining parenteral nutrition[1]. The most common etiology of IF is short bowel syndrome (SBS), where surgical resections have minimized the length of small intestine due to conditions such as necrotizing enterocolitis, gastroschisis, and intestinal atresias[2]. Children with short bowel syndrome represent a heterogeneous group with differences in small intestine and colon length, segment of resection, and preservation of the ileocecal valve. The goal for a child with IF is for intestinal adaptation and to achieve enteral autonomy with subsequent discontinuation of parenteral nutrition (PN). The prognosis for individual children may be different dependent on anatomic differences such as small intestinal length, the number of abdominal laparotomies, gestational age, and the underlying disease such as gastroschisis[3]. The composition of the fecal microbiome may represent an independent risk factor for parenteral nutrition dependence.

The Human Microbiome Project has demonstrated that the complex bacterial ecosystem residing in the human gastrointestinal tract can have a profound impact on human health and disease [4]. As the composition of a person's microbiome influences intestinal epithelial barrier integrity and bacterial translocation, it is of critical importance in the SBS population to further characterize patterns of dysbiosis [5-7]. Recent publications of microbial diversity in those with SBS have identified a reduction in alpha diversity and abundance of the *Proteobacteria* phylum[8, 9]. Our prospective study sought to characterize fecal differences in the composition of the microbial communities between children with SBS who have successfully achieved enteral autonomy and those who have remained on parenteral nutrition.

Short chain fatty acids (SCFA) are the products of dietary fibre and resistant starch fermentation by anaerobic colonic bacteria[10, 11]. Butryate, specifically, is central to colonic epithelial integrity and is the primary energy source for these cells[10, 11]. We sought to evaluate the SCFA quantity in stool samples and correlate with butyrate-producing *Clostridia* species by 16s rRNA sequencing. To our knowledge, this is the first prospective cohort study of children with SBS to evaluate SCFA quantity in combination with bacterial profiling by 16s rRNA gene sequencing.

METHODS:

We conducted a prospective cohort study of children with SBS followed by McMaster Children's Hospital Pediatric Short Bowel Syndrome Rehabilitation Team. The first cohort consisted of children with SBS and intestinal failure for Group 1 who were less than 16 years of age with SBS (defined as those who required monitoring by the Pediatric Short Bowel Syndrome Rehabilitation Team) and who, at the time of enrolment, had required a minimum of twice-weekly PN for a component of caloric intake for a minimum of three months. The second cohort were children less than 16 years of age with SBS (defined above) who had previously required home parenteral nutrition for a minimum of three months but at the time of enrollment had discontinued PN and achieved enteral autonomy. The third cohort were control children, age- and sex- matched who had no underlying gastrointestinal illnesses and were recruited from a pediatric hematology clinic. Ethics approval was obtained from the Hamilton Integrated Research Ethics Board and consent was obtained from each participant.

Data Collection:

Demographic information was collected at each clinic visit following enrolment, including age and anthropometry (*z*-scores calculated through STAT GrowthCharts Lite). Medical records of study participants were reviewed and the following information abstracted: etiology of short bowel, gestational age, presence of ileocecal valve, degree of preservation of the colon and total number of indwelling vascular catheter days. The shortest measured length of small intestine was obtained from operative reports. These were compared to previous published norms [12]. Stool samples were obtained at each clinic visit for up to six visits. Six stools were collected for patients in Group 1 and a minimum of 2 samples were collected for patients in Group 2. A 24-hour dietary recall was submitted at the time of stool sample collection and was reviewed by a Registered Dietitian. Each dietary recall was analyzed by Health Canada's Canada Nutrient File or associated nutrition profile. Total fibre consumption was calculated. Antibiotic use in previous 2 weeks, PN use, and PN constituents were recorded at each visit. Growth was reviewed over the data collection period with weight and height expressed as *z*-scores.

Stool Studies:

Stool specimens were stored in a sterile sample container and kept frozen until delivered to the lab where samples were transferred into four 2 mL cryovials and frozen with liquid nitrogen for 16s rRNA sequencing of microbiota. Bacterial community profiling of 16S rRNA genes was carried out using 250 nt paired end reads of the V3 region using the MiSeq Illumina sequencer. Reads were processed into an OTU table using the sl1p pipeline[13]. In brief, reads were trimmed of adapters and low-quality bases using cutadapt, and merged using PANDAseq[14, 15]. Reads were

then clustered into OTUs at a 97% cutoff using AbundantOTU, and OTUs are assigned taxonomy using the RDP Classifier via QIIME[16-19]. Each participant's first stool sample submitted for this study was also analyzed for total SCFA quantity by mass spectrometry. To extract short chain fatty acids, 30 mg stool samples for each subject were acidified with equivalent 3.7% HCl. Samples were extracted twice with ether, derivatized, and analyzed by GCMS. Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, pentanoic acid, and lactic acid were quantified and reported as nmol/mg. Calibration curves were reviewed. Duplicates were completed for three samples and percentage recovery was calculated. One subsequent stool was analyzed by qPCR for total bacterial load. Only bacteria were analyzed, and each sample was diluted 1/10 to produce reasonable standardized curves. Each sample was analyzed in duplicate and the mean result was used in downstream analysis.

DATA ANALYSIS

Microbiome Analysis:

All microbiome analyses were conducted in R [20-22]. The phyloseq package (version 1.19.1), and the tidyverse packages (dplyr v. 0.7.2, tidyr v. 0.6.1, and readr v. 1.1.1), were used for data curation and manipulation in R[23-26]. Plots of microbiome data and statistical models were generated using ggplot2 [27]. Statistical models used the lmerTest wrapper for the lme4 package [28] The 'vegan' package was used to model beta diversity [29].

Shannon diversity scores were calculated on samples that had been rarefied (without replacement) to 21,016 reads (the smallest library size). Using ImerTest and Ime4, we fit a linear mixed model to investigate the effects of illness severity and the presence of ileocecal valve and intact colon on Shannon index. We used a random effect of individual to correct for non-independence of multiple

samples from the same patient. Bray-Curtis values were computed on rarefied samples. A permanova test was run using the 'vegan' package in R to determine the contribution of illness severity to among-sample variation. Tests of differential OTU abundance for a number of taxa of interest were conducted with the lmerTest and lme4 packages in R, on arcsin-sqrt-transformed relative abundance data, with a fixed effect of illness severity and random effect of individual. The dominant OTU (OTU 1, assigned to genus *Escherichia*) was assigned to species using the GreenGenes 2013 database and subjected to oligotyping to identify internal structure within that OTU[19].

Abundance of obligate aneorobes in the *Clostridia* class were investigated, as these bacteria are known to have anti-inflammatory properties via T regulatory cell expansion and short chain fatty acid production[30]. As previous pediatric papers have investigated non-pathogen *Clostridia* such as *Clostridiaceae*, *Erysipelotrichaceae*, *Eubacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae* in the context of short bowel syndrome and pediatric GVHD, we similarly assessed the differential abundance of these families in our population[31, 32].

Short Chain Fatty Acids and Bacterial qPCR

Quantity of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, pentanoic acid and lactic acid were analyzed for each subject from one stool sample. Five samples were submitted for Group 1, six for Group 2 and 5 for Group 3. Differences between groups were analyzed by non-parametric Kruskal Wallis test. Differences in fecal bacterial load in nmol/L were analyzed between groups for all samples collected (n=53) by non-parametric Kruskal Wallis

test testing. Results were re-analyzed by presence/ absence of ileocecal valve and intact colon to assess if any statistical difference existed between these groups.

Statistical analysis of demographics, fibre quantity, SCFA concentration and qPCR was completed using GraphPad Prism (GraphPad software, San Diego, CA). Statistical significance is accepted at $p<0.05$. Descriptive data is presented as medians with interquartile range or mean with confidence interval. Demographic analysis between groups assessed by Mann-Whitney or Kruskal Wallis tests.

RESULTS:

Demographics**:**

Seventeen children were recruited in our study (Group 1 *n=6*; Group 2 *n=6*; Group 3 *n=5*) and 53 stool samples were submitted over 6-15 months (Figure 1). Up to six samples were submitted by each patient in Group 1 (total 34), 2-3 samples were submitted by each patient in Group 2 (total 14) and one sample was submitted by each child in Group 3 (total 5). The median age of children in group 1 was 67 months old (5.6 years) compared to 47.5 months in group 2 and 65 in group 3 (p=NS). At the onset of the study, the median catheter days in Group 1 was 2023 (IQR 811- 2947) in Group 1 compared to 484 (IQR 274, 588) in Group 2 ($p<0.05$). All children in Group 1 had a central line and all central lines had been removed in Group 2. Shortest measured length of residual small bowel in Group 1 measured 26.5cm (IQR 24.75, 40) compared to 55cm in Group 2 (IQR 31.2, 89; $p<0.01$). These differences were to be expected as the groups were defined by the ongoing need for PN and persistent IF. The children in our control group on average had a higher *z*-score for both weight and height than the children in Groups 1 and 2. There was no statistical

difference in weight or height *z*-score between Group 1 and Group 2. The etiology of intestinal failure included necrotizing enterocolitis, gastroschisis, and malrotation with mid-gut volvulus. In Group 1, three patients had a preserved ileocecal valve and full colon compared to 4 in Group 2 (p=NS). There were no differences in gestational age or date of resection between Groups 1 and 2.

No patient was taking a probiotic during the time of study; however, 83% (5 of 6) of those with IF (Group 1) had been treated with an antibiotic during the study period for small bowel bacterial overgrowth (SIBO) or previous sepsis. These antibiotics included trimethoprim, metronidazole, vancomycin, rifaximin and ciprofloxacin. Two of six children in Group 1 remained on SIBO treatment throughout the study period alternating 1 week on, 1 week off, while the other three children were treated intermittently for SIBO. There was a significant difference in antibiotic use between groups, as only one patient each in Groups 2 and 3 had been treated with antibiotics within 2 weeks for indications other than SIBO ($p<0.05$). These antibiotics included Amoxicillin and Metronidizole. Full demographics are presented in Table 1.

Microbiome Diversity

A total of 1853 OTUs were detected across 53 samples. Children with IF + SBS (Group 1) and SBS (Group 2) had significantly reduced Shannon indices compared to control children (Figure 2) ($p<0.05$). Those with IF appeared to have the greatest reduction in Shannon indices, however there was no significant effect between Group 1 and 2. Presence or absence of a colon and ileocecal valve did not have a significant effect (p=0.3) in the linear mixed model.

Permanova testing of Bray-Curtis distances found that 18% of among-sample variation was explained by Group. Although the effect is significant ($p<0.001$), it is small. Principal coordinate analysis of the Bray-Curtis values (Figure 3) suggests that the tight clustering of the healthy controls likely accounts for most of this effect. The results shown are the outcome of a single rarefaction; however, the results were robust to multiple rarefactions (data not shown).

Taxonomic Abundance Pattern: *Escherichia* Abundance

There was a significant increase in the abundance of *Proteobacteria* in children with SBS-IF (Group 1) and SBS (Group 2) over controls. In our controls, *Proteobacteria* only represent 5% of the total abundance, while three stools samples from Group 1 contained *Proteobacteria* abundance greater then 94%. Furthermore, we analyzed abundance patterns within the *Proteobacteria* phylum at the class, order, family, genus level, and the same trend was seen in *Gammaproteobacteria*, *Enterobacteriaceae* and *Escherichia* (Figures 4-7). This investigation down the taxonomic tree revealed that this increase was the result of very high levels of *Escherichia*, so our further analysis focused on that genus. In healthy controls, *Escherichia* had a mean abundance of 0.25%, with a maximum abundance of 0.66%. In Group 1, the mean abundance of *Escherichia* was 42%, with four samples consisting of more than 90% *Escherichia*, while Group 2 had a mean *Escherichia* abundance of 24% and a maximum of 66% (Figure 7). This represents a 168-fold increase in *Escherichia* between control children and those with IF + SBS. A significant difference was detected between all SBS groups and controls however no significant difference was detected between Groups 1 and 2. The *Escherichia* genus was dominated by a single OTU, OTU 1, which assigned to species *E. coli* in the GreenGenes 2013 database with 54% certainty according to the RDP classifier. A single oligotype made up 89% of

the sequences in this OTU, with no apparent structure to the distribution of the remaing 11% (3 oligotypes) (Figure 8).

Clostridia genera with anti-inflammatory properties

Of the *Clostridia* investigated generally associated with anti-inflammatory properties, *Clostridiaceae*, *Eubacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were present in our samples. The proportion of *Ruminococcaceae* were significantly reduced in children with SBS-IF and SBS (means 0.2% and 2.6% respectively) compared to controls (Group 3; 18.6%) (Figure 9). No significant difference between Groups 1 and 2 was detected. The proportion of *Lachnospiraceae* were also significantly reduced (Group 1 mean 11%, Group 2 mean 34%) vs. healthy controls (mean 51%). In controls, there was a 5-fold increase in *Lachnospiraceae* and a 93-fold increase in Ruminococcaceae compared to the children with IF (Group 1).

Short Chain Fatty Acids:

There was a significant difference in fecal butyrate quantity between Group 1 (median 0.37nmol/mg), Group 2 (6.51nmol/mg) and controls Group 3 (10.92nmol/mg) with higher butyrate production in the microbiome of healthy controls ($p<0.0001$) (Figure 10). Quantities of other short chain fatty acids including acetic acid, propionic acid, isobutyric acid, lactic acid, pentanoic acid, and isovaleric acid were not statistically different between groups (Table 2). Interestingly, the one stool obtained from a children in Group 2 who was on antibiotics had the lowest quantity of acetic acid, isobutryic acid, propionic acid, pentanoic acid and isovaleric acid, however the highest quantity of lactic acid (22.8nmol/mg compared to median 0.35nmol/mg stool).

Fecal Bacterial Load:

Children with IF had only 10% of the bacterial load of children in the control group. Children with IF had the lowest bacterial load by qPCR, with a median of 124.2nmol/L (IQR 69, 270), compared to 279nmol/L in Group 2 (IQR 230, 845) and 1225nmol/L in controls (IQR 460, 1753) (p=0.006) (Table 2). When qPCR data was re-analyzed by presence or absence of an ileocecal valve, the observed effect size was much smaller and there was no statistical difference (ICV valve present median 152nmol/L, ICV absent 192nmol/L; p 0.83). Therefore, the overall quantity of bacteria in stool samples appears to be associated with disease and not presence of absence of an ileocecal valve.

DISCUSSION:

Children with IF and short bowel syndrome require long term PN to survive[2]. Complications of this rare diagnosis are severe, including frequent sepsis, PN-associated liver disease, and feeding intolerance[2]. Despite ongoing advancements in bowel adaptation strategies, a proportion of children require liver and/or intestinal transplants and the overall mortality rate remains 25%[2]. While children with IF may represent a heterogeneous cohort with varying intestinal lengths and etiologies for short bowel syndrome, we sought to further investigate a common microbial signature. Taxon abundance differences between children with SBS who achieved enteral autonomy and those with IF may play a role in the physiology of this disease. Furthermore, microbial products such as short chain fatty acids are important to the host metabolism and for energy harvest, and could influence clinical outcomes for children with SBS.

Children with IF have a reduction of bacterial diversity compared to that of controls. This is consistent with a recent publication demonstrating a similar reduction in Shannon diversity index of a subset of their cohort with SBS on PN as compared to those who had weaned from PN [9]. We failed to find any effect of colon resection or absence of an ileocecal valve to explain the difference in Shannon diversity indices. A number of factors could predispose children with SBS to the identified dysbiosis, including short- and long-term enteral deprivation, recurrent antibiotic courses, elemental formula diets, repeated surgical intervention and dysmotility [5, 33].

While this study demonstrates association, we cannot conclude if the microbial composition is a by-product of the disease or if it contributes to the underlying physiology for the reliance of PN. In piglet models of SBS, a reduction in bacterial diversity has been reported as early as six weeks post-intestinal resection demonstrating the early influence surgical resection has on dysbiosis [34]; however, long term continuation of PN is secondary to many factors, including the length of remaining bowel and the presence of an ileocecal valve[2]. Microbial composition is likely important to the natural history of this disease. One of the limitations to weaning PN is whether feeds are tolerated. Tolerability of feeds can be influenced by gas production, diarrhea, small bowel bacterial overgrowth, and dysmotility, all of which can be influenced by intestinal bacterial composition[5]. Furthermore, bacterial by-products have been associated with improved bowel adaptation post-surgical resection, including electrolyte and water balance[10]. These suggest that the microbiome may play in an important role in SBS physiology.

The *Proteobacteria* phylum, to which *Escherichia* belongs, usually represent less than 1-2% of the microbial community of the intestine [33]. Our study identified the lipopolysaccharide-containing

and often pro-inflammatory *Escherichia* (likely *E. coli*) as an important distinguishing feature between healthy controls and children with SBS, with up to 95% relative abundance in SBS-IF, compared to a maximum of 0.66% in controls. Our IF group exhibited higher proportion of *Proteobacteria* than those described by Davidovics *et al*.; however, in their study some children had discontinued PN, which may explain the lower abundance[5]. Previous microbiome studies have studied varying numbers of PN-independent and -dependent children with SBS [5, 9, 31, 33]. While some studies have reported an abundance to the Phylum level of *Proteobacteria*, others have also described the trend through to the *Gammaproteobacteria* class and *Enterobacteriaceae* family [8, 31 5, 9]. In our study, we have identified that the abundance of *Proteobacteria* was almost entirely driven by the over-abundance of the genus *Escherichia* and likely to the species *E.coli*.

Proteobacteria and/or *Enterobacteriaceae* abundance has been associated with prolonged PN use, recent and current antibiotic use, enteral deprivation, and liver fibrosis and steatosis[35 5, 33 8]. Korpela *et al*. have proposed the bacterial overgrowth in PN-dependent children with SBS as due to *Proteobacteria* and that in children weaned off PN, as due to *Lactobacilli*[33], however, we did not demonstrate a similar trend of *Lactobacilli* in our groups. Group 2 had an incremental increase in the proportion of *Enterobactericeae* as well as *Lactobacilli* compared to controls; however, they had less than the IF group (data not shown).

While some trends in microbial composition may have minimal functional impact, overpopulation of *Proteobacteria* has been associated with important clinical outcomes. In multiple PN-dependent mouse models, *Proteobacteria* abundance has been associated with loss of epithelial barrier function and bacterial translocation[7, 36]. Direct effects of enteral deprivation included an

increase in the relative proportion of of *Proteobacteria*, increased Toll-like Receptor (TLR) signaling through LPS and pro-inflammatory cytokine expression of IFN-γ and TNF-α with subsequent villous atrophy, and loss of tight junction integrity[7, 36]. Of significant importance to the SBS population, these PN-dependent mouse models subsequently experienced bacterial translocation, with cultured bacteria found in spleen and lymph node isolates[7]. The similar increase in abundance of *Proteobacteria* in our intestinal failure group, may contribute to physiological changes that influence bacterial translocation, central line-associated bloodstream infections, and sepsis.

Prospective long term studies would help to understand the cause and timeline of the shift to a dominant *Escherichia* microbiome in children with SBS. Current research has provided some associations to these microbial signatures specifically with antibiotic use. Antibiotics can have drastic and, at times, lasting effects on microbial abundance which can reduce resistance to colonization of opportunistic pathogens[37]. Metronidizole has been found to have a similar effect on increased abundance of *Enterobactericeae* [31]. One can hypothesize a combination of enteral deprivation and antibiotic use may decrease diversity and abundance of tolerogenic commensal bacteria. This may provide the ideal host for opportunistic pathogens to flourish given the lack of microbial competition. Antimicrobials are frequently used for small bowel bacterial overgrowth in children with SBS. In this cohort, all children in the IF group had been exposed to antibiotics during the study period while few in Group 2 and 3 had recent antibiotic use. In addition to changing colonization of commensals, antibiotics can also increase resistance over time. The most common antibiotic for SIBO, metronidazole, would not influence *Escherichia* resistance in the GI

tract; however, others such as Trimethoprim could. Further research is required to study the resistance of abundant microbes in SBS.

Microbial communities influence the absorption of nutrients, metabolism, and short chain fatty acid production. SCFA are monocarboxylic hydrocarbons produced by aneorobic fermentation of complex carbohydrates by colonic bacteria[10, 11]. SCFA promote healthy intestinal epithelial barrier function, regulate proliferation of cells, increase mucus production, increase Na absorption through the Na-SCFA cotransporter, and decrease pro-inflammatory cytokines[10, 38]. These beneficial effects are thought to improve bowel adaptation due to enhanced electrolyte and water absorption. Butryate is the primary energy source for colonic epithelial cells and is transported into cells via monocarboxylate transporters[10]. Here it influences gene expression via inhibition of histone deacetylases and pro-inflammatory transcription factor NF-κB[11, 38-41]. Butyrate also stimulates cytoprotective heat shock protein HSP25 in rat intestinal models promoting mucosal barrier function as well as influence colonic paracellular permeability [10, 42]. Commensal anaerobes of the intestine, including *Lachnospiraceae* and *Ruminococcaceae*, both deficient in Group1 and Group2, are known to produce butyrate. They predominate in the mucus layer near the epithelial cells, and have a role in modulating immune tolerance within the GI tract[39]. Many bacteria belonging to the *Clostridia* class are commensal bacteria belonging to the *Clostridia clusters IV* and *XIVa* [30, 43]. They are important for IgA production, butyrate production, and anti-inflammatory properties via expanded colonic regulatory Foxp3$^+$ T regulatory cells[30, 39 33]. *Roseburia* genus belongs to the *Lachnospiraceae* family, and is a known butyrate producer with Butryl CoA:acetate CoA transferase activity[44 43]. Previous research has also identified a reduction in similar anti-inflammatory *Clostridia* in children with SBS [31]. Given the benefits of butyrate in maintenance

of a healthy intestinal epithelium, the low quantity of butyrate in the IF Group 1 could have implications in paraceullar permeability, inflammatory cytokine production, lack of cyroprotection, and the production of the mucus layer. This is turn could influence bacterial translocation.

The cause of this reduction in anti-inflammatory *Clostridia* in children with SBS is unknown. In surgical SBS piglet models, within two weeks of intestinal resection, *Ruminococcaccae* was significantly reduced[34]. Other studies have identified a negative association of *Clostridium cluster XIVa* or *anti-inflammatory Clostridia* with antibiotic use over the preceding three months[31, 33]. Since fermentable fibres are the complex carbohydrates hydrolyzed to SCFA, we reviewed fibre consumption in 24 hour dietary recalls. While fibre supplementation has had inconsistent results in modulation of microbiome composition in clinical trials, a previous study has reported that a high fibre meal leads to increased detectable butyrate in the serum of humans [45, 46]. In our results, fibre intake during the 24 hours before stool sample collection and average fibre intake over the study period were not significantly different between groups. We suspect other variables such as enteral deprivation, surgical resection, and recurrent antibiotic use may drive the reduction in anti-inflammatory *Clostridia* class and butyrate concentration.

As with many pediatric IF studies, limitations of our study included the small sample size and lack of consistency of stool samples between groups. While no statistically significant difference in age existed between groups, the children in SBS Group 2 were younger than those with IF. Microbial communities are known to change over time and with age; however, they are thought to be stable by early childhood[47]. Multiple samples over 1 year should help to ensure that a

representative overview of the microbiome was captured. Fecal samples are representative of distal intestine population and do not represent the mucosal microbiota, which may be more critical in mucosal immunity[48]. Our study strengths included controls who were age and sex matched; however, only 5 control participants were obtained during the study period. While exclusion criteria included any gastrointestinal illness, we are not able to confirm that our small control group from the hematology clinic is representative of all children in this age bracket. In addition, we have studied an older population of children with well-established IF and SBS to increase our confidence in clinical outcomes between these two groups. As this is a human study, we are limited to collection and measurement through stool. While 95% of butyrate produced is transported in colonic epithelial cells, remaining fecal butyrate quantities are the best available sample[43]. Previous researched has also documented higher butyrate levels in stool samples in healthy control groups[49, 50].

In summary, children with SBS and IF have significant dysbiosis with a reduction of total bacterial load, alpha diversity indices, and abnormal abundance with *Proteobacteria* shift. Commensal *Clostridia* are reduced known to produce SCFA and have implications in colonic health, energy harvest, and host metabolism. The microbial signature of children with IF are characterized by an over-abundance of pro-inflammatory *Escherichia,* likely *E.coli*. Further research is required to investigate fecal transplantation, probiotic use, and short chain FA supplementation for SBS patients. Furthermore, the full effects of cycled antibiotics for small bowel bacterial overgrowth should be evaluated given the association with *Enterobacteriaceae* abundance and its possible effects on anti-inflammatory butyrate-producing commensals.

Table 1. Demographic characteristics of study participants

Variable	SBS-IF Group 1	SBS Group 2	Controls Group 3	*p* value
Age, *months,* median *(IQR)*	67 (30.75, 89.5)	47.5 (34.75, 101.3)	65 (55, 101.3)	NS
Gender, n *(%)*				
Male	5 (83.3%)	3 (50%)	4 (80%)	NS
Number of indwelling vascular catheter days, median *(IQR)*	2023 (811, 2947)	484 (274, 588)	N/A	0.041
Etiology of intestinal Failure, n *(%)*				
Necrotizing enterocolitis	2 (33.3%)	3 (50.0%)	N/A	
Gastroschisis	3 (50.0%)	1 (16.7%)		
Malrotation with mid-gut volvulus	1 (16.7%)	1 (16.7%)		
Isolated jejunal atresias	0	1 (16.7%)		
Gestational age, *weeks,* median *(IQR)*	33.9 (25.8, 37)	33.9 (31.2, 38.6)	N/A	NS
Age at initial intestinal resection, *days* median *(IQR)*	27.5 (1.8,63.3)	10.5 (1.75, 32)	N/A	NS
Shortest measured length of small intestine, *cm* Median *(IQR)*	26.5 (24.8, 40)	55 (31.2, 89)	N/A	<0.01
Predicted bowel length at shortest measure, mean *(SD)*	18.35% (13.8, 27.5)	35.3% (18.2, 52.7)	N/A	NS
Preserved ileocecal valve, n *(%)*	3 (50%)	4 (66.7%)	N/A	NS
Colon Anatomy, n *(%)*				
Full colon intact	3 (50.0%)	4 (66.7%)	5 (100%)	NS
Partial or hemi-colon	3 (50.0%)	1 (16.7%)		
Sub-total colectomy	0	1 (16.7)		
Weight z-score at initial Visit, median *(IQR)*	-0.895 (-0.98, -0.35)	-0.92 (-1.79, -0.62)	-0.17 (-0.47, 1.0)	0.02
Height z-score at initial visit, median *(IQR)*	-0.315 (-1.33, 0.16)	-1.575 (-2.64, -0.16)	1.72 (0.4, 14.5)	0.01

Antibiotic use during study period, n *(%)*	5 (83%)	1 (17%)	1 (20%)	0.05

Abbreviations: Standard Deviation; IQR, Interquartile range at 25% and 75%

TABLE 2. Fibre intake, Short chain fatty acid analysis and Bacterial Load Results

	Group 1	Group 2	Group 3	P-value
Total Fibre Consumption of stool for SCFA analysis, median *(IQR)*	12.6 g/day (5.1, 23.0)	14.0 g/day (4.5, 21)	10.2 g/ day (8.6, 13.5)	NS
Total Fibre Consumption over collected clinic visits, median *(IQR)*	9.5 g/day (3.5, 18.1) (n= 20 samples)	11.3 g/day (7.2, 21.9) (n=12 samples)	10.2 g/day (8.6, 13.5) (n=4 samples)	NS
Butryic acid (nmol/mg of stool)	**0.37 (0.03, 1.85)**	**6.51 (4.08, 8.5)**	**10.92 (8.43, 15.79)**	**<0.0001**
Acetic Acid (nmol/mg of stool)	9.48 (7.96, 36.83)	29.53 (23.43, 31.3)	36.75 (26.59, 44.09)	NS
Propionic Acid (nmol/mg of stool)	2.0 (0.55, 11.36)	9.025 (4.43, 15.79)	8.05 (5.49, 18.18)	NS
Isobutryic acid (nmol/mg of stool)	0 (0, 1.4)	1.44 (0.62, 1.64)	1.38 (0.58, 2.47)	NS
Isovaleric Acid (nmol/mg of stool)	0 (0, 1.07)	1.06 (1.53, 1.63)	0.9 (0.29, 1.72)	NS
Pentanoic Acid (nmol/mg of stool)	**0 (0, 0.045)**	**0.07 (0.02, 2.0)**	**1.45 (0.52, 3.2)**	**0.005**
Lactic Acid (nmol/mg of stool)	0.38 (0.24, 9.92)	0.58 (0.13, 7.09)	0.21 (0.16, 0.24)	NS
Bacterial Load (nmol/L), median *(IQR)*	**124.2 (69, 270)**	**279 (230, 845)**	**1225 nmol/L (460, 1753)**	**0.0006**

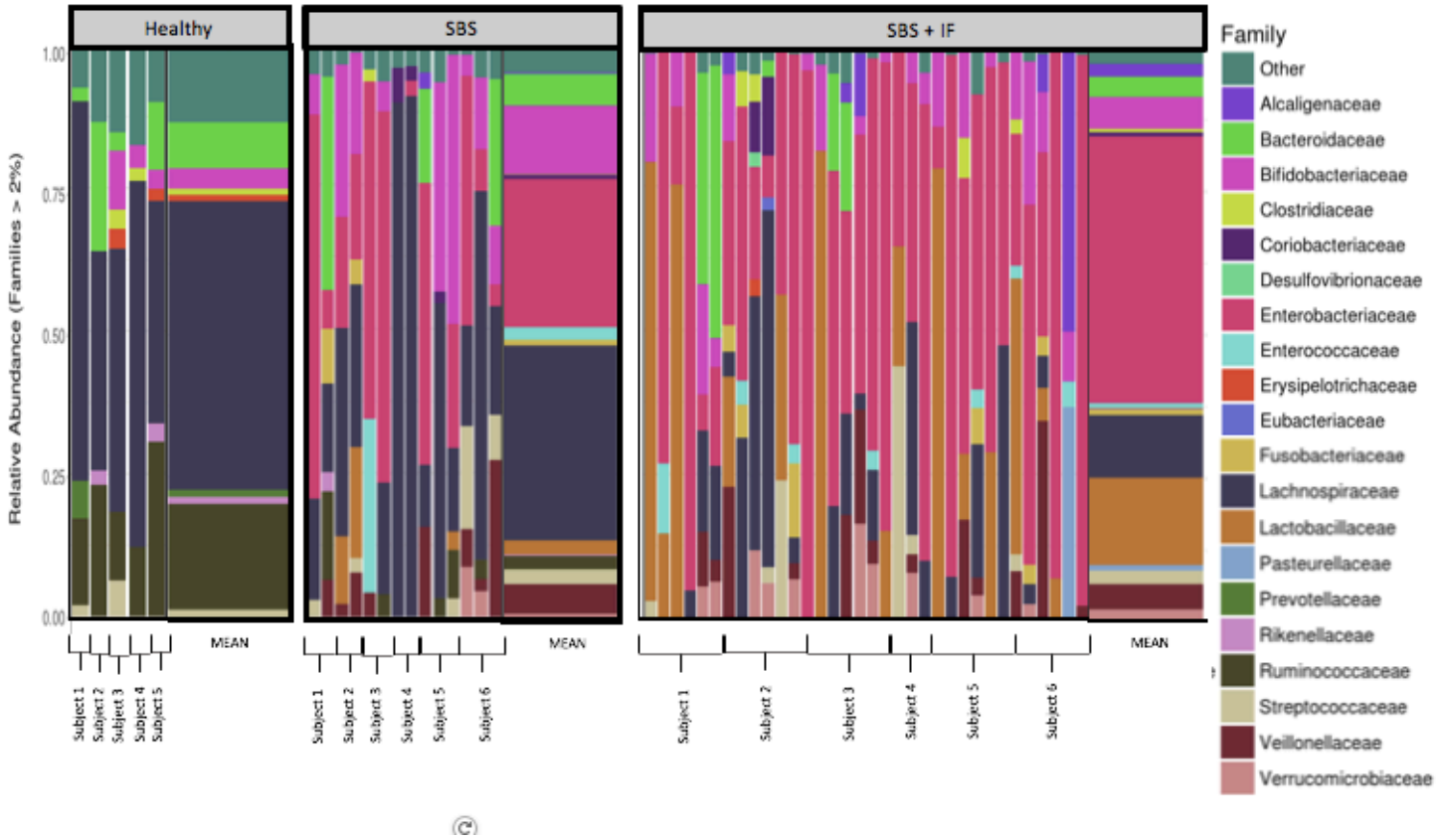

Figure 1. Relative Abundance at the Family level between control children, children with SBS and children with Intestinal Failure.

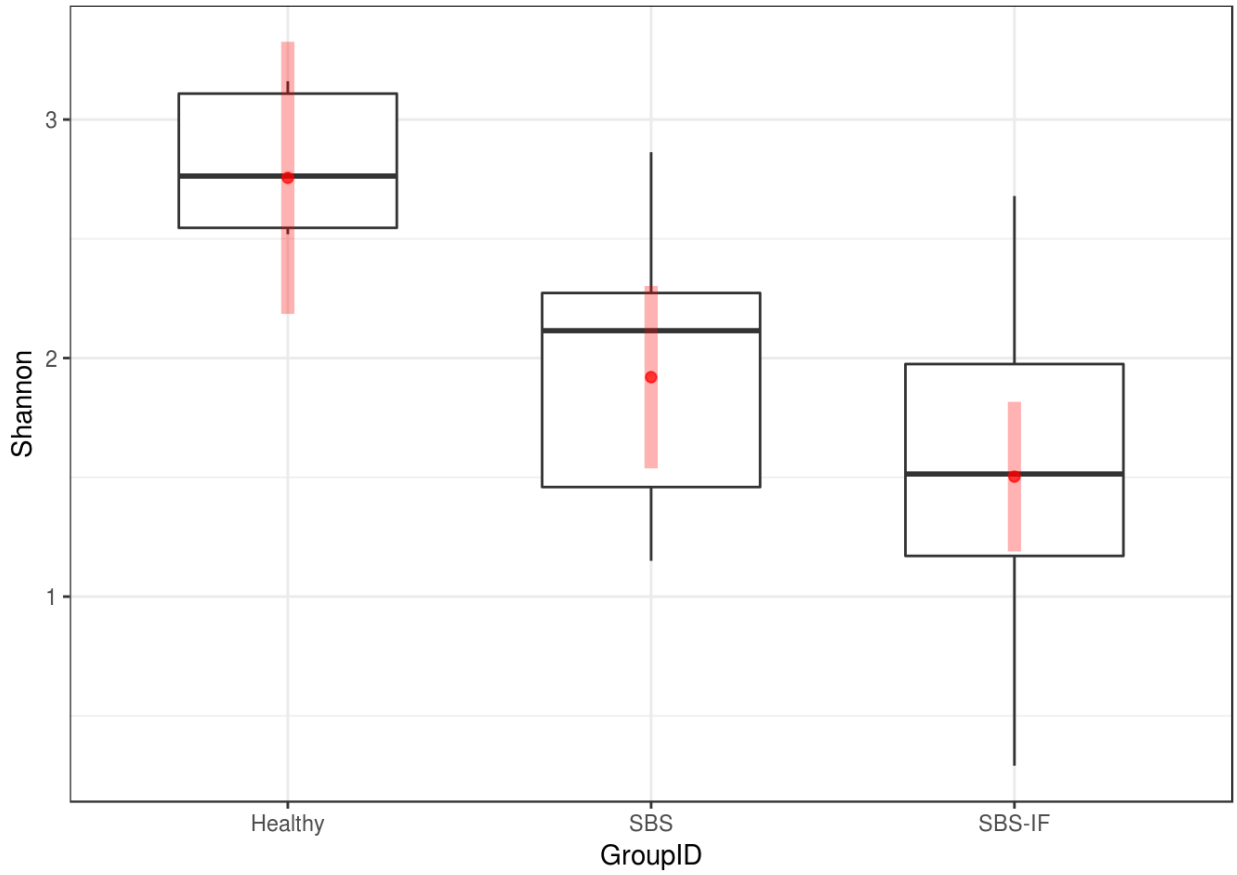

Figure 2. Predicted and observed Shannon diversity in control children, children with short bowel syndrome, and children with short bowel syndrome and intestinal failure. Red points and ranges represent predicted values and 95% confidence intervals from the statistical model, while boxplots show the observed data's mean, quartiles, and 5th and 95th quantiles.

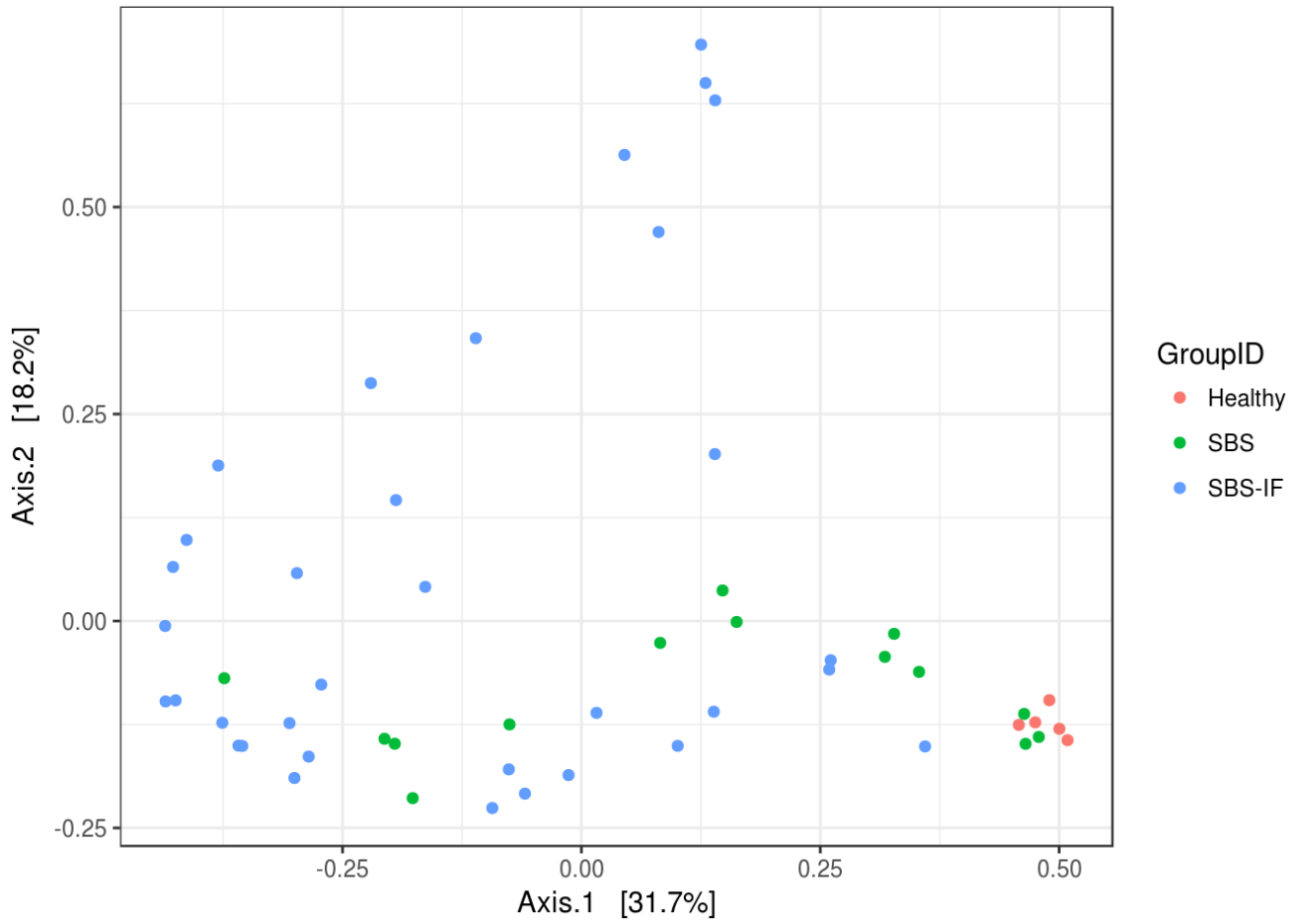

Figure 3A. PCoA of Bray-Curtis distances among samples. The control samples appear to cluster together, while there is minimal clustering between samples of children with SBS and children with SBS and IF. Approximately 17.8% of the among-sample variation is the result of group membership. Although the effect is significant, it is small.

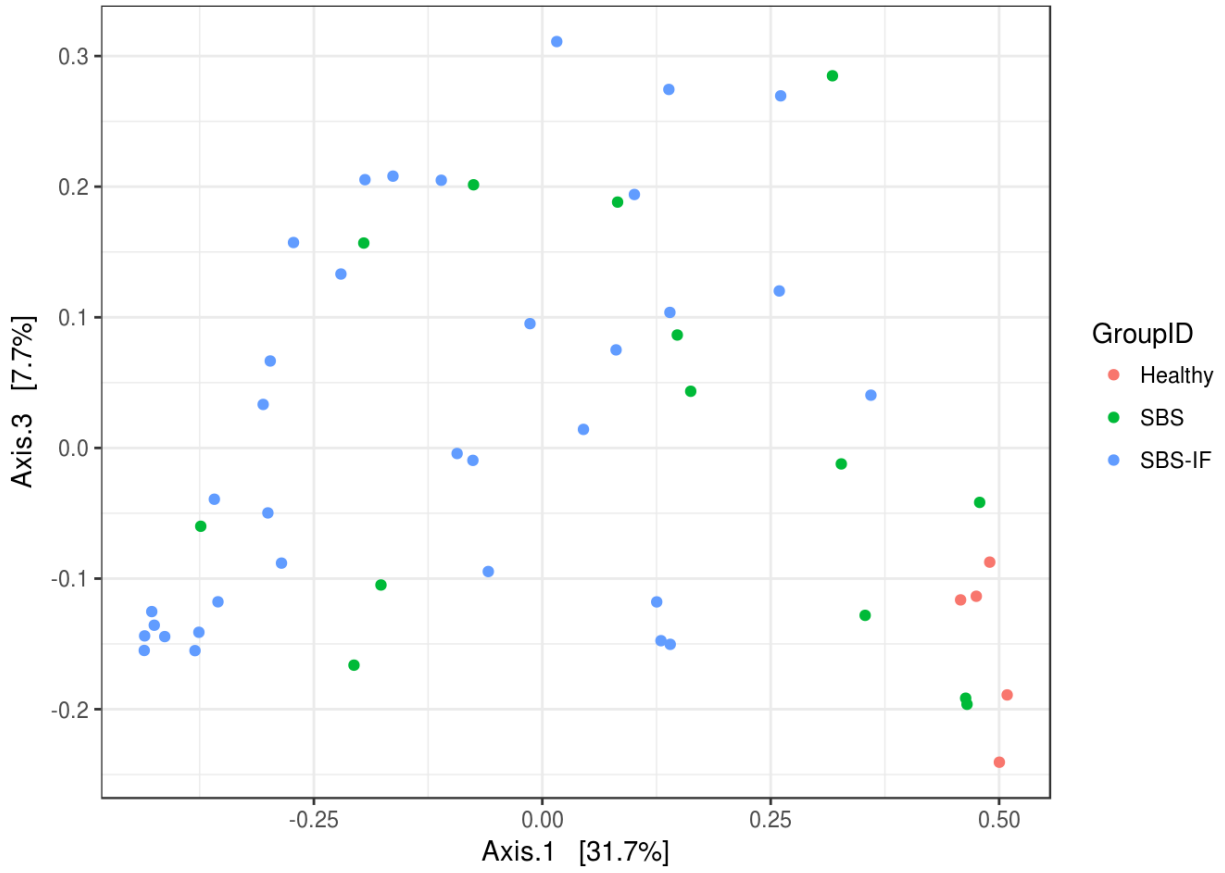

Figure 3B. PCoA of Bray-Curtis distances among samples. The control samples appear to cluster together, while there is minimal clustering between samples of children with SBS and children with SBS and IF. Approximately 17.8% of the among-sample variation is the result of group membership. Although the effect is significant, it is small.

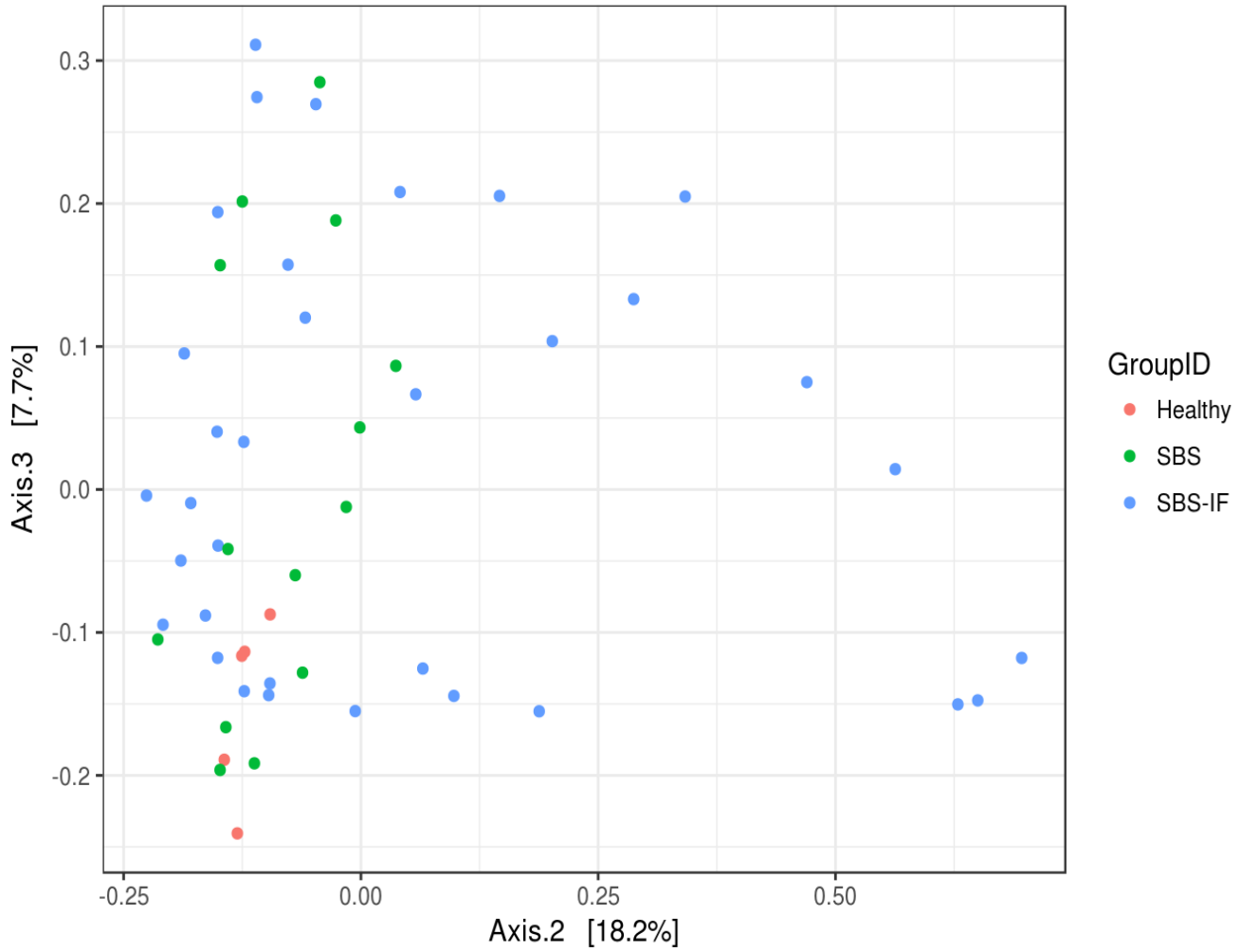

Figure 3C. PCoA of Bray-Curtis distances among samples. The control samples appear to cluster together, while there is minimal clustering between samples of children with SBS and children with SBS and IF. Approximately 17.8% of the among-sample variation is the result of group membership. Although the effect is significant, it is small.

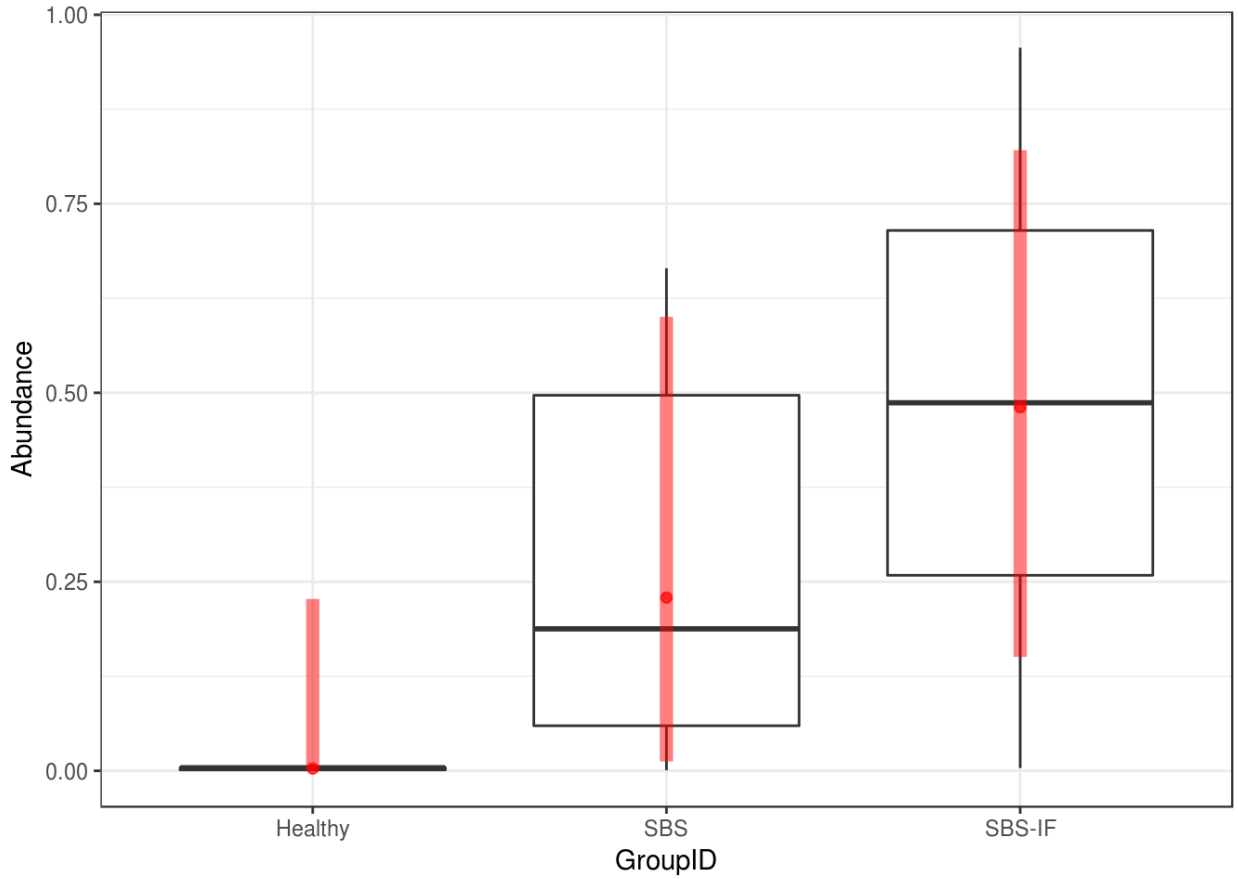

Figure 4. Predicted and observed relative abundance of *Proteobacteria* in control children, children with SBS, and children with SBS and IF. Red points and ranges represent predicted values and 95% confidence intervals from the statistical model, while boxplots show the observed data's mean, quartiles, and 5th and 95th quantiles.

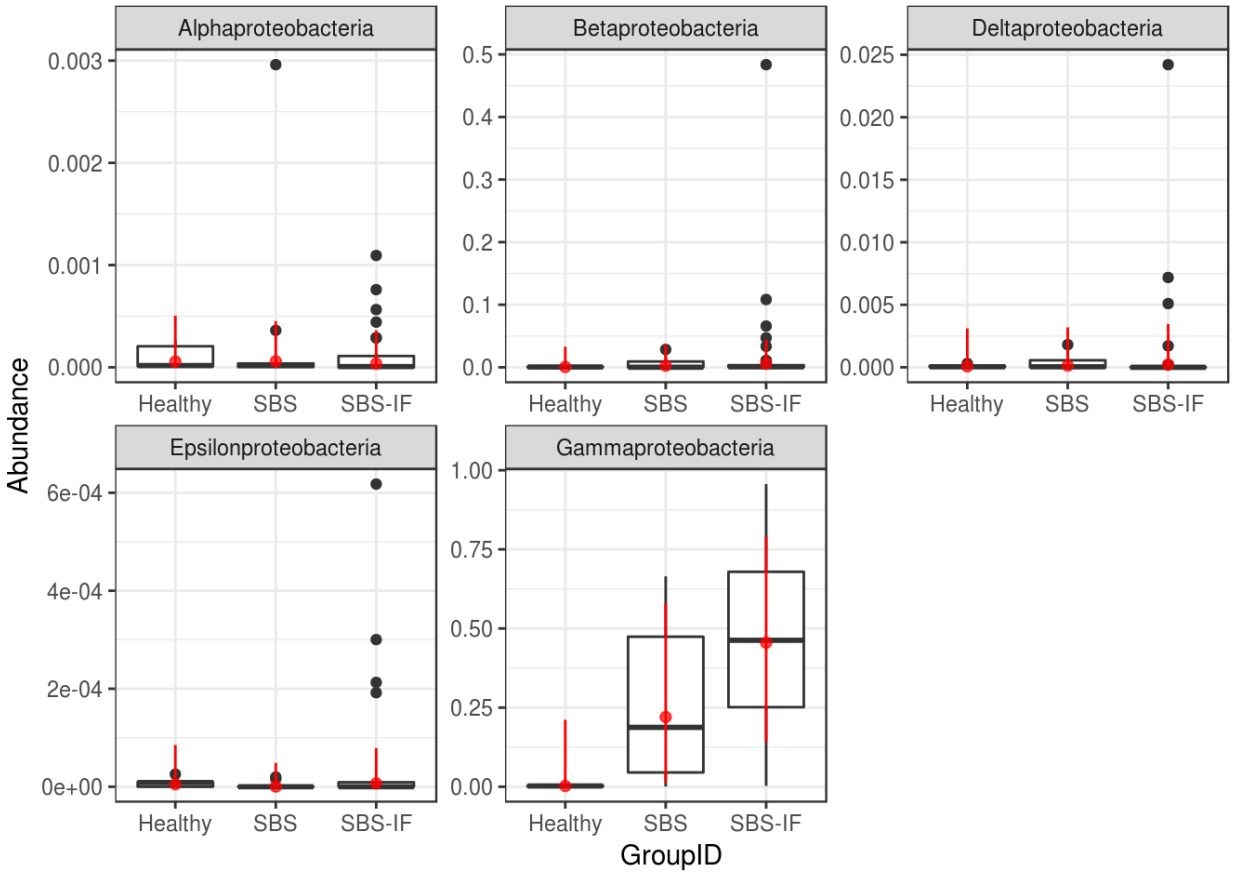

Figure 5. Predicted and observed relative abundance of *Proteobacteria* class in control children, children with SBS, and children with SBS and IF. Red points and ranges represent predicted values and 95% confidence intervals from the statistical model, while boxplots show the observed data's mean, quartiles, and 5th and 95th quantiles. Statistical significance of *Gammaproteobacteria*.

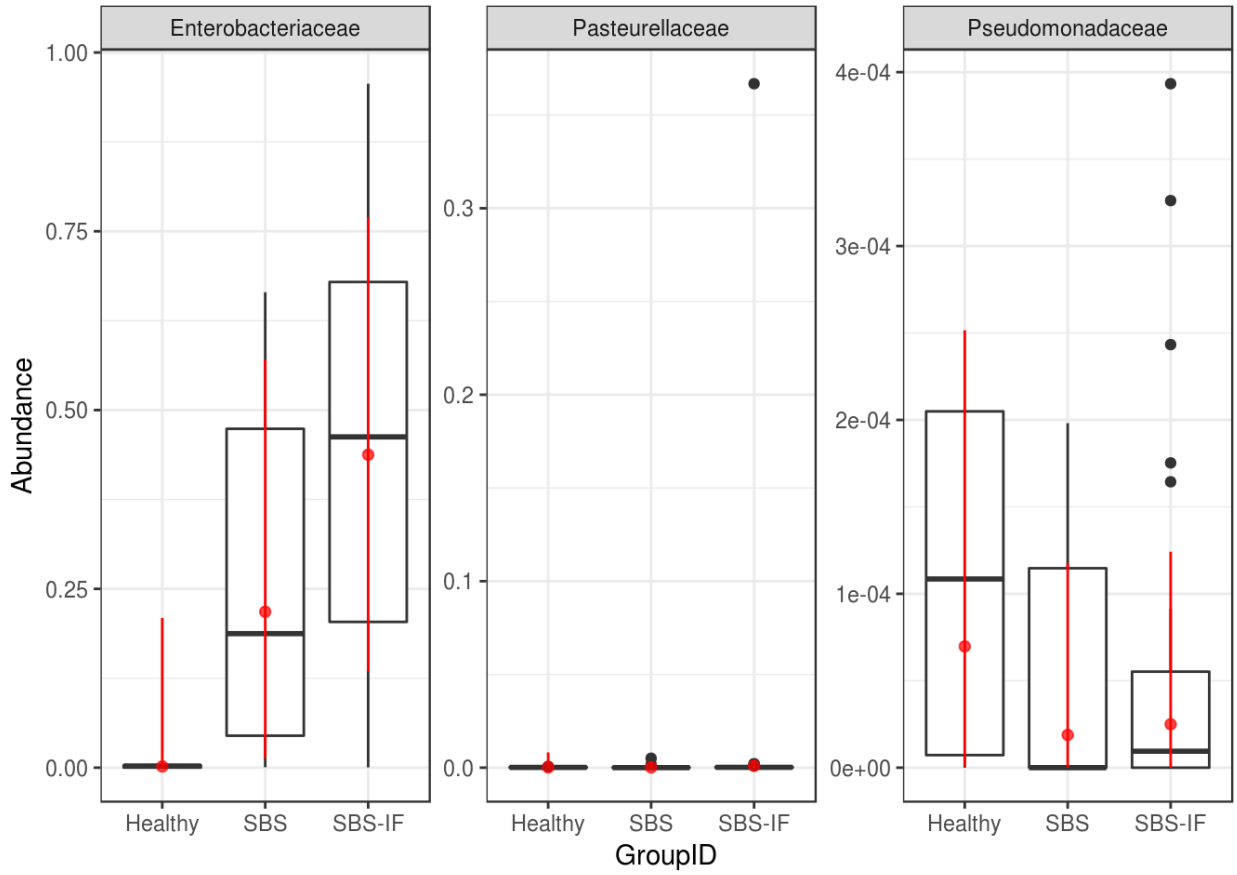

Figure 6. Predicted and observed relative abundance of *Gammaproteobacteria* family in control children, children with SBS, and children with SBS and IF. Red points and ranges represent predicted values and 95% confidence intervals from the statistical model, while boxplots show the observed data's mean, quartiles, and 5th and 95th quantiles. Statistical significance of *Enterobacteriacae*.

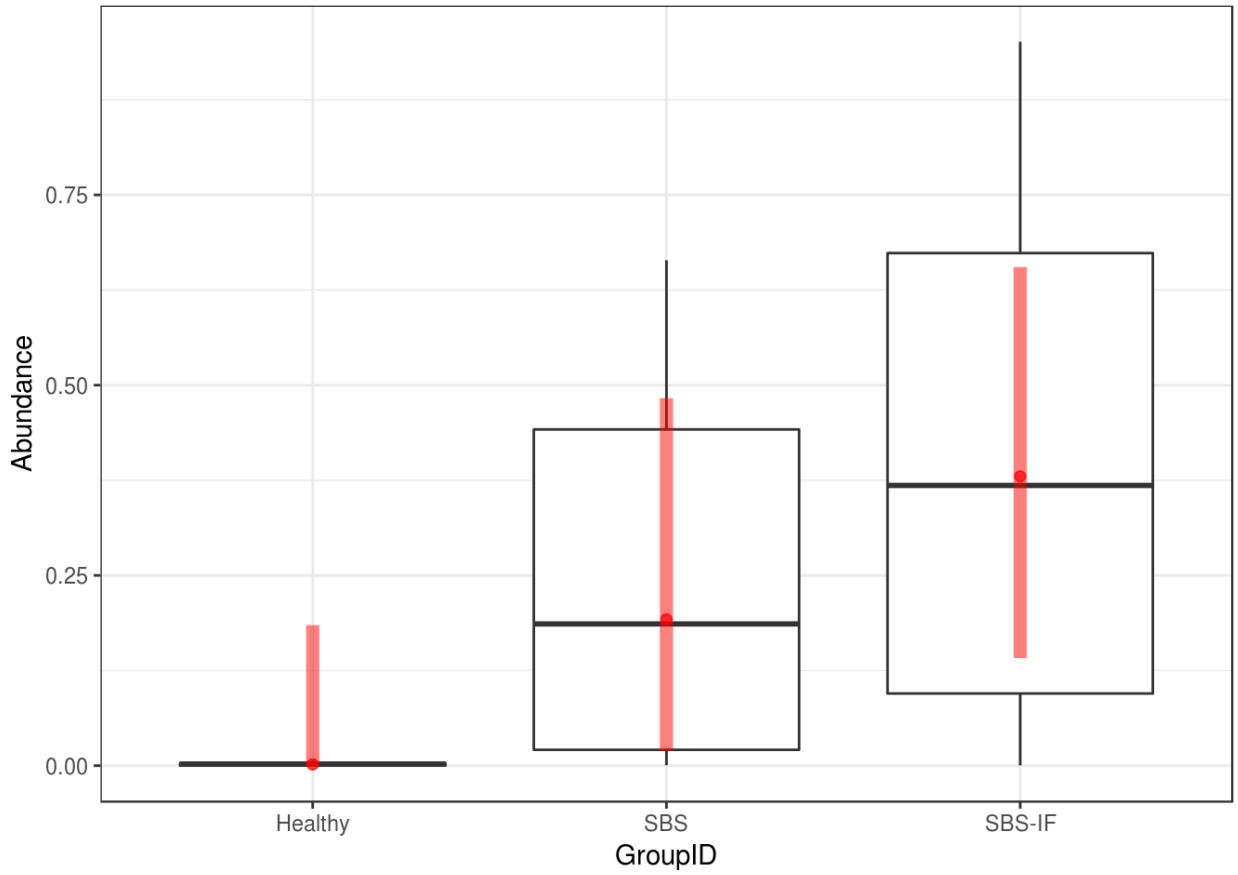

Figure 7. Predicted and observed relative abundance of *Escherichia* in control children, children with SBS, and children with SBS and IF. Red points and ranges represent predicted values and 95% confidence intervals from the statistical model, while boxplots show the observed data's mean, quartiles, and 5th and 95th quantiles.

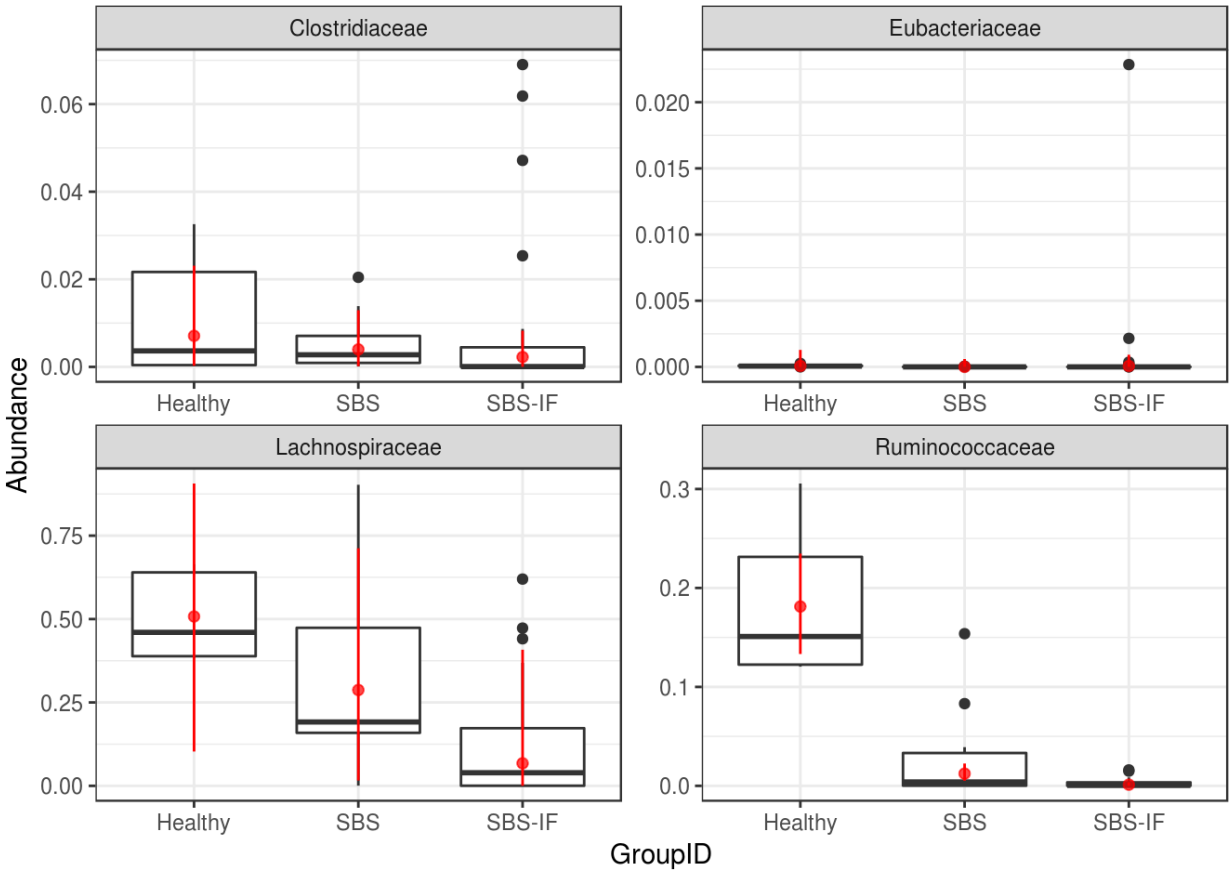

Figure 8. Predicted and observed relative abundance of specific families within *Clostridia* genera in control children, children with SBS, and children with SBS and IF. Red points and ranges represent predicted values and 95% confidence intervals from the statistical model, while boxplots show the observed data's mean, quartiles, and 5th and 95th quantiles. Statistical significance of *Lachnospiraceae* and *Rumnococcus*.

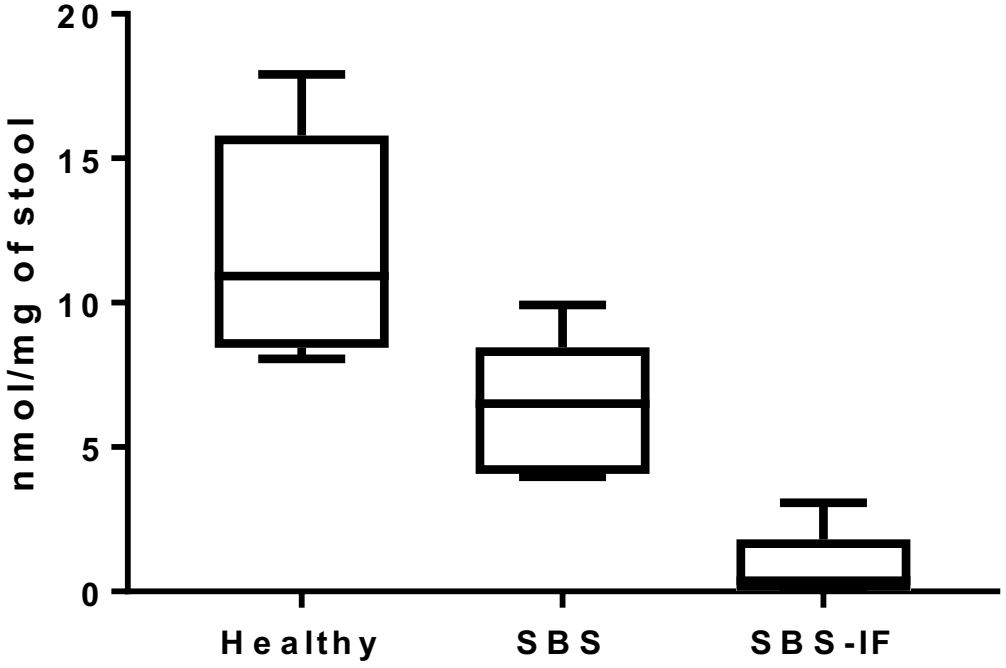

Figure 9. Short chain fatty acid Butryric Acid concentration in stool samples between control group, children with SBS and children with SBS and IF.

REFERENCES:

1. O'Keefe SJ, Buchman AL, Fishbein TM, et al. Short bowel syndrome and intestinal failure: consensus definitions and overview. Clin Gastroenterol Hepatol 2006;4:6-10.
2. Squires RH, Duggan C, Teitelbaum DH, et al. Natural history of pediatric intestinal failure: initial report from the Pediatric Intestinal Failure Consortium. J Pediatr 2012;161:723-8.e2.
3. Fallon EM, Mitchell PD, Nehra D, et al. Neonates with short bowel syndrome: an optimistic future for parenteral nutrition independence. JAMA Surg 2014;149:663-70.
4. Turnbaugh PJ, Ley RE, Hamady M, et al. The human microbiome project. Nature 2007;449:804-10.
5. Davidovics ZH, Carter BA, Luna RA, et al. The Fecal Microbiome in Pediatric Patients With Short Bowel Syndrome. JPEN J Parenter Enteral Nutr 2016;40:1106-1113.
6. Pereira-Fantini PM, Byars SG, Pitt J, et al. Unravelling the metabolic impact of SBS-associated microbial dysbiosis: Insights from the piglet short bowel syndrome model. Sci Rep 2017;7:43326.
7. Demehri FR, Barrett M, Ralls MW, et al. Intestinal epithelial cell apoptosis and loss of barrier function in the setting of altered microbiota with enteral nutrient deprivation. Front Cell Infect Microbiol 2013;3:105.
8. Huang Y, Guo F, Li Y, et al. Fecal microbiota signatures of adult patients with different types of short bowel syndrome. J Gastroenterol Hepatol 2017.
9. Engstrand Lilja H, Wefer H, Nyström N, et al. Intestinal dysbiosis in children with short bowel syndrome is associated with impaired outcome. Microbiome 2015;3:18.

10. Kles KA, Chang EB. Short-chain fatty acids impact on intestinal adaptation, inflammation, carcinoma, and failure. Gastroenterology 2006;130:S100-5.

11. Lührs H, Gerke T, Schauber J, et al. Cytokine-activated degradation of inhibitory kappaB protein alpha is inhibited by the short-chain fatty acid butyrate. Int J Colorectal Dis 2001;16:195-201.

12. Struijs MC, Diamond IR, de Silva N, et al. Establishing norms for intestinal length in children. J Pediatr Surg 2009;44:933-8.

13. Whelan FJ, Surette MG. A comprehensive evaluation of the sl1p pipeline for 16S rRNA gene sequencing analysis. Microbiome 2017;5:100.

14. Martin M. Cut adapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 2011;17:10-12.

15. Masella AP, Bartram AK, Truszkowski JM, et al. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics 2012;13:31.

16. Ye Y. Identification and Quantification of Abundant Species from Pyrosequences of 16S rRNA by Consensus Alignment. Proceedings (IEEE Int Conf Bioinformatics Biomed) 2011;2010:153-157.

17. Wang Q, Garrity GM, Tiedje JM, et al. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261-7.

18. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335-6.

19. Eren AM, Maignien L, Sul WJ, et al. Oligotyping: Differentiating between closely related microbial taxa using 16S rRNA gene data. Methods Ecol Evol 2013;4.

20. Computing RCTRFfS. A language and environment for statistical computing. : Vienna, Austria. , 2016.

21. Langille MG, Zaneveld J, Caporaso JG, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 2013;31:814-21.

22. Paradis E, Claude J, Strimmer K. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics 2004;20:289-90.

23. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 2013;8:e61217.

24. Francois HWaR. dplyr: A Grammar of Data Manipulation. R package version 0.5.0. ed, 2016.

25. Wickham H. tidyr: Easily Tidy Data with 'spread()' and 'gather'() Functions. R package 0.6.1. ed, 2017.

26. Wickham H, Hester J, Francois R. readr: Read Rectangular Text Data. R package version 0.6.1. ed, 2017.

27. Wickham H. ggplot2: Elegant Graphics for Data Analysis. : Springer-Verlag New York, 2009.

28. Alexandra Kuznetsova PBBaRHBC. lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-33, 2016.

29. Oksanen J, Blanchet FG, Friendly M, et al. Vegan: Community Ecology Package. R package version 2.4-3., 2017.

30. Atarashi K, Tanoue T, Oshima K, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature 2013;500:232-6.

31. Piper HG, Fan D, Coughlin LA, et al. Severe Gut Microbiota Dysbiosis Is Associated With Poor Growth in Patients With Short Bowel Syndrome. JPEN J Parenter Enteral Nutr 2016.

32. Simms-Waldrip TR, Sunkersett G, Coughlin LA, et al. Antibiotic-Induced Depletion of Anti-inflammatory Clostridia Is Associated with the Development of Graft-versus-Host Disease in Pediatric Stem Cell Transplantation Patients. Biol Blood Marrow Transplant 2017;23:820-829.

33. Korpela K, Mutanen A, Salonen A, et al. Intestinal Microbiota Signatures Associated With Histological Liver Steatosis in Pediatric-Onset Intestinal Failure. JPEN J Parenter Enteral Nutr 2017;41:238-248.

34. Lapthorne S, Pereira-Fantini PM, Fouhy F, et al. Gut microbial diversity is reduced and is associated with colonic inflammation in a piglet model of short bowel syndrome. Gut Microbes 2013;4:212-21.

35. Jakobsson HE, Jernberg C, Andersson AF, et al. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. PLoS One 2010;5:e9836.

36. Feng Y, Ralls MW, Xiao W, et al. Loss of enteral nutrition in a mouse model results in intestinal epithelial barrier dysfunction. Ann N Y Acad Sci 2012;1258:71-7.

37. Clemente JC, Ursell LK, Parfrey LW, et al. The impact of the gut microbiota on human health: an integrative view. Cell 2012;148:1258-70.

38. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. Nat Immunol 2011;12:5-9.

39. Lopetuso LR, Scaldaferri F, Petito V, et al. Commensal Clostridia: leading players in the maintenance of gut homeostasis. Gut Pathog 2013;5:23.

40. Csordas A. Butyrate, aspirin and colorectal cancer. Eur J Cancer Prev 1996;5:221-31.

41. Segain JP, Raingeard de la Blétière D, Bourreille A, et al. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut 2000;47:397-403.

42. Kinoshita M, Suzuki Y, Saito Y. Butyrate reduces colonic paracellular permeability by enhancing PPARgamma activation. Biochem Biophys Res Commun 2002;293:827-31.

43. Pryde SE, Duncan SH, Hold GL, et al. The microbiology of butyrate formation in the human colon. FEMS Microbiol Lett 2002;217:133-9.

44. Duncan SH, Barcenilla A, Stewart CS, et al. Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. Appl Environ Microbiol 2002;68:5186-90.

45. Kuo SM. The interplay between fiber and the intestinal microbiome in the inflammatory response. Adv Nutr 2013;4:16-28.

46. Nilsson AC, Östman EM, Knudsen KE, et al. A cereal-based evening meal rich in indigestible carbohydrates increases plasma butyrate the next morning. J Nutr 2010;140:1932-6.

47. Spor A, Koren O, Ley R. Unravelling the effects of the environment and host genotype on the gut microbiome. Nat Rev Microbiol 2011;9:279-90.

48. Van den Abbeele P, Belzer C, Goossens M, et al. Butyrate-producing Clostridium cluster XIVa species specifically colonize mucins in an in vitro gut model. ISME J 2013;7:949-61.

49. Weir TL, Manter DK, Sheflin AM, et al. Stool microbiome and metabolome differences between colorectal cancer patients and healthy adults. PLoS One 2013;8:e70803.

50. Hester CM, Jala VR, Langille MG, et al. Fecal microbes, short chain fatty acids, and colorectal cancer across racial/ethnic groups. World J Gastroenterol 2015;21:2759-69.

PRO-INFLAMMATORY, ANTI-INFLAMAMTORY AND INTESTINAL PERMEABILITY MARKERS IN CHILDREN WITH SHORT BOWEL SYNDROME

[1]Jenna Katherine **Dowhaniuk** (MD, FRCPC), [2]Sam **Chorlton** (MD), Grace Teskey (BSc.), Dessi **Loukov** (BSc.), Chris Verschoor (PhD), [5]Jillian **Owens** (RD), [5]Rose-Frances **Clause** (NP) [1]Jeffery **Pernica** (MD, FRCPC), [4]Dawn **Bowdish** (MSc, PhD), [3]Mike **Surette** (PhD), [1]Elyanne **Ratcliffe** (MD, FRCPC)

[1]Department of Pediatrics, McMaster University, Hamilton, Ontario

[2]Department of Undergraduate Medicine, McMaster University, Hamilton, Ontario

[3]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario

[4]Department of Pathology & Molecular Medicine, McMaster University, Hamilton, Ontario

[5]Division of Pediatric Gastroenterology and Nutrition, McMaster Children's Hospital

Correspondence to: Dr. Jenna Dowhaniuk, Division of Pediatric Gastroenterology, Department of Pediatrics, McMaster University 3A61,1280 Main Street West, Hamilton, Ontario, L8S 4K1, Canada. Tel.: 905-527-2299 x73455; fax: 905-521-2655; e-mail: jenna.dowhaniuk@medportal.ca

Funding and Supports: Source of funding includes Regional Medical Associates of Hamilton and Canadian Association of Gastroenterology

Conflicts of Interest: None.

PREFACE:

PRO-INFLAMMATORY, ANTI-INFLAMAMTORY AND INTESTINAL PERMEABILITY MARKERS IN CHILDREN WITH SHORT BOWEL SYNDROME

J. Dowhaniuk: Involved in the following aspects of study: Study concept, completion of study design, research ethics submission and revisions, data abstraction, RNA primer optimization, RNA extraction and qPCR, statistical analysis and first author of the manuscript.

Dr. Elyanne Ratcliffe, Dr. Dawn Bowdish and Dr. Surette were involved in study concept and design, statistical analysis and revisions and edits of the manuscript.

Grace Teskey completed analysis of circulating bacterial products. Chris Verschoor and Dessi Loukov assisted with RNA extraction and qPCR.

Cytokine analysis was completed at Eve Technologies in Calgary, AB Canada.

SUBMISSION: drafted for submission

INTRODUCTION

Short bowel syndrome is a complex medical diagnosis following massive intestinal surgical resection with an incidence of 21.5 per 100 000 births per year and a mortality rate of 25%[1, 2]. It is the leading cause of intestinal failure (IF) defined as a functional intestinal mass below what is required to sustain electrolyte, nutrient or water balance without parenteral nutrition[2, 3]. Long-term parenteral nutrition requires central venous access. Children with IF have frequent central-line associated bloodstream infections (CLABSI) that can lead to sepsis which is the leading cause of death in this patient population. As children with SBS have small bowel bacterial overgrowth and dysmotility, increased intestinal permeability may predispose them to recurrent CLABSI[4, 5]. CLABSI occurrence in this patient cohort is well documented, however little research on the immune system in children with short bowel syndrome has been completed.

Up to two thirds of cultured CLABSI microbes are of the *Enterobactericae* family or *Enterococcus species* and considered enteric bacteria with a likely gastrointestinal origin [6, 7]. This family of bacteria, which includes *Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Proteus* and *Serratia*, has also been documented in significant abundance in the microbiome of children with SBS via 16s rRNA high-throughput sequencing[8, 9]. Furthermore, the class *Escherichia* and species *E. coli* has been identified as a dominant microbe in the intestine of children with IF (manuscript in preparation). These pro-inflammatory pathogenic bacteria may have a role in inflammation of the gastrointestinal tract, intestinal permeability, systemic inflammation and bacterial translocation.

This prospective cohort study is designed to further investigate intestinal permeability and inflammation in children with established short bowel syndrome. This includes differences in gene expression of pro- and anti-inflammatory transcription factors and cytokine concentrations. As previous research has identified that there are differences in the composition of the microbial community in children with short bowel syndrome (manuscript in preparation), we sought to investigate the permeability of the intestine. As children with IF and central line access exhibit the highest rates of sepsis, we opted to investigate differences in inflammatory markers and intestinal permeability between those with IF and those with SBS who have achieved enteral autonomy. We, furthermore, compared to control groups of both children and adults.

METHODS:

We conducted a prospective cohort study of children with SBS followed by McMaster Children's Hospital Pediatric Short Bowel Syndrome Rehabilitation Team. The first cohort consisted of children with SBS and IF for Group 1 who were less than 16 years of age with SBS (defined as those who required monitoring by the Pediatric Short Bowel Syndrome Rehabilitation Team) and who, at the time of enrolment, had required a minimum of twice-weekly PN for a component of caloric intake for a minimum of three months (Table 1). The second cohort were children less than 16 years of age with SBS (defined above) who had previously required home parenteral nutrition for a minimum of three months but at the time of enrollment had discontinued PN and achieved enteral autonomy. We opted to include pediatric controls with the insertion of a central indwelling catheter to minimize any confounding effect of central venous access on inflammatory markers. Therefore, the third cohort were control children, age- matched who had no underlying gastrointestinal illnesses, recruited from a pediatric hematology clinic with a central indwelling

catheter. The fourth cohort were control children, age-matched who had no underlying gastrointestinal illnesses and no indwelling venous catheter. The fifth cohort included adult controls. Ethics approval was obtained from the Hamilton Integrated Research Ethics Board and consent was obtained from each participant including adult controls.

DATA COLLECTION :

We abstracted from previous medical records demographic data including: gestation age at birth, current age, number of indwelling vascular catheter days, and surgical resection details. The shortest measured length of small intestine was obtained from operative reports. These were compared to previous published normal values[10]. Blood samples were obtained following enrollment for serum analysis and a stool sample was provided in sterile containers on ice for analysis of fecal calprotectin for those in Group 1 and 2 as normal reference ranges are published.

Cytokine Multiplex ELISA assays

To determine pro- and anti-inflammatory cytokine profiles we quantified IFNγ, IL-1β, IL-8, IL-12 (p70), IL-17A, IL-10 and TNFα using the Human High Sensitivity T-Cell Discovery Array 14-Plex Discovery Assay through Eve Technologies Corp Calgary AB, Canada. The multiplex assay was performed at Eve Technologies by using the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and a Milliplex Human High Sensitivity T-Cell panel (Millipore, St. Charles, MO, USA) according to their protocol[11]. The assay sensitivities of these markers range from 0.11 – 3.25 pg/mL. Samples were analyzed in duplicate and mean values were included in final analysis.

Stool inflammatory markers:

To determine colonic inflammation, fecal calprotectin was quantified from a single stool sample. Extractions were performed with Smart Prep Extraction device from Roche Diagnostics[12]. Extracts were homogenized and centrifuged at 3000 ×g for 5 minutes. Fecal calprotectin was measured by ELISA with PhiCal Calprtoectin-EIA (Nova Century) kit according to their protocol[12]. The laboratory technologist was blinded to patient history and cohort.

Gene expression Analysis using Real-time quantitative PCR

To determine gene expression of transcription factors FoxP3+ and RORγT as well as TLR2, and TLR4, RNA extraction and quantitative PCR was completed. RNA was extracted with Genezol RNA minikit and the RNA concentration was determined using a Nanodrop Spectrophotometer. cDNA synthesis was performed with Maxima reverse transcriptase using oligonucleotide primers and gene expression was measured with the GoTaq qPCR Master Mix (promega, WI, USA) and StepOne Plos Real-Time PCR System (Life Technologies, CA, USA). Primers can be found in Table 4. Annealing temperatures for FoxP3+, RORγT was 60 degrees Celsius while TLR 4 and TLR2 at 55 degrees Celsius. Relative expression values were calculated using ΔCT relative to the standard housekeeping gene HPRT. Statistical analysis by non-parametric analysis was performed with Kruskall-Wallis ANOVA.

Intestinal Permeability

To evaluate intestinal permeability, we quantified circulating bacterial products lipopolysaccharide (LPS) and muramyl dipeptide (MDP) using a bioassay in which the binding of their respective receptors, toll-like receptor (TLR)-4 and nucleotide oligomerization domain 2 (NOD2) leading to the activation of Nuclear factor kappa-light-chain of B cells (NF-κB). Two HEK293T cell lines were used, one transfected with the pNifty2-SEAP (Invivogen, CA, USA)

reporter plasmid and expressing the NOD2 receptor, the other transfected with the pNifty2-SEAP reporter plasmid and expressing TLR4, CD14 and MDP. Cells were seeded at $4x10^3$ cells/well in a 96 well plate in complete DMEM medium overnight. DMEM was removed prior to the addition of heat inactivated serum (diluted 1:10) in HEK Blue Detection medium (Invivogen, CA, USA). A standard curve of increasing concentrations of both MDP and LPS (Invivogen, CA, USA) were used as a positive control. Absorbance was read at 630nm after 24 hours with a higher absorbance value indicating higher quantities of bacterial products.

Statistical Analysis

Statistical analysis was completed using GraphPad Prism (GraphPad software, San Diego, CA). Statistical significance is accepted at $p<0.05$. Descriptive data is presented as medians with interquartile range or mean with confidence interval. Demographic analysis between groups assessed by Mann-Whitney or Kruskal Wallis tests.

RESULTS:

Demographics:

Twenty-two children were recruited in our study (Group 1 *n=6*; Group 2 *n=6*; Group 3 *n=5;* Group 4 *n=5*) as well as 10 adult control samples were obtained. The median age of children in Group 1 was 67 months compared to 47.5 months in Group 2, 51 months in Group 3 and 55 months in Group 4. At the onset of the study, the total days with an indwelling vascular catheter was 2023 (IQR 811, 2947) in Group 1 compared to 484 (IQR 274, 588) in Group 2 ($p<0.05$). At the time of enrollment, all children in Group 1 and 3 had an indwelling vascular catheter while all catheters

had been removed in Group 2. The shortest measured length of residual small bowel in Group 1 measured 26.5 cm (IQR 24.75, 40) compared to 55 cm in Group 2 (IQR 31.2, 89; $p<0.01$). The children in our control groups on average had a higher *z*-score for both weight and height than the children in groups 1 and 2. There was no statistical difference in weight or height *z*-score between Group 1 and Group 2. The etiology of intestinal failure included necrotizing enterocolitis, gastroschisis, and malrotation with mid-gut volvulus. In Group 1, three patients had a preserved ileocecal valve and full colon compared to 4 in Group 2 (p=NS). Full demographics are presented in Table 2.

Cytokine Multiplex ELISA assays

Serum analysis of pro-inflammatory cytokine TNFα was statistically significant by non-parametric ANOVA testing between groups ($p<0.005$). Cytokine results can be found in Table 3 and Figure 1. Children with IF (Group 1) had concentrations of 12.7pg/mL (IQR 6.9, 16.6) while adult controls had significantly lower concentration 6.2pg/mL (IQR 4.5, 8.1) ($p<0.05$). However, within group analysis by Mann-Whitney testing did not identify a difference between those with IF (Group 1) and Groups 2, 3 or 4. Other cytokines IFNγ, IL-1β, IL-8, IL-12 (p70), IL-10 and IL-17A were not statically significant between groups (Table 3).

Stool inflammatory markers:

Fecal calprotectin levels were statistically lower in children with IF (median 12.8mg/kg; IQR 9.3, 34.9) compared to those with SBS who no longer required PN (median 96mg/kg, IQR 71.6, 188.2) $p<0.01$ (Figure 2). However, the median results remained in the normal range.

Gene expression analysis using real-time quantitative PCR

Relative quantification of RNA expression of FoxP3+ and RORγT were similar between groups (Figure 3 and 4). Median values of 2^ (-ΔCT) of FoxP3+ was lowest in those with intestinal Failure (Group 1) median 0.217 however no statistical differences were detected between groups.

Intestinal Permeability

Circulating bacterial products of LPS and MDP were not statistically different between groups by analysis of binding of their receptors TLR 4 and NOD2. The majority of samples detected absorbance at 630nM below zero. Only Group 5 adult controls had an average detectable LPS (Figure 5 and 6). A similar pattern was seen in RNA expression of TLR 4 with 2^ (-Δ CT) values highest in adult controls (43.71) compared to 7.108 in children with IF (Group 1); $p<0.005$ (Figure 7). No difference between relative quantification of TLR 2 was detected.

CONCLUSIONS:

A major cause of morbidity and mortality for children with SBS and IF is frequent blood stream infections. Recurrent CLABSI can result in life threatening sepsis and loss of venous access which remains an indication for intestinal transplantation[13]. It has been hypothesized that the frequency of infections may be due to increased intestinal permeability and bacterial translocation especially given the frequency of enteric microbes cultured from central line catheters. We sought to investigate for systemic activation of the inflammatory cascade in children with short bowel syndrome and specifically examine any differences between those with IF and those who have achieved enteral autonomy. In addition, inflammation within the colon was assessed by fecal

calprotectin as well as circulating bacterial products to further investigate for markers of intestinal permeability.

The barrier between host and microbe in the gastrointestinal tract is tightly regulated by intestinal epithelial cells, secretory IgA as well as the innate and adaptive mucosal immune system. Regulation of the intestinal barrier requires tight junction proteins, epithelial cells and cell processes which regulate secretion and absorption[14]. Maintenance of the semi-permeable barrier of the GI tract is critical. LPS, translocated bacteria and pathogen associated molecular patterns (PAMPs) can activate the mucosal inflammatory cascade. LPS specifically binds to TLR 4 found on the basolateral surface of intestinal epithelial cells thereby signaling the inflammatory cascade in the setting of translocation or epithelial cell damage [15]. As children with SBS have had a disruption in the intestinal epithelial barrier with early surgical resection as well as continued insults including enteral deprivation and dysbiosis, they could be predisposed to longstanding mucosal inflammation.

Animal models have investigated inflammation in SBS. Previous zebrafish models have identified 1346 upregulated genes following extensive intestinal resection[16]. These included those associated with acute phase signaling as well as both innate and adaptive immunity including expression of stat4, IL-6, IL6 Receptor, TNFα, IL-22, complement system and IL-1β[16]. Piglet models following surgical resection have also identified elevated pro-inflammatory cytokines such as IL-1β and TNF-α as well as iNOS and macrophage in colonic epithelium [17]. In the piglet model, significant changes occurred to microbial diversity as early as two weeks post-surgical resection however no immune dysregulation was appreciated at the same time point. However, at six weeks,

dysbiosis was maintained and the development of pro-inflammatory cytokine expression including IL-1β and TNF-α was isolated in colonic specimens.[17] Applying these results, this model suggests a potential causative role that dysbiosis has on inflammation in the GI tract.

Previous research has also identified similar elevations in pro-inflammatory cytokines in children under aged two with SBS on PN for three months where, at baseline, TNF-α was increased compared to controls however subsequently decreased over time[18]. Despite these results, our study did not identify similar upregulation of inflammatory cytokines or decrease in anti-inflammatory markers. Our contrasting results may be due to the age of the patient and timing of surgical resection. Piglet and human studies of SBS with elevation in TNF-α were conducted closer to initial surgical resection and, in human studies, improved over time. As the children in this study are, on average, 5 years of age, the initial pro-inflammatory response may have resolved.

Regulatory T cells (Tregs) are critical in regulation of the inflammatory cascade via suppression of T cell proliferation[19]. FoxP3+ is a transcription factor required to regulate T helper cells to differentiate to Tregs[19]. Treg cells further regulate immune tolerance via secretion of IL-10[19]. In contrast RORγT transcription factor is regulated by IL-6 and differentiates T helper cells to Th 17 cells with subsequent secretion of pro-inflammatory IL-17, IL-6 and IL-23[19]. Neither transcription factor appeared to be upregulated in children with SBS compared to control.

While our study did not appreciate any significant differences in inflammatory or anti-inflammatory cytokines, transcription factors, RNA gene expression or circulating bacterial

products, the venous samples may not represent mucosal inflammation at the level of intestinal epithelial cells. Tissue biopsies were not feasible in this study, however, may be of use in future studies to examine for inflammation. Despite frequent septic events, we did not detect elevation in circulating bacterial products to indicate an altered intestinal barrier or intestinal permeability. LPS has been assessed in children with SBS previously and similarly not detected differences between healthy controls[18]. However, other methods of intestinal permeability have not been assessed in this study such as anti-flagellin and anti-LPS antibody concentration or lactulose-mannitol testing. Lactulose-mannitol testing was not feasible in this study design. Other studies have detected abnormal permeability studies by these methods [5, 18]. Given the lack of a single validated test for intestinal permeability it is difficult to apply these results and further studies should be dedicated to further investigate intestinal permeability in children with SBS.

In contrast to a previous study of children under aged 2 with IF, our study did not detect elevation in fecal calprotectin[18]. Calprotectin is a protein complex expressed by activated macrophages and monocytes and within neutrophil cytosol [14]. Activated neutrophils, which migrate to the gastrointestinal epithelium in times of disease, release calprotectin into feces[14]. In our study, children with IF had a statically lower amount of calprotectin in their stools samples compared to children with SBS who had achieved enteral autonomy. The children with SBS did not present with elevation in fecal calprotectin and all values remained in the normal range. Therefore, these results indicate that at the time of sampling there was no activation of macrophages, monocytes and neutrophils in the mucosa of the intestine to indicate ongoing intestinal inflammation.

Despite frequent bloodstream infections and risk factors for a disruption in the delicate balance of the gastrointestinal mucosal barrier, this prospective study of children with SBS did not identify ongoing intestinal inflammation by measurement of fecal calprotectin or concentration of inflammatory cytokines or gene expression analysis using qPCR. There also was no elevation in circulating bacterial products which may have indicated increased intestinal permeability. Further research should investigate intestinal permeability in states of health and sepsis as well as the adaptive and innate immune system of a child with SBS to investigate if any acquired differences may account for the frequency of infections.

Table 1. Study Cohorts

Group	Inclusion Criteria	Age- matched to Group	Indwelling vascular catheter
1	Short Bowel Syndrome Intestinal Failure	NA	Yes; requiring minimum of twice weekly PN for three months
2	Short Bowel Syndrome Achieved enteral autonomy and discontinued previous PN	NA	No
3	Pediatric controls Hematology clinic No gastrointestinal illnesses	1	Yes
4	Pediatric controls Hematology clinic No gastrointestinal illnesses	2	No
5	Adult controls	NA	No

Abbreviations: PN, parenteral nutrition

Table 2. Demographic characteristics

Variable: statistics	SBS-IF Group 1	SBS Group 2	Controls Group 3	Control Group 4	*p* value
Age, *months,* median *(IQR)*	67 (30.75, 89.5)	47.5 (34.75, 101.3)	51 (42, 91)	55 (46.5, 110)	NS
Gender, n *(%)*					
Male	5 (83.3%)	3 (50%)	5 (100%)	5 (100%)	NS
Number of indwelling vascular catheter days, median *(IQR)*	2023 (811, 2947)	484 (274, 588)	N/A	N/A	0.041
Etiology of intestinal Failure, n *(%)*					
Necrotizing enterocolitis	2 (33.3%)	3 (50.0%)	N/A	N/A	NS
Gastroschisis	3 (50.0%)	1 (16.7%)			
Malrotation with mid-gut volvulus	1 (16.7%)	1 (16.7%)			
Isolated jejunal atresias	0	1 (16.7%)			
Gestational age, *weeks,* median *(IQR)*	33.9 (25.8, 37)	33.9 (31.2, 38.6)	N/A	N/A	NS
Age at initial intestinal resection, *days* median *(IQR)*	27.5 (1.8,63.3)	10.5 (1.75, 32)	N/A	N/A	NS
Shortest measured length of small intestine, *cm* Median *(IQR)*	26.5 (24.8, 40)	55 (31.2, 89)	N/A	N/A	<0.01
Predicted bowel length at shortest measure, mean *(SD)*	18.35% (13.8, 27.5)	35.3% (18.2, 52.7)	100%	100%	<0.01
Preserved ileocecal valve, n *(%)*	3 (50%)	4 (66.7%)	5 (100%)	5 (100%)	NS
Colon Anatomy, n *(%)*					
Full colon intact	3 (50.0%)	4 (66.7%)	5 (100%)	5 (100%)	NS
Partial or hemi-colon	3 (50.0%)	1 (16.7%)			
Sub-total colectomy	0	1 (16.7)			
Weight z-score at initial Visit, median *(IQR)*	-0.895 (-0.98, -0.35)	-0.92 (-1.79, -0.62)	0.12 (-0.6, 0.9)	1.12 (0.6, 1.4)	0.005

Height z-score at initial visit, median *(IQR)*	-0.315 (-1.33, 0.16)	-1.575 (-2.64, -0.16)	0.04 (-0.7, 0.95)	1.1 (-0.1, 2.1)	0.05

Abbreviations: SD, Standard Deviation; IQR, Interquartile range at 25% and 75%

TABLE 3. Cytokine analysis observed concentration

	Group 1	Group 2	Group 3	Group 4	Adult Controls	P-value
IFN-γ, pg/mL, median *(IQR)*	13.4 (11.2, 159.2)	19.6 (16.9, 23.2)	19.4 (15.7, 21.3)	20.1 (18.0, 24.7)	18.4 (14.7, 30.2)	NS
IL-Iβ, pg/mL, median *(IQR)*	1.1 (0.6, 2.5)	2.1 (1.4, 3.5)	2.7 (1.7, 3.7)	4.0 (1.6, 6.6)	2.4 (1.2, 5.8)	NS
IL- 8, pg/mL, median *(IQR)*	15.2 (8.3, 35.6)	10.8 (6.7, 20.0)	8.6 (6.5, 15.8)	10.4 (5.7, 13.5)	7.82 (5.0, 10.3)	NS
IL-10, pg/mL, median *(IQR)*	9.7 (2.8, 20.1)	10.2 (7.2, 18.2)	9.9 (8.7, 13.1)	7.8 (5.8, 11.4)	7.5 (5.6, 10.7)	NS
IL – 17A, pg/mL, median *(IQR)*	18.6 (12.5, 379.7)	30.0 (26.7, 39.4)	28.3 (22.9, 33.5)	25.3 (20.9, 34.0)	24.3 (19.7, 32.5)	NS
TNF-α, pg/mL, median *(IQR)*	12.7 (6.9, 16.6)	16.5 (13.3, 20.8)	13.1 (6.9, 18.3)	11.3 (9.7, 19.8)	6.2 (4.5, 8.1)	0.0047

Abbreviations: SD, Standard Deviation; IQR, Interquartile range at 25% and 75%; IL, interleukin; TNF, tumour necrosis factor; IFN, interferon

Table 4. Primers used for qPCR of specific genes

Gene	Forward Primer	Reverse Primer	Gene ID	PMID
TLR2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACCT	NM_003264	12829592
TLR4	CTGCAATGGATCAAGGACCA	TTATCTGAAGGTGTTGCACATTCC	XM_057452	12829592 74bp
RORγT	GCA GCG CTC CAA CAT CTT CT	ACG TAC TGA ATG GCC TCG GT		25552913 Amplicon: 111bp
FoxP3+	CAC CTG GCT GGG AAA ATG G	GGA GCC CTT GTC GGA TGA		25552913 Amplicon: 63bp

Abbreviations: SD, Standard Deviation; IQR, Interquartile range at 25% and 75%; IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; qPCR, quantitative real-time polymerase chain reaction

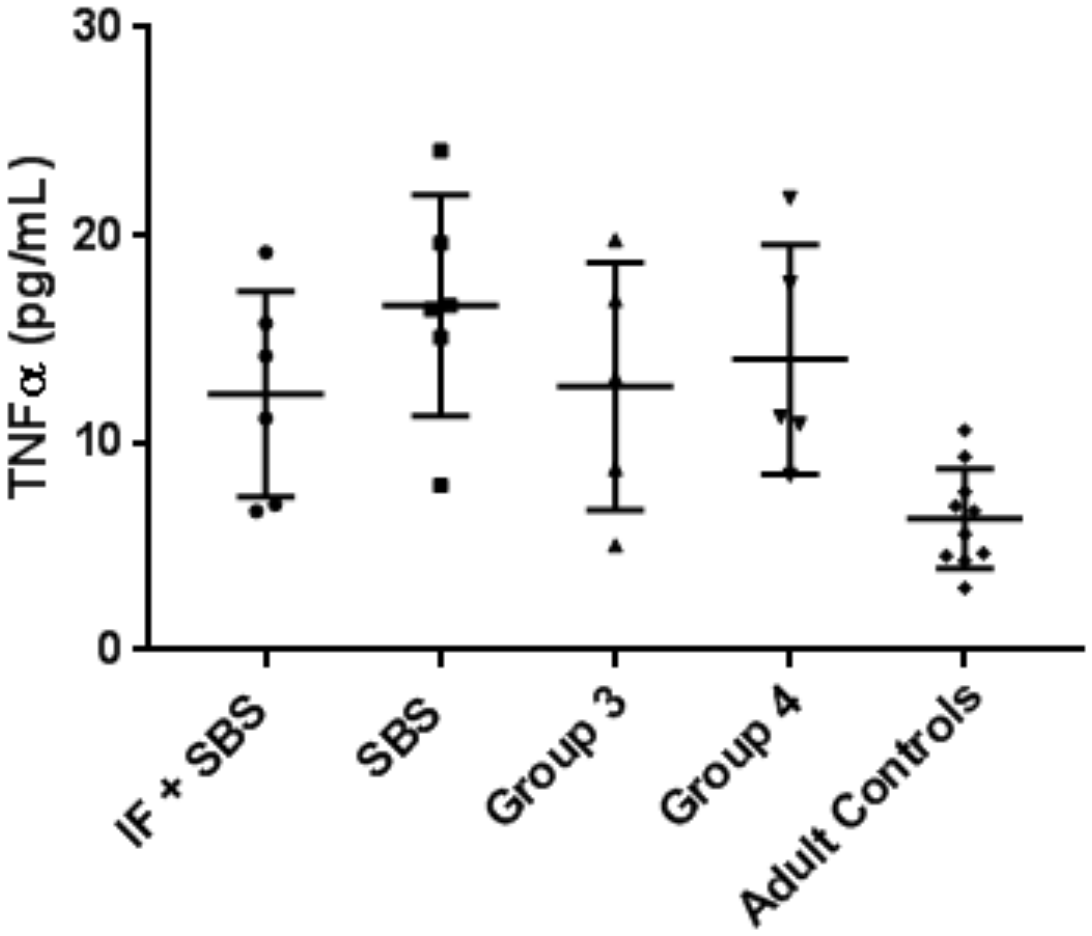

Figure 1. ELISA quantification of cytokine tumour necrosis factor alpha between children in Group 1 with intestinal failure and short bowel syndrome, those in Group 2 with short bowel syndrome, and pediatric aged- matched control groups 3, 4, and adult controls. Abbreviations: TNF, tumour necrosis factor; IF, intestinal failure; SBS, short bowel syndrome

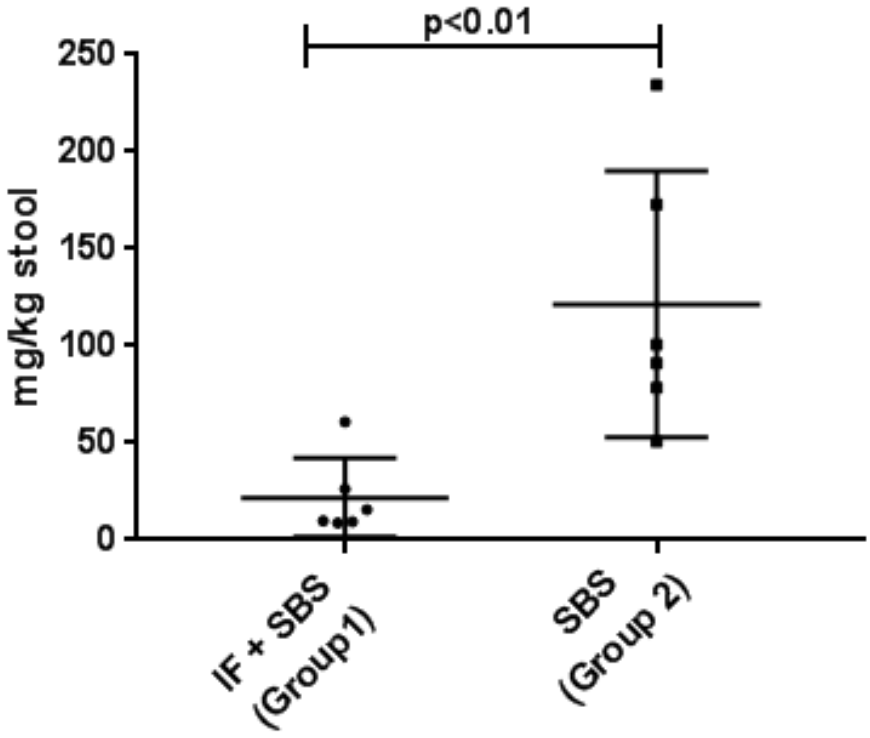

Figure 2. Fecal calprotectin quantity by ELISA to determine intestinal inflammation between children in Group 1 with intestinal failure and short bowel syndrome, and those in Group 2 with short bowel syndrome who have achieved enteral autonomy. Abbreviations: IF, intestinal failure; SBS, short bowel syndrome

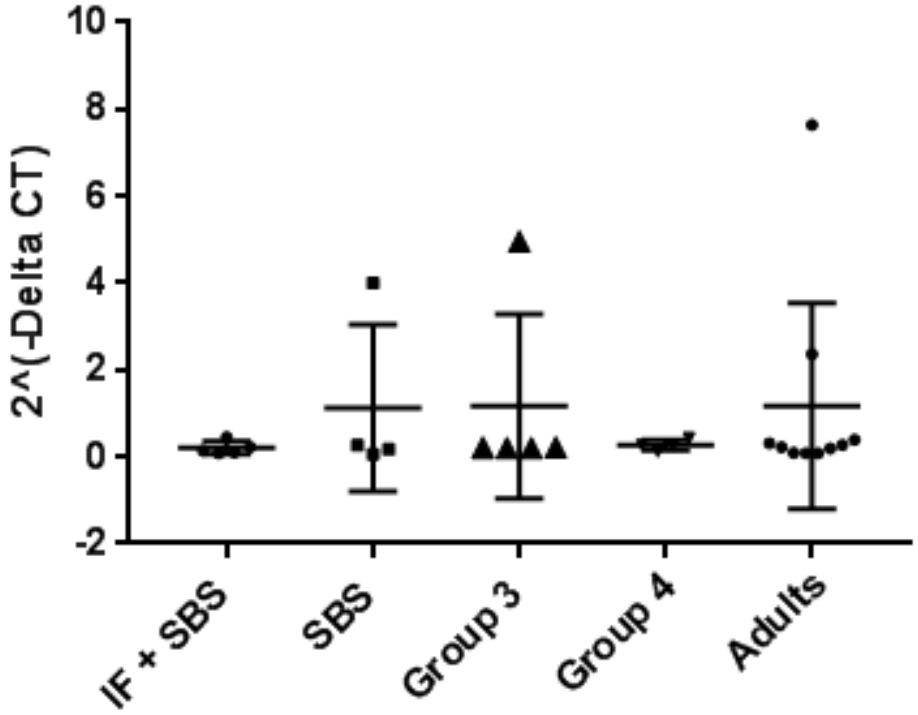

Figure 3. FoxP3+ transcription factor relative quantification by quantitative real-time polymerase chain reaction between children in Group 1 with intestinal failure and short bowel syndrome, those in Group 2 with short bowel syndrome, and pediatric aged- matched control groups 3, 4, and adult controls. Abbreviations: CT, cycle threshold; IF, intestinal failure; SBS, short bowel syndrome

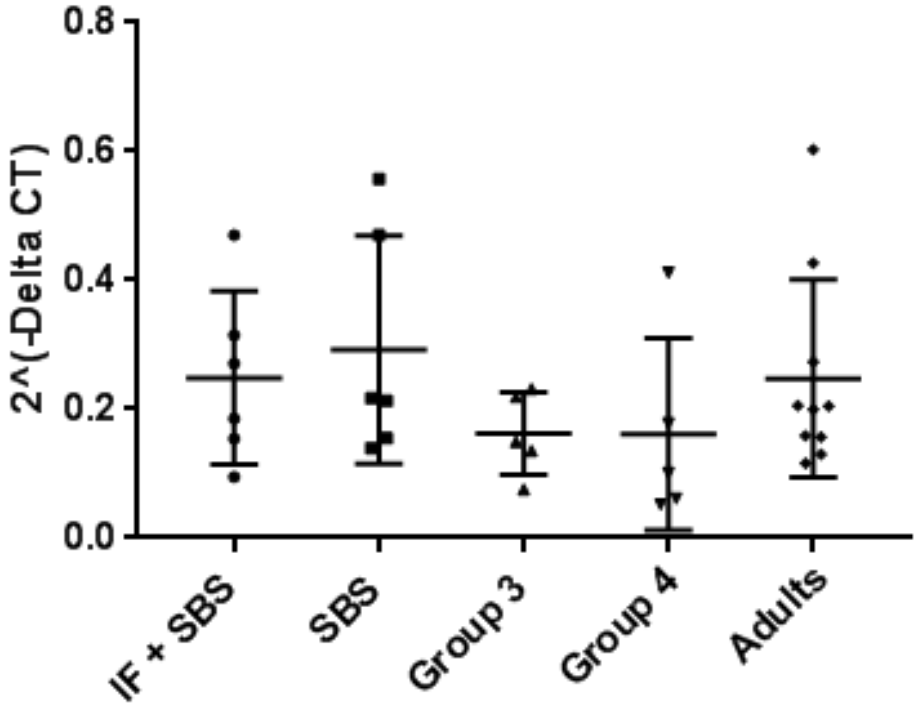

Figure 4. RORγT transcription factor relative quantification by quantitative real-time polymerase chain reaction between children in Group 1 with intestinal failure and short bowel syndrome, those in Group 2 with short bowel syndrome, and pediatric aged- matched control groups 3, 4, and adult controls. Abbreviations: CT, cycle threshold; IF, intestinal failure; SBS, short bowel syndrome

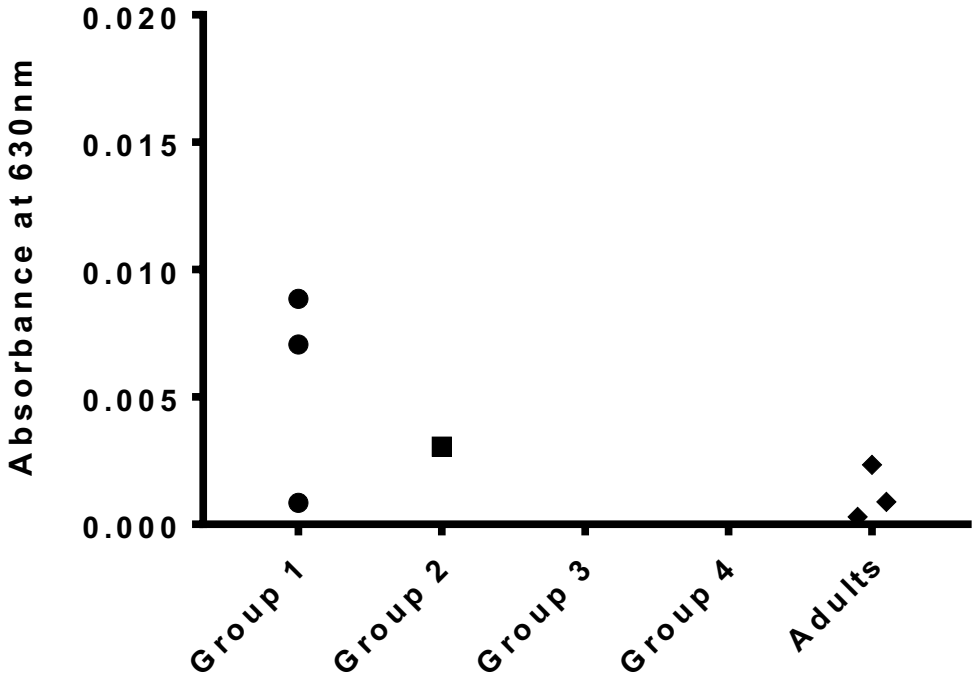

Figure 5. Circulating bacterial product, lipopolysaccharide, as measured with a bioassay in which binding of toll-like receptor 4 leads to the activation of NF-κB with absorbance read at 630nm. Comparison between children in Group 1 with intestinal failure and short bowel syndrome, those in Group 2 with short bowel syndrome, and pediatric aged- matched control groups 3, 4, and adult controls

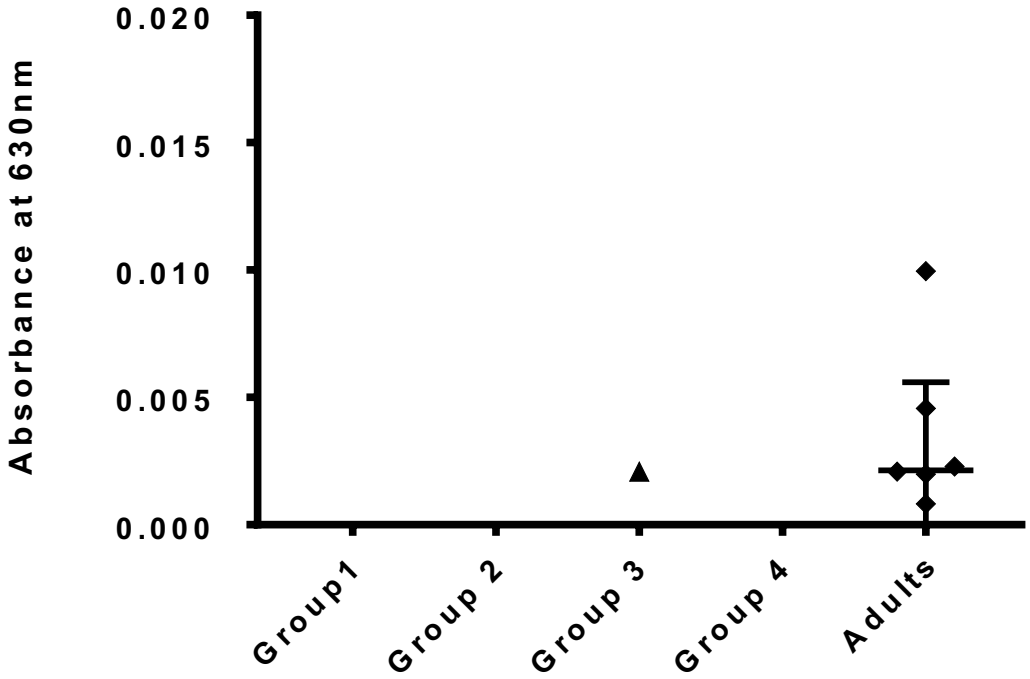

Figure 6. Circulating bacterial product, muramyl dipeptide (MDP), as measured with a bioassay in which binding of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) leads to the activation of NF-κB with absorbance read at 630nm. Comparison between children in Group 1 with intestinal failure and short bowel syndrome, those in Group 2 with short bowel syndrome, and pediatric aged- matched control groups 3, 4, and adult controls

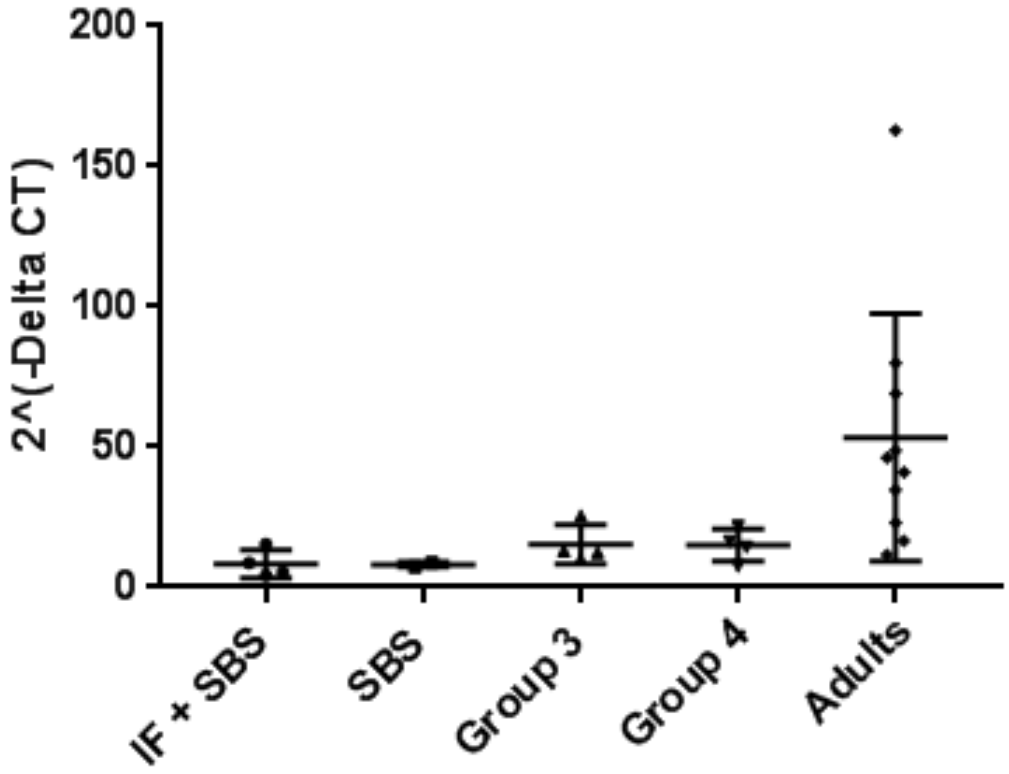

Figure 7. Toll-like receptor 4 relative quantification by quantitative real-time polymerase chain reaction between children in Group 1 with intestinal failure and short bowel syndrome, those in Group 2 with short bowel syndrome, and pediatric aged- matched control groups 3, 4, and adult controls. Abbreviations: CT, cycle threshold; IF, intestinal failure; SBS, short bowel syndrome

REFERENCES:

1. Squires RH, Duggan C, Teitelbaum DH, et al. Natural history of pediatric intestinal failure: initial report from the Pediatric Intestinal Failure Consortium. J Pediatr 2012;161:723-8.e2.
2. Goulet O, Ruemmele F, Lacaille F, et al. Irreversible intestinal failure. J Pediatr Gastroenterol Nutr 2004;38:250-69.
3. O'Keefe SJ, Buchman AL, Fishbein TM, et al. Short bowel syndrome and intestinal failure: consensus definitions and overview. Clin Gastroenterol Hepatol 2006;4:6-10.
4. Galloway DP, Troutt ML, Kocoshis SA, et al. Increased Anti-Flagellin and Anti-Lipopolysaccharide Immunoglobulins in Pediatric Intestinal Failure: Associations With Fever and Central Line-Associated Bloodstream Infections. JPEN J Parenter Enteral Nutr 2015;39:562-8.
5. D'Antiga L, Dhawan A, Davenport M, et al. Intestinal absorption and permeability in paediatric short-bowel syndrome: a pilot study. J Pediatr Gastroenterol Nutr 1999;29:588-93.
6. Alexander T, Blatt J, Skinner AC, et al. Outcome of Pediatric Gastroenterology Outpatients With Fever and Central Line. Pediatr Emerg Care 2016;32:746-750.
7. Chang MI, Carlson SJ, Nandivada P, et al. Challenging the 48-Hour Rule-Out for Central Line-Associated Bloodstream Infections in the Pediatric Intestinal Failure Population: A Retrospective Pilot Study. JPEN J Parenter Enteral Nutr 2016;40:567-73.
8. Davidovics ZH, Carter BA, Luna RA, et al. The Fecal Microbiome in Pediatric Patients With Short Bowel Syndrome. JPEN J Parenter Enteral Nutr 2016;40:1106-1113.

9. Korpela K, Mutanen A, Salonen A, et al. Intestinal Microbiota Signatures Associated With Histological Liver Steatosis in Pediatric-Onset Intestinal Failure. JPEN J Parenter Enteral Nutr 2017;41:238-248.

10. Struijs MC, Diamond IR, de Silva N, et al. Establishing norms for intestinal length in children. J Pediatr Surg 2009;44:933-8.

11. Eve Technologies Discovery Assays, 2017.

12. Kittanakom S, Shajib MS, Garvie K, et al. Comparison of Fecal Calprotectin Methods for Predicting Relapse of Pediatric Inflammatory Bowel Disease. Can J Gastroenterol Hepatol 2017;2017:1450970.

13. Sudan D. The current state of intestine transplantation: indications, techniques, outcomes and challenges. Am J Transplant 2014;14:1976-84.

14. Wang L, Llorente C, Hartmann P, et al. Methods to determine intestinal permeability and bacterial translocation during liver disease. J Immunol Methods 2015;421:44-53.

15. Dheer R, Santaolalla R, Davies JM, et al. Intestinal Epithelial Toll-Like Receptor 4 Signaling Affects Epithelial Function and Colonic Microbiota and Promotes a Risk for Transmissible Colitis. Infect Immun 2016;84:798-810.

16. Schall KA, Thornton ME, Isani M, et al. Short bowel syndrome results in increased gene expression associated with proliferation, inflammation, bile acid synthesis and immune system activation: RNA sequencing a zebrafish SBS model. BMC Genomics 2017;18:23.

17. Lapthorne S, Pereira-Fantini PM, Fouhy F, et al. Gut microbial diversity is reduced and is associated with colonic inflammation in a piglet model of short bowel syndrome. Gut Microbes 2013;4:212-21.

18. Cole CR, Frem JC, Schmotzer B, et al. The rate of bloodstream infection is high in infants with short bowel syndrome: relationship with small bowel bacterial overgrowth, enteral feeding, and inflammatory and immune responses. J Pediatr 2010;156:941-7, 947.e1.

19. Karthikeyan B, Talwar, Arun KV, et al. Evaluation of transcription factor that regulates T helper 17 and regulatory T cells function in periodontal health and disease. J Pharm Bioallied Sci 2015;7:S672-6.

4.0 CONCLUSION:

This health translational study explored host-microbe interactions within the gastrointestinal tract of children with SBS. We sought to explore microbiome diversity and abundance patterns as well as total bacterial load analyzed by qPCR. Through quantification of the bacterial fermentation product short chain fatty acids by mass spectrometry, we investigated a link between the microbial community to the mucosal immunity. Furthermore, we characterized any systemic inflammation with ELISA assay of cytokine concentrations, gene expression analysis by real-time qPCR as well as intestinal mucosal inflammation with fecal calprotectin. These results were applied to clinical epidemiologic data of bloodstream infection history and presenting leukocyte response at the time of infection.

4.1 PATIENT POPULATION:

Our cohort included twelve children with short bowel syndrome; six with IF with a median age of 67 months, and six with SBS no longer requiring PN with a median age of 47.5 months (p=NS). The IF group represented children with well-established disease with a median number of indwelling venous catheter days of 2023 per patient (IQR 811, 2947 days). Studies early in infancy may not reflect sustained differences between groups, therefore this older cohort improves the reliability of our results. The etiology of SBS was representative of this disease with 41.7% with necrotizing enterocolitis, 33% with gastroschisis and 16% with malrotation and volvulus. Both SBS study groups included children with IC valve resection and hemi-colectomy which should control for this variable (50% vs 66%, p=NS). Small intestine lengths in those with IF were a median length of 26.5cm (IQR 24.8, 40) at surgical resection compared to 55cm (IQR 31.2, 89)

in those who discontinued PN ($p<0.01$). This is to be expected as children with shorter lengths of remnant intestine require prolonged PN.

4.2 MICROBIOME

A healthy microbial community, dominated by the *Firmicutes* and *Bacteroidetes* phyla, provide colonization-resistance of enteric pathogens (Hughes et al., 2017). Disruption of this process can allow the *Proteobacteria* phylum and specific bacteria species to dominate (Hughes et al., 2017). The *Proteobacteria* abundance identified in this study has been described in other disease states of inflammation including inflammatory bowel disease and HIV enteropathy as well as in the setting of short term insults such as dietary changes, antibiotics and medications (Hughes et al., 2017; Stecher, 2015). Children with IF experience recurrent antibiotic exposure, enteral deprivation and surgical procedures which have been described to influence a *Proteobacteria* bloom (Stecher, 2015). An explanation for this shift in major phyla may be explained by a disruption in processes which maintain colonization-resistance. Mouse models have suggested increased availability of oxygen at the intestinal epithelium influence formate deyhydrogenase and promote facultative anaerobic pathogen expansion (Hughes et al., 2017). In contrast, colonocytes will use butyrate for beta-oxidation to deplete mucosal oxygen and promote anaerobic micro-environments which can contribute to colonization-resistance (Hughes et al., 2017). The microbiome our cohort with IF was characterized by a massive overgrowth of *Escherichia* and presumed *E. coli* abundance which indicates a significant loss of colonization-resistance. As our studies were descriptive, cause and effect could not be established from this research. However, we hypothesize, that the observed reduction in butyrate in this patient population could contribute to alterations in oxygen respiration and loss of colonization-resistance.

While 83% of our cohort with IF was on an antibiotic during our study period, the direct implications of this on the microbial community is only correlational. In previous research of immunodeficient mice who were treated with antibiotics, even blooming of non-pathogen *E. coli* strains could trigger inflammation and lead to *E. coli*-induced sepsis (Ayres, Trinidad, & Vance, 2012). The *Escherichia* abundance in our patient fecal samples could also predispose to bacterial translocation and possibly infectious outcomes. While most bloodstream isolates cultured in this cohort could be considered enteric, *E. coli* remained the sixth most common isolate despite its abundance within the fecal microbiota. This indicates that expansion of pathogenic or non-pathogenic facultative anaerobic microbiota alone, does not solely influence bacterial translocation.

4.3 MUCOSAL IMMUNITY

As gastrointestinal microbiota prime and regulate mucosal immunity, the significant dysbiosis in this patient population could contribute to an altered immune defense. With a bloodstream infection frequency of 5.84 per 1000 catheter days and a frequent occurrence of candidemia in our cohort, we question both the intestinal permeability of children with SBS and the typical immune response to antigen exposure. Our studies did not detect a difference in circulating bacterial products LPS and MDP as a measure of intestinal permeability however other studies have through the application of alternative experiments (Cole et al., 2010; D'Antiga et al., 1999).

This research has identified a significant reduction in butyric acid concentration as well as the abundance of *Ruminococcaceae* and *Lachnospiraceae* known to produce short chain fatty acids.

A reduction in butyric acid concentration can influence bacterial translocation through paracellular permeability and enhancement of the intestinal epithelial barrier (Kinoshita et al., 2002; Kles & Chang, 2006). Furthermore, this reduction of butyric acid and the commensal obligate anaerobes belonging to *Clostridia clusters IV and XIV,* can influence colonic T-regulatory cells and inhibition of NF-κB (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013). Despite this, in this cohort, measures of systemic venous sampling, Foxp3+ transcription factor gene expression and IL-10 concentration was unchanged between groups.

Pro-inflammatory bacteria in the gastrointestinal tract could promote inflammation of the mucosa. However, pro-inflammatory cytokine concentrations in children with SBS in our studies were unchanged between groups despite other reports of elevation in both human and animal studies (Cole et al., 2010; Lapthorne et al., 2013; Schall et al., 2017). Furthermore, fecal calprotectin remained normal in children with SBS minimizing the likelihood of chronic intestinal inflammation.

4.4 FUTURE DIRECTIONS

This body of research promotes further study of the mucosal immunity and the microbiome of children with SBS. With animal models, a cause-and-effect model for microbiota shifts in SBS can be studied and to allow for further assessment of inflammatory markers within tissue sampling. Tissue samples would allow for local quantification of cytokine concentration within the mucosa compared to systemic venous samples. This research also highlights the impact that enteral deprivation and antibiotic use may have in the microbe-host interactions. While only speculative for our patients, the high frequency of antibiotic use in IF may influence some of the shift to

facultative aneorobic organisms as seen in other studies (Hughes et al., 2017). This would highlight the need for clinicians to carefully select those who may require long term cycled antibiotics to minimize potential harm. Larger multi-centered prospective studies of dysbiosis in children with SBS should be completed longitudinally, starting from initial surgical resection, to investigate such factors and the impact they have on the microbiome in this population.

While there was no statistical difference in inflammatory cytokine quantity, our retrospective epidemiologic review of BSI identified leukocyte and neutrophil response at the onset of BSI was in the normal range with no significant leukocytosis or leukopenia. Additional evaluation of the immune response should be assessed at the time of sepsis to investigate if cell-signaling differences occur in the setting of bloodstream infection. Furthermore, alternative measures of intestinal permeability should be investigated during fevers and BSI.

Canadida is known to colonize the gastrointestinal tract however the frequency of candidemia in patients with SBS is surprising. Specific research into gastrointestinal colonization and immune defense to candidemia should be further investigated to understand the frequency of such infections. These results should also be considered in guidelines in the management of presumed sepsis in SBS to prompt early broad spectrum antibiotic coverage and the consideration of prophylactic anti-fungals.

Re-establishment of colonization-resistance, improvement in microbial diversity and promotion of butyrate-producing commensals should be a further goal for research and clinical focus. Probiotic use, fecal transplantation and short chain fatty acid enemas all offer potential therapeutic options

for improved microbial diversity. Clinical outcomes including diarrhea, bloating and transit time could be evaluated with these therapies as well as intestinal permeability and bacterial translocation. In summary, our research has added to a developing clinical field of microbe-host interactions in a rare pediatric disease with significant morbidity and mortality. We hope our descriptive microbiota data and SCFA analysis results will promote further research into therapeutic options for children with IF.

REFERENCES:

Alexander, T., Blatt, J., Skinner, A. C., Jhaveri, R., Jobson, M., & Freeman, K. (2016). Outcome of Pediatric Gastroenterology Outpatients With Fever and Central Line. *Pediatr Emerg Care, 32*(11), 746-750. doi:10.1097/PEC.0000000000000541

Amin, S. C., Pappas, C., Iyengar, H., & Maheshwari, A. (2013). Short bowel syndrome in the NICU. *Clin Perinatol, 40*(1), 53-68. doi:10.1016/j.clp.2012.12.003

Ayres, J. S., Trinidad, N. J., & Vance, R. E. (2012). Lethal inflammasome activation by a multidrug-resistant pathobiont upon antibiotic disruption of the microbiota. *Nat Med, 18*(5), 799-806. doi:10.1038/nm.2729

Cole, C. R., Frem, J. C., Schmotzer, B., Gewirtz, A. T., Meddings, J. B., Gold, B. D., & Ziegler, T. R. (2010). The rate of bloodstream infection is high in infants with short bowel syndrome: relationship with small bowel bacterial overgrowth, enteral feeding, and inflammatory and immune responses. *J Pediatr, 156*(6), 941-947, 947.e941. doi:10.1016/j.jpeds.2009.12.008

D'Antiga, L., Dhawan, A., Davenport, M., Mieli-Vergani, G., & Bjarnason, I. (1999). Intestinal absorption and permeability in paediatric short-bowel syndrome: a pilot study. *J Pediatr Gastroenterol Nutr, 29*(5), 588-593.

Diamanti, A., Basso, M. S., Castro, M., Calce, A., Pietrobattista, A., & Gambarara, M. (2007). Prevalence of life-threatening complications in pediatric patients affected by intestinal failure. *Transplant Proc, 39*(5), 1632-1633. doi:10.1016/j.transproceed.2007.02.083

Duggan, C. P., & Jaksic, T. (2017). Pediatric Intestinal Failure. *N Engl J Med, 377*(7), 666-675. doi:10.1056/NEJMra1602650

Engstrand Lilja, H., Wefer, H., Nyström, N., Finkel, Y., & Engstrand, L. (2015). Intestinal dysbiosis in children with short bowel syndrome is associated with impaired outcome. *Microbiome, 3*, 18. doi:10.1186/s40168-015-0084-7

Fallon, E. M., Mitchell, P. D., Nehra, D., Potemkin, A. K., O'Loughlin, A. A., Gura, K. M., & Puder, M. (2014). Neonates with short bowel syndrome: an optimistic future for parenteral nutrition independence. *JAMA Surg, 149*(7), 663-670. doi:10.1001/jamasurg.2013.4332

Goulet, O., Olieman, J., Ksiazyk, J., Spolidoro, J., Tibboe, D., Köhler, H., . . . Beattie, R. M. (2013). Neonatal short bowel syndrome as a model of intestinal failure: physiological background for enteral feeding. *Clin Nutr, 32*(2), 162-171. doi:10.1016/j.clnu.2012.09.007

Hughes, E. R., Winter, M. G., Duerkop, B. A., Spiga, L., Furtado de Carvalho, T., Zhu, W., . . . Winter, S. E. (2017). Microbial Respiration and Formate Oxidation as Metabolic Signatures of Inflammation-Associated Dysbiosis. *Cell Host Microbe, 21*(2), 208-219. doi:10.1016/j.chom.2017.01.005

Kaufman, S. S., Atkinson, J. B., Bianchi, A., Goulet, O. J., Grant, D., Langnas, A. N., . . . Transplantation, A. S. o. (2001). Indications for pediatric intestinal transplantation: a position paper of the American Society of Transplantation. *Pediatr Transplant, 5*(2), 80-87.

Kinoshita, M., Suzuki, Y., & Saito, Y. (2002). Butyrate reduces colonic paracellular permeability by enhancing PPARgamma activation. *Biochem Biophys Res Commun, 293*(2), 827-831. doi:10.1016/S0006-291X(02)00294-2

Kles, K. A., & Chang, E. B. (2006). Short-chain fatty acids impact on intestinal adaptation, inflammation, carcinoma, and failure. *Gastroenterology, 130*(2 Suppl 1), S100-105. doi:10.1053/j.gastro.2005.11.048

Lapthorne, S., Pereira-Fantini, P. M., Fouhy, F., Wilson, G., Thomas, S. L., Dellios, N. L., . . . Bines, J. E. (2013). Gut microbial diversity is reduced and is associated with colonic inflammation in a piglet model of short bowel syndrome. *Gut Microbes, 4*(3), 212-221. doi:10.4161/gmic.24372

Lopetuso, L. R., Scaldaferri, F., Petito, V., & Gasbarrini, A. (2013). Commensal Clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathog, 5*(1), 23. doi:10.1186/1757-4749-5-23

Lucas, K., & Maes, M. (2013). Role of the Toll Like receptor (TLR) radical cycle in chronic inflammation: possible treatments targeting the TLR4 pathway. *Mol Neurobiol, 48*(1), 190-204. doi:10.1007/s12035-013-8425-7

Lührs, H., Gerke, T., Schauber, J., Dusel, G., Melcher, R., Scheppach, W., & Menzel, T. (2001). Cytokine-activated degradation of inhibitory kappaB protein alpha is inhibited by the short-chain fatty acid butyrate. *Int J Colorectal Dis, 16*(4), 195-201.

Maslowski, K. M., & Mackay, C. R. (2011). Diet, gut microbiota and immune responses. *Nat Immunol, 12*(1), 5-9. doi:10.1038/ni0111-5

O'Keefe, S. J., Buchman, A. L., Fishbein, T. M., Jeejeebhoy, K. N., Jeppesen, P. B., & Shaffer, J. (2006). Short bowel syndrome and intestinal failure: consensus definitions and overview. *Clin Gastroenterol Hepatol, 4*(1), 6-10. doi:10.1016/j.cgh.2005.10.002

Rosner, A. J., & Keren, D. F. (1984). Demonstration of M cells in the specialized follicle-associated epithelium overlying isolated lymphoid follicles in the gut. *J Leukoc Biol, 35*(4), 397-404.

Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol, 9*(5), 313-323. doi:10.1038/nri2515

Sant'Anna, A. M., Altamimi, E., Clause, R. F., Saab, J., Mileski, H., Cameron, B., . . . Sant'Anna, G. M. (2012). Implementation of a multidisciplinary team approach and fish oil emulsion administration in the management of infants with short bowel syndrome and parenteral nutrition-associated liver disease. *Can J Gastroenterol, 26*(5), 277-280.

Schall, K. A., Thornton, M. E., Isani, M., Holoyda, K. A., Hou, X., Lien, C. L., . . . Grikscheit, T. C. (2017). Short bowel syndrome results in increased gene expression associated with proliferation, inflammation, bile acid synthesis and immune system activation: RNA sequencing a zebrafish SBS model. *BMC Genomics, 18*(1), 23. doi:10.1186/s12864-016-3433-4

Segain, J. P., Raingeard de la Blétière, D., Bourreille, A., Leray, V., Gervois, N., Rosales, C., . . . Galmiche, J. P. (2000). Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut, 47*(3), 397-403.

Squires, R. H., Duggan, C., Teitelbaum, D. H., Wales, P. W., Balint, J., Venick, R., . . . Consortium, P. I. F. (2012). Natural history of pediatric intestinal failure: initial report from the Pediatric Intestinal Failure Consortium. *J Pediatr, 161*(4), 723-728.e722. doi:10.1016/j.jpeds.2012.03.062

Stanger, J. D., Oliveira, C., Blackmore, C., Avitzur, Y., & Wales, P. W. (2013). The impact of multi-disciplinary intestinal rehabilitation programs on the outcome of pediatric patients with intestinal failure: a systematic review and meta-analysis. *J Pediatr Surg, 48*(5), 983-992. doi:10.1016/j.jpedsurg.2013.02.070

Stecher, B. (2015). The Roles of Inflammation, Nutrient Availability and the Commensal Microbiota in Enteric Pathogen Infection. *Microbiol Spectr, 3*(3). doi:10.1128/microbiolspec.MBP-0008-2014

Struijs, M. C., Diamond, I. R., de Silva, N., & Wales, P. W. (2009). Establishing norms for intestinal length in children. *J Pediatr Surg, 44*(5), 933-938. doi:10.1016/j.jpedsurg.2009.01.031

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The human microbiome project. *Nature, 449*(7164), 804-810. doi:10.1038/nature06244

Wales, P. W., de Silva, N., Kim, J., Lecce, L., To, T., & Moore, A. (2004). Neonatal short bowel syndrome: population-based estimates of incidence and mortality rates. *J Pediatr Surg, 39*(5), 690-695.

Wang, L., Llorente, C., Hartmann, P., Yang, A. M., Chen, P., & Schnabl, B. (2015). Methods to determine intestinal permeability and bacterial translocation during liver disease. *J Immunol Methods, 421*, 44-53. doi:10.1016/j.jim.2014.12.015

Weale, A. R., Edwards, A. G., Bailey, M., & Lear, P. A. (2005). Intestinal adaptation after massive intestinal resection. *Postgrad Med J, 81*(953), 178-184. doi:10.1136/pgmj.2004.023846