Gut Microbes 4:3, 212–221; May/June 2013; © 2013 Landes Bioscience

Gut microbial diversity is reduced and is associated with colonic inflammation in a piglet model of short bowel syndrome

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Keywords: short bowel syndrome, mucosal immunology, colonic microbiota, high-throughput sequencing, bacterial diversity, small bowel resection

Abbreviations: ANOVA, analysis of variance; cDNA, complementary deoxyribonucleic acid; DAB, 3,3-diaminobenzidine; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NOC, non-operation control; qPCR, quantitative polymerase chain reaction; qRTPCR, real-time reverse-transcription polymerase chain reaction; SBR, small bowel resection; SBS, short bowel syndrome; TNF, tumor necrosis factor

Background and objectives: Following small bowel resection (SBR), the luminal environment is altered, which contributes to clinical manifestations of short bowel syndrome (SBS) including malabsorption, mucosal inflammation and bacterial overgrowth. However, the impact of SBR on the colon has not been well-defined. The aims of this study were to characterize the colonic microbiota following SBR and to assess the impact of SBR on mucosal inflammation in the colon.

Results: Analysis of the colonic microbiota demonstrated that there was a significant level of dysbiosis both two and six weeks post-SBR, particularly in the phylum *Firmicutes*, coupled with a decrease in overall bacterial diversity in the colon. This decrease in diversity was associated with an increase in colonic inflammation six weeks post-surgery.

Methods: Female (4-week old) piglets (5–6/group) received a 75% SBR, a transection (sham) or no surgery. Compositional analysis of the colonic microbiota was performed by high-throughput sequencing, two- and six-weeks post-surgery. The gene expression of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8, IL-18 and tumor necrosis factor (TNF)-α in the colonic mucosa was assessed by qRT-PCR and the number of macrophages and percentage inducible nitric oxide synthase (iNOS) staining in the colonic epithelium were quantified by immunohistochemistry.

Conclusions: SBR significantly decreased the diversity of the colonic microbiota and this was associated with an increase in colonic mucosal inflammation. This study supports the hypothesis that SBR has a significant impact on the colon and that this may play an important role in defining clinical outcome.

Introduction

Short bowel syndrome (SBS) is the clinical state of malabsorption and malnutrition that occurs following small bowel resection (SBR).[1] Surgical resection of the small bowel may be required for the treatment of a range of conditions including congenital bowel abnormalities and necrotising enterocolitis in children and Crohn disease, trauma and malignancy in adults.[2] Although the underlying reason for SBR between adults and children may differ, the clinical manifestations and consequences of SBS are similar. The symptoms of SBS reflect the loss of absorptive surface area and functional disturbance of the remaining intestine and include diarrhea, vomiting, malabsorption, dehydration and electrolyte imbalance, malnutrition and bacterial overgrowth.

Most cases of SBS occur in the neonatal age group and the mortality associated with the condition ranges from 15–25%.[3,4] The gut microbiota is known to be a major factor in determining the clinical outcome in children.[5] The effects of an alteration in the microbiota on the small intestine are well-studied in this cohort and include increased risk of bacterial overgrowth, bacteraemia, villous atrophy and small intestinal mucosal inflammation, which can result in a loss of intestinal epithelial barrier function and can lead to sepsis.[6-8] In both adult patients with SBS

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Submitted: 01/04/13; Revised: 03/13/13; Accepted: 03/19/13
http://dx.doi.org/10.4161/gmic.24372

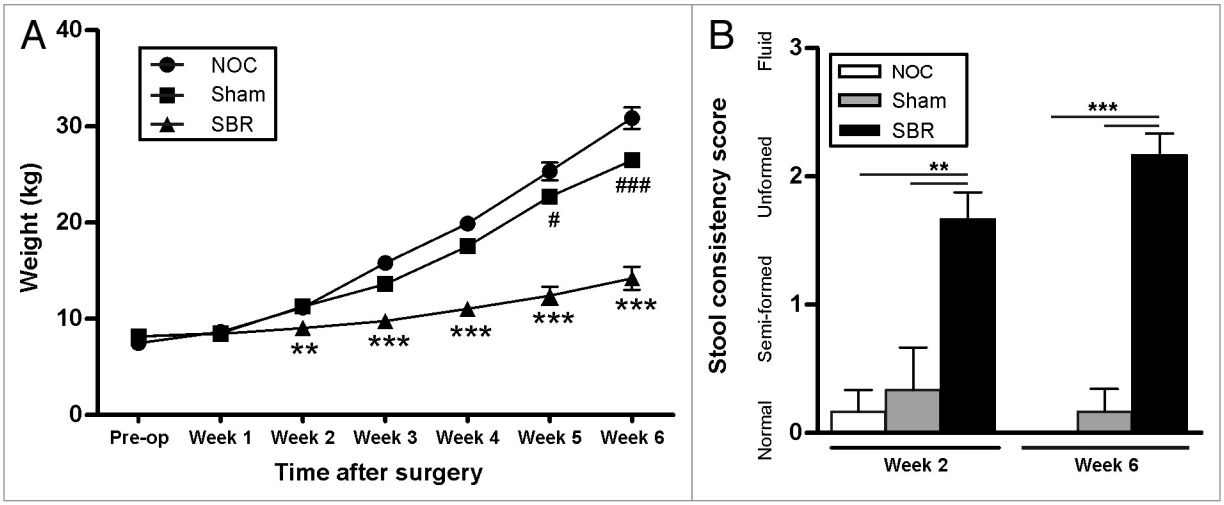

Figure 1. Clinical effects of small bowel resection. (**A**) The weight of piglets that received a small bowel resection (SBR) was significantly lower two weeks post-surgery than those that received a sham surgery (Sham) or no surgery (NOC; **p < 0.01). This suboptimal weight gain was sustained for the duration of the experiment (***p < 0.001 at week 3, p < 0.0001 at weeks 4, 5 and 6). The sham piglets also had a significantly lower weight gain at weeks 5 (#p < 0.05) and 6 (###p < 0.0001) compared with the NOC group. Values are expressed as mean ± SEM; n = 12 NOC, n = 10 sham, n = 12 SBR at pre-op, week 1 and week 2; n = 6 NOC, n = 5 sham, n = 6 SBR at weeks 3, 4, 5 and 6. (**B**) Piglets in the SBR group had a significantly higher stool consistency score than the sham and NOC groups two weeks (**p < 0.01) and six weeks (***p < 0.001) post-surgery. Values are expressed as mean ± SEM; n = 5–6/group/time-point.

and a piglet SBS model, the colon is known to play a critical role in determining clinical outcome, and significant morphological adaptation of the colonic mucosa has been observed following resection.[9-11] However, the actual impact of changes in the microbiota on the colon itself is poorly understood. Anecdotal evidence suggests that changes in the composition of the colonic microbiota contribute to the generation of symptoms including malnutrition due to decreased bile acid deconjugation, insufficient breakdown of nutrients and diarrhea and play a key role in development of serious complications such as septicaemia. However, very little is known about specific changes to the colonic microbiota following SBR or the impact these changes may have on colonic mucosal inflammation, the adaptive response and severity of symptoms in SBS.

Despite an obvious clinical need, there are a number of barriers to the study of gut function and immune regulation in SBS. Patients with SBS are a complex, heterogeneous group and access to tissue is limited. Thus, many studies are based on animal models. Rodent SBS models, although useful for molecular and cellular analysis, may not accurately reflect the physiology and pathophysiology of the human intestine. The pig is accepted as the best model for the study of human intestinal biology and diet[12-14] and, with a similar intestinal microbiota to that of humans,[15-17] it is an ideal model for the study of complex gastrointestinal diseases.

The aims of this study were the following: (1) to utilize high-throughput sequencing to characterize changes in the colonic diversity and microbial composition following SBR, (2) to examine the influence of surgical resection on colonic mucosal inflammation and (3) to examine whether there is a correlation between microbial differences and inflammation in a pre-clinical piglet model of SBS in children.

Results

Piglets that received a small bowel resection had a poorer clinical outcome. Food intake was monitored throughout the study and all diets were isocaloric and isonitrogenous. Piglets that received a small bowel resection (SBR) had suboptimal weight gain compared with the non-operation control (NOC) and sham-operated piglets (**Fig. 1A**). This was significant from the two-week time-point (p = 0.004), up until and including the six-week time-point (p < 0.0001). The sham group also had a significantly lower mean weight than the NOC group at the five-week and six-week time-points (p = 0.041 and p < 0.0001).[18] Examination of feed intake showed that, although piglets in the SBR group consumed equal energy per kg per day, their total energy consumption was reduced when compared with the NOC and sham groups due to their decreased weight. Piglets in the SBR group failed to resolve diarrhea at either the two-week (p = 0.002 and p = 0.005) or six-week (p < 0.0001) time-points (**Fig. 1B**).

Total colonic bacterial number is unaltered following small bowel resection. Absolute quantification, using qPCR, was used to determine the impact of surgery on total numbers of bacteria. The results indicate that there were no significant differences in the total 16S rRNA gene copies (representative of total bacteria numbers) between any of the groups at either week two (p = 0.7845) or week six (p = 0.8784; data not shown).

Surgical resection of the small intestine decreases bacterial diversity in the colon. The colonic content from NOC, sham and SBR piglets was sequenced two- and six-weeks post-surgery. A total of 318,784 V4 16S sequence reads were generated, averaging at 9,376 per sample. Species richness, coverage and diversity estimations were calculated for each data set (**Table 1**). Rarefaction curves for each sample were parallel or approaching parallel, with

Table 1. Surgical resection decreases the diversity of colonic bacteria

Data set	NOC Week 2	Sham Week 2	SBR Week 2	NOC Week 6	Sham Week 6	SBR Week 6
Chao 1 richness estimation	905 ± 41	892 ± 123	718 ± 59	1224 ± 82	1276 ± 97	716 ± 34
Shannon's index for diversity	6.9 ± 0.2	6.5 ± 0.6	5.7 ± 0.3	7.1 ± 0.1	7.4 ± 0.2	5.5 ± 0.2
Number of observed species	552 ± 16	571 ± 80	475 ± 44	726 ± 46	777 ± 63	428 ± 15
Simpson's diversity index	0.96 ± 0.01	0.95 ± 0.02	0.93 ± 0.01	0.98 ± 0.01	0.98 ± 0.00	0.93 ± 0.01

Estimation of diversity within the non-operation control (NOC), sham and small bowel resection (SBR) groups at two- and six-weeks post surgery as assessed by Chao 1 richness estimation, Shannon's index for diversity, number of observed species and Simpson's diversity index.

the x axis indicating total bacteria diversity was well represented (**Fig. S1A**). There was a trend toward a decrease in the level of diversity in the SBR group compared with the NOC and sham groups at the two-week time-point as assessed by the Chao 1 calculation (**Fig. 2A and B; Table 1**) and the Simpson and Shannon indices (**Table 1**). By six weeks, however, there was a significant decrease in diversity in the SBR group compared with the NOC and sham groups as demonstrated by the Chao 1 calculation ($p = 0.004$; **Fig. 2A and B**), the Simpson index ($p = 0.008$) and the Shannon index ($p = 0.003$; **Table 1**). Principle component analysis revealed that the SBR groups at week two and week six clustered together and were distinct from the NOC and sham groups, which clustered together at both times (**Fig. S1B**).

The composition of the pig microbiota is altered two weeks after small bowel resection. Two weeks post-SBR, the majority of changes were detected at the family and genus levels. There were significant differences at the family level between the SBR group and the NOC and sham groups (**Fig. 2C; Table 2**). These included a significant increase in *Veillonellaceae* ($p = 0.0018$ and $p = 0.0115$) and a significant decrease in *Ruminococcaceae* ($p = 0.0012$ and $p = 0.0244$) in the SBR group compared with the NOC and sham groups (**Fig. 2C; Table 2**). *Peptostreptococcaceae* was undetectable in any of the piglets in the SBR group at week two (**Table 2**). At the genus level, there was a significant increase in *Acidaminococcus* in the SBR group compared with the NOC and sham groups ($p = 0.0015$ and $p = 0.0033$; **Table 2**).

Small bowel resection leads to dysbiosis of the gut microbiota six weeks post-surgery. Six weeks post-surgery, there were significant decreases in the proportions of *Bacteroidetes* ($p = 0.0018$ and $p = 0.0013$) and significantly increased proportions of *Fusobacteria* ($p = 0.0072$ and $p = 0.0032$) in the SBR group compared with the NOC and sham groups at this time-point (**Fig. 2C**; **Table 2**). The majority of differences were observed at the level of the family, particularly in members of the *Firmicutes* phylum (**Fig. 2C; Table 2**). At six weeks, there was a significant decrease in members of the *Peptostreptococcaceae* ($p = 0.0477$ and $p = 0.0154$) and *Ruminococcaceae* ($p = 0.00001$ and $p = 0.0002$) families in the six-week SBR group compared with the six-week NOC and sham groups. *Peptococcaceae* were undetectable in the majority of SBR piglets at week six. Conversely, there was a significant increase in the proportions of *Veillonellaceae* ($p = 0.00001$ and $p = 0.0007$) and *Fusobacteriaceae* ($p = 0.0072$ and $p = 0.0032$) in the SBR group compared with the NOC and sham groups at the six-week time-point. Increases at the family level were reflected at the genus level, with increases in the proportion of *Megasphaera* ($p = 0.0156$) in the SBR group compared with the NOC group, and *Acidaminococcus* ($p = 0.0006$ and $p = 0.0063$) and *Fusobacterium* ($p = 0.0073$ and $p = 0.0032$) compared with both the NOC and sham groups. *Clostridium* proportions were significantly decreased in the SBR group compared with the sham group ($p = 0.0433$).

There is an increase in colonic inflammation following small bowel resection, which is associated with decreased diversity. The gene expression of a panel of pro-inflammatory cytokines was assessed in the colon. Two weeks after surgery, there was no difference in inflammatory gene expression among groups (**Fig. 3A–D**). There was, however, a significant increase in the expression of IL-1β ($p = 0.045$), IL-18 ($p = 0.006$) and TNF-α ($p = 0.036$) in the SBR group compared with the sham group six-weeks post-surgery (**Fig. 3A, C and D**), and IL-8 was significantly increased in the SBR group compared with both the NOC and sham groups ($p = 0.025$ and $p = 0.034$; **Fig. 3B**).

In the case of IL-1β and IL-8, the increase in these pro-inflammatory cytokines correlated with the observed decrease in colonic bacterial diversity ($r = -0.829$ and $r = -0.771$, respectively; **Table 3**). The increase in colonic pro-inflammatory cytokines was also associated with a concomitant increase in the total number of macrophages present in the colonic epithelium of the SBR group compared with the NOC and sham groups at six weeks (**Fig. 4A**). At this time-point, there was also a significant increase in the amount of the inflammatory marker iNOS in the colonic epithelium of the SBR group compared with both the NOC and the sham ($p = 0.03$; **Fig. 4B**).

Discussion

This study provides the first comprehensive description of the changes in the colonic microbiota following small bowel resection. Not only does this study take advantage of high-throughput

Figure 2 (See opposite page). Surgical resection of the small intestine decreases the diversity of colonic bacteria and alters the relative proportion of families in the *Firmicutes* phylum. (**A**) There is a decrease in the number of distinct operational taxonomic units (OTUs) representing bacterial genera two and six weeks post-surgery in the small bowel resection (SBR) group compared with the non-operation control (NOC) and sham group. This is translated into a decrease in overall bacterial diversity as calculated by the Chao 1 richness estimation (**B**). (**C**) Pie charts representing the major bacterial phyla and the relative proportion of families in the *Firmicutes* family at the two- and six-week time-point in the NOC, sham and SBR groups.

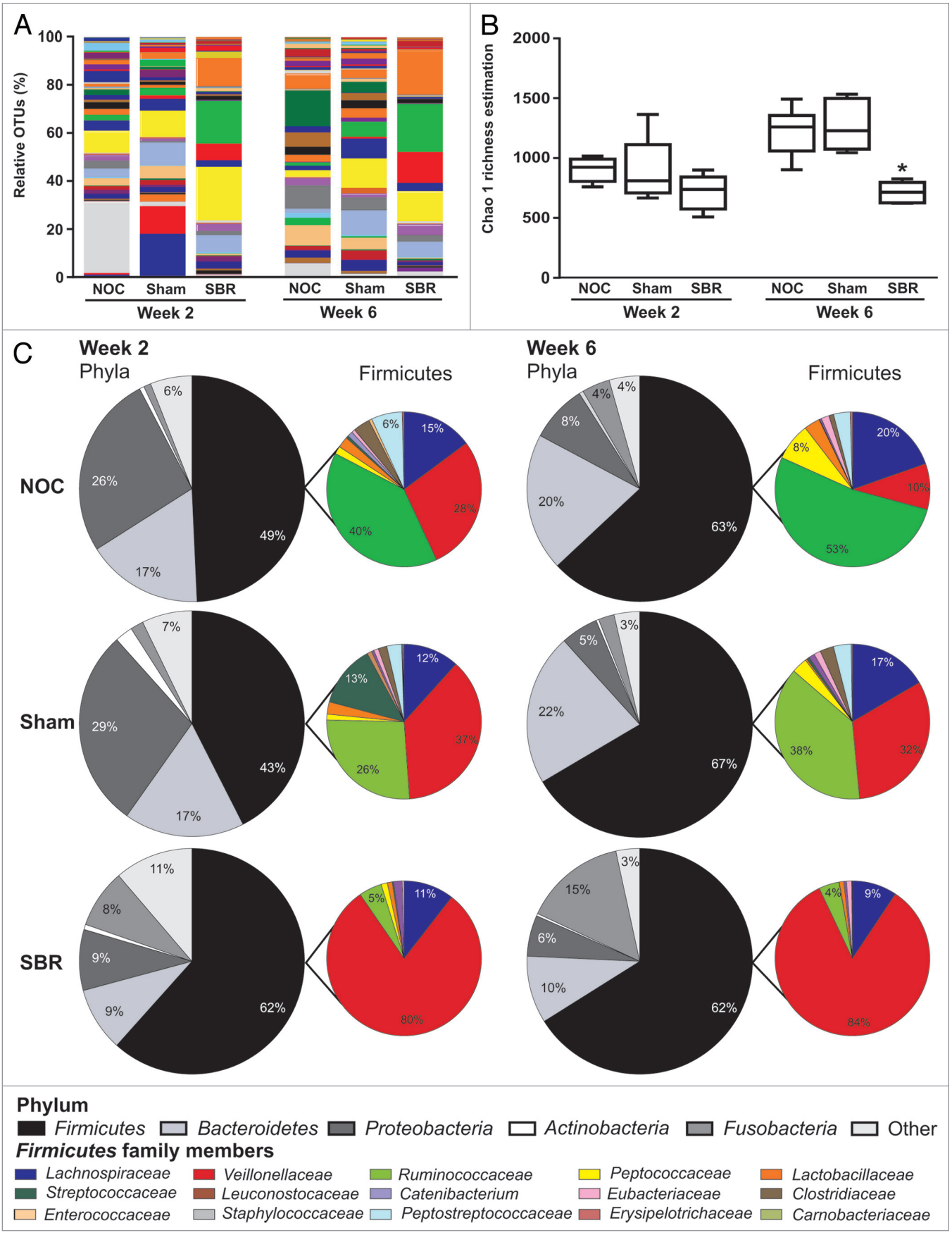

Figure 2. For figure legend, see page 214.

Table 2. Small bowel resection alters the composition of the colonic microbiota over time

Weeks post-surgery		Week 2			Week 6	
Group	**NOC**	**Sham**	**SBR**	**NOC**	**Sham**	**SBR**
Phylum:						
Firmicutes	52.6 ± 5.0	52.1 ± 10.6	68.5 ± 6.1	61.7 ± 6.5	67.7 ± 3.6	68.2 ± 4.2
Proteobacteria	27.8 ± 5.1	23.1 ± 14.6	11.9 ± 4.6	10.6 ± 3.3	6.0 ± 0.6	6.2 ± 1.8
Bacteroidetes	17.9 ± 2.5	19.1 ± 5.8	10.1 ± 1.7	22.3 ± 2.6	23.2 ± 1.9	10.0 ± 1.6**††
Fusobacteria	0.9 ± 0.4	3.8 ± 3.1	8.5 ± 2.7	4.7 ± 2.3	2.8 ± 1.4	15.4 ± 2.4**††
Actinobacteria	0.7 ± 0.2	2.0 ± 1.5	1.0 ± 0.4	0.7 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
Family:						
Enterobacteriaceae	31.8 ± 6.9	5.8 ± 2.5**	1.0 ± 0.5***	6.7 ± 2.7	1.9 ± 0.7	4.0 ± 2.1
Ruminococcaceae	28.1 ± 3.1	27.0 ± 4.2	5.2 ± 2.5**†	41.5 ± 5.2	36.1 ± 4.1	3.9 ± 1.6***†††
Veillonellaceae	19.5 ± 4.9	36.7 ± 8.4	70.5 ± 7.1**†	9.9 ± 2.9	28.0 ± 6.5	64.2 ± 6.6***†††
Peptostreptococcaceae	4.6 ± 1.5	2.1 ± 1.5	0	2.8 ± 0.6	3.7 ± 1.4	0*†
Prevotellaceae	3.9 ± 0.6	14.4 ± 2.8*	7.1 ± 0.5	2.0 ± 0.9	9.7 ± 1.8**	5.8 ± 0.7**
Bacteroidaceae	3.5 ± 0.8	1.1 ± 0.6	2.4 ± 1.1	9.0 ± 2.1	6.0 ± 0.9	2.9 ± 1.2
Erysipelotrichaceae	2.5 ± 0.9	1.4 ± 0.7	0.4 ± 0.2	10.0 ± 6.0	3.8 ± 1.3	0
Clostridiaceae	2.1 ± 0.5	1.0 ± 0.5	0	0.7 ± 0.1	2.7 ± 1.3	0.1 ± 0.0
Lactobacillaceae	1.5 ± 0.6	2.1 ± 1.1	1.2 ± 0.3	2.9 ± 0.8	0.2 ± 0.1*	0.8 ± 0.4
Fusobacteriaceae	1.4 ± 0.6	7.5 ± 3.9	11.9 ± 4.1	8.3 ± 4.3	4.8 ± 2.5	18.2 ± 3.0**††
Peptococcaceae	1.1 ± 0.5	0.9 ± 0.3	0.4 ± 0.4	6.3 ± 1.9	3.2 ± 0.7	0**
Genus:						
Enterobacteriaceae family members	48.8 ± 8.7	9.4 ± 3.7***	1.4 ± 0.7***	11.6 ± 2.8	3.6 ± 1.1	5.7 ± 2.9
Bacteroides	5.8 ± 1.3	2.1 ± 1.1	3.5 ± 1.5	21.2 ± 3.2	11.5 ± 1.6	4.3 ± 1.7
Parabacteroides	5.1 ± 1.2	10.9 ± 3.3	1.6 ± 1.1	19.2 ± 4.5	10.9 ± 3.4	0.6 ± 0.2*
Prevotella	6.7 ± 1.6	23.2 ± 4.5*	10.4 ± 1.2	3.1 ± 1.5	18.9 ± 3.4**	9.6 ± 0.9*
Clostridium	3.4 ± 0.7	1.8 ± 0.9	0	1.8 ± 0.6	5.1 ± 2.6	0.1 ± 0.1†
Fusobacterium	2.7 ± 1.4	12.4 ± 6.6	17.7 ± 6.2	11.9 ± 5.1	9.0 ± 4.8	29.9 ± 4.3**††
Megasphaera	17.1 ± 6.0	27.3 ± 4.4	31.4 ± 6.1	6.2 ± 2.5	23.5 ± 7.7	18.3 ± 3.0*
Acidaminococcus	4.5 ± 1.2	8.0 ± 2.8	31.7 ± 6.7**††	3.6 ± 2.3	11.1 ± 6.5	30.2 ± 5.1***††
Peptococcus	1.9 ± 0.9	1.5 ± 0.6	0.7 ± 0.6	14.9 ± 5.7	6.1 ± 1.7	0.1 ± 0.1
Lactobacillus	4.0 ± 1.8	3.4 ± 1.8	1.7 ± 0.4	6.4 ± 1.9	0.4 ± 0.2*	1.3 ± 0.6

The composition of the colonic microbiota is altered two and six weeks post-surgery in the small bowel resection (SBR) group. Values are relative proportions of operational taxonomic units (OTUs) ± SEM, n = 5–6/group/time-point. Statistical significance was determined using a general linear model ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001 compared with non-operation control (NOC) group; †p < 0.05, ††p < 0.01, †††p < 0.001 compared with sham group.

sequencing to identify and characterize the dysbiosis that occurs following SBR using an established preclinical piglet model of SBS in children, it also links these changes with local mucosal inflammatory responses.

Following SBR, the luminal environment becomes altered due to rapid shunting of luminal content from the upper intestine into the lower intestine, due to shortened bowel length and increased intestinal transit.[19] This exposes the colon and its resident bacteria to both digesta and intestinal and pancreatico-biliary secretions that would normally be broken down or absorbed in the upper intestinal tract, which could potentially impact on the composition of bacteria in the lumen.[20] Evidence on the impact of SBR on bacterial composition is largely anecdotal; however any changes in bacterial composition are likely to have important implications for patients with SBS, in terms of management of their condition.

This study has established that there is a significant decrease in overall bacterial diversity in the colon in a piglet model of SBS that is particularly evident six weeks post-resection. This is consistent with other inflammatory conditions including Crohn disease,[21] ulcerative colitis[22] and antibiotic-associated diarrhea[23] and has previously been described in adult patients with SBS.[24] A decrease in microbial diversity also corresponds to decreased metabolic diversity, which has potential implications for SBS patients including insufficient breakdown of dietary components such as complex polysaccharides leading to malabsorption, reduced energy availability and decreased production of short-chain fatty acids.[25]

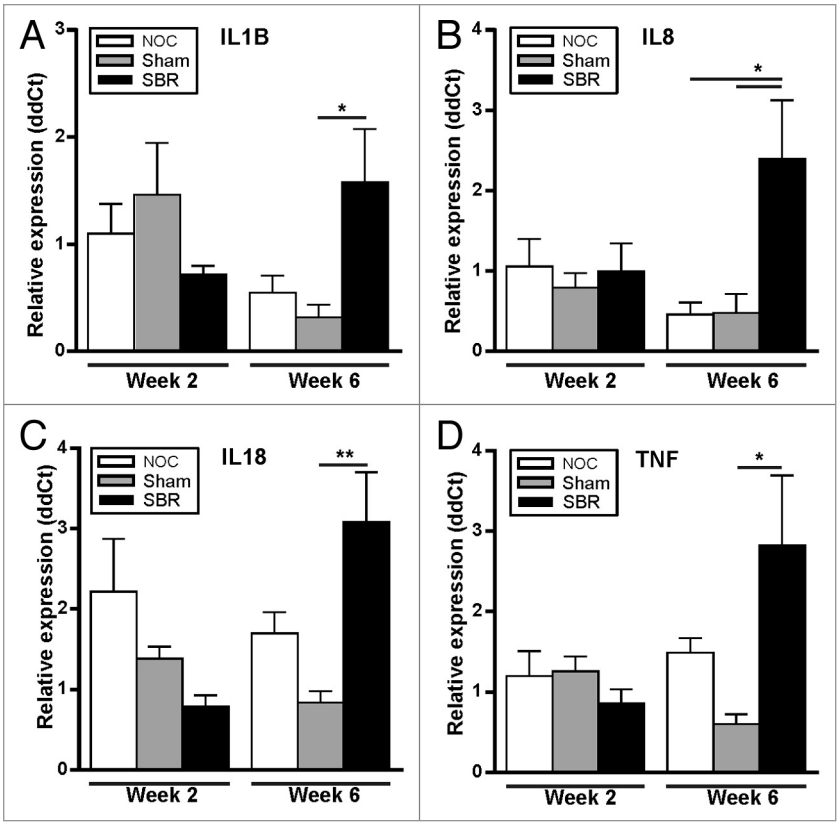

Figure 3. Surgical resection alters the expression of pro-inflammatory cytokines in the colon. In the colon, the gene expression of IL-1β (**A**), IL-18 (**C**) and TNF-α (**D**) was significantly higher in the SBR group compared with the sham group at six weeks (*p < 0.05, **p < 0.01). IL8 (**B**) was significantly higher in the colon in the small bowel resection (SBR) group compared with the non-operation control (NOC) and sham group at six weeks (*p < 0.05). Values are expressed as mean ± SEM; n = 5–6/group/time-point.

Table 3. A decrease in colonic bacterial diversity is associated with an increase in inflammatory cytokines

Bacterial α diversity	IL-1β	IL-8	IL18	TNF-α
Spearman r	-0.8286	-0.7714	-0.2571	-0.4857
p value	0.0583	0.1028	0.6583	0.3556

The expression of colonic IL-1β and IL-8 negatively correlate with colonic bacterial diversity as assessed by Spearman's rank correlation coefficient.

This decrease in diversity is due to certain species becoming dominant in the colon, to the detriment of others. At two weeks post-surgery, members of the *Firmicutes* phylum underwent the most significant alterations, with an increase in the proportion of the *Veillonellaceae* family and a decrease in the proportion of the *Ruminococcaceae* family in the SBR group relative to the NOC and sham group. Major differences in bacterial composition were observed six weeks post-surgery. The phylum *Fusobacteria* was significantly increased in the SBR group relative to the NOC and sham groups. Gram negative bacteria in this phylum express pro-inflammatory molecules such as lipopolysaccharide, which is known to trigger inflammation in the gut.[26-28] At the family level, the majority of changes were again within the *Firmicutes* phylum, with a decrease in the proportion of *Clostridiaceae*, *Peptostreptococcaceae* and *Peptococcaceae* in the SBR group compared with the sham group at six weeks. These Gram positive anaerobic bacteria may provide CpG DNA with immunomodulatory activities and their decrease or absence is postulated to disrupt the crosstalk between the host mucosal immune system and the microbiota.[29] The increase in the *Veillonellaceae* family and the decrease in *Ruminococcaceae* family observed in the SBR group at two weeks was sustained at the six week time-point. Several studies have linked alterations in each of these families with systemic and local inflammation.[30,31] As the delicate balance between host epithelial cells and resident bacteria is already disturbed in patients with SBS due to the unfavorably altered luminal environment, these specific changes in key bacterial families may further exacerbate the clinical manifestations of SBS.

Interestingly, there were also alterations in some bacterial families in the sham surgery group. The proportion of *Enterobacteriaceae* was significantly decreased and the proportion of *Prevotellaceae* was significantly increased in the sham group compared with the NOC group at the two week time-point. This increase in *Prevotellaceae* was sustained at the six week time-point and there was also a decrease in the proportion of *Lactobacillaceae* at this time. It is known that surgical trauma often results in postoperative ileus and in the activation of resident macrophages and the upregulation of functional activity of pro-inflammatory cytokines.[32] Thus, the sham surgery, although relatively minor compared with the massive small bowel resection surgery, would also have an effect on the gut microbiota.

A limited number of studies have examined the bacterial community in SBS patients using culture- and microscopy-based techniques. These studies suggest that there is a shift in the microbiota and that the microbiota of SBS patients is composed primarily of Gram positive organisms such as lactobacilli.[33] More recent studies that used quantitative PCR to identify changes in the microbiota in adult patients with SBS, confirmed that there is enrichment in lactobacilli in these patients.[24,34] Using high-throughput DNA sequencing, we observed no significant difference in the proportion of *Lactobacillus* spp at either time-point. Given that a purported 70–80% of gut bacteria are unculturable, our results may reflect the more comprehensive identification of previously unidentified bacterial content[35] and may also indicate that there are important differences between adult and pediatric SBS patients in terms of their microbiota.

Despite significant differences in pro-inflammatory bacteria in the SBR group two weeks post-surgery, there was no increase in pro-inflammatory colonic cytokine expression at this time-point. However, six weeks post-SBR, there were significant increases in

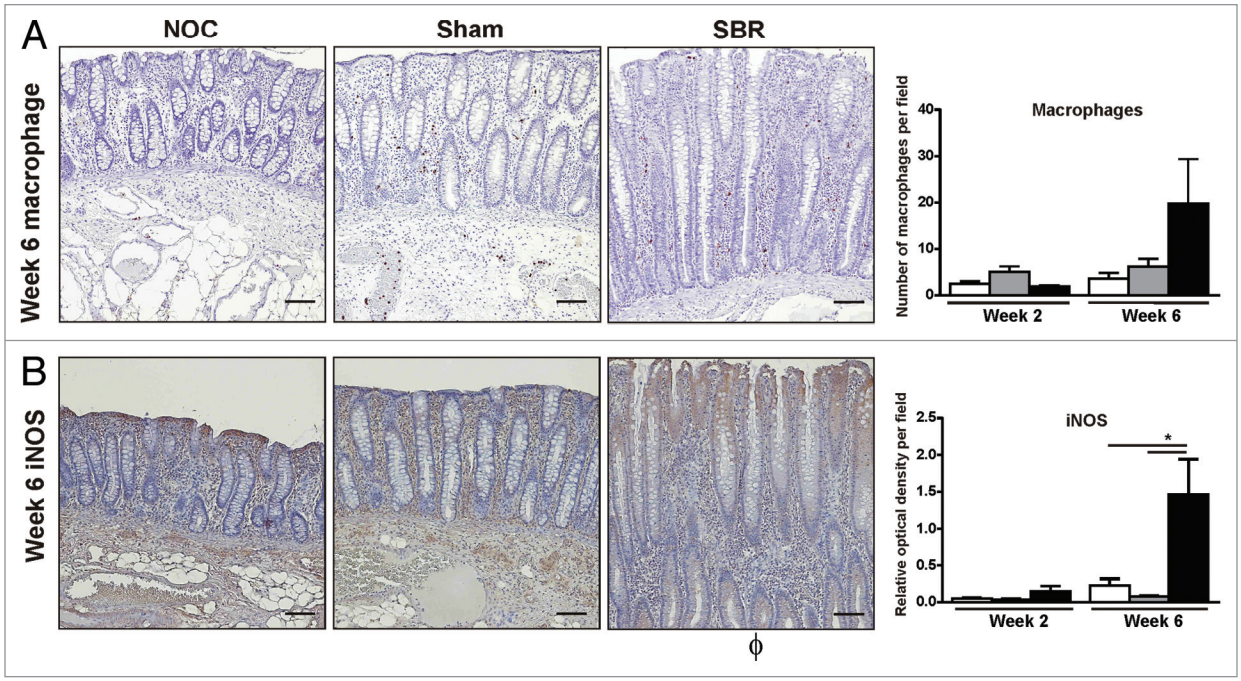

Figure 4. There is an increase in inflammatory cells and mediators in the colonic epithelium following small bowel resection. There was an increase in the number of macrophages (**A**) and the percentage of cytoplasmic inducible nitric oxide synthase (iNOS) staining (**B**) in the colonic epithelium six weeks post-surgery. Immunohistochemistry images illustrate representative staining six weeks post-surgery. Scale bar represents 100 μm. Values are expressed as mean ± SEM; n = 5–6/group.

the pro-inflammatory cytokines IL-1β, IL-8, IL-18 and TNF-α in the SBR group. This was coupled with an increase in the number of macrophages in the colon, as well as an increase in colonic iNOS in the SBR group. This increase in mucosal inflammation coincided with a marked decrease in diversity and dysbiosis at the later time-point. Consistent with this observation, there was a correlation between a decrease in bacterial diversity and an increase in colonic IL-8 and IL-1β. Several studies have shown a link between diversity and inflammation, although there is debate as to which is the causative agent.[21,29,36] In our study, the decrease in diversity was evident two weeks post-surgery, but significant markers of inflammation did not occur until six weeks post-SBR. Thus, it is our hypothesis that the colonic microbiota is negatively impacted by the altered luminal environment following the surgical resection, resulting in a decrease in diversity, which initiates a pro-inflammatory response in the colon.

This study has identified and characterized dysbiosis in the colon that occurs following SBR and challenges current accepted theories regarding specific alterations in the microbiota of patients with SBS. We have shown a reduction in diversity following SBR due to certain bacterial species establishing dominance and described inflammation in the colon that persists for six weeks after SBR. We suggest that this inflammation is a consequence, rather than a trigger of the dysbiosis and that the overall composition of the microbiota may be more relevant than the presence or absence of a single species. This study has highlighted that the colon is significantly impacted by proximal SBR and the role that the colon may play in determining the clinical outcome following a resection has been underappreciated.

Materials and Methods

Animals. This study was approved by the Animal Ethics Committee of the Murdoch Childrens Research Institute. Weaned female 3-week-old piglets (Landrace/Large White cross; Aussie Pride Pork) were transported to The University of Melbourne Centre for Animal Biotechnology and acclimatised prior to surgery. Piglets were fed a polymeric infant formula diet (Karicare De-Lact, Nutricia) supplemented to meet the daily requirements for piglets as described previously.[11,37,38] The diets were isocaloric and isonitrogenous among the groups and were administered on a per kilogram basis. Water was given twice daily. Piglets were housed separately throughout the study to allow accurate daily monitoring of food and water intake and stool output.

Clinical assessment and growth. Piglet weight was measured weekly before feeding. Fecal samples were collected weekly and stool consistency was scored by the Royal Children's Hospital Laboratory Services, Melbourne, Australia using the following scale: 0 = formed, 1 = semi-formed, 2 = unformed and 3 = fluid.

Experimental design. At four weeks of age, piglets underwent either a 75% proximal small bowel resection (SBR, n = 12) or a transection and re-anastomosis (sham, n = 10) operation. One group of piglets did not receive any surgery (non-operation control; NOC, n = 12). The 75% SBR included the removal of the small bowel from 90 cm distal to the ligament of Treitz to 225 cm proximal to the ileocecal valve. During the sham procedure the intestine was transected and re-anastomosed at a site 225 cm proximal to the ileocecal valve. Piglets received intramuscular amoxicillin (70 mg/kg; CSL Limited) 24 h pre-surgery. On the

day of surgery, piglets were anesthetized and given amoxicillin. Piglets received amoxicillin and oral rehydration salts (Sanofi-Aventis Australia) for three days post-surgery in line with current clinical practice. Water and the polymeric infant formula diet were re-introduced from the third day post-operation. All piglets in the NOC group followed the same feeding regime, with piglets in the week 2 NOC group receiving antibiotics in line with the surgical groups.

Sample collection. Animals in the SBR and sham groups were sacrificed either two- or six-weeks post-surgery and at age-matched times in the NOC group. Colonic tissue was collected 3 cm and 10 cm distal to the cecum in the two-week and six-week groups, respectively, at locations optimised for age, as based on a previous study.[37] A 3 cm section from each site was divided in half longitudinally and fixed in 10% neutral buffered formalin (Australian Biostain Pty. Ltd.) or snap frozen in liquid nitrogen. Colonic content was collected from the excised colonic tissue sample.

DNA extraction and amplicon sequencing. DNA was extracted from colonic content using the standard QIAamp DNA Stool Mini Kit protocol (Qiagen, 51504), with the addition of an initial bead beating step. The 16S rRNA amplicons were generated using a previously outlined approach.[39] Amplicons were generated using one forward primer and a combination of four reverse primers as described previously.[40] Each primer contained a distinct multiple identifier (MID) allowing pooling of the amplicons and subsequent separation of the results for analysis. Duplicate PCR products were pooled and cleaned using Agencourt AMPure kit (Beckman Coulter, A63880). Quantification was completed using Quant-iT Picogreen quantification kit (Invitrogen, P7589) and the Nanodrop 3300 (Thermo Scientific). The V4 region of the 16S rRNA was sequenced at the Teagasc 454-Sequencing facility on a Genome Sequencer FLX platform (Roche Diagnostics Ltd.).

Bioinformatic analysis. Raw sequencing reads were quality trimmed using the RDP Pyrosequencing Pipeline applying the following criteria: (1) exact matches to primer sequences and barcode tags, (2) no ambiguous bases (Ns) and (3) read-lengths no shorter than 150 base pairs. Trimmed FASTA sequences were then BLASTED[41] against the SILVA (v100) database for 16S reads.[42] Phylum, family and genus counts were extracted from MEGAN[43] using a bit score cut-off of 86.[42] Clustering into operational taxonomical units (OTUs), alignments, chimera-checking and alpha diversities were implemented using the Qiime suite of tools.[44] A phylogenetic tree was generated using the FastTree package[45] and principal coordinate analysis (PCoA), measuring dissimilarities at phylogenetic distances based on unweighted Unifrac analysis, was performed with Qiime suite of tools.[44] PCoA plots were visualized with KiNG software package (http://kinemage.biochem.duke.edu/software/king.php).

Quantitative PCR. Absolute quantification was completed using the Roche LightCycler 480 platform (Roche Diagnostics). Samples consisted of 2 μl PCR grade water, 1 μl forward primer F1 (5'-AYT GGG YDT AAA GNG; 0.15 μM), 1 μl reverse primer R1 (5'-TAC CRG GGT HTC TAA TCC; 0.15 μM), 1 μl template DNA and 5 μl SYBR green (Roche Diagnostics, 04887352001). Bacteria were quantified using 16S rRNA counts based on a standard curve, using a previously outlined calculation.[46] All reactions were run in triplicate.

Real-time reverse-transcription PCR (qRT-PCR). The muscle layer was stripped from 100 mg of colonic tissue leaving the colonic mucosa, which comprised the epithelium and the lamina propria. Total RNA was extracted from the mucosa using TRIzol (Invitrogen, 15596–026). cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, 04897030001). PCR primers were designed against pig gene sequences using Roche Universal ProbeLibrary Assay Design Centre (Roche Applied Science, 04683633001). Primer sequences and probe combinations are listed in **Supplemental Table 1**. PCR reactions were performed in triplicate on the LightCycler 480. The $2^{-\Delta\Delta Ct}$ method[47] was used to calculate relative changes in gene expression using RPL32 as a housekeeping gene and relative to a pre-operation group (n = 6).

Microscopic assessment of inflammation. Immunohisto-chemistry was performed on formalin-fixed, paraffin-embedded colon tissues sectioned at 5 μm. To identify macrophages, antigen retrieval was performed with proteinase K followed by addition of primary antibody (MAC387; 1:1000; Abcam, ab22506) for two hours at room temperature. Staining of inducible nitric oxide synthase (iNOS) was performed by performing antigen retrieval in citrate buffer (pH 6.0) and adding primary antibody (iNOS; 1:100; Abcam, ab15323) overnight at 4°C. Secondary staining and 3,3-Diaminobenzidine (DAB) detection was performed using Histostain-Plus 3rd Gen IHC Detection Kit (Invitrogen, 85–9073). Negative controls were included in which the primary antibody was substituted with antibody diluent. Slides were viewed under an apochromat 10x objective lens using a Nikon Eclipse 80i microscope (Nikon Instruments Inc.), equipped with a DS-Ri1 CCD camera (Nikon) and controlled by NIS-Elements acquisition software version 4.00 (Nikon). For each set of analyses, ten fields of view of the epithelium were collected. Macrophages were quantified by counting the number of positively-stained cells in the colonic epithelium using ImageJ.[48] The amount of iNOS epithelial cytoplasmic staining was quantified by color deconvolution using ImageJ.[49]

Statistical analysis. Data are presented as mean values with their standard error (SEM). Statistical analysis was performed using one-way ANOVA at each time-point, followed by Tukey's post-hoc test (GraphPad Prism Software 5.0). Sequencing analysis was completed using Minitab Release 15.1.1.0 (Minitab Inc. 2007). To identify if significant differences occurred at phylum, family and genus levels between the three groups, non-parametric Kruskal-Wallis or Mann-Whitney tests were performed followed by Tukey's post-hoc test. This test was also used for comparing the changes in total bacterial counts in the three groups. The non-parametric Spearman rank correlation was used to determine relationships between diversity and inflammation. Statistical significance was accepted at $p < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgments

This work was supported by the Victorian Government's Operational Infrastructure Support Program. F.F. is in receipt of an Irish Research Council for Science, Engineering and Technology EMBARK scholarship and is a Teagasc Walsh fellow. Research in the Ross, Fitzgerald, Stanton and Cotter laboratories is funded by Science Foundation Ireland (in the form of a research center grant to the Alimentary Pharmabiotic Centre and a PI award to P.C.). The authors wish to thank Magdy Sourial, Shane Osterfield and Dr Andrew French for expert technical assistance with the animals and Dr Jean-Pierre Scheerlinck and The University of Melbourne Centre for Animal Biotechnology for the use of their facilities. The authors would also like to thank Dr Eva Rosberg-Cody for high-throughput DNA sequencing services.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/gutmicrobes/article/24372

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