Changes in the colon microbiota and intestinal cytokine gene expression following minimal intestinal surgery

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Abstract

AIM: To investigate the impact of minor abdominal surgery on the caecal microbial population and on markers of gut inflammation.

METHODS: Four week old piglets were randomly allocated to a no-surgery "control" group (n = 6) or a "transection surgery" group (n = 5). During the transection surgery procedure, a conventional midline incision of the lower abdominal wall was made and the small intestine was transected at a site 225 cm proximal to the ileocaecal valve, a 2 cm segment was removed and the intestine was re-anastomosed. Piglets received a polymeric infant formula diet throughout the study period and were sacrificed at two weeks post-surgery. Clinical outcomes including weight, stool consistency and presence of stool fat globules were monitored. High throughput DNA sequencing of colonic content was used to detect surgery-related work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

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disturbances in microbial composition at phylum, family and genus level. Diversity and richness estimates were calculated for the control and minor surgery groups. As disturbances in the gut microbial community are linked to inflammation we compared the gene expression of key inflammatory cytokines (TNF, IL1B, IL18, IL12, IL8, IL6 and IL10) in ileum, terminal ileum and colon mucosal extracts obtained from control and abdominal surgery groups at two weeks post-surgery.

**RESULTS:** Changes in the relative abundance of bacterial species at family and genus level were confined to bacterial members of the *Proteobacteria* and *Bacteroidetes* phyla. Family level compositional shifts included a reduction in the relative abundance of *Enterobacteriaceae* (22.95 ± 5.27 vs 2.07 ± 0.72, \(P < 0.01\)), *Bacteroidaceae* (2.54 ± 0.56 vs 0.86 ± 0.43, \(P < 0.05\)) and *Rhodospirillaceae* (0.40 ± 0.14 vs 0.00 ± 0.00, \(P < 0.05\)) following transection surgery. Similarly, at the genus level, changes associated with transection surgery were restricted to members of the *Proteobacteria* and *Bacteroidetes* phyla and included decreased relative abundance of *Enterobacteriaceae* (29.20 ± 6.74 vs 2.88 ± 1.08, \(P < 0.01\)), *Alistipes* (4.82 ± 1.73 vs 0.18 ± 0.13, \(P < 0.05\)) and *Thalassospira* (0.53 ± 0.19 vs 0.00 ± 0.00, \(P < 0.05\)). Surgery-associated microbial dysbiosis was accompanied by increased gene expression of markers of inflammation. Within the ileum IL6 expression was decreased (4.46 ± 1.60 vs 0.24 ± 0.06, \(P < 0.05\)) following transection surgery. In the terminal ileum, gene expression of TNF was decreased (1.51 ± 0.13 vs 0.80 ± 0.16, \(P < 0.01\)) and IL18 (1.21 ± 0.18 vs 2.13 ± 0.24, \(P < 0.01\)), IL12 (1.04 ± 0.16 vs 1.82 ± 0.32, \(P < 0.05\)) and IL10 (1.04 ± 0.06 vs 1.43 ± 0.09, \(P < 0.01\)) gene expression increased following transection surgery. Within the colon, IL12 (0.72 ± 0.13 vs 1.76 ± 0.28, \(P < 0.01\)) and IL10 (0.96 ± 0.02 vs 1.95 ± 0.14, \(P < 0.01\)) gene expression were increased following transection surgery.

**CONCLUSION:** This study suggests that minor abdominal surgery in infants, results in long-term alteration of the colonic microbial composition and persistent gastrointestinal inflammation.

**Key words:** Surgery; Intestinal; Microbiota; Dysbiosis; Inflammation; Surgery; Bacteria; Infant; Pediatric; Transection

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**Core tip:** Early colonization of the infant gut is increasingly recognized as impacting on health due to the influence of resident microbes on nutritional, immunological and physiological functions. However, medical and surgical interventions required during infancy, including abdominal surgery, have the potential to modify the microbiome. Using 454-pyrosequencing technology we have described microbial dysbiosis within the *Proteobacteria* and *Bacteroidetes* phylum and increased gut inflammatory cytokines following transection surgery in a juvenile pig model. This is the first study examining minor surgery-associated changes in the gut microbiome and inflammation and provides new insights into potential long-term consequences of abdominal surgery in infancy.

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**INTRODUCTION**

The gut microbiota is essential to human health, yet the acquisition of this microbial community during infancy remains poorly understood. At birth, humans are essentially free of bacteria, with colonization of the gastrointestinal tract beginning during the birthing process as the newborn is exposed to maternal and environmental microbes\(^1\). The infant microbiota is marked by heterogeneity and instability until approximately 2-4 years of age\(^2,3\), when it becomes more stable, resembling an adult microbiota\(^4\). The development of the microbiota is known to be strongly influenced by early extrinsic factors including mode of infant delivery\(^5\), type of feeding\(^6,7\) and antibiotic therapy\(^8-10\). Acute intestinal conditions including intestinal obstruction, perforation and intussusception may necessitate exploratory abdominal surgery and/or surgical intervention during the neonatal or infant period; however the impact of abdominal surgery on the microbiota has not previously been studied.

Colonization of the newborn intestine plays a key role in the development and fine-tuning of intestinal immune responses\(^11\), and disruption of the gut microbiota has been linked to an increasing number of immune-related diseases, including inflammatory bowel disease, necrotizing enterocolitis (NEC), eczema, allergies and asthma\(^12\). Given the previously demonstrated impact of early life bacterial dysbiosis on future adult health, characterization of the impact of laparotomy in infancy on the development of the microbiome and inflammation is clinically relevant. The neonatal piglet surgical model is an excellent alternative model for the study of human gastrointestinal disease due to physiological\(^13,14\), bacteriological\(^15\) and immunological\(^16,17\) similarities between pigs and humans. In addition, the accelerated ageing rate of pigs when compared with humans allows us to study the equivalent human timeframe of infancy to early childhood within a two week study period.

The primary aim of the present study was to use...
454-pyrosequencing technology to assess the impact of minor abdominal surgery on the colonic microbiota in a juvenile pig model of intestinal transection surgery. Our secondary aim was to perform a multi-site assessment of molecular alterations in key gut inflammatory markers to assess if surgery-related microbial dysbiosis was associated with intestinal inflammation at two weeks following surgery.

MATERIALS AND METHODS

Animals
This study was approved by the Animal Ethics Committee of the Murdoch Childrens Research Institute. Weaned female three-week-old piglets (Landrace/Large White cross; Aussie Pride Pork) were transported to the University of Melbourne Centre for Animal Biotechnology and acclimatized 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[18-23]. 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 collection of stool (analysed for consistency, presence of fat globules and presence of fatty acid crystals). Piglet weight was measured weekly before feeding.

Experimental design
Four week old piglets were randomly allocated to a no-surgery “control” group (n = 6) or a “transection” surgery group (n = 5). During the surgery procedure, a conventional midline incision of the lower abdominal wall was made and the small intestine was transected at a site 225 cm proximal to the ileocaecal valve, a 2 cm segment was removed and the intestine was re-anastomosed. The removal of 2 cm of intestine was equivalent to a mean of 0.11% of the initial intestinal length. Both groups received intramuscular amoxicillin (70 mg/kg; CSL Limited) 24 h pre-surgery, and for three days post-surgery in line with current clinical practice. In addition, both groups received oral rehydration salts (Sanofi-Aventis Australia) for three days post-surgery or equivalent date, with water and the polymeric infant formula diet re-introduced from the third day post-operation.

Sample collection
Animals in the transection group were sacrificed two weeks post-surgery and at an age-matched time in the control group. Ileum tissue was collected 8 cm distal to the caecum in both groups. Samples from each site were snap frozen in liquid nitrogen. Colonic content was collected from the proximal colon.

High-throughput sequencing
The 16S rRNA amplicons from colonic content were generated using a previously outlined approach[24]. Amplicons were generated using one forward primer and a combination of four reverse primers as described previously[24]. 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[25] against the SILVA (v100) database for 16S reads[26]. Phylum, family and genus counts were extracted from MEGAN using a bit score cut-off of 86[26]. Clustering into operational taxonomical units (OTUs), alignments, chimera-checking and alpha diversities were implemented using the Qime suite of tools. The relative abundance was determined for individual pigs as the number of reads for each species as a proportion of the total number of reads for that pig at the relevant level of phylum, family or genus.

Real-time reverse transcription PCR
The muscle layer was stripped from the ileum, terminal ileum and colon tissue leaving the mucosa, which comprised the epithelium and the lamina propria. Total RNA was extracted from 100 mg of intestinal mucosa using TRIzol (Invitrogen). Complementary DNA (cDNA) was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). PCR primers were designed against pig gene sequences using Roche Universal ProbeLibrary Assay Design Centre (Roche Applied Science). Primer sequences and probe combinations are listed in Table 1. PCR reactions were performed in triplicate on the LightCycler 480. The 2^−ΔΔCT method[27] was used to calculate relative changes in gene expression in the surgical group relative to the non-surgical control group, using RPL32 as a housekeeping gene.

Statistical analysis
Sequencing analysis was completed using Minitab
Microbial composition is significantly altered following transaction surgery

High throughput DNA sequencing was used to detect disturbances in microbial composition following transaction surgery. Diversity and richness estimates were calculated for the control and transaction surgery groups. The Chao 1 calculation is an estimator of phylotype richness in a dataset and the Shannon index of diversity reflects both the richness and the community evenness (i.e., proportional phylotype abundance)\(^{28}\). The overall alpha diversity of the colonic microbiota was unchanged following surgery as calculated by either the Chao 1 richness estimate (905 + 41 vs 892 ± 123) or the Shannon’s index for diversity (6.9 ± 0.2 vs 6.5 ± 0.6), and there was no difference in the number of observed species.

16S rRNA sequence data obtained from colonic content samples was analyzed to determine if transaction surgery was associated with changes in the proportion of assignable reads at the phylum, family or genus level when compared with no surgery controls. There was no difference in composition at a phylum level between the control group and the transaction group. Changes were observed however, at family and genus level (Tables 2 and 3). Changes in the relative abundance of bacterial species at family and genus level were confined to members of the Proteobacteria and Bacteroidetes phyla, with no difference in the relative abundance at family or genus level of species belonging to the Firmicute, Actinobacteria, Fusobacteria or Spirochaetes phyla.

A tissue-specific pattern of inflammation occurs following transaction surgery

Disturbances of the gut microbial community are closely linked to inflammation\(^{29}\), therefore given the detected bacterial dysbiosis in the transaction surgery model we next examined changes in the mucosal gene expression of key inflammatory cytokines within the ileum, terminal ileum and colon (Figure 1). Within the ileum, a decrease in interleukin 6 (IL6) gene expression followed surgery (P = 0.0383; Figure 1A). In the terminal ileum, gene expression of the pro-inflammatory cytokine tumor necrosis factor (TNF) was decreased (P = 0.0072), and interleukin 18 (IL18) and interleukin 12 (IL12) gene expression was increased following surgery when compared with controls (P = 0.0123 and P = 0.0430 respectively; Figure 1B). Gene expression of the anti-inflammatory cytokine interleukin 10 (IL10) was increased in terminal ileum...
Table 2  Relative abundance of bacterial species at the family level in colonic content samples obtained from no-surgery control animals ($n = 6$) or animals who have undergone a transaction surgery ($n = 5$)

| Phylum level | Family level | Control (Rel. Abundance) | Transection (Rel. Abundance) | $P$ value |
|--------------|--------------|--------------------------|----------------------------|----------|
| Proteobacteria | Enterobacteriaceae | 22.95 ± 5.27 | 2.07 ± 0.72 | 0.0104 |
| | Desulfovibrionaceae | 2.89 ± 1.26 | 2.95 ± 1.16 | 0.9716 |
| | Moraxellaceae | 0.69 ± 0.55 | 8.81 ± 8.72 | 0.4501 |
| | Pseudalteromonadaceae | 0.55 ± 0.43 | 1.42 ± 1.42 | 0.5844 |
| | Vibrionaceae | 0.52 ± 0.37 | 6.03 ± 5.91 | 0.4038 |
| Bacteroidetes | Rikenallaceae | 6.00 ± 1.87 | 3.70 ± 1.31 | 0.3416 |
| | Porphyromonadaceae | 2.33 ± 0.76 | 4.50 ± 1.78 | 0.3880 |
| | Prevotellaceae | 2.76 ± 0.39 | 8.05 ± 2.60 | 0.1105 |
| | Bacteroidaceae | 2.54 ± 0.56 | 0.86 ± 0.43 | 0.0432 |
| Firmicutes | Ruminococcaceae | 19.97 ± 2.01 | 15.26 ± 4.71 | 0.3962 |
| | Veillonellaceae | 14.19 ± 4.12 | 22.40 ± 7.73 | 0.3834 |
| | Lachnospiraceae | 7.63 ± 2.31 | 6.19 ± 2.00 | 0.6475 |
| | Pseudalteromonadaceae | 0.55 ± 0.43 | 1.42 ± 1.42 | 0.5844 |
| | Lactobacillaceae | 0.13 ± 0.06 | 1.56 ± 1.54 | 0.4056 |
| | Lactocestaceae | 4.17 ± 1.47 | 3.17 ± 1.36 | 0.1843 |
| | Streptococcaceae | 0.13 ± 0.06 | 1.56 ± 1.54 | 0.4056 |
| | Erysipelotrichales Incertae Sedis | 1.78 ± 0.61 | 1.03 ± 0.53 | 0.3809 |
| | Moraxellaceae | 1.45 ± 0.35 | 0.73 ± 0.36 | 0.1843 |
| | Lachnospiraceae Incertae Sedis | 2.50 ± 1.02 | 1.10 ± 0.47 | 0.2548 |
| | Erysipelotrichales Incertae Sedis | 2.28 ± 0.77 | 1.48 ± 0.78 | 0.4859 |
| | Lactobacillaceae | 1.96 ± 0.74 | 2.15 ± 1.16 | 0.8958 |
| | Clostridium | 1.86 ± 0.45 | 1.05 ± 0.52 | 0.2732 |
| | Anaerotruncus | 2.23 ± 0.71 | 0.97 ± 0.31 | 0.1501 |
| | Orbibacterium | 0.63 ± 0.25 | 1.88 ± 0.88 | 0.2341 |
| | Lactococcus | 0.06 ± 0.06 | 1.60 ± 1.58 | 0.3860 |
| | Mitsuokella | 0.05 ± 0.05 | 1.90 ± 1.82 | 0.3664 |
| | Other | 6.49 ± 1.09 | 5.84 ± 1.41 | 0.7245 |

Data are expressed as mean ± SEM (%). *P < 0.05 vs control. Bacterial species whose relative abundance was < 1% in both the control and transaction surgery group are compiled within the “other” category. Significant changes in bacteria whose relative abundance was < 1% are detailed within the text.

Table 3  Relative abundance of bacterial species at the genus level in colonic content samples obtained from no-surgery control animals ($n = 6$) or animals who have undergone a transaction surgery ($n = 5$)

| Phylum level | Genus level | Control (Rel. Abundance) | Transection (Rel. Abundance) | $P$ value |
|--------------|-------------|--------------------------|----------------------------|----------|
| Proteobacteria | Members of the Enterobacteriaceae | 29.20 ± 6.74 | 2.88 ± 1.08 | 0.0108 |
| | Desulfovibrio | 2.08 ± 0.68 | 2.74 ± 0.78 | 0.5389 |
| | Bilophila | 1.48 ± 0.81 | 1.13 ± 0.95 | 0.7847 |
| | Psychrobacter | 0.90 ± 0.72 | 9.13 ± 9.01 | 0.4156 |
| | Vibrio | 0.62 ± 0.46 | 5.89 ± 5.70 | 0.4105 |
| Bacteroidetes | Alistipes | 0.11 ± 0.07 | 1.47 ± 1.47 | 0.4192 |
| | Prevotella | 3.61 ± 0.59 | 11.11 ± 3.68 | 0.1110 |
| | Bacteroides | 3.25 ± 0.71 | 1.24 ± 0.63 | 0.0625 |
| | Parabacteroides | 2.88 ± 0.65 | 5.59 ± 2.41 | 0.3313 |
| | Lachnospiraceae Incertae Sedis | 2.50 ± 1.02 | 1.10 ± 0.47 | 0.2548 |
| Firmicutes | Megaspheara | 9.71 ± 3.87 | 14.18 ± 4.01 | 0.4434 |
| | Phascolarctobacterium | 4.07 ± 1.00 | 6.90 ± 1.89 | 0.2330 |
| | Rumminococcaceae Incertae Sedis | 2.94 ± 0.71 | 1.78 ± 0.47 | 0.2106 |
| | Peptostreptococcaceae Incertae Sedis | 2.79 ± 1.23 | 1.80 ± 1.49 | 0.6208 |
| | Subdoligranulum | 2.49 ± 0.99 | 1.28 ± 0.57 | 0.3264 |
| | Acidaminococcus | 2.40 ± 0.71 | 3.53 ± 1.82 | 0.5866 |
| | Erysipelotrichales Incertae Sedis | 2.28 ± 0.77 | 1.48 ± 0.78 | 0.4859 |
| | Lactobacillus | 1.96 ± 0.74 | 2.15 ± 1.16 | 0.8958 |
| | Clostridium | 1.86 ± 0.45 | 1.05 ± 0.52 | 0.2732 |
| | Anaerotruncus | 2.23 ± 0.71 | 0.97 ± 0.31 | 0.1501 |
| | Orbibacterium | 0.63 ± 0.25 | 1.88 ± 0.88 | 0.2341 |
| | Lactococcus | 0.06 ± 0.06 | 1.60 ± 1.58 | 0.3860 |
| | Mitsuokella | 0.05 ± 0.05 | 1.90 ± 1.82 | 0.3664 |
| | Other | 8.05 ± 0.69 | 6.66 ± 1.21 | 0.3549 |

Data are expressed as mean ± SEM (%). *P < 0.05 vs control. Bacterial species whose relative abundance was < 1% in both the control and transaction surgery group are compiled within the “other” category. Significant changes in bacteria whose relative abundance was < 1% are detailed within the text.
plays a key role in the development and fine-tuning of the intestinal immune responses, it is essential to detail the effect of minor abdominal surgery performed during the infant period, on the major microbial community and associated inflammation.

Our current study details alterations in the colonic microbial community in response to abdominal surgery. We chose to focus on the colonic microbiota as (1) the colonic microbial community represents the largest, and most widely studied microbial community in both health and disease states; and (2) any alterations to the colonic microbial community are likely to influence the immune response via interactions with the gut-associated lymphoid tissue. In the current study, whilst there was no observed effect on the overall richness or diversity of the colonic microbiota, there were alterations in specific bacterial genera that are likely to be of clinical relevance. Similarly, studies of irritable bowel syndrome also describe significant associations between quantitative differences in specific bacterial components of the gut microbiome and disease symptoms in the face of unchanged overall microbial diversity. In particular, we observed alterations only within the Proteobacteria and Bacteroidetes phylum and report reductions in the relative abundance of family level members Enterobacteriaceae, Rhodospirillaceae and Bacteroidaceae and genus level reductions in the relative abundance of Enterobacteriaceae, Thalassospira, Alistipes and Bacteroides following transection surgery.

The reduction in the relative abundance of Bacteroides sp. is of particular clinical relevance as, in early life, the intestinal microbiota of healthy infants displays a large abundance of Bacteroides sp. Bacteroides sp. play a fundamental role in host metabolic processes and prevents the colonisation of the gut by potential pathogens. Depletions in Bacteroides species are observed in children with inflammatory conditions including IgE mediated food allergy and inflammatory bowel disease. Furthermore, Bacteroides is postulated to play a crucial role in the development of gastrointestinal-associated lymphoid tissues and in the modulation of T-helper Th1/Th2/T-regulatory balance. Therefore reduction in relative abundance of Bacteroides in the gut following intestinal transection surgery may, at least in-part, explain the observed increase in IL12 and IL18 gene expression within the terminal ileum. IL12 and IL18 form a link between innate resistance and adaptive immunity and may be produced by a wide range of immune cells in response to bacteria and bacterial products.

Interestingly, we observed markers of an active gut inflammatory response at two weeks post-surgery that was not limited to the area within the immediate proximity of the intestinal transection and re-anastomosis. Specifically, we observed increased gene expression of IL12 and IL10 in both terminal ileum and colon, and increased IL18 and decreased TNF gene

Figure 1 Relative gene expression of pro- and anti-inflammatory cytokines in (A) ileum, (B) terminal ileum and (C) colon from no-surgery control and transection surgery groups. Mean ± SEM. aP < 0.05, bP < 0.01 vs control.

samples from the surgery group when compared with controls (P = 0.0054). Similar to the terminal ileum IL12 and IL10 gene expression was increased within the colon following surgery (P = 0.0055 and P = 0.0001 respectively; Figure 1C).

**DISCUSSION**

Immediately after birth, the newborn gut environment is colonized by facultative anaerobic bacteria such as Enterobacteriaceae and Streptococcaceae. These bacteria gradually consume the oxygen in the intestine and produce new metabolites, preparing the intestinal environment for the establishment of a strict anaerobic environment for the establishment of a strict anaerobic conditions including IgE mediated food allergy and inflammatory bowel disease. Furthermore, Bacteroides is postulated to play a crucial role in the development of gastrointestinal-associated lymphoid tissues and in the modulation of T-helper Th1/Th2/T-regulatory balance. Therefore reduction in relative abundance of Bacteroides in the gut following intestinal transection surgery may, at least in-part, explain the observed increase in IL12 and IL18 gene expression within the terminal ileum. IL12 and IL18 form a link between innate resistance and adaptive immunity and may be produced by a wide range of immune cells in response to bacteria and bacterial products.

Interestingly, we observed markers of an active gut inflammatory response at two weeks post-surgery that was not limited to the area within the immediate proximity of the intestinal transection and re-anastomosis. Specifically, we observed increased gene expression of IL12 and IL10 in both terminal ileum and colon, and increased IL18 and decreased TNF gene.
expression in terminal ileum. Whilst it is not possible to ascertain the underlying mechanism responsible for the wide-spread increase in inflammatory mediators observed in our juvenile pig model, we postulate surgery-related alterations in the small intestine microbiota may influence inflammatory marker expression within the ileum and terminal ileum. Alternatively, surgery-related factors including bowel handling could have contributed to the inflammatory response observed. However, bowel handling has been reported to induce TNF, IL6 and IL1B production\(^{39,40}\), cytokines that remained unchanged or were decreased in this study (Figure 1).

Early colonization of the infant gut is increasingly recognized as impacting on health due to the influence of resident microbes on nutritional, immunological and physiological functions. However, medical and surgical interventions required during infancy, including abdominal surgery, have the potential to modify the microbiome, sometimes permanently. We have described microbial dysbiosis within the Proteobacteria and Bacteroidetes phylum and increased gut inflammatory cytokines following surgery in a juvenile pig model. This is the first study examining changes in the gut microbiome and in gut inflammation that occur following transaction surgery in infancy and provides new insights into potential long-term consequences of abdominal surgery in infancy.

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