Evaluation of fecal microbial transplant as a treatment method for disruption of the gut microbiota associated with short bowel syndrome in neonates.

by

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Abstract

Background

Short bowel syndrome (SBS) is the leading cause of intestinal failure (IF), wherein either the function of the intestines is suboptimal, or length has been reduced due to congenital or acquired conditions, such that parenteral nutrition (PN) is required for >6 weeks. The most common etiology in neonates is necrotizing enterocolitis (NEC), a condition resulting from disruption or poor establishment of the microbiota that allows inflammatory species to proliferate and cause tissue damage. Repeated rounds of antibiotics to treat central line infections and small intestinal bacterial overgrowth are necessary, but further exacerbate dysbiosis. Sepsis with gut-derived organisms and IF-associated liver disease, which is partly caused by bacterial translocation, are now the leading causes of mortality in children with SBS. Treatment for dysbiosis must replace beneficial organisms, rather than simply remove harmful ones, in order to support normal growth and development of children with SBS. Probiotics are one option that have had success in preventing NEC, but some *Lactobacilli* have been associated with inhibition of adaptation in some instances of single species supplementation, and in others with an increase in sepsis. In this experiment, we looked at using fecal microbial transplant (FMT) as a way of restoring a healthy diverse microbiota in a single treatment in neonatal piglets with SBS.

Methods

Neonatal male Duroc piglets aged 3-7 days were assigned to saline (SAL, n=12), fecal microbial transplant (FMT, n=12), or sow-fed control (SOW, n=6) treatment groups. SAL and FMT underwent 75% distal SBS surgery on day 0, and treatment on day 2 (100 mL saline or FMT material, respectively). Donor FMT material was collected from a healthy female gilt, filtered, stored at -80 °C, and thawed at 4 °C overnight before use. SOW piglets remained with their litter,

and were terminated on day 7 along with their treatment piglet littermates. Tissue samples were collected at baseline and termination surgeries, as well as daily stool samples pre-treatment, post-treatment, and on the mornings of day 3-6. Microbial DNA was extracted, analyzed for 16S rRNA, and compared to established databases for taxonomy assignment. Percent relative composition of bacterial taxa was analyzed and compared between groups, as well as alpha and beta diversities.

Results

No piglets developed sepsis or experienced disease-specific mortality, and FMT was well-tolerated. FMT piglets showed increased Actinobacteria and Firmicutes families, and increased alpha diversity, compared with SAL post-treatment and on day 3, but these increases in general did not persist afterwards. FMT and SAL piglets had significantly different beta diversity post and day 3, but the Donor material was significantly different at all time points. SOW piglet term tissue sample alpha diversity in jejunum and colon, and beta diversity in the colon, was significantly higher than treatment piglets.

Conclusion

Piglets in this trial demonstrated no disease-specific mortality and no increase in sepsis rates compared with our previous SBS trials. Differences in bacterial taxa were measurable in stool samples only through the day after FMT treatment. It is possible this was due to inadequate dietary fiber sources for establishment of the donor communities, given the neonatal piglets were fed enteral nutrition (EN) that contained only simple carbohydrates. Differences in beta diversity between SAL and FMT were present in the colonic mucosal-associated bacteria at termination, indicating small numbers of some species were able to establish and survive. Supplementation

with complex fiber sources accessible by butyrate-producing species should be added to EN in the future to aid in persistence of beneficial species.

Preface

This thesis is the work of Tierah Hinchliffe, which received ethical approval #AUP00000155 from University of Alberta Faculty of Agriculture, Life and Environmental Sciences Animal Policy and Welfare Committee.

Dedication

Beliefs should conform to one's best scientific understanding of the world. One should take care never to distort scientific facts to fit one's beliefs.

- The Satanic Temple, Tenet V

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Now, in the immortal words of Paul Wales, "Let's get the poo ball rolling!"

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Chapter One: Literature Review

1.1. Introduction to Intestinal Failure

The classical definition of intestinal failure (IF) is a reduction in gastrointestinal function below the minimum threshold for sufficient absorption of nutrients, water, and electrolytes to support growth in children and sustain life in adults[1,2]. IF is most commonly caused by a loss of absorptive surface area either due to a congenital malformation or after surgical resection due to an acquired condition or injury[3,4]. IF can also be present functionally, wherein the patient does not have sufficient absorptive capacity but maintains all or the majority of their intestinal surface area[3,5]. The impairment leading to loss of function may be temporary, for example if caused by an ileus after abdominal surgery, or it may be acute, lasting weeks to months, usually caused by obstructions (ie. adhesions, hernias, volvulus, cancer, etc[6]), fistulae, mesenteric infarction, or traumatic injuries[7]. A third category is chronic IF (CIF), where acute patients have become stable, but management of their condition is months to years in duration[7].

Once almost certainly fatal in patients with CIF, the advent of parenteral nutrition (PN) in the early 1970s and refinements to its administration have increased survival rates in infants with IF[8]. Patients with CIF are dependent upon PN for their survival[9], but this long-term (>60 days[10,11]) requirement often leads to other serious complications, including central venous catheter (CVC) infections, sepsis, thromboses, and IF-associated liver disease (IFALD)[12,13]. With a multidisciplinary intestinal rehabilitation program in place, and further advances to PN formulations[14], mortality rates of PN-dependent patients have shrunk from 29.4% in the early 1990s[15] to less than 10% as of 2016[16].

1.1.1. Short Bowel Syndrome

Short bowel syndrome (SBS) is the leading cause of IF in adults[17] and infants[11], with the overall reported incidence of neonatal SBS in Canada being 24.5 per 100,000 live births[4]. For premature infants, this increases over 14-fold to 353.7 per 100,000 live births[4]. It is difficult to diagnose SBS in terms of absolute intestinal length, as it is the quality of bowel remaining that is more important[5]. SBS is better defined as a post-surgical need for PN that lasts longer than 6 weeks, or a relative short bowel length that is less than 25% of expected for age or height[4]. Length of remaining colon and presence or absence of the ileocecal valve are critical determinants of the chronicity of IF in patients with SBS[3,5]. In one study[16], infants with SBS who retained <50% of the expected length for both small and large intestine had an 8.3% probability of returning to enteral autonomy. If >50% of either small or large intestine remained, probability of autonomy rose to over 50%, and those with >50% of expected length for both intestines had an 83.3% probability of independent nutrition. These differing remnant anatomies can be broadly classified into three categories: jejunoileal (JI), where some ileum remains and the ileocecal valve (ICV) and colon are in continuity; jejunocolic (JC), where the entire ileum is removed along with the ICV and a portion of the colon; and resections that leave a jejunostomy[13]. Each anatomy presents its own challenges, though JI anatomy has been associated with better outcomes in both humans[13] and neonatal piglets[18,19].

The etiologies of pediatric SBS are diverse, encompassing the IF-related causes above, congenital malformations including gastroschisis, atresia, or Hirschsprung's Disease, as well as acquired conditions such as necrotizing enterocolitis (NEC), volvulus, and obstructions[4,15]. NEC is the leading cause of SBS in children, being the primary diagnosis in 34-43% of cases[4,20]. NEC primarily occurs in preterm infants, partly due to the immature intestinal epithelium and

diminished barrier function in this group, and partly due to some as-yet unidentified disruption in the pre- or perinatal establishment of the infant microbiota[21]. Use of antibiotics and antacids for preterm infants in the neonatal intensive care unit (NICU) alters the microbiota in its most vulnerable stage of development[21], while mode of delivery does not appear to affect the incidence of NEC[22]. As NEC most frequently affects the distal ileum and proximal colon[13,18], the JC models of residual anatomy are more common post-surgery, and are also the most challenging with worse outcomes. Loss of the ileum and part of the colon means loss of the enteroendocrine L-cells responsible for releasing important factors such as trophic hormone glucagon-like peptide 2 (GLP-2), and peptide YY, which slows intestinal transit time allowing adequate absorption of nutrition[17].

1.1.2. Adaptation after surgery

Children under the age of 5 are still growing their intestines as an innate process, and this has traditionally been viewed as an advantage in their post-surgical care. Following extensive resection, the intestine naturally goes through a process called adaptation, which involves functional and structural changes to compensate for the loss of length[23]. Studies done in animals show that structurally there is local angiogenesis[24] and increased crypt cell hyperplasia[25] following resection. In adult human patients, there is evidence of mucosal hyperplasia in the form of increased villus height and crypt depth, bowel lengthening, and dilatation of isolated intestinal loops[26]. Infants with SBS due to NEC have also shown taller villi and deeper crypts at the time of ostomy takedown compared with initial surgery[27].

Functionally, studies in rats have shown that ileal enterocytes increase expression of the Na^+/glucose transporter SGTL1 within 6 hours following resection[28]. Data on this effect in humans is sparse; however, there is some data that show increased mRNA expression of protein

transporters in the colon[29]. Adult patients with colon in continuity experience lowered fecal wet-weight output[30] and higher levels of peptide YY in the blood[31] than those with jejunostomy, indicating the importance of this hormone in maintaining adequate fluid balance, and highlighting how residual anatomy affects prognosis.

1.1.3. Care and management

The most effective factor in improving survival rates of patients with SBS is implementation of a comprehensive intestinal rehabilitation program (IRP) that focuses on communication and continuity of care across many dedicated professionals. In one 20-year study, when IRPs were implemented the 2-year mortality rate dropped from 29.4% to 10.0%, a 66% reduction, with increasing survival trends as time went on[15]. This is reflected in another study, where ultra-short residual bowel (≤ 20 cm) was shown to be no longer an adequate predictor of poor outcome, with mortality of this group reducing from 100% pre-2005 to just 9% from 2006 – 2012[32]. The main facets of the IRP include lipid optimization in PN formulations, bowel lengthening procedures, and rotating antibiotic regimens to control bacterial overgrowth. Other aspects of all-around care involve use of enteral nutrition where tolerated, monitoring of vitamin and electrolyte levels, gastric acid suppression, motility agents, and soluble fiber supplementation[33,34].

One of the more effective components of an IRP has been improvement of PN delivery and formulations. Conventionally, PN formulations were made with soy lipids, but this has been found to contribute to the development of IFALD due, in part, to a large proportion of omega-6 (n-6) fatty acids, the metabolites of which create a pro-inflammatory state[14]. One study indicated that patients who received PN containing >1g/kg/day of n-6 lipids were at significantly increased risk

of developing cholestasis, with 65% of patients displaying chronic symptoms in a median of 6 months[35]. Reduction of soy-based lipids below 0.5 g/kg/d and replacement with a combination of soy, medium-chain triglycerides, olive oil, and fish oil (SMOFlipid) that is high in omega-3 (n-3) fatty acids has the potential to reduce or reverse the development of cholestasis[33,36], and is associated with a significant decrease in SBS-related mortality[37]. The best ratio of n-6:n-3 lipids has not yet been experimentally proven. However, a ratio between 1:1 and 2:1 has been proposed as it provides a balance similar to breast milk that may reduce inflammation and help to prevent glucose overfeeding with increased lipogenesis[14].

Trophic therapy involving the administration of a long-lasting glucagon-like peptide-2 (GLP-2) analogue, teduglutide, has had success in decreasing the need for PN through supporting mucosal adaptation[38,39]. Teduglutide is approved for use in patients >1 year old and although it causes mucosal proliferation and reduced apoptosis, it requires daily (potentially long-term) injection to maintain its effect in animal models[40] and in humans[41]. In one follow-up study, nearly half of adult patients who had been receiving teduglutide and shown reduced PN requirements demonstrated increased needs for PN a year after stopping the drug[42]. Another GLP-2 analogue, apraglutide, is currently in clinical trials, and has shown longer-lasting intestinal growth in length in piglets[43] and has been well tolerated in humans[44]. If approved, this latest analogue could encourage a lasting regrowth in length that reduces lifetime need for the intervention.

Although these methods have improved outcomes for children with IF, some patients do not improve enough and require intestinal transplantation[34]. Current indications for transplant are the presence of two of the following: ≥ 2 ICU admissions, persistently high conjugated bilirubin absent of infection and in spite of lipid management strategies, or loss of ≥ 3 CVC sites[32]. Although sepsis or number of septic episodes are no longer on the list recommendations for transplant listing,

loss of access to CVC sites is frequently caused by recurrent sepsis, so it is an indirect indicator for potential transplant requirement.

1.1.4. Complications of SBS

The most common complications of SBS are IFALD and catheter-associated sepsis[45]. In one study, liver failure was responsible for 60% of SBS-related deaths and sepsis for 20%, with the disease-specific mortality rate of SBS patients reaching 5-fold that of non-SBS patients[4]. Another, multi-center study reported a cumulative mortality of 26% at 3 years follow-up, with 82% of these attributable to either multi-organ failure or sepsis[10]. Soon after the advent of total parenteral nutrition (TPN) in the 1970s, hepatobiliary damage was identified as a major iatrogenic injury[46]. Infants on TPN are more likely than adults to develop hepatocellular injuries and fibrosis, and also progress to this condition more rapidly[47]. IFALD is multifactorial, however, and not solely caused by TPN usage. The major patient-related risk factors for development of IFALD include gestational age at birth, the underlying diagnosis that lead to the development of SBS, site and frequency of sepsis, tolerance of EN, and small intestinal bacterial overgrowth[48].

While the exact mechanisms are unknown, there are also potentially relevant pathways to IFALD in SBS patients related to sepsis and the disruption of the microbiota. Loss of the ileum during resection can alter the flow of bile acids due to lack of reabsorption. This altered distal bile flow affects the relative proportions of certain bacterial phyla, increasing aerobic and aerotolerant Proteobacteria while decreasing anaerobic Bacteroidetes compared with healthy patients[49]. Lack of enteral feeding causes intestinal muscular atrophy and decreased barrier function, allowing bacteria to translocate and their cell wall components like lipopolysaccharide (LPS) and flagellin[50]

to stimulate toll-like receptors (TLRs) on innate immune cells in the lamina propria and eventually the liver, inducing inflammation and fibrosis[46].

Other complications include gastric acid hypersecretion, which is likely due to loss of an intestine-secreted gastrin inhibitor[51] or loss of the small intestinal site of gastrin breakdown[33]. These increased acid levels lead to damage in the stomach and small bowel, alteration of digestion, and precipitation of bile salts[51]. Children with SBS tend to have greater proportions of Lactobacilli, some species of which can produce the neurotoxic D-lactate enantiomer of L-lactate, a key metabolic intermediate, when high amounts of undigested carbohydrates reach the colon[52]. This can cause neurological symptoms which are frequently mistaken for inebriation, including slurred speech, ataxia, fatigue, and confusion[52,53]. Lactobacilli are also associated with steatosis due to excessive bile acid deconjugation[46], which may also play a role in multiple vitamin deficiencies and impaired bone mineralization that are also seen in children with IF[45].

1.1.5. Small intestinal bacterial overgrowth and sepsis

In addition to the above complications, small intestinal bacterial overgrowth (SIBO) is a common condition that is present in 50%[50] to 70%[54] of those with SBS and leads to a 7-fold increase in risk of septicemia[50]. Sepsis and end-stage IFALD are now the leading causes of mortality associated with SBS, with the main predictive variables being underlying diagnosis, failure to wean from PN, and peak bilirubin concentrations[55]. Sepsis with gut-derived organisms contributes to CVC infections[56], which are difficult to cure and can lead to eventual loss of access, which removes the ability of the patient to receive lifesaving PN[57].

The gastric and duodenal environments have a low pH due to gastric and bile acids, which, when coupled with rapid movement of contents, contributes to the low microbial biomass in these

areas, typically on the order of 10^{2-3} cells/mL[58]. The jejunum and ileum are a more neutral pH, allowing the microbe numbers to increase to between 10^4 and 10^8 cells/mL, with higher numbers possible the more distal the location. Currently, an overgrowth of bacteria is considered as counts over 10^3 cells/mL in the proximal bowel, although for other locations within the bowel, and other etiological conditions, this number may be higher[59]. Previously a value of 10^5 cells/mL was used as the diagnostic limit, however over time this figure has been questioned and the lower value has replaced it[56,59]. SIBO can be present not only in post-surgical SBS patients, but also those with radiation enteropathy, scleroderma, and post-operative Crohn's Disease[56], as well as in individuals living with poor sanitation[60], and those treated with proton-pump inhibitors, which, when used for as little as 4 weeks[61], can lower the gastric and duodenal pH allowing bacteria to proliferate.

Suspicion of SIBO is typically based on post-surgical risk factors including abdominal distension or discomfort, anemia, vitamin deficiencies, excess byproducts of alternative biosynthetic pathways (like D-lactic acidosis)[62] or malnutrition[59]. Diagnosing SIBO is not straight-forward, as culture-based methods of enumerating bacterial populations are not entirely representative of true cell counts, and the samples may be expensive and invasive to obtain[59]. Non-invasive tests include hydrogen (H_2) breath tests after ingestion of one of several different mono- or disaccharides, as well as high serum D-lactate or urine indicant measurements[60]. Hydrogen is solely a byproduct of microbial saccharide fermentation[60], and is carried from the intestines to the lungs where it is exhaled, the amount and time of detection being changed from baseline in those with an overgrowth of bacteria. H_2 tests are the most commonly used in older children or adults, where the time from ingestion of saccharide to emission of H_2 in the breath is assumed to be longer than 90 minutes[60]. Those with shorter transit time, such as very young children and neonates, or

those with higher proportions of methanogenic bacteria that use H_2 to produce methane (CH_4), may produce false positive test results[60].

SIBO is associated with villus atrophy and epithelial damage leading to an inflammatory response and translocated bacteria[63]. Those patients in which the ICV is resected are at increased risk for SIBO due to the loss of this critical barrier[51], although some studies[15,54] have found that absence of the ICV was not predictive of development of SIBO. In these cases, the initiating factor may be adaptation-induced dilatation, which leads to stasis of the luminal contents and creates an environment where resident bacteria can proliferate[64]. Overgrowth of bacteria in the jejunum in turn leads to a significant increase in septic episodes that is worse in those with shorter (<50cm) remaining small bowel length[65]. Specifically, abnormal growth of aerobic gram-negative bacilli (AGNB) is associated with a significant increase in both sepsis and septicemia in neonates receiving PN[66]. This overgrowth of AGNB is allowed due to lack of gut motility and impaired gut-associated lymphoid tissue[66], and is also thought to stimulate hepatic Kupffer cells via the portal circulation and prime them to an over-reactive state that creates liver damage and systemic inflammation[67].

This complex relationship between PN-dependence, SIBO, sepsis, and IFALD, makes the goal of attaining enteral autonomy ever more pressing for these patients. While ability to wean from PN may largely depend upon remnant surgical anatomy, advancement of enteral nutrition (EN) stimulates adaptation and prevents the intestinal muscular and functional atrophy that occurs with PN-only treatment[65,68]. Use of breast milk where possible has been associated with a shorter duration of PN, which may be partly due to humoral factors such as IgA antibodies and leukocytes carried in the milk that support the neonate's immune defenses[20]. EN-induced adaptation and preservation of the bowel musculature could help maintain the epithelial barrier and prevent

stagnation of the food bolus, thereby reducing the ability of the microbiota to proliferate beyond normal levels. Increased barrier function lowers bacterial translocation, meaning a reduction in sepsis and systemic inflammation, and a reduced need for antibiotics. In piglets, we have previously shown that repeated use of antibiotics and lack of EN administration exert a greater effect upon the microbiota than surgery does alone[69]. One must be careful with EN usage, however, as use of high-carbohydrate low-fat diets can facilitate overgrowth of lactobacilli, as carbohydrates are their preferred energy source[70]. This can then lead to complications of D-lactic acidosis and excess CO_2 production[70]. Large amounts of CO_2 can cause abdominal pain, flatulence, and lowered absorption of lipids and dissolved vitamins, and so appropriate formulations must be used[70].

While rotating antibiotic regimens to treat SIBO have been used successfully as part of a comprehensive IRP, there is a risk that comes with over-prescription of these therapies. Emergence of single- and multi-drug resistant organisms is of great concern, particularly amongst AGNB[66]. Some SBS patients with SIBO may be non-responsive to antibiotics[71], or may require continuous antibiotic administration in order to keep symptoms at bay[56]. In those cases, patients are then subject to continuous diarrhea, are at risk for *Clostridioides difficile* infections and the expansion of other opportunistic and antibiotic-resistant pathogens[72], and experience a heavy burden of healthcare costs as well as reduced quality of life[56]. Constant use of antibiotics merely removes organisms or reduces overall numbers without pro-actively filling the empty gut with a beneficial protective population.

1.2. Our Gut Microbiota

Inside our bodies is a flourishing microbial ecosystem that has co-evolved with us in an intricate, mutualistic network. Originally roughly estimated at 10-fold more than our somatic cell

count, latest calculations place the population of our microbial partners at 1.3x the number of our own cells[73]. This ratio skewed against us begs the question of whether we are each a human with microbial symbionts, or colony-living microbes with a *Homo sapiens*-shaped motility appendage. The microbiota are the organisms themselves, whereas the microbiome is comprised of their genomes[74], a collection which also outnumbers our own contributions, this time by a staggering factor of 150[75]. This internal ecosystem has been referred to as a neglected[76], forgotten[77], or invisible[78] organ, though some disagree with this designation[79]. Unofficial organ or not, the microbiota and microbiome have been implicated in wide-reaching effects on obesity[80,81], Alzheimer's Disease[77], autism[82], inflammatory bowel disease[83], malnutrition[84] and more.

Concurrently as we have come to understand that our health can be affected by the microbes living in our guts, we have also realized the importance of describing what makes up a healthy microbiota as this allows us to identify when something goes wrong. Understanding how an individual's microbiota varies day to day, or year to year, is crucial to identifying what changes might be contributing to disease when they occur. Trying to understand what defines a healthy microbiota brings up the question of whether any decided parameters would be the same for all individuals, or to what degree a healthy composition would vary person to person[74]. As methods used for measuring and classifying the microbiota have evolved, and so, too, has our overall understanding of its function. Before the advent of culture-independent methods of analysis, detection of microorganisms was limited to those that were amenable to culturing and identification by comparison of attributes to reference texts[85]. Unsurprisingly, this lead to much uncertainty and lack of specificity. The discovery of the highly conserved nature of the bacterial and archaeal 16S rRNA gene allows for construction of phylogenetic relationships between most genera and species, though other genes such as those for virulence factors or metabolic enzymes

may be more useful in some clades, or for distinction at the strain level[85]. High throughput sequencing and the construction of next generation sequencing libraries based on 16S rRNA genes now allows for the processing and filtering of highly complex community data through the use of software and analysis tools such as quantitative insights into microbial ecology (QIIME) and mothur[86].

The advent of molecular technologies has allowed us to investigate further than was previously possible into the composition of our microbiota. These studies have revealed that most of our internal and external surfaces are colonized by microbes, with the vast majority being present in the colon at a density of 100 times that of other areas[87]. More than 90% of our colonic microbes belong to just 2 of the known bacterial phyla, the Firmicutes and Bacteroidetes[88]. Other phyla such as Actinobacteria, Proteobacteria, and Fusobacteria also appear in highly individualized relative proportions, with dominant genera tending to cluster around *Bacteroides*, *Prevotella,* and *Ruminococcus* enterotypes[89].

1.2.1. Diversity and composition

The diversity of species detected can be as different from site-to-site on a single individual as it is between individuals themselves. Alpha diversity measures these intrasample differences, whereas beta diversity measures both intersite and interindividual diversity[88]. Saliva and various niches within the oral cavity tend to show high alpha diversity but low beta diversity, whereas the vagina is the opposite: low alpha, high beta diversity[88]. The GI tract displays high alpha and beta diversities measured in stool[88], and when subdivided into stomach, duodenum, jejunum, ileum and colonic microenvironments, further differences are revealed[58]. The stomach and proximal duodenum, with their highly acidic gastric secretions, are typically sparsely populated with counts

around 10^{2-4} cells/mL. The pH increases as one moves distally along the GI tract, and once the ileocecal valve is crossed, it reaches ~7, and the colon becomes home to ~10^{11} cells/mL[58].

In addition to differences in microbial composition between locations along the GI tract's length, there are multiple spatial niches from the lumen to the mucosa as well. The lumen is home to planktonic species that metabolize molecules in the digesta, such as simple sugars or plant fiber, whereas the loose outer layer of mucosa provides substrate for mucin-degraders[90]. The thick inner mucosa is sterile, a necessary physical layer of defense illustrated beautifully in the murine colon by Tropini and colleagues[90]. The mucosa provides not only metabolic substrate, but also scaffolding for the creation of biofilms that allow the microbes to resist being washed out by the passing digesta[91].

There are few microbial residents that truly colonize the GI tract (autochthonous) versus simply passing through (allochthonous). *Helicobacter pylori* is perhaps the best example of autochthony, as it is commonly found in the stomach, but is present neither in food nor in the various niches of the oral cavity[58]. In contrast, *Blastocystis spp* have been commonly found in the GI tract, but are a known environmental contaminant usually present due to poor sanitation[92]. Whether resident or tourist, the microbiota is a community dominated by bacteria, with smaller contributions from archaea[93], viruses[94], and fungi[92]. Enzymes and acids secreted by the stomach, pancreas, and liver provide us with access to the majority of our ingested nutrients, but it is the bacterial complement of enzymes that digest the inaccessible fibers and resistant starches to produce essential short chain fatty acids (SCFAs) we cannot otherwise obtain[58]. Archaeal hydrogenotrophic species such as *Methanobrevibacter smithii* are a minor but important subset of the microbiota that engage in a complex cross-feeding economy with hydrogen-producers like *Roseburia spp*, *Ruminococcus spp,* and *Bacteroides spp*[95]. The production and metabolism of

hydrogen, methane, and hydrogen sulfide is a critical indicator of microbiota health, to the extent that breath tests for these gases may be used as a test in the diagnosis of dietary intolerances, SIBO, and irritable bowel syndrome (IBS)[95].

Viruses are an often-overlooked aspect of the microbiota. Many human viruses are known to interact in negative and positive ways with the microbiota[96]. Bacteriophages and siphophages may make up around 6% of genomic reads[89], and exert a form of population control on their bacterial and archaeal hosts[94]. Fungi are also present, collectively known as the mycobiota, though predominantly as yeasts, food-borne molds, and parasites[92]. The most common fungal species are *Saccharomyces cerevisiae, Malassezia restricta,* and *Candida albicans*[97], though this fluctuates rapidly with changes to the diet[98]. Fungi have been shown to have a protective role in monoculture against colitis and influenza viral infections[99], but have also been implicated in many different disease pathogeneses[100,101], particularly Crohn's Disease[78].

The GI tract of a fetus was initially thought to be sterile; however, maternal microbes have been detected in amniotic fluid and meconium, indicating that colonization begins even before birth[102]. Postnatally, the infant microbiota is simple and composed of few members, mainly *Enterobacteriaceae*, but this undergoes rapid maturation. Changes occur with exposure to breast milk, formula, complimentary feeding, and various environmental factors, including microbial and antibiotic exposure, taking up to 3 years to stabilize and reflect an adult composition[102,103]. The microbes that colonize us, and in what proportions, are partly affected by the order in which we encounter them[104]. In this comparison of two wild-caught murine microbiotas transplanted into germ-free lab mice in different orders, some species from donor B were only able to successfully colonize the recipient mouse if they were transplanted first, whereas they were less able to survive if the mouse had received a transplant from donor A previously. In addition to variables such as

order of encounter, host genetics also play a role in the establishment of our microflora. In humans, the microbiota of healthy monozygotic twins has less beta diversity than that of dizygotic twin pairs[105], though twin pairs and their mothers have been found to display more similarity in their microbiomes than their microbiotas[106]. In mice, variations between families and within litters are only minimally explained by environmental factors, with the majority of variation thus being due to intrinsic genetic factors[107].

1.2.2. The core microbiome

With representatives from all three domains of life, and over a thousand detectable species across a cohort[75], it is perhaps surprising that a core microbiota common to all individuals may be comprised of only between 18 to 40 species[75,108]. Just 8 of the known bacterial lineages have been detected in humans: 3 are highly abundant (Cytophaga-Flavobacterium-Bacteroides [CFB], Firmicutes, Proteobacteria) and 5 are less abundant (Spirochaeates, Actinobacteria, Fusobacteria, Verrucomicrobia, VadinBE9 7)[109]. Compared with soil-based communities (the terrestrial digestive tract[109]), which have been found to harbour between 21 and 31 different bacterial phyla[110,111], we are much less diverse.

Measures of diversity are further complicated by difficulties in assigning taxonomy. Sequencing and analysis of the variable segments of the 16S rRNA gene is the standard method, with 95% similarity denoting a genus and 98% similarity being the limitation of a species[109]. This is not without fault, however, as evolutionary variation of the 16S rRNA gene may occur at different rates between taxa, meaning that some organisms that are currently described as different species may be >98% similar, and some assigned to the same genus may be <95% identical[112]. Proportions of microbiota composition are usually described in relative abundances within a given

sample or testing point only, giving the comparison of results from different experiments an extra layer of complexity. Interpreting relative increases or decreases of one genus or species from one experiment to another may lead to subjective inferences that are not correct, in cases where the microbiome is not measured. Further, the different variable segments of the 16S rRNA gene (V1-V9) have different abilities to make distinctions between species and genera[113]. As such, species-level resolution is not usually reported for whole microbiota surveys, with genus and family being more common.

There is evidence to suggest that rather than a core microbiota, a core microbiome, a set of necessary genes present in functionally redundant species, might be the reality[106]. A community made of diverse species with the same or similar traits allows for stability in the face of stochastic events that may alter the community structure at the individual level. Stated another way, there are several important ecological niches within our gut, and the species that fills the niche is not as important as the fact that the niche itself is filled. Some of these niches are saccharolysis, aerobic sugar fermentation, and production of butyrate[58] as well as other SCFAs that are the products and substrates of complex cross-feeding networks[114].

One species that is important to have in our microbiota is *Faecalibacterium prausnitzii*, a member of the Firmicutes phylum that is associated with an anti-inflammatory protective phenotype in Crohn's Disease[115]. *F. prausnitzii* produces large amounts of butyrate[115], the primary metabolic substrate for intestinal epithelial cells (IECs), which enhances barrier function by suppressing high-permeability tight junction proteins[116], and preferentially stimulates intestinal regulatory T cells (Tregs) to differentiate[117]. Butyrate production is a trait shared by other members of the *Clostridium leptum* group[118], which emphasizes this group's importance for gut homeostasis.

Bacteroidetes member *Bacteroides thetaiotamicron* is also protective as it is associated with increased barrier function, immunostimulation, and nutrient production[119]. *B. thetaiotamicron* increases luminal butyrate via complex interactions with *Eubacterium rectale*[120]. *B. thetaiotamicron* first stimulates the host to produce glycan substrates that only it can utilize, then produces byproducts that *E. rectale* takes up and converts to butyrate, which is, in turn, absorbed by host IECs. While this is a simplified gnotobiotic model, it demonstrates the complex ways in which we and our microbiota have co-evolved for mutual benefits.

1.2.3. Dysbiosis in SBS

A healthy microbiota is naturally plastic, with relative proportions of species and genera shifting up and down in response to the foods we eat[98] and environmental factors[102]. Shifts or losses that contribute to the instigation or propagation of disease states are termed dysbiosis. Dysbiosis is likely to be almost universal in children with SBS, and has been characterized by a loss of diversity relative to both healthy siblings[72] and unrelated infant controls.[121] In SBS, an increase in gram-negative *Enterobacteriaceae* and *Lactobacillus*, as well as decreased *Ruminococcus* associated with more frequent stool output may be common[122]. NEC, the most common cause of SBS, also shows this pattern, with one study[123] finding that the dominant phyla in all infants with NEC was Proteobacteria, and in almost all non-NEC controls, Firmicutes were the most abundant. Interestingly, two control infants without NEC who showed high proportions of *Escherichia* at the time of sampling went on to develop NEC soon after.

With pediatric SBS patients, these significant microbiota changes are related to remnant anatomy, proportion of parenteral calories, and antibiotic usage[124]. Patients with more advanced disease showed a loss of Firmicutes with a commensurate increase in *Lactobacilli, Proteobacteria,*

Actinobacteria, and other species that are typically minor constituents, while those with the less severe disease exhibited a microbiota signature that more closely resembled a healthy adult diversity. This loss of diversity in association with PN usage is detectable as early as the first week of usage, becoming more significant over time[125]. Antibiotic therapies in the face of frequent sepsis are necessary for patient survival, but facilitate the colonization and expansion of opportunistic pathogens, which further perpetuate the use of antibiotic therapies, in a "snowball"-type effect[72].

These changes to the microbiota are not only associated with the genesis of disease, as in the case of NEC, but also with detrimental effects on other aspects of the patient's health. More severe dysbiosis is associated with worse growth compared to SBS patients with good growth and healthy controls[126]. Those patients with poor growth were deficient in Firmicutes, which translated into a deficiency in key metabolic processing and synthesis pathways. One such set of pathways are those dependent upon vitamin B_{12}, a deficiency of which is commonly seen in SBS[127]. Impairment of the B_{12} pathways may be obscured by over-production of other SCFAs due to SIBO, and lead to potentially irreversible neurological effects, which emphasizes the need to effectively control SIBO as soon as possible[62]. Though the dysbiosis leading to these health effects may be instigated and perpetuated by use of PN-only nutrition, weaning from PN may not be immediately possible and comes with significant challenges[128]. An overabundance of Lactobacilli has been associated with progression to steatosis, even after weaning from PN[124], demonstrating that fixing the dysbiosis is key to preventing SIBO and the advancement of IFALD.

1.2.4. Treatment for dysbiosis

The options for initial treatment of dysbiosis and bacterial overgrowth is an individually tailored process dependent upon each patient's anatomy and requirements. Those with adequate

remaining bowel length may be able to slowly wean from PN, use motility agents to prevent stasis-induced SIBO, or rely on antibiotics to reduce overgrowth when it is present. Various doses and rotating courses of antibiotics must be chosen in order to prevent the development of resistance[56]. Antibiotic therapy may be successful at reducing pathogenic or opportunistic organisms, but its drawback is that it cannot restore or replace beneficial or helpful populations.

As we know that a diet deficient in substrate for microbes results in a deficiency in their beneficial effects[91], dietary supplementation with probiotics or prebiotics could be a first step to modulate the microbiota when dysbiosis occurs. Probiotics are live microorganisms ingested for their beneficial effects upon the microbiota[129] and were first proposed to treat antibiotic-associated dysbiosis in the 1950s[130]. Probiotics must be capable of surviving the gastric environment, able to adhere to the intestinal mucosa, and deliver some benefit to the host, such as inhibition of pathogenic organisms via antimicrobial peptides or niche pre-emption[130]. Added bacterial species may not persist in the gut, however, if they are not also provided with an appropriate nutritional substrate. Such microbial foods are termed prebiotics[131] or microbiota-accessible carbohydrates (MAC)[91]. Both terms carry the definition that the substrate must be digestible only by bacteria, not the host, and that such species then provide a known health benefit to the host. Breast milk is the original prebiotic as it contains many unique oligosaccharides that are indigestible by infants but support a subspecies of *Bifidobacterium longum* that retains the necessary glycosidases that are absent in adult-associated species[132].

A combination of pro- and prebiotics administered together is known as a synbiotic[130]. The prebiotic ingredient may selectively feed only the probiotic ingredient, or it may be a more broadly accessible substrate that also nourishes the host's resident populations as well. An early study in healthy adult men showed that only prebiotics, either with or without probiotics, had a significant

impact on the microbiota, and that probiotic *Bifidobacteria* alone had no effect upon culture-based detection of gut residents[133]. Subsequent studies have been less robust, however, and lack of appropriate method designs have obscured the ability to draw meaningful conclusions from the data[130].

1.2.5. Prebiotics and probiotics in SBS

Prebiotic and probiotic therapy may be a viable option in adults or in individuals with intact guts, but they may not be a safe choice for post-surgical neonates, whose immune systems are underdeveloped and whose microbiota is unstable. In some cases of older children with SIBO secondary to SBS, probiotic *Lactobacilli* has produced some positive results such as improved stool consistency or reduced arthritic inflammation[71], but they have also been associated with sepsis in some SBS patients[134] and in non-SBS patients with other diagnoses[135]. These risks may be mitigated by use of a synbiotic containing galactooligosaccharides (GOS) with some success[136], including increased height and weight gain, but small sample sizes restrict the ability to draw firm conclusions. A systematic review of therapeutic use of probiotics in children with SBS found no appreciable benefit[137], and a double-blind crossover study looking specifically at probiotics to improve intestinal permeability also found no consistent effects[138]. Yeasts and fungi are occasionally used as probiotic organisms due to their ability to withstand antibiotic attack; However, supplementation with the yeast *Saccharomyces boulardii* in monoculture has also been associated with four cases of fungemia (one with neonatal SBS)[139].

In animals, *Lactobacillus rhamnosus* supplementation was able to reduce NEC severity in newborn mice and piglets[140]. NEC was induced in non-surgical mouse and piglet models using stool from a human infant with NEC, and when also given the live or dead probiotic, or with

extracted *L. rhamnosus* DNA, NEC severity was attenuated. This indicates that the benefit is dependent on TLR-9 innate sensing, and could also be created using other probiotic species or their DNA. Other lactobacilli species, such as *L. plantarum*, are also able to improve weanling piglet gut health by reducing incidence of diarrhea and increasing relative proportions of beneficial *Prevotellaceae* and *Bifidobacteriaceae*[141]. *In vitro* experiments show that intestinal mucosa from pigs treated with a combination therapy of *B. lactis* and *L. rhamnosus* can significantly reduce adherence of pathogens *Clostridium perfringens, C. difficile, E. coli,* and *Salmonella enterica*, whilst also increasing their own adhesion allowing for prolonged protection[142]. *In vivo*, healthy pigs receiving treatments of probiotic *Entercoccus faecium*, prebiotic lactulose, or the synbiotic, showed decreased *Enterobacteriaceae* across all treatments, with the lactulose-treated group showing a 10-fold reduction[143]. All groups also showed an increased proportion of Firmicutes, indicating that probiotic and prebiotic supplementation can support healthy microbiome functions.

These examples are encouraging, and yet the data from surgical and SBS models are less promising. In a mouse model of induced colitis, supplementation with fructooligosaccharides (FOS) has been associated with a loss of barrier function and overall decrease in diversity and richness in mice with ileocecal resection (ICR)[144]. ICR mice had significantly decreased *Bifidobacteria*, a complete lack of fecal butyrate, and increased serum inflammatory cytokines compared with control. In SBS piglets, FOS significantly increases adaptation measured by villus height, but this effect was negated entirely by probiotic *L. rhamnosus* on its own, and reduced by the synbiotic[145].

As shown in these examples, supplementing the growth of only a single phylum or species may be ineffective or even too dangerous for consideration in SBS infants with SIBO. Animal models also support the conclusion that probiotics may hamper the adaptation after surgery that is

necessary for a good prognosis, which may then inhibit their ability to wean from PN-dependence. Instead, reconstitution of the entire microbiota with healthy diversity and richness may be the more prudent choice, and potentially the administration of prebiotic oligosaccharides without a corresponding probiotic. Engstrand Lilja, et al[72], noted that fecal microbial transplant (FMT) may be a viable treatment option to counteract the disruptive effects that frequent antibiotic use to treat SIBO have upon the microbiota.

1.3. Fecal Microbial Transplant

Fecal microbial transplant (FMT) is the complete replacement of a dysbiotic microbiota with one from a healthy donor in an effort to treat disease[146]. As such, it represents a potential solution to the risk of sepsis as discussed above with use of probiotics. Originally described in the 4th and 16th centuries in China to treat severe diarrhea, fever, vomiting, and constipation[146], the first modern published uses were documented in 1958 to treat pseudomembranous colitis, and in 1981 a series of 16 patients receiving fecal enemas to cure recurrent *C. difficile* infection (RCDI) was reported[147]. Modern recurrence rates for CDI are between 20-35%, with 45-65% of patients having multiple relapsing episodes[148]. As most patients with RCDI are treated with many rounds of rotating antibiotics, their susceptibility to further bouts of CDI may be due to the lack of colonization resistance provided by other bacteria that have also been eliminated by the antimicrobials. Current clinical guidelines in the US recommend use of FMT after the third round of CDI, however it may be considered as a first step in those who cannot tolerate or cannot receive antibiotics[149]. One systematic review of 27 reports covering 317 adult patients from 8 countries found an overall cure rate of 92% for cases of CDI or pseudomembranous colitis, with 89% of patients being cured after a single infusion[150]. In all included studies, relapse (3.9%), adverse events (2.5%), or deaths (4.1%) were all rare, with no deaths being directly attributable to the FMT, which

showcases the high rate of success and low risk nature of this treatment. In a small study of children with IBD (median age 13y) receiving FMT to treat RCDI, 6 of 8 (25%) patients had not experienced a recurrence of CDI at 60 days follow-up, but a further 3 patients (37.5%) did develop a CDI between 60 days and 6 months post-FMT[151]. The authors noted that reasons for the low efficacy rate may have been disease severity coupled with the number of immunomodulatory drugs being used to treat the children. In these cases, regular use of FMT every 2-3 months may provide longer-lasting relief of symptoms. FMT for RCDI has also shown the ability to treat colonization by antibiotic resistant organisms and maintain this effect through a year-long follow-up period[152]. With the promising results shown for treatment of RCDI, clinicians are increasingly studying this therapy for use with other conditions as well. Other GI disorders include ulcerative colitis and Crohn's Disease, IBS, and chronic constipation, with other studies exploring its use in non-GI conditions such as Parkinson's disease, chronic fatigue syndrome, insulin resistance, and autism[146].

In the context of short bowel syndrome, FMT has already been used successfully in two patients with SBS to treat recurrent D-lactic acidosis[53,153], one a 15-year-old male and one a 7-year-old female. Lactic acid is produced in two enantiomeric forms, D-lactate and L-lactate, by many lactobiotic species, with not all species producing the same ratio of D:L forms[52]. L-lactate is a key metabolic intermediate in gut fermentation and cross-feeding food webs, as well as being an energy source for neurons, while D-lactate is a neurotoxic byproduct. Accumulation of D-lactate is caused by an overgrowth of the species that produce that enantiomer in higher quantities. The overgrowth can be due to lack of colonization resistance or simply due to excess oxygen exposure during surgery that favours the lactate-producers and harms the beneficial anaerobes. Symptoms of D-lactate intoxication include slurred speech, ataxia, confusion, fatigue, and other encephalopathies that may be irreversible if the condition persists long-term. The patients in question had both had

multiple episodes of severe acidosis in a 3 to 4-month span, and the male was experiencing abdominal discomfort even when other D-lactic acidosis symptoms were not present, in addition to lack of satiety after adequate oral nutrition intake. Both patients experienced no recurrence of acidosis after a single infusion of donor feces, experiencing improvement of all symptoms, and did not have a relapse within the follow-up periods (6 months for the male, 12 months for the female). While these patients were not a part of the vulnerable neonatal population, this nevertheless demonstrates that FMT can be used successfully in patients with SBS.

In addition to successfully resolving an issue of overgrowth of D-lactate producing bacteria, FMT could be successful at resolving total small intestinal bacterial overgrowth as well. SIBO, as described above, becomes an issue when beneficial species are eliminated by antibiotics or oxygen exposure, allowing the damaging aerobes and Proteobacteria to grow in their place. This leads to a breakdown of barrier function, increased liver damage leading to IFALD, and an increase in septic episodes[66]. Patients with SIBO often experience oral feeding intolerance and a delay in progression to weaning from PN[71]. FMT could eliminate these issues all at once, by replacing the dysbiotic microbiota with a healthy diversity and richness that restores colonization resistance, and necessary digestive functions like SCFA production[72].

1.3.1. Methods of Delivery

Given that FMT is still a relatively new form of medical treatment, a standard protocol has not yet been universally decided upon, though general sets of recommended guidelines have been put together by The European FMT (EFMT) Working Group[154] and by Health Canada[155]. These recommendations allow for some flexibility in administration according to the individual patient's needs and preferences. The first step is to obtain informed consent from the patient, and then after

selection of a donor, they should be screened for a wide range of viral, bacterial, and parasitic pathogens[155]. As many bacterial, viral, and parasitic diseases are transmitted through fecal material, this is essential and is discussed further below.

One of the great advantages of FMT as a treatment is that it is extremely flexible in how it must to be administered. Microbiota can be fresh, administered on the same day as collection after homogenization and filtration, or it can be frozen and stored until needed, then thawed and used in the same manner. One systematic review that looked at fresh vs. frozen usage found no significant difference between the modes of preparation[156]. This is supported by another study that found storage and preparation methods only minimally explained differences in human and canine fecal samples stored over an 8-week period[157], and a third study that found no difference in cure rates based on fresh (92%) versus frozen (90%) methods after one infusion[158]. Another available method for storage and delivery is to make the fecal extract into capsules[149], and this method has been found to be just as efficacious[156,159]. This option would provide convenience and flexibility in terms of the timeline of material collection before administration. Lastly, the option to lyophilize prepared stool for storage before reconstitution and use appears to have a significantly lowered success rate, compared with fresh or frozen, and should not be considered as a first-choice method[160].

The next main difference in FMT delivery is whether to choose a proximal or distal delivery route. Proximal options include nasogastric tube, upper endoscopy, or capsule, and distal routes include retention enema, colonoscopy, or sigmoidoscopy[149]. Oral routes tend to be easier on the patient and do not require sedation, but they also bear the risks of nausea, vomiting, and potential aspiration of feculent material that may make distal routes more attractive in the neonate. Distal routes may be easier to deliver, depending on the bowel location chosen, despite requiring

sedation and cause less distress or discomfort to infants. Khan, et al[156], found that there was no significant difference between upper versus lower delivery for frozen FMT when comparing two studies that looked at these comparisons in adults.

1.3.2. Factors Affecting Success

The main factor influencing the success of human biotherapy is survival of the microbiota through the preparation and storage of the donated material. The EFMT Working Group recommends that processing and storage of fecal material should be as quick as possible (within 6 hours) to protect anaerobes, and that anaerobic preparation conditions should be used if possible[154]; however, as none of the above studies[156,159,160] prepared the fecal material under anoxic conditions and yet still found high cure rates seems to suggest that this is not of utmost importance. General EFMT guidelines advise that when preparing material for freezing, up to 10% glycerol should be added to protect against cellular damage, and that -80 °C is preferable to -20 °C as the lower temperature ensures all digestive or degradative enzymes are inactive.

One of the first literature reports of fecal bacteriotherapy by Tvede and Rask-Madsen reported[161] that 5 patients were cured of RCDI after having been administered a synthetic bacterial microbiota. There were 6 patients studied in total; one was cured after fecal transplant, one received fecal transplant and did not experience remission but did after then receiving the bacterial transplant, and the remaining 5 were cured after a single enema with the synthetic mixture. The authors noted that success of the therapy was likely due to the restoration of the essential *Bacteroides* species which were absent in all patients before biotherapy because these species prevent colonization by pathogens and restore essential fatty acid production. A further study[162] has also shown that a synthetic microbiota comprised of 33 species also cures RCDI, and noted

the additional benefit of low risk of pathogen transmission and higher patient acceptance for aesthetic reasons.

In terms of composition of the transplant material, the bacterial species present may not be the only important aspect. Bacteriophages (phages) are often ignored when speaking of the microbiota, perhaps justifiably as they are not technically alive, but nevertheless they exhibit an important aspect of population control upon their bacterial hosts. Phages are currently under exploration as antibiotics and food preservatives[163], uses which are allowed due to their lack of ability to infect human cells. Phages have been implicated in the success of FMT to treat RCDI with sterile fecal filtrate[164], with another retrospective study finding that higher phage alpha diversity and lower abundance correlated with better success[165]. In the human microbiota, phages primarily exist as integrated prophages which may be awakened and stimulated by the use of antibiotics[165]. As RCDI patients have generally experienced frequent antibiotic use, it is intuitive that their phage content would be higher, which would then also enable the phages to target the desirable species, further perpetuating dysbiosis. Potential FMT donors are recommended to be excluded if they have had recent (<3 months) exposure to antibiotics, but phage content or diversity has not yet been considered an important factor in donor screening[154]. These unknown aspects warrant further investigations into the rise and fall of bacteriophage populations in stool after antibiotic treatments.

1.3.3. Cautions and risks

The largest and most pressing concern of treatments involving fecal or other biological material is the presence of pathogens or transmission of microbiota-associated diseases such as obesity[80], autoimmune conditions such as myocarditis[166] or rheumatoid arthritis, and others like

celiac disease and Type 1 Diabetes that have been associated with increased intestinal permeability[167]. A post-FMT patient's microbiota displays characteristics that are a mix of both donor and pre-FMT patient, with some previously undetectable species becoming detectable[168]. This would open an avenue for any potential low-abundance opportunistic pathogens that are not causing harm in the donor to expand and harm the recipient, as in the recent case of a patient who died after ingesting FMT capsules that contained a strain of extended-spectrum beta-lactamase (ESBL)-producing *E. coli*[169]. In this case, donors had been screened for suitability in terms of weight and medical history, however, at the time of the donation that contained the drug-resistant pathogen, donated stool was not itself tested for this specific organism. Such requirements came into effect upon US Food and Drug Administration recommendation, but previously donated and prepared biotherapy capsules were not retroactively tested.[154] This case is tragic and serves to emphasize the importance of extensively screening and testing both donors and their donations.

In addition to a broad range of general health inquiries, donor blood should be tested for a range of viruses (including cytomegalovirus, Epstein-Barr virus, hepatitis, and human immunodeficiency virus [HIV]), bacterial infections (syphilis), and parasites (*Entamoeba histolytica, Strongyloides stercoralis*), as well as overall indicators of health[154]. Health Canada also recommends testing donors for Creutzfeldt-Jakob disease and other prion-related diseases[155]. The donated fecal matter should be equally screened for presence of *C. difficile* and its toxins, *Vibrio cholera* and its toxin, and other bacterial pathogens with special attention for drug-resistant strains. Stool should also be tested for protozoan and helminthic pathogens, as well as fecal-borne viruses like Adenovirus and Rotavirus. Although the EFMT Working Group does not specifically mention coronaviruses (CoV), sudden acute respiratory syndrome (SARS-CoV) and Middle Eastern respiratory syndrome (MERS-CoV) have been demonstrated to shed viral particles in feces[170].

Given that SARS-CoV-2 has also been detected in fecal material of patients, regardless of severity of disease, for 6-10 days after infection and after pharyngeal swabs became negative[171], the most recent Health Canada guidelines[155] recommend that it is also prudent to test for this virus in fecal matter of potential donors.

Extensive screening of donor and donation still may not reduce risk of complication to zero in the case of patients with a history of inflammatory bowel disease (IBD). In one case, a patient with a history of ulcerative colitis (UC) that had been refractory for 20 years developed an RCDI, and upon receiving an FMT using material that had been screened from a healthy donor, he experienced a flare of his UC[172]. The UC responded well to treatment and went back into remission. In another IBD cohort given FMT for RCDI, 11 of 43 patients (25.6%) experienced an IBD flare, including two hospitalizations[173]. In this instance, the authors noted that the patients who experienced an IBD flare may have only been colonized with *C. difficile* and that the IBD flare may have been the true cause of their discomfort. Together, these cases provide supporting data that caution is warranted when considering FMT for use in patients with IBD, but taken in the broader context of the overall success rate of FMT for RCDI, these risks are likely manageable and should not be considered prohibitive. The experience gained from these studies indicates that FMT is overall a safe and well-tolerated therapy, and should be investigated for its use in the resolution of gut dysbiosis associated with short bowel syndrome.

1.4. Piglets as a Model

Studying human disease in an animal model is fraught with challenges that can hamper the external validity of the experiments. Species-related differences in development, anatomy, physiology, and immunology must be accounted or controlled for if one hopes to make useful

inferences and conclusions. Still, studying in humans is often more prohibitive, with ethical concerns and heterogeneity in the participant pool limiting fidelity. Short bowel syndrome's status as an orphan disease means that the population from which to draw participants is, by definition, extremely small, disparate in location, and often divergent in etiology, therefore animal models which seek to remedy these drawbacks are essential.

As SBS is primarily a condition involving loss of all or part of an organ, rather than one generally originating in malfunctioning cellular or biochemical processes (though those do play a part in the disease as well), a mammalian model organism that provides the best approximation of the human GI tract is more applicable than a cell culture or *in vitro* design. When choosing a model, the least complex with the greatest degree of homology to the organ or system being studied is often the best choice[174]. Non-human primates may be appropriate for the study of neurological or social sciences, but their sentience and capacity for self-determination means the moral cost of using them in experiments involving the potential for harm, and ultimately termination, is too high[175]. Rodent models are frequently used to approximate human gastrointestinal disease, as their small size, low maintenance costs, short reproductive cycle, homogenous genetic background, and multiparous nature make them attractive options[176]. There are, however, several drawbacks to using rodents to study human GI diseases or microbiota. Humans and rodents have vastly different body sizes and life spans, gestational terms, designation of premature birth, and intestinal maturity at full term[177,178]. Importantly, for the study of the microbiota, rodents have different eating styles than humans (meals vs. grazing) including nutrition tolerance at birth, and rodents engage in coprophagia, whereas humans do not[177]. Lastly, as our model of SBS requires major open abdominal surgery, neonatal rodents are disqualified from being used as they cannot tolerate such insult[177]. Despite their larger size and comparatively more expensive care, the gastrointestinal

morphology and physiology of pigs provides a high degree of homology with humans and makes them an appropriate model[176].

1.4.1. Gestation and development

Porcine and human embryological development is similar, including cellular origin of the gastrointestinal tissue, and gestational stage at which the GI tract begins to form[177]. The intestinal epithelial cells form in crypts and migrate outwards along the forming villi, achieving recognizable morphology to adult intestines at 49% gestation in piglets and 53% gestation in humans[177]. Whereas humans and other primates are viable from approximately 70% gestation, piglets are not generally viable until ~90% gestation, even with significant intervention[179]. In both humans and piglets, major developmental maturation of the GI tract occurs rapidly in response to fetal swallowing and intake of amniotic fluid, though piglets are born with a slightly less mature GI system than human infants[179]. This immaturity makes them more similar to the premature human infant, which is ideal for the study of SBS[177], as this is a disease with an incidence rate inversely proportional to gestational age. Taken together with rapid growth rates and weight gain postnatally, a longer gestational period than rodents, and large litters, piglets are well-placed to serve as an accelerated model for the study of human neonatal GI disorders.

1.4.2. Gastrointestinal physiology

Similarities between the porcine and human GI tract begin in the esophagus, as both species have submucosal glands along its length, followed by a completely glandular stomach[176]. Rodents share neither of these traits, with rats and mice having large aglandular portions of the stomach which may cause large differences in their digestion that potentially impact their microbiotas[180]. Distally to the stomach, pigs and humans both have a duodenum, jejunum, and ileum, though adult

pigs average between 15-22 m in total length of these sections. Humans make do sufficiently with only 5.5-7 m in total length, though this does work out to approximately the same length-to-bodyweight ratio (0.1) for both species[176]. Pigs have no gallbladder and have separate pancreatic and bile ducts, while humans employ a common bile duct[177]. At full term birth, piglets have 215-380 cm small intestine and 75-80 cm large intestine, while human infants have on average 200 cm small and 50 cm large intestine[177]. Once the ileocecal valve (ICV) is crossed, more differences become apparent; chiefly, pigs do not have an appendix and are possessed of a spiral-shaped colon, whereas humans do have appendices and have square-shaped colons[176,177]. Piglets have similar longitudinal intestinal muscles to humans, regardless of colon shape, which results in very similar transit times[176]. Notably, rats make poor models in this regard, as they can voluntarily alter their transit time in periods of low food availability through reclamation of nutrients lost in fecal matter, which reduces their fiber fermentation capacity and increases transit time[181]. Piglets have a very high rate of intestinal growth postnatally, doubling their length in just 10 days, whereas human infants take 2-3 years to perform the same feat[177].

In regard to appropriateness for studying neonatal NEC, the piglet is the only animal model to spontaneously develop NEC dependent on the same risk factors of premature birth, formula feeding, and microbiota disruption as human infants[182]. Azcarate-Peril and colleagues showed that piglet NEC was characterized by a dominance of Proteobacteria, an increase in diversity of ileal mucosal-associated bacteria, and an over-representation of three *Clostridium* species that were absent in piglets without NEC (*C. butyricum, C. proteolyticum*, and *C. neonatale*). A second study also found that neonatal piglet NEC developed spontaneously after premature birth and formula feeding, and was associated with bacterial overgrowth, high densities of *C. perfringens*, and that NEC was prevented in formula-fed piglets by keeping them germ free[183].

Post-surgically, adaptation after 75% small bowel mid-resection in the juvenile pig is characterized by bowel dilatation and increase in length, as well as increased villus surface area, when compared with animals who received a transection only[184]. This resected group also showed lowered protein, fat, and carbohydrate absorption than their transection-only peers. In neonatal piglets, those who received a 75% JI resection had significantly taller ileal villi and deeper crypts than non-surgical piglets, as well as a dilated small bowel[18], which is also seen in infants with jejunostomy[27]. Piglets with a JC remnant anatomy adapt poorly, evidenced by shorter villi and crypt depth, less weight gain, and more pronounced malabsorption, which is also reflective of human infants[13].

1.4.3. Porcine Immunology

The porcine immune system bears many similarities with humans, beginning with the expression of pathogen recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs), common indicators of non-self expressed by beneficial and harmful microbes. Pigs express TLRs1-10 that share between 73% - 85% homology with their human counterparts, with the tissue expression patterns and intracellular pathways also bearing many of the same adaptors and intermediates[185]. Swine also have a robust humoral complement system that has a high degree of polymorphism, suggesting an effective first line of pathogen defense[185,186]. The porcine repertoire of white blood cells is comprised of mostly monocytes, neutrophils, and lymphocytes, with eosinophils and basophils making up smaller fractions[187]. Piglets are born without maternal immunoglobulins due to the impermeable placental barrier and depend on receiving them through suckling and ingestion of colostrum after birth[188].

Despite the initial similarities in receptor and cell types, there are some differences between pigs and humans in how they respond to pathogen challenge. In humans, monocytes challenged with LPS or lipoteichoic acid (LTA) respond by upregulating expression of TLR-4, which amplifies the cycle of inflammation, whereas in pigs only LTA produces this response[189]. In humans, interleukin-4 (IL-4) is a key stimulatory cytokine in the Type II immune response against extracellular pathogens and production of antibodies, but in pigs it has an inhibitory effect on B cells[190]. IL-4 in pigs has been shown to suppress B cell activation and antibody production, as well as reducing the effects of other inflammatory cytokines IL-2 and IL-6, though it does also appear to stimulate monocytes to differentiate into dendritic cells[190]. This indicates a change in the way the porcine inflammatory response is initiated in response to bacterial infection, and might indicate a Type I intracellular immune response bias in the pig, meaning a greater disposition to severe disease in models of sepsis, but the full meaning of these differences is unclear at present.

1.4.4. Microbiota

The piglet microbiota is similar to the infant microbiota at birth in several respects. Firstly, the alpha diversity is generally lower than their older, weaned peers, and secondly, it is typically dominated by *Enterobacteriaceae*, with contributions from *Bacteroidaceae*, *Clostridiaceae, Lachnospiraceae,* and *Lactobacillaceae*[191]. Early life microbe exposure is important in overall piglet health, just as in humans, with piglets dependent on maternal humoral factors obtained from nursing to prevent pathogen establishment in their guts[192]. Sow milk typically contains different oligosaccharides with lower diversity than does human breast milk, but the total abundance of such oligosaccharides is comparable[193]. The structures present varies during the lactation and nursing period, and in doing so they modulate the piglet microbiota both taxonomically and functionally[193]. Diversity gradually increases day by day from birth to weaning, with no one day being significantly

different from the previous or following days[191]. Weaning generally happens around 21 days of age and is a pivotal moment in a young pig's development. The switch from sow milk to solid feeds results in swift changes in the dominant taxa that are visible within one week of weaning: *Enterobacteriaceae, Bacteroidaceae*, and *Clostridiaceae* are reduced and replaced by increased fractions of *Lactobacillaceae, Ruminococcaceae*, *Prevotellaceae*, and *Streptococcaceae*[191]. At this time, the makeup of the juvenile pig microbiota closely resembles a more adult-like distribution, as described in the colon and cecum of ~3.5-4-year-old Göttingen minipigs[194]. The process of microbiota stabilization into that resembling an adult's progresses much faster in pigs than in humans, which can take up to three years, despite humans generally transitioning to solid foods by 1 year of age[102].

In addition to the above similarities, young piglets are also vulnerable to dysbiosis, particularly around the time of weaning and the dramatic microbial shifts that accompany it. Diarrhea commonly occurs at weaning, possibly due in part to both the transient drop in nutritional intake as piglets adjust to solid food, and to the loss of maternal antibodies that prevent expansion of harmful species[192]. Piglets can be more or less susceptible to infection by enterotoxigenic *E. coli* (ETEC) diarrhea if the ETEC strain expresses a fimbriae gene that matches a receptor on piglet IECs[192], so host genetics play a role the development of piglet dysbiosis. In an SBS model, piglets that undergo a 75% proximal small intestinal resection have reduced diversity compared with both transected and non-operational piglets that is more pronounced at 6 weeks after surgery, compared with 2 weeks[195]. Phylum-level analysis shows a similar proportion of Firmicutes as a whole, with reduced levels of Bacteroidetes and Fusobacteria, compared with both sham and non-operational piglets. Within the Firmicutes group, however, resected pigs were dominated by gram-negative *Veillonellaceae* at 84% of OTUs, while non-operational piglets only have 10% of this family and

significantly higher Chao1 richness. All pigs in this study were fed the same elemental infant formula and given the same medication, to minimize the effect of those variables. In a model more similar to human infants that are not allowed enteral nutrition and experience multiple rounds of antibiotics, our work in SBS piglets shows that these two common aspects of intestinal failure have a greater effect on the piglet microbiota than surgery does alone[69]. Overall, these attributes of the piglet microbiota serve as supporting evidence that neonatal piglets make an excellent accelerated model to study the human infant microbiota.

1.4.5. FMT in pigs

Fecal transplant has been explored in pigs, primarily as a way to reduce diarrhea incidence without the use of antibiotics[196-200], and as an attempted method of increasing growth and feed-use efficiency[201,202]. One study[200] found lowered incidence of diarrhea was associated with higher relative proportions of *Lactobacillus spp*, lower *E. coli*, and increased SCFAs, but that this benefit was only conferred when using fecal donations from one species of pig (out of three tested). Another study[199] found that higher Bacteroidetes:Firmicutes ratio was associated with resistance to diarrhea induced by enterotoxigenic *E. coli* (ETEC), and that this resistance was transferrable via FMT with jejunal contents from non-diarrheal pigs. Diarrheal piglets had more *Lactococcus* (a Firmicutes member) and lower *Prevotella* (Bacteroidetes), while piglets who recovered from their symptoms had more *Lactobacillus* species than those that did not recover. A third study[196] investigated weaning stress-induced diarrhea and found that a specific secretory peptide, gassericin A, released by *L. gasseri* LA39 and *L. frumenti* associated closely with the IECs and enhanced fluid absorption, thus lowering diarrhea.

Use of FMT for enhanced growth performance in pigs has more mixed results. One study found that while certain Families were enriched in FMT-treated pigs (*Streptococcus, Clostridiaceae*) and growth performance improved, the overall composition of the microbiota was unchanged[202]. A second study found many compositional changes post-FMT, but this was associated with negative impacts on intestinal morphology, poor nutrient absorption, and a reduction in growth and feed-use efficiency[201]. A third study[198] found the opposite, that intestinal barrier function and growth were improved in recipient pigs, and was associated with increases in Firmicutes and decreases in inflammatory groups like *Proteobacteria, Fusobacteriaceae, Clostridiaceae, Veillonellaceae,* and *Escherichia*. This last study did also find a decrease in *Bacteroidaceae* and *Bacteroides*, members of the Bacteroidetes phylum typically thought of as desirable species, in improved piglets, indicating the complicated nature of microbiota studies and the difficulty in defining the ideal healthy microbiota.

Low or inconsistent response in these experiments could potentially be explained due to the highly heterogenous nature of the different treatment regimens. In the study by Wang and colleagues[202] that overall found no changes to the microbiota, 3-week-old pigs were gavaged with 3 mL of suspended fecal material on two consecutive days. McCormack and colleagues[201] found large changes to the microbiota, and gave 8 mL fecal suspension to newborn piglets (one group once, one group four times), and 100 mL to sows that had received a bowel preparation solution prior to transplant. In other studies, 10 mL was given multiple times and increased to 20 mL as the piglets aged[200], high and low doses were given in 2 mL aliquots multiple times[196], 20 mL was given every day for 6 days[199], and 1.5 mL was given every day for 11 days[198]. Interestingly, while few of these studies dosed the animals based on concentration of live bacterial cells, one that did[196] found that a lower dose of FMT conferred greater resistance to diarrhea than a dose 10-fold higher. These

highly heterogenous results indicate that amount of material infused, the composition of species within it, as well as the nature of both recipient and donor play a part in determining the efficacy of an FMT treatment in pigs. Hu and colleagues[203] have proposed a standardized procedure for porcine FMT that may help to bring consistency to future experiments and allow for easier comparison between studies.

1.5. Summary

Short bowel syndrome is a heterogenous disease that places enormous burden on our healthcare system, disproportionate to its incidence[204]. Not all costs are covered by the Canadian national healthcare system, as many families must travel for care and spend time away from work. Some costs are intangible, with losses to schooling, stress, and loss of life being impossible to quantify. Long-term dependence on parenteral nutrition due to intestinal failure brings with it an increase in risk of liver damage and of sepsis due to disruptions of the gut microbiota associated with the disease itself, and with the methods used to manage it. There are many ways of altering the microbiota by using probiotics and prebiotics that have met with some success, but these have been associated with an inhibition of post-surgical adaptation in some cases, and with an increase in sepsis in others. Fecal microbial transplant represents a unique opportunity to replace the entire dysbiotic microbiota with a healthy one in a single treatment, thereby potentially reducing chances of sepsis and increasing the ability to wean from parenteral nutrition. Our already established neonatal piglet model of short bowel syndrome is an ideal setting with which to test this theory.

Chapter Two: Rationale for Research

As sepsis and end-stage liver failure are now the leading cause of death in those with intestinal failure, we must focus on remediating the factors that lead to their development. Much work has already been done to optimize parenteral nutrition formulas and delivery as a key part of reducing intestinal failure-associated liver disease (IFALD). However, the etiology of this condition is not purely based on the ingredients in PN, as reduced intestinal barrier function and endotoxin from translocated bacteria has the potential to create an inflammatory state in the liver, which leads to fibrosis and cholestasis. A method that maintains the appropriate balance of beneficial species of bacteria in the gut in absence of oral nutrition, their traditional source of substrate, is essential to keeping the integrity of the mucosal layer and epithelial barrier intact, thus potentially reducing the occurrence of both sepsis and IFALD.

Beneficial species in the gut have the duties not only of filling space and crowding out an overgrowth of harmful species, but they are also vital to our gut homeostasis as they provide essential digestive functions. Food components that are otherwise indigestible by our own enzymes are broken down by members of the Firmicutes and Bacteroidetes phyla who then produce the essential short chain fatty acids like butyrate that are required for our gut integrity. When not provided with these components, the species quickly die out and need replacing, else the minor populations of opportunistic pathogens be allowed to expand in their place[205]. Probiotics have been explored in this context and have had some positive results, but certain species have been associated with reduced mucosal adaptation, and may not be appropriate in a vulnerable post-surgical neonatal population.

Fecal microbial transplant (FMT) has been eagerly explored as a therapy for recurrent *Clostridioides difficile* infections (RCDI), with success rates as high as 92%, and with minimal

adverse events[150]. FMT has also already been used to reverse colonization with antibiotic-resistant bacteria[152], and to treat D-lactic acidosis[53], a common complication of SBS due to overgrowth of D-lactate-producing bacteria. In light of these successes, we feel the time is right to investigate the applicability of FMT to treat dysbiosis and sepsis in short bowel syndrome, using our neonatal piglet model as a proxy for human infants.

2.1. Objectives

1. In a neonatal piglet model of short bowel syndrome, determine if fecal microbial transplant can be safely administered without increase in mortality.
2. Determine if a dysbiotic gut microbiota can be made to resemble the composition of a healthy Donor after a single infusion of feces.
3. Investigate if this treatment leads to a change in incidence of septic episodes.

2.2. Null Hypotheses

1. FMT administration will be associated with no change in overall mortality.
2. There will be no significant difference in the microbiota of piglets before and after FMT treatment, and there will be no difference in the microbiotas of FMT-treated or saline-treated piglets.
3. There will be no change in the incidence of sepsis based on FMT treatment.

We will be testing these hypotheses in our neonatal piglet model of short bowel syndrome as this model is already well-established to study both SBS[18,177] and the neonatal piglet

microbiota[69]. Neonatal piglets have a high degree of homology with human neonates in terms of gastrointestinal physiology and development, and in terms of microbiota establishment, progression, and composition. This research is a necessary step in finding novel therapies to combat the growing concern of sepsis and IFALD in a vulnerable neonatal population. Piglets provide a more genetically homogenous, faster-growing, and adaptable model that answers the issues inherent with orphan disease research in humans, and also provide a more ethically viable avenue of experimentation.

Chapter Three: Evaluation of fecal microbial transplant for the treatment of short bowel syndrome-associated dysbiosis and sepsis in neonatal piglets as a model for human infants

3.1. Introduction

Short bowel syndrome (SBS) is the most common cause of intestinal failure in both adults and children, and is classified as a loss of >75% of the intestinal length for age or height[4]. In children, necrotizing enterocolitis is the most common cause, while gastroschisis, atresia, and volvulus are also important contributors[13]. These congenital and acquired conditions result in massive resection of the large and small bowels, leading to a dependence upon parenteral nutrition (PN) for survival. While life-saving, PN also comes with the risk of developing IF-associated liver disease (IFALD) and catheter-associated sepsis, two complications that together are the causes of 80% of disease-specific mortality[4].

The exact pathways by which SBS leads to IFALD and sepsis are not currently known, however disruption of the gut microbiota presents a plausible avenue. A peri-surgical exposure to oxygen and a post-surgical lack of enteral nutrition lead to loss of essential fibre-fermenting Bacteroidetes species and an increase in aerotolerant Proteobacteria[49]. Use of antibiotics further disrupts the balance, allowing inflammatory species and opportunistic pathogens to overgrow. In the absence of their normal substrate, fiber fermenters die off and mucin-degraders devour the barrier that keeps them at bay. Once the mucus layer is depleted, remaining bacteria damage the thin epithelial layer and translocate to the bloodstream. After invasion, they release endotoxin which travels to the liver and produces inflammation and fibrosis[46,50].

A way to prevent this progression would be to restore the lost species so they can maintain their protective and beneficial functions. Fecal microbial transplant (FMT) has already been

approved by Health Canada[155] for use to treat recurrent *Clostridioides difficile* infections, has been used successfully to treat colonization with antibiotic resistant organisms[152], and twice to treat D-lactic acidosis due to SIBO in pediatric patients with SBS[53,153]. Other microbiota-affecting treatments have been investigated, including supplementation with beneficial organisms (probiotics), substrate for the organisms (prebiotics), or combinations of the two (synbiotics).

In some cases, probiotics have been associated with an increase in septic episodes[134,135,139], raising safety concerns for use in neonates. Therefore, the aim of this study was to investigate the use of FMT in a neonatal piglet model of SBS. We wanted to see if using FMT is safe in neonates, whether or not it can restore a healthy balance of bacteria to the gut, and if this also will lead to a change in the rate of sepsis.

3.2. Methods

Procedures used in this study received approval from the University of Alberta Care and Use Committee for Livestock and were carried out in adherence to the guidelines set forth by the Canadian Council of Animal Care, Ethics #AUP00000155.

3.2.1. Fecal donor selection

A healthy female gilt (unfarrowed female ~6 months old) was selected from the Swine Research and Technology Centre (SRTC) population for fecal donation based on optimum weight, absence of fever, presence of normal behaviours (including breathing, feeding, and excreting), and lack of wounds, contusions, or hematomas on the skin[203]. The gilt was moved to a separate, freshly cleaned pen for duration of the collection period. A second similarly screened healthy gilt was placed in the adjacent pen in order to reduce stress related to social isolation. Both gilts were

provided water *ad libitum* and fed standard chow according to the schedule of the SRTC. Neither gilt was in estrus at time of collection. Feces were collected upon defecation after visual examination to ensure no trampling had occurred and no urine or blood contamination. After collection, fecal samples were refrigerated and processed within two hours.

3.2.2. FMT solution preparation

The FMT preparation protocol was based on a previously published method[206]. In brief, a 1:2 (w/w) ratio of feces to sterile water was weighed into one side of a new stomacher blender bag (VWR, CA89085-572, pore size 0.33 mm), air was manually removed, and the bag sealed with a closure clip. The bag was manually agitated for 3 – 5 minutes to homogenize. Once all large clumps had been dispersed, the opposite side of the bag was cut open and the filtrate was poured into a new HDPE bottle (Fisher Scientific, 028962D), whereupon 10% (v/v) sterile glycerol was added. The bottle was sealed tightly, mixed, and then stored at -80 °C. Prior to administration, a portion was thawed overnight at 4 °C.

3.2.3. Animals and treatments

Neonatal male Duroc piglets, ages 3 – 7 days and weighing 2.0 – 2.8 kg, were selected from the SRTC and assigned to either saline control (SAL, n=12), fecal microbial transplant (FMT, n=12),or sow-fed control (SOW, n=6) treatment groups **(Fig. 1)**. Piglets were litter-blocked where possible to minimize inherent differences. Sow control piglets received the same pre-surgical antibiotics as treatment piglets but remained with their mother until termination to serve as the ideal piglet microbiome. Only male piglets were studied as female piglet anatomy makes it impossible to attach the fecal collection appliances used to acquire uncontaminated samples.

3.2.4. Surgical procedures and aftercare

SAL and FMT piglets underwent surgical intestinal resection as previously described[18]. Briefly, piglets were placed under general anesthesia and given buprenorphine (Chiron, Guelph, ON, Canada) for pain management. A 5-French (5F) central venous catheter was placed in the left external jugular vein for parenteral nutrition (PN) delivery. A midline laparotomy was performed, and the small intestinal length was carefully measured using a 3-0 silk suture along the anti-mesenteric border with minimal traction or stretching. Piglets underwent removal of 75% small intestinal length, including distal jejunum, entire ileum, ileocecal valve and 3cm of colon, and creation of a jejunocolic anastomosis. Tissue and digesta samples were taken immediately distal to transection for jejunum and immediately proximal for colon. A 10-F Stamm gastrostomy tube was inserted into the stomach for administration of enteral nutrition (EN), and finally, a stoma appliance (Hollister, Aurora, ON, Canada) was attached for collection of daily stool samples.

After surgery, piglets were kept according to in-house standard procedures[18]. Both surgical groups received a single dose of florfenicol (Intervet Canada Corp., Kirkland, QB, Canada), which has a 48-hour half-life, immediately post-surgery, and then no further antibiotics. Piglet weight gain was monitored daily and furosemide (Intervet Canada Corp. Kirkland, QB, Canada) was given to any that appeared edematous or had excessive weight gain for 2 consecutive days. Central lines were locked with a 4% tetrasodium ethylenediaminetetraacetic acid (T-EDTA) lock solution (KiteLock 4%, SterileCare, Markham, Ontario, Canada) daily for 2 hours to ensure patency[207].

3.2.5. Nutrition

All piglets were sow-fed until inclusion in the study, with sow control piglets remaining as above. Solid chow was not provided to any piglets in selected litters until after termination of all

study piglets from that litter. Surgical piglets were provided with PN immediately post-surgery using an in-house formula[18,208]. Initially PN was introduced at 50% nutritional requirements, advanced to 75% in the evening of day 0, maintained at 100% for day 1, and then reduced to 80% requirements from day 2 until termination in accordance with our standard procedures to reduce fluid overload[209]. EN was commenced on day 2 after FMT transplant (see below) at 20% nutritional requirements to stimulate adaptation as described[18,210].

3.2.6. FMT and fecal collections

On day 2, a 100mL portion of FMT solution that had been thawed overnight at 4 °C was delivered to FMT piglets **(Fig. 1)**. An equivalent 100mL portion of sterile saline was given to SAL piglets. Delivery was paced over 1 hour to prevent vomiting. One hour after FMT or saline was administered, EN was commenced, and bags were attached to the stoma appliance to collect the post-treatment stool samples. Daily stool samples were taken pre-treatment, post-treatment, and every morning through day 6, when possible unless piglets did not provide stool.

3.2.7. Termination and tissue collection

On day 7, piglets from all groups were placed under general anesthesia and a laparotomy was performed. Small intestinal length was measured in the same manner as initial surgery and portal blood was collected prior to humane euthanasia by intravenous injection of pentobarbital sodium (Schering, Pointe-Claire, Quebec, Canada). Digesta was taken from the proximal jejunum before the entire small intestine was removed, emptied, and weighed. Mucosa from the first 20cm distal to the ligament of Treitz was scraped and weighed, and jejunal tissue immediately distal to this location was collected. Digesta was also collected from the first 10cm of colon, as well as

colonic tissue immediately distal to the anastomosis. Mesenteric lymph nodes and splenic tissue were collected for potential future analyses.

3.2.8. Assessment of structural adaptation

Jejunal tissue was stored in formalin until it was embedded in paraffin, sectioned, and then stained with hematoxylin and eosin prior to histological assessment by a board-certified veterinary pathologist (P.N. Nation) who was blinded to treatment assignments as described[18]. Mucosal adaptation was measured via villus height and crypt depth using a micrometer eyepiece (Nikon Eclipse 80i; Nikon, Tokyo, Japan). Villi were measured longitudinally with crypts from the same area also being recorded. Ten duplicate measurements of height or depth were used to calculate the mean for each piglet. Upon histological examination, one piglet from the FMT group was found to have severely damaged jejunal epithelium, so villi and crypt measurements could not be taken (see 3.4.3.6).

3.2.9. DNA extraction and analysis

Tissue and stool samples were outsourced for genomic DNA extraction using DNeasy 96 PowerSoil Pro QIAcube HT Kit (Qiagen, Toronto, Ontario, Canada) to the Centre d'Expertise et de Service Génome Québec (Montréal, Québec, Canada). Homogenization was done using the TissueLyser II method with PowerBead Pro Plates (Qiagen, Toronto, Ontario, Canada). Microbiota composition was characterized by 16S DNA tag sequencing using the MiSeq Illumina PE300 technology (paired-end), targeting the V3-V4 regions with forward primer 341F and reverse primer 805R.

3.2.10. Bioinformatics

Bases with a quality score (Q) smaller than 20 were trimmed, and only reads that were at least 150 bp in length were kept for further analyses. The QIIME2 pipeline was used for analysis of 16S libraries[211]. Sequences were denoised using the DADA2 pipeline[212], using a truncated length of 250 bp at each end. Multiple alignments and generation of a phylogenetic tree were conducted with the pipeline align-to-tree-mafft-fasttree (mafft alignment, and fasttree phylogeny). Principal coordinated analysis and estimations of beta diversity were conducted with the pipeline core-metrics-phylogenetic using a sampling depth of 5000 sequences. Alpha diversity estimations were derived with the alpha-group-significance pipeline. Rarefaction curves were produced using a sampling depth of 4000 sequences. For taxonomic classification, aligned sequences in the greengenes database[213] clustered at 99% identity were used to crop the V3-V4 regions with primers forward: CCTACGGGNGGCWGCAG and reverse: GACTACHVGGGTATCTAATCC. A Machine learning classifier was trained to assign taxonomy to the denoised representative sequences.

The Phyla and Families selected for further investigation and statistical analyses were those that aligned with previously published short bowel piglet microbiota data[195], and those in which non-zero results were consistently observed across samples.

3.3. Statistics

Data are reported as mean (standard deviation). For biometric characteristics, all characteristics were found to approximate a normal distribution using the Shapiro-Wilk test[215], and student t-tests or one-way ANOVA with post-hoc Tukey HSD were performed using SPSS for Windows (version 26; SPSS Inc, and IBM Company, Chicago, IL, USA). For comparison of

microbiota composition, independent samples Mann-Whitney U test was performed in the case of two groups being compared, and where more than two groups were compared, the independent samples Kruskal-Wallis test was reported with Bonferroni correction for multiple comparisons. For baseline to termination comparisons within groups, the related samples Wilcoxon signed rank test was performed. Diversity measures were tested using permanova or independent samples Kruskal-Wallis as noted. An alpha value of <0.05 was considered significant in all cases. Samples taken and included in the analyses are named: jejunum base (JB) and termination (JT), colon base (CB) and termination (CT) tissue samples, and pre-treatment (pre), post-treatment (post), and days 3 through 6 (d3 – d6) daily stool samples.

3.3.1. Power Estimations

In order to detect a relative mean composition difference of 5% in bacterial abundances at the given alpha level using a Wilcoxon or Mann-Whitney test with two independent sample groups (FMT and SAL), assuming standard deviations (SD) of 3 in each group, the effect size measured by Cohen's D is 1.67, required sample size per group is 6, and actual power is 83%. If the relative mean composition difference is 10% and SD is 3, then Cohen's D is 3.33, required sample size is 3, and power is 94%. If an SD of 5 is assumed, then for a relative mean difference of 5%, Cohen's D is 1.0, required sample size per group is 14, and power is 81%. For a relative mean difference of 10% and SD of 5, Cohen's D is 2.0, required sample size is 5, and power is 88%. For related samples tests (ie. comparisons within a single group) and the Cohen's D calculations as above, an effect size of 1.67 requires a total sample size of 8 and provides 97% power, effect size of 2.0 requires total n=6 and has 96% power, and an effect size of 3.33 requires total n=4 and provides 98% power. With our final group numbers of SAL (n=11), FMT (n=11), and SOW (n=6), we have more than satisfied the requirements for adequate statistical power to reduce the chance of Type I

and II errors. True estimations of power for the Kruskal-Wallis test are complex to the point of being prohibitive to perform[216,217]; However, a generic F-test with degrees of freedom (df) = 27, an alpha value of 0.05, and a critical test statistic of 3.3541 will have 80% power to detect differences between groups. All a priori power calculations performed using G*Power 3.1.9.7[218].

3.4. Results

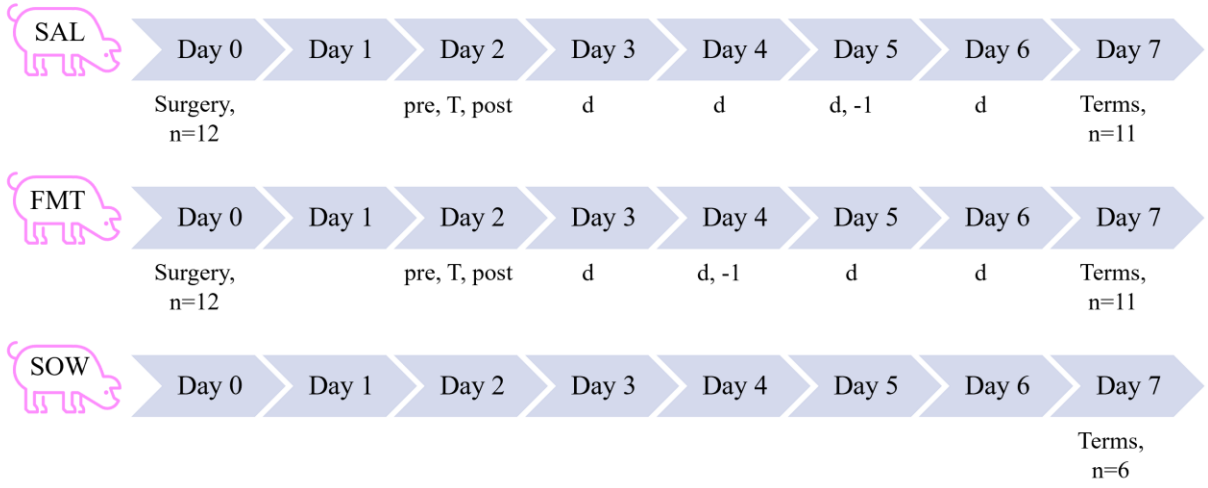


Figure 1 – Treatment Flowchart. Shown above is the outline of experimental events for each treatment group. Male piglets, 3-7 days old, were assigned to one of these treatments with allocation concealment: saline (SAL, n=12), fecal microbial transplant (FMT, n=12), or non-surgical sow-reared control (SOW, n=6). SAL and FMT piglets underwent 75% proximal jejunocolic short bowel surgery on day 0, during which baseline tissue samples were obtained. Pre-treatment stool samples were collected day 2 (pre) prior to treatment (T) with either 100mL sterile saline or 100mL FMT material, as appropriate. Post-treatment stool samples (post) were then collected. Daily stool samples were collected on days 3 – 6, as indicated (d). Termination surgeries were performed on day 7, during which tissue samples were collected. One piglet each group were removed from the study early as indicated (-1): from SAL due to a broken gastrostomy tube, and from FMT due to bowel obstruction. One SOW piglet was selected of the remaining piglets in each litter from which treatment piglets were taken, except for one litter as noted in the text. Final group numbers are SAL n=11, FMT n=11, and SOW n=6.

3.4.1. Clinical outcomes

FMT and SAL groups had a target sample size of 12, with one piglet being excluded from FMT on day 4 due to bowel obstruction leading to humane euthanasia, and one piglet being excluded from SAL on day 5 due to a broken gastrostomy tube that prevented delivery of enteral nutrition. Final group numbers were thus FMT (n=11), SAL (n=11) **(Fig. 1)**. Sow-fed control (SOW) target was one per litter and this was achieved for all except for the first, due to the litter contracting scours (bacterial diarrhea) after the first two treatment piglets were selected (see 3.4.3.6). Final SOW inclusions were n=6. One piglet from the FMT group was suspected of sepsis on day 4 due to slightly elevated ear canal temperature (40.6 °C, normal range 39.5 °C – 40.5 °C) and one incidence of vomiting, but piglet recovered completely by day 5 and remained in the study (see 3.4.3.6).

3.4.2. Biometric data

At initial surgery, treatment piglets were equivalent in age, weight, baseline short bowel length, and resected remnant short bowel length **(Table 1)**. At termination, there were no significant differences between age, weight, short bowel length or weight, mucosal weight, or mean villus height or crypt depth between SAL and FMT piglets. SOW piglets were on average about a day older than treatment piglets at termination, but this was not statistically different. SOW piglets had greater weight, short bowel length, and short bowel weight compared to both FMT and SAL piglets. SOW mucosal weight and mean villus height were greater compared to FMT but not SAL piglets; however, SOW piglets had significantly shorter crypt depth compared to both treatment groups.

Table 1 – Biometric Characteristics

	SAL n=11	FMT n=11	SOW n=6	p-value
Characteristic at initial surgery				
Age (d)	4.9 (1.0)	4.9 (1.1)	-	1.00
Weight (kg)	2.4 (0.2)	2.4 (0.3)	-	0.85
Baseline SBL (cm)	583 (50.7)	557 (64.1)	-	0.31
Resected SBL (cm)	145 (12.7)	139 (15.8)	-	0.32
Characteristic at termination				
Age (d)	11.9 (1.0)	11.9 (1.1)	13.2 (1.0)	0.06
Weight (kg)	3.5 (0.4)[a]	3.4 (0.5)[a]	4.8 (0.6)[b]	<0.001
Weight Gain (kg)	1.1 (0.3)	1.0 (0.3)	-	0.58
Termination SBL (cm)	145 (15.7)[a]	142 (17.4)[a]	892 (103)[b]	<0.001
SB weight (g)	26.4 (5.3)[a]	24.6 (5.0)[a]	177 (21)[b]	<0.001
SB weight (g/kg)	7.7 (1.4)[a]	7.2 (1.0)[a]	36.8 (2.8)[b]	<0.001
Mucosal weight (g)	1.98 (0.40)[a,b]	1.78 (0.35)[a]	2.38 (0.36)[b]	0.015
Villus height (mm)	0.63 (0.16)[a,b]	0.56 (0.14)[a,#]	0.81 (0.14)[b]	0.009
Crypt depth (mm)	0.16 (0.02)[a]	0.16 (0.02)[a,#]	0.13 (0.02)[b]	0.008

Data are presented as mean (SD), comparisons used student's t-test for SAL-FMT, and one-way ANOVA for SAL-FMT-SOW comparisons. Superscripts (a,b) indicate Tukey post hoc differences, # indicates n=10 as noted in the text. Mucosal weight was measured per 20 cm length of jejunum. SBL=short bowel length, SB=short bowel, SAL=saline, FMT=fecal microbial transplant, SOW=sow-fed control. All piglets were kept for 7 days.

3.4.3. Microbiota

Results were analyzed to investigate differences based on litter, and by which batch of FMT material was administered. This experiment was performed 3 times in groups of 10 piglets, with FMT piglets from each round receiving doses from the same FMT batch, resulting in 3 different batches being used. There were no measurable statistical differences detected based either on litter or FMT batch, and so all comparisons were made between treatment groups alone.

3.4.3.1. Phylum-level differences in mucosal-associated bacteria

At baseline and termination, mucosal-associated bacteria in the jejunum and colon were dominated by Bacteroidetes, Firmicutes, and Proteobacteria, with Actinobacteria and Fusobacteria contributing other major proportions **(Fig. 2)**. Within SAL piglets, Bacteroidetes significantly increased between base and term in the jejunum ($p<0.05$), but significantly decreased in the colon ($p<0.05$), while Proteobacteria also significantly increased in the colon ($p<0.01$) **(Fig. 3A, Table S1)**. Within FMT piglets, Bacteroidetes significantly increased in the jejunum ($p<0.01$) and significantly decreased in the colon ($p<0.05$), Fusobacteria increased in the jejunum ($p<0.05$), and Proteobacteria significantly increased in the colon ($p<0.01$) **(Fig. 3B, Table S1).** There were no significant differences between SAL and FMT mucosal-associated bacteria at baseline or termination **(Table S1)**. In comparing SOW to surgical piglets, SOW had significantly more Bacteroidetes (SAL $p<0.05$; FMT $p<0.05$) and significantly less Proteobacteria (SAL $p<0.05$; FMT $p<0.01$) **(Fig. 3C)**.

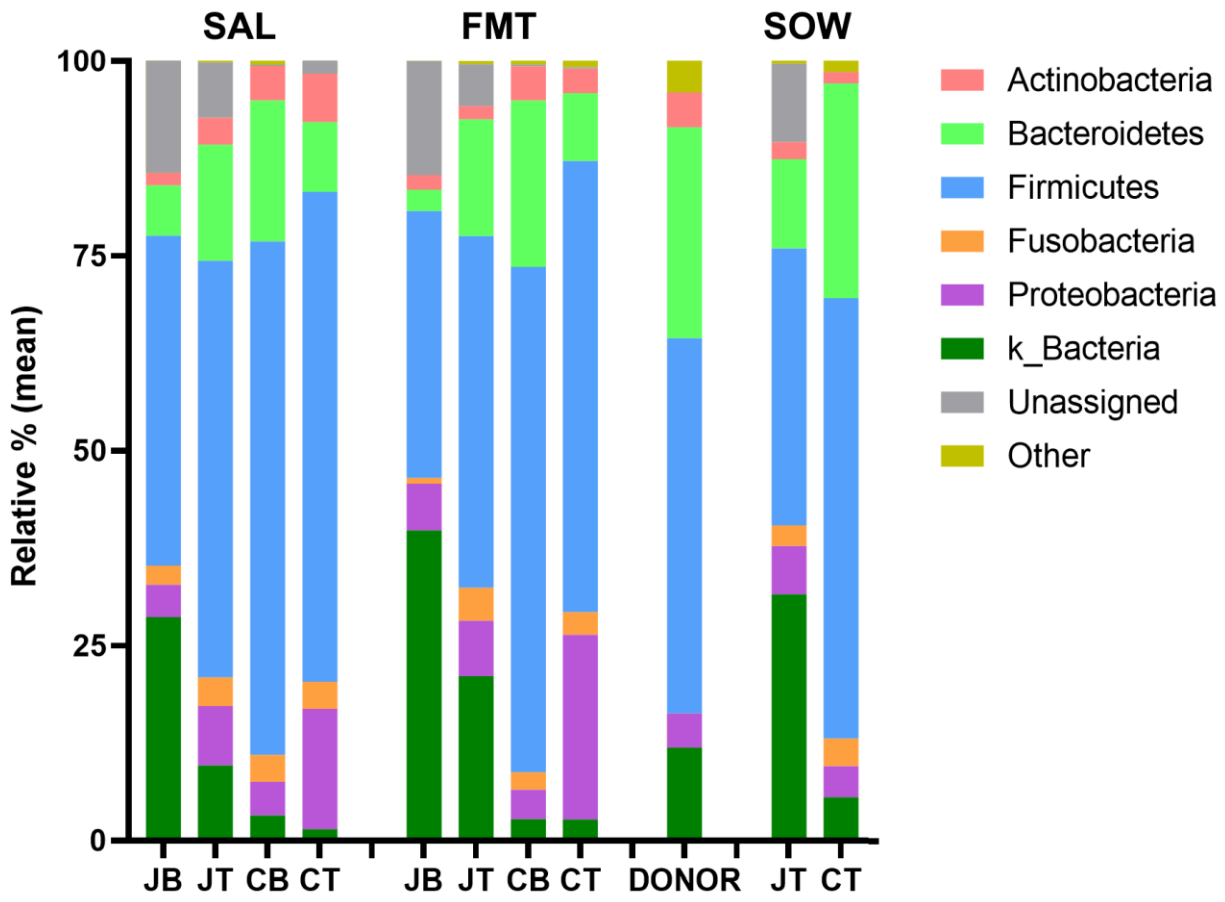


Figure 2 – Phylum level microbiota comparisons from base and term tissue samples. Neonatal short bowel piglets given fecal microbial transplants (FMT) experience limited changes in their intestinal microflora at the phylum level of classification. Results are given as relative % (mean) composition. Phyla of mucosa-associated bacteria in jejunum (J) and colon (C) tissue samples taken at baseline (B) and termination (T) surgeries, from saline (SAL, n=11) and fecal microbial transplant (FMT, n=11) treated piglets, compared to FMT material from the Donor (n=3). JB = jejunum base, JT = jejunum term, CB = colon base, CT = colon term. See full quantification of phyla proportions in Tables S1.

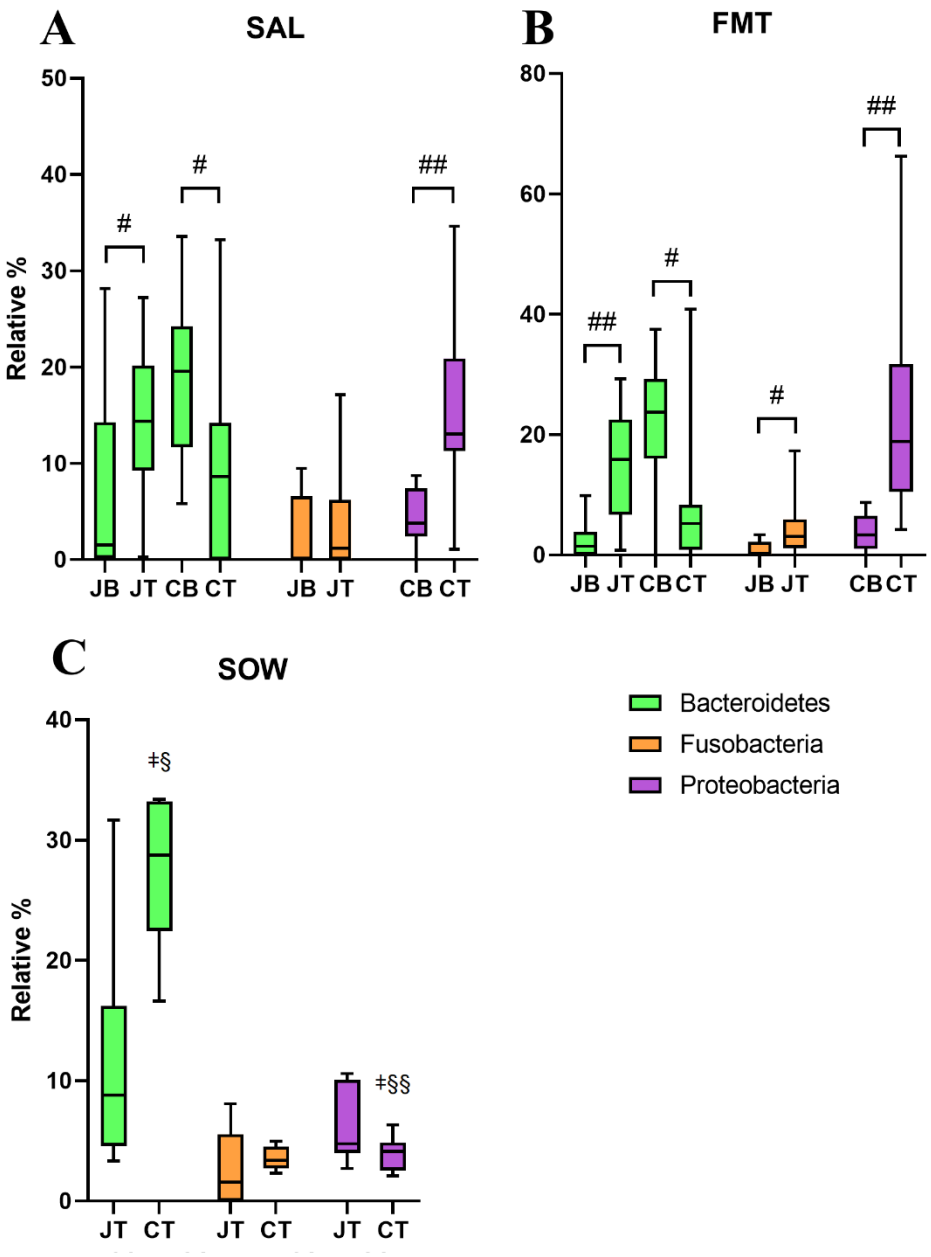


Figure 3 – Significant differences in base to term comparisons of phyla from SAL, FMT, and SOW piglets. Shown above are statistically significant differences between phyla (Fig. 2) of samples taken from saline (SAL, n=11), fecal microbial transplant (FMT, n=11), and non-surgical sow-reared (SOW, n=6) piglets. See full quantification of significant and non-significant results in Tables S1. Results are displayed as relative % composition. Base (B) to term (T) comparisons of jejunum (J) and colon (C) tissue samples, within SAL piglets **(A)** and FMT piglets **(B)**. Comparisons made using related samples Wilcoxon Signed Rank tests, #p<0.05, ##p<0.01. **C)** Base and term tissue sample significant differences between SOW and SAL (ǂp<0.05, ǂǂp<0.01) and SOW and FMT (§p<0.05, §§p<0.01) phyla. Comparisons made using independent samples Kruskal-Wallis tests. Boxplots show median and interquartile range with whiskers showing min and max values. JB = jejunum base, JT = jejunum term, CB = colon base, CT = colon term.

3.4.3.2. Phylum analysis of daily stool samples

Daily stool samples were likewise dominated by Bacteroidetes, Firmicutes, and Proteobacteria, with limited proportions of Actinobacteria or Fusobacteria **(Fig. 4)**. FMT piglets had significantly increased Actinobacteria compared with SAL piglets in their post-treatment stool sample ($p<0.01$) and in their d3 stool sample ($p<0.05$) **(Fig. 5A, Table S2)**. SAL piglets had more Proteobacteria than FMT piglets in their d4 sample ($p=0.051$), but this was of marginal statistical significance.

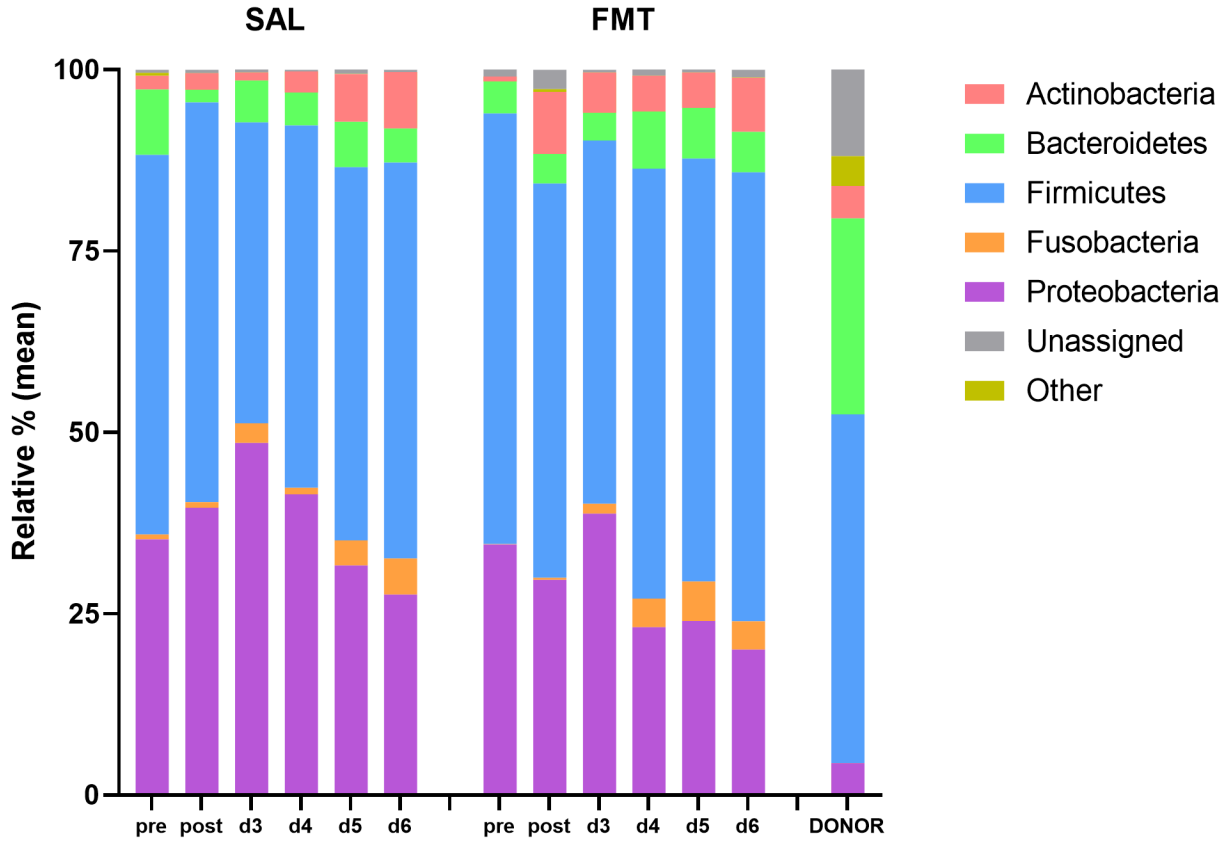


Figure 4 – Phylum level microbiota comparisons from daily stool samples. Neonatal short bowel piglets given fecal microbial transplants (FMT) experience limited transient changes in their intestinal microflora at the phylum level of classification. Results are given as relative % (mean) composition. Phyla of daily stool samples from saline (SAL, n=11) and fecal microbial transplant (FMT, n=11) treated piglets beginning day 2 pre-treatment (pre), day 2 post-treatment (post), and days 3 – 6 (d3 – d6), compared with Donor FMT material (n=3). See full quantification of phyla proportions in Tables S2.

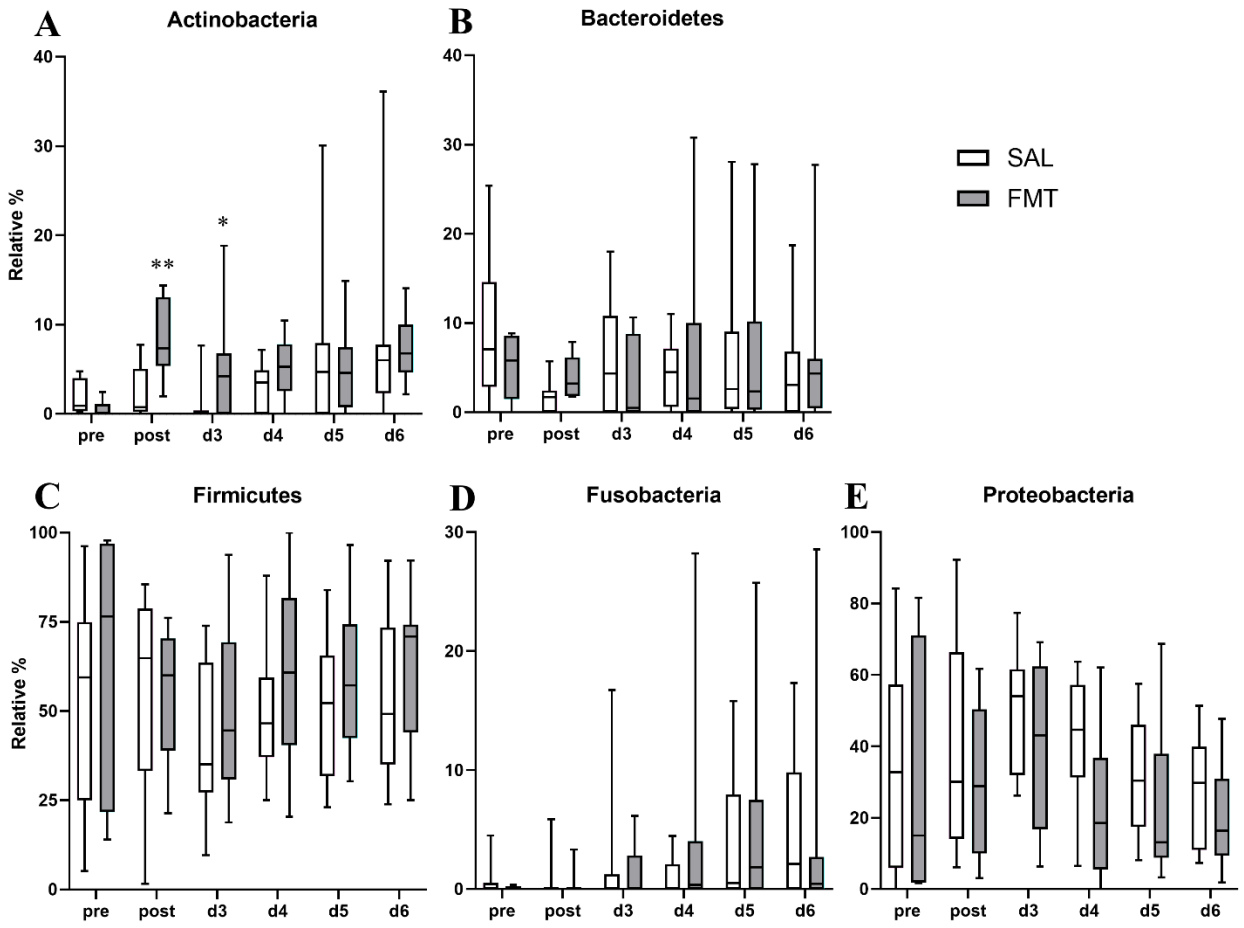


Figure 5 – Statistical comparisons of Phylum level differences in daily stools. Shown above are statistically significant differences between phyla (Fig. 2) of samples taken from saline (SAL, n=11), and fecal microbial transplant (FMT, n=11). See full quantification of significant and non-significant results in Tables S2. Results are displayed as relative % composition. Comparisons made using independent samples Mann-Whitney U test, *p<0.05, **p<0.01.

3.4.3.3. Family-level differences in mucosal-associated bacteria

At baseline and termination, the main Families represented in the mucosal-associated bacteria were the Firmicutes *Lactobacillaceae, Clostridiaceae*, and *Lachnospiraceae* **(Fig. 6, Table S3)**. Within SAL piglets, *Bacteroidaceae* ($p<0.05$) and *Lachnospiraceae* ($p<0.01$) increased significantly in the jejunum **(Fig 7A)**, *Streptococcaceae* ($p<0.05$), *Peptostreptococcaceae* ($p<0.05$) and *Ruminococcaceae* ($p<0.01$) decreased in the colon, and *Campylobacteraceae*

($p<0.05$) and *Enterobacteriaceae* ($p<0.01$) increased in the colon **(Fig. 7D)**. Within FMT piglets, *Lachnospiraceae* ($p<0.05$), *Peptostreptococcaceae* ($p<0.05$), *Ruminococcaceae* ($p<0.05$), and *Fusobacteriaceae* ($p<0.05$) all increased in the jejunum **(Fig. 7B)**, *Lachnospiraceae* ($p<0.05$), *Peptostreptococcaceae* ($p<0.05$), *Ruminococcaceae* ($p<0.05$), and *Erysipelotrichaceae* ($p<0.05$) all decreased in the colon, and *Veillonellaceae* ($p<0.05$), *Campylobacteraceae* ($p<0.05$) and *Enterobacteriaceae* ($p<0.01$) increased in the colon **(Fig. 7E)**. FMT piglets had significantly more *Veillonellaceae* in the colon ($p<0.01$) at termination than SAL piglets **(Table S3)**. SOW piglet colons had more *Peptostreptococcaceae* (SAL $p<0.01$; FMT $p<0.01$) and *Ruminococcaceae* (SAL $p<0.01$; FMT $p<0.01$) but less *Enterobacteriaceae* than SAL piglets ($p<0.01$) **(Fig. 7C, Table S3)**.

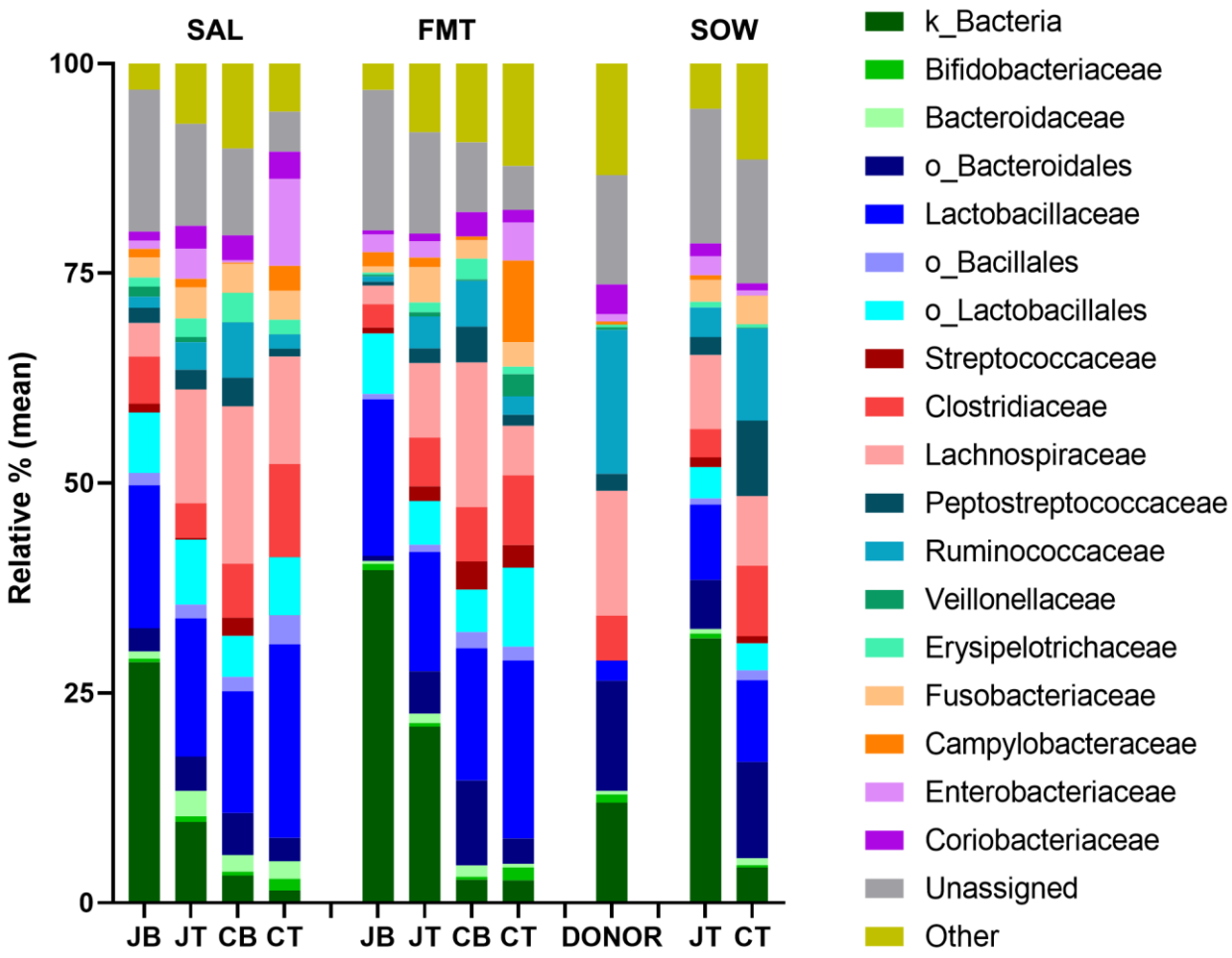


Figure 6 - Family level microbiota comparisons from baseline and termination tissue samples. Neonatal short bowel piglets given fecal microbial transplants (FMT) experience only transient changes in their intestinal microflora by Family. **A)** Family taxonomy of mucosa-associated bacteria in jejunum (J) and colon (C) tissue samples taken at baseline (B) and termination (T) surgeries, from saline (SAL, n=11) and fecal microbial transplant (FMT, n=11) treated piglets, compared to FMT material from the Donor (n=3). JB = jejunum base, JT = jejunum term, CB = colon base, CT = colon term. See full quantification of phyla proportions in Table S3.

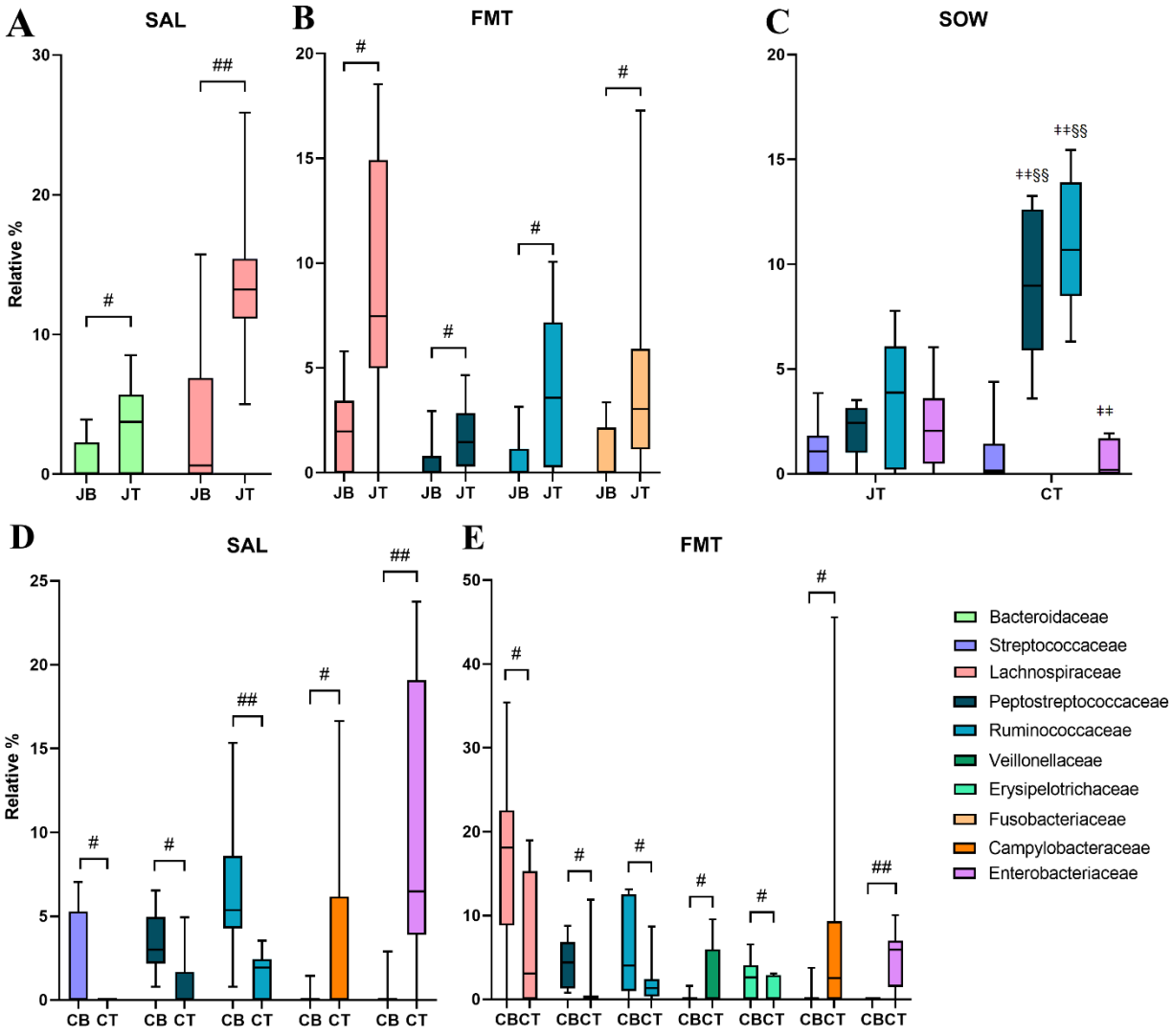


Figure 7 - Statistical comparisons between baseline and termination of Family level differences. Shown above are statistically significant differences between base and term tissue samples (Fig. 5) taken from saline (SAL, n=11), fecal microbial transplant (FMT, n=11), and non-surgical sow-reared control (SOW, n=6) piglets. See full quantification of significant and non-significant results in Table S3. All results are displayed as relative % composition. Significant differences within jejunum tissue for **A)** SAL and **B)** FMT piglets, and within colon tissue for **D)** SAL and **E)** FMT piglets. Comparisons made between base and term using related samples Wilcoxon Signed Rank tests, #p<0.05, ##p<0.01. **C)** Base and term tissue sample significant differences between SOW and SAL (ǂp<0.05, ǂǂp<0.01), SOW and FMT (§p<0.05, §§p<0.01), and SAL and FMT (*p<0.05, **p<0.01) phyla made using independent samples Kruskal-Wallis tests. Boxplots show median and interquartile range with whiskers showing min and max values. JB = jejunum base, JT = jejunum term, CB = colon base, CT = colon term.

3.4.3.4. Family analysis of daily stool samples

Daily stool samples from SAL and FMT piglets were mostly comprised of *Lactobacillaceae* and *Enterobacteriaceae* **(Fig. 8)**. In the post-treatment stool sample, FMT piglets had more *Bifidobacteriaceae* ($p<0.01$), *Lactobacillaceae* ($p<0.05$), *Clostridiaceae* ($p<0.01$), *Peptostreptococcaceae* ($p<0.01$), and *Coriobacteriaceae* ($p<0.01$) than SAL piglets **(Fig 9)**. By day 3, the FMT stool samples had more *Bifidobacteriaceae* ($p<0.05$), *Lactobacillaceae* ($p<0.05$), *Streptococcaceae* ($p<0.05$), *Erysipelotrichaceae* ($p<0.05$) and *Coriobacteriaceae* ($p<0.05$); However, by day 4, the only significant difference was in *Enterobacteriaceae*, of which SAL piglets had more than FMT ($p<0.05$). There were no significant differences on days 5 or 6 **(Table S4)**.

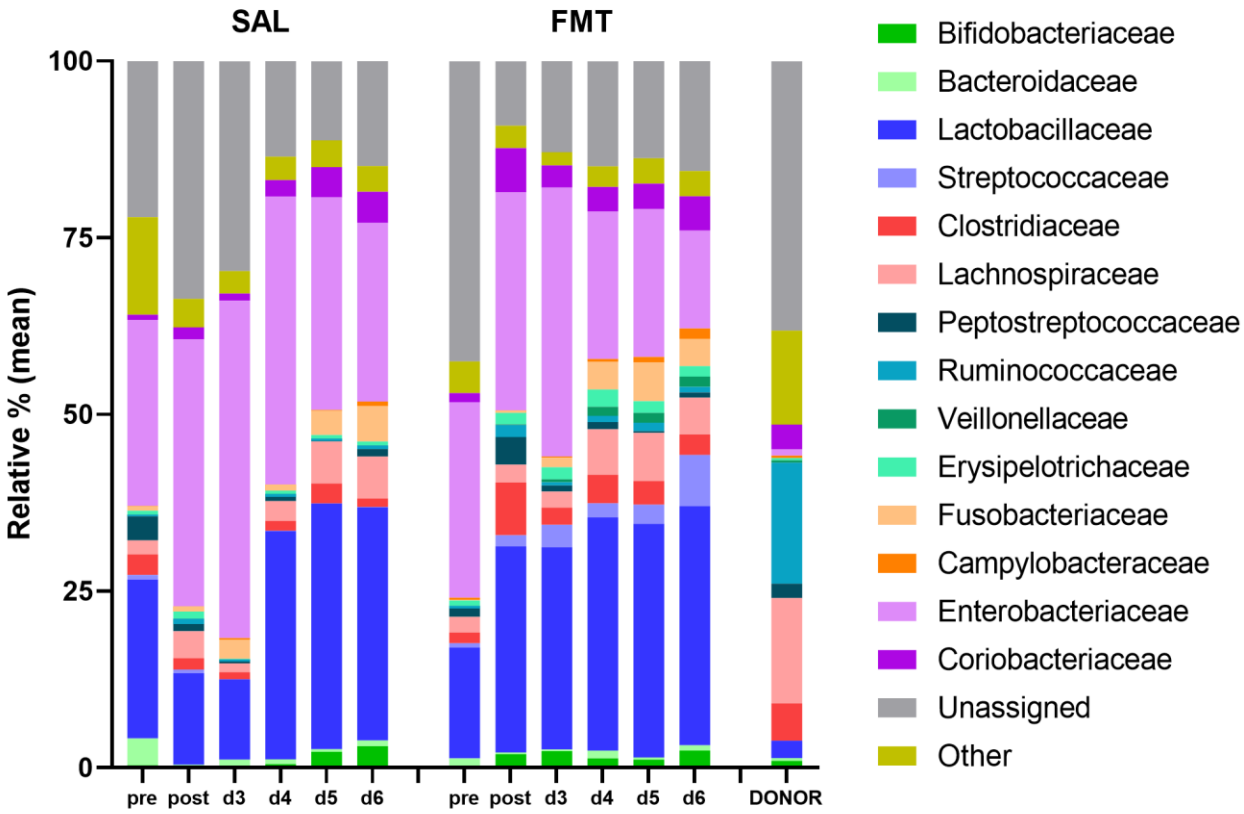


Figure 8 - Family level microbiota comparisons from daily stool samples. Neonatal short bowel piglets given fecal microbial transplants (FMT) experience only transient changes in their intestinal microflora by Family. Family taxonomy of daily stool samples from saline (SAL, n=11) and fecal microbial transplant (FMT, n=11) treated piglets beginning day 2 pre-treatment (pre), day 2 post-treatment (post), and days 3 – 6 (d3 – d6), compared with Donor FMT material. See full quantification of phyla proportions in Table S4.

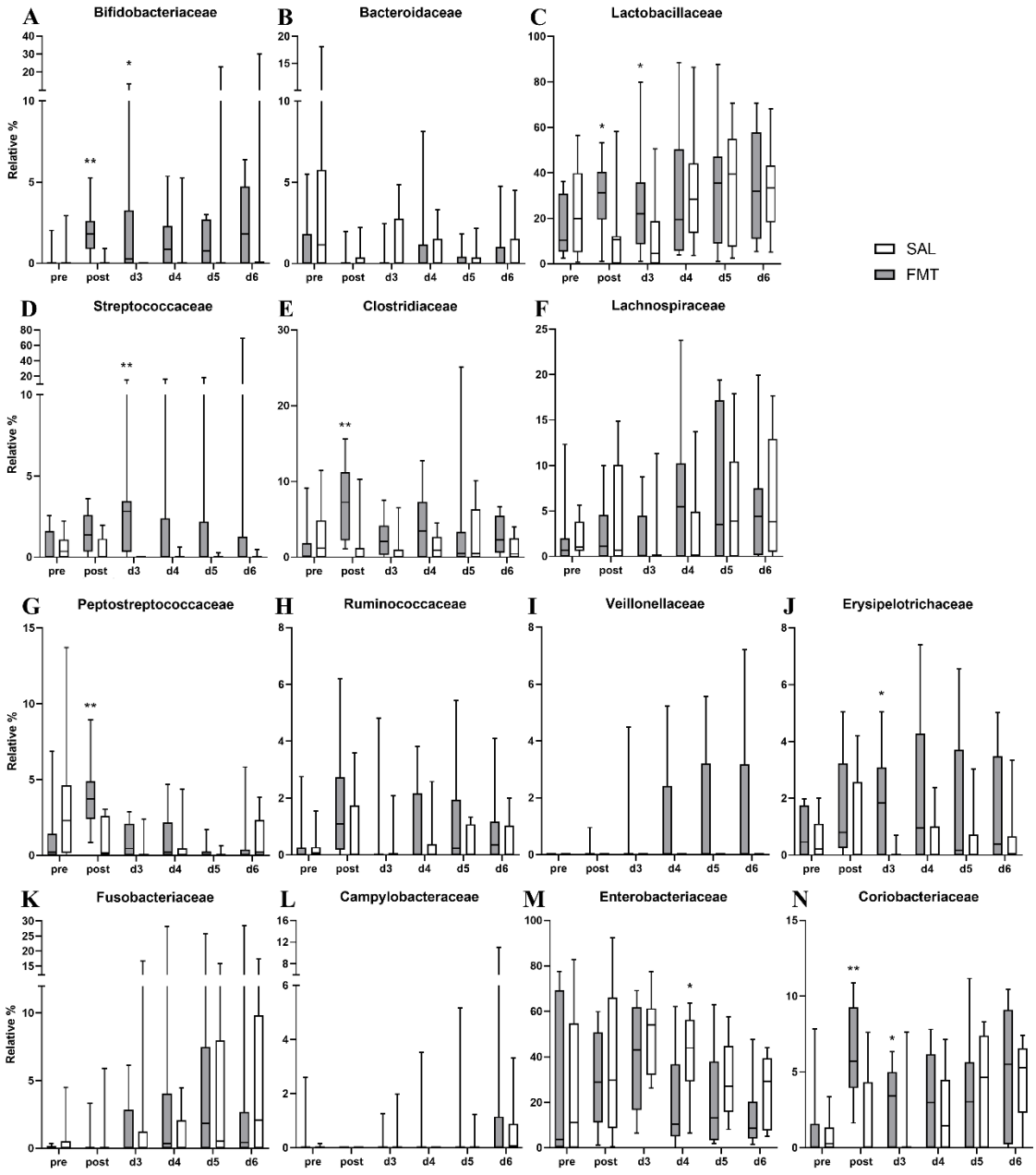


Figure 9 – Statistical comparisons of Family level differences in daily stool samples. Shown above are comparisons between family-level analyses of daily stool samples taken from saline (SAL) and fecal transplant (FMT)-treated piglets. See full quantification in Table S4. All results are displayed as relative % composition. Comparisons made using independent samples Mann-Whitney U test, *$p<0.05$, **$p<0.01$. Samples were taken day 2 pre-treatment (pre), day 2 post-treatment (post), and days 3 – 6 (d3 – d6).

3.4.3.5. Piglet daily stools compared with Donor

After fecal transplant on day 2, FMT-treated piglets had no difference in Actinobacteria than Donor material in their post-treatment stool samples, but did have significantly more compared to SAL piglets ($p<0.01$) and compared to their own pre-treatment samples ($p<0.05$) **(Fig. 10A)**. These differences in Actinobacteria did not persist to day 3 or beyond. FMT-treated piglets had significantly less Bacteroidetes than Donor material on day 3 ($p<0.01$), day 5 ($p<0.05$), and day 6 ($p<0.05$), while SAL piglets had less post-treatment ($p<0.01$), on day 3 ($p<0.05$), and day 6 ($p<0.05$) **(Fig. 10B)**. Bacteroidetes were not different from pre- to post-treatment in either FMT or SAL. There were no significant differences in Firmicutes between the three groups **(Fig. 10C)**, but neither was there a difference between pre- and post-treatment for either SAL or FMT. Fusobacteria **(Fig. 10D)** had no significant differences between FMT and SAL on any day, however both groups experienced a steady day-to-day increase. There was no Fusobacteria at all present in the Donor material **(Table S5)**. Proteobacteria was not different between FMT and SAL piglets **(Fig. 10 E)**, and while FMT piglets had more than Donor material, only SAL piglets were significantly higher on days 3 to 6 (all $p<0.05$) **(Fig. 10E)**.

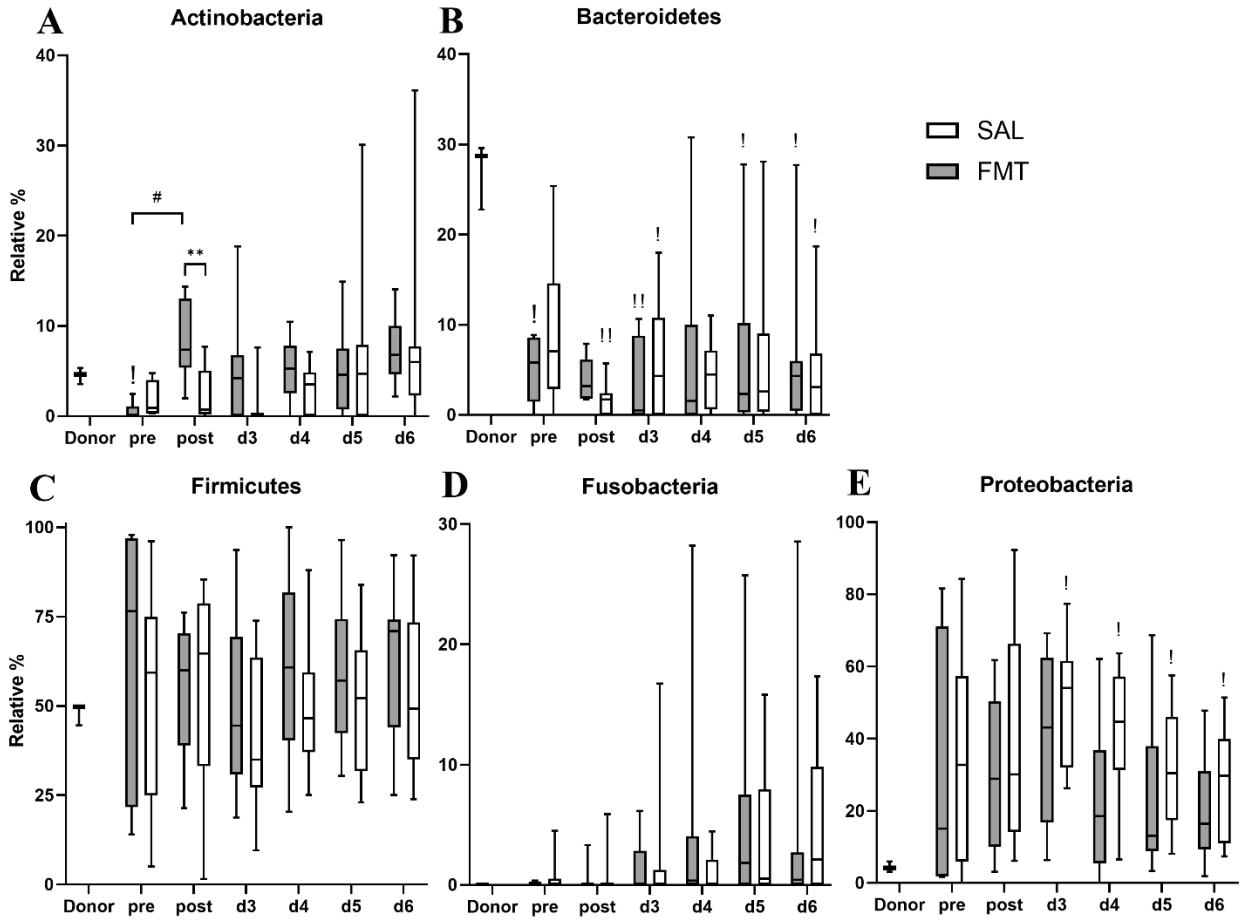
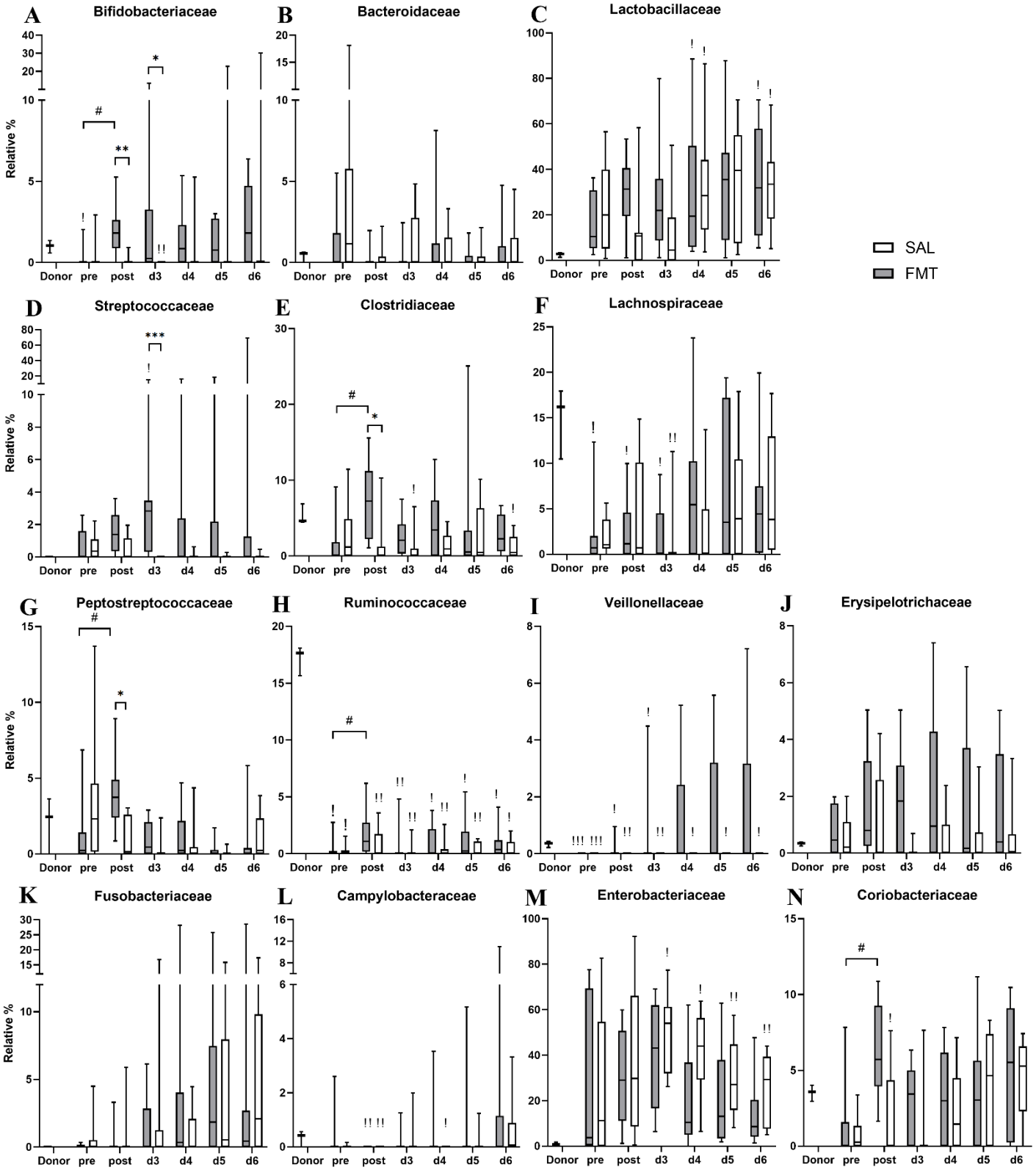


Figure 10 – Comparisons of Phyla in Donor material to FMT and SAL piglet daily stool samples. Shown above are statistical differences between bacterial phyla in Donor FMT material, FMT, and SAL piglet daily stool samples. See full quantification in Table S5-S6. All results are displayed as relative % composition in daily stool samples pre-treatment (pre), post-treatment (post), and days 3 to 6 (d3-d6). Comparisons between Donor, FMT, and SAL made using independent samples Kruskal-Wallis test and reported with Bonferroni correction for multiple comparisons. FMT to Donor, and SAL to Donor post-hoc significance shown by !p<0.05, !!p<0.01, and FMT to SAL shown by *p<0.05, **p<0.01. Comparisons between pre and post within FMT made using related samples Wilcoxon Signed Rank test, #p<0.05.

FMT-treated piglets had more *Bifidobacteriaceae* than SAL-treated piglets in their post-treatment stool sample (p<0.01) and on day 3 (p<0.05), as well as experiencing a significant increase over their pre-treatment levels (p<0.05) **(Fig. 11A)**. FMT piglets were significantly lower than the Donor in this family pre-treatment (p<0.05), and then were not different at any post-

transplant measurement. SAL piglets had significantly less *Bifidobacteriaceae* on day 3 ($p<0.01$) only. There were no significant differences between FMT, SAL, and Donor in *Bacteroidaceae* **(Fig. 11B)**, although FMT and SAL experienced a nonsignificant but noticeable drop from pre- to post-treatment **(Table S5-S6)**. FMT and SAL piglets were not different day to day nor from each other at any collection timepoint for *Lactobacillaceae* **(Fig. 11C)**. FMT and SAL were consistently higher than Donor *Lactobacillaceae*, but only significantly so on days 4 and 6 (all $p<0.05$). FMT piglets had more *Streptococcaceae* on day 3 than SAL piglets ($p<0.001$) and the Donor ($p<0.05$), but there were no other significant measurements of this Family **(Fig. 11D)**. *Clostridiaceae* significantly increased from pre to post in FMT piglets ($p<0.05$), but these levels did not persist to day 3 and no stool measurement was significantly different to the Donor **(Fig. 11E)**. FMT post-treatment *Clostridiaceae* was significantly more than SAL ($p<0.05$), and SAL was significantly less than Donor on days 3 and 6 (both $p<0.05$). Both FMT and SAL has less *Lachnospiraceae* than the Donor, with FMT reaching significance both pre and post-treatment and day 3 sample (all $p<0.05$), and SAL reaching significance on day 3 only ($p<0.01$) **(Fig. 11F)**.

Figure 11 (Following Page) – Comparisons of Families in Donor material to FMT and SAL piglet daily stool samples. Shown above are statistical differences between bacterial families in Donor FMT material, FMT, and SAL piglet daily stool samples. See full quantification in Table S5-S6. All results are displayed as relative % composition in daily stool samples pre-treatment (pre), post-treatment (post), and days 3 to 6 (d3-d6). Comparisons between Donor, FMT, and SAL made using independent samples Kruskal-Wallis test and reported with Bonferroni correction for multiple comparisons. FMT to Donor, and SAL to Donor post-hoc significance shown by !$p<0.05$, !!$p<0.01$, and FMT to SAL shown by *$p<0.05$, **$p<0.01$. Comparisons between pre and post within FMT made using related samples Wilcoxon Signed Rank test, #$p<0.05$.


A
Bifidobacteriaceae
B
Bacteroidaceae
C
Lactobacillaceae
D
Streptococcaceae
E
Clostridiaceae
F
Lachnospiraceae
G
Peptostreptococcaceae
H
Ruminococcaceae
I
Veillonellaceae
J
Erysipelotrichaceae
K
Fusobacteriaceae
L
Campylobacteraceae
M
Enterobacteriaceae
N
Coriobacteriaceae
Relative %
Donor
pre
post
d3
d4
d5
d6
SAL
FMT

There was a brief increase in the *Peptostreptococcaceae* present in FMT piglets from pre- to post-treatment ($p<0.05$), which made the FMT also greater than the SAL post-treatment ($p<0.05$), but this increase did not persist beyond day 2 **(Fig. 11G)**. *Ruminococcaceae* were almost uniformly lower than the Donor in both FMT and SAL piglets, except for FMT post-treatment **(Fig. 11H)**. FMT piglets were less than Donor pre-treatment ($p<0.05$), and the post-treatment sample was greater than pre ($p<0.05$), but this Family returned to low levels by day 3 ($p<0.01$), which was then maintained on days 4 to 6 (all $p<0.05$). SAL was less pre ($p<0.05$) and post-treatment ($p<0.01$), on days 3 to 5 (all $p<0.01$), and day 6 ($p<0.05$). FMT piglets initially had no *Veillonellaceae* **(Table S5)**, and were significantly less than the Donor ($p<0.001$) **(Fig 11I)**. This significance was maintained, but less so, post-treatment and day 3 (both $p<0.05$), but by days 4 to 6 this population had expanded slightly and was not significantly different. SAL piglets had no *Veillonellaceae* at any time point and were significantly less than Donor throughout **(Table S6)**. There were no significant differences in *Erysipelotrichaceae* **(Fig. 11J)** or *Fusobacteriaceae* **(Fig. 11K)**. *Campylobacteraceae* was initially not significantly different from Donor for either FMT or SAL pre-treatment, and despite the Donor having a measurable level of this Family, detection levels fell significantly post-treatment for both groups ($p<0.01$), but recovered a little by days 3 to 6, and only SAL on day 4 was significantly less ($p<0.05$). *Enterobacteriaceae* in the Donor was very low, though not significantly different to the FMT piglets at any timepoint **(Fig 11M)**. SAL piglets began not significantly different, but as their levels of the Family expanded slightly, they were significantly higher by days 3 and 4 (both $p<0.05$) which was further increased on days 5 and 6 (both $p<0.01$). FMT piglets were not different than Donor *Coriobacteriaceae* levels for any timepoint **(Fig 11N)**, however did increase from pre- to post-treatment ($p<0.05$), making FMT greater than SAL post-treatment also ($p<0.05$). FMT *Coriobacteriaceae* were not maintained at

higher levels on days 3 to 6 and none of these measurements were significantly different from Donor or SAL.

3.4.3.6. Individual piglets with unique cases

The piglet from the FMT group that was suspected of sepsis and returned blood cultures positive for *C. coli* had no detectable levels of this organism in any of the daily stool samples obtained, nor any significant amounts of any other *Campylobacteraceae* or *Campylobacter* species. Donor FMT material likewise did not contain any detectable *Campylobacteraceae* species.

The first two treatment piglets taken were from the same litter, and this litter subsequently developed scours after treatment piglet selection. Scours is a transmissible gastroenteritis caused in the neonatal stage (hours to days after birth) by a bacterial pathogen, most often *E. coli* or *C. perfringens*[219]. Neither piglet was different from their group means for relative % composition of *Enterobacteriaceae* or *Escherichia* species, nor for *Peptostreptococcaceae Clostridium spp* as measured in the baseline jejunum and colon tissue samples. For *Clostridiaceae*, the two piglets were not different from their respective group means in baseline jejunal tissue, but were significantly higher in their baseline colon tissue. The SAL piglet measured 20.9% *Clostridium* genera, compared to a group mean of 6.5% (SD=5.8; $p<0.001$), and the FMT piglet measured 10.2% versus a group mean of 6.5% (SD=3.7; $p<0.01$). In both cases, however, neither piglet was the only group outlier. SAL also had another group outlier that measured 12.0% *Clostridium* ($p<0.01$), and FMT had an outlier measure 15.5% *Clostridium* ($p<0.001$). As such, we do not think that these two piglets should have been excluded from the experiment.

3.4.3.7. Diversity of Daily Stool samples

The alpha diversity of each piglet was measured in the daily stool samples by investigating the number of observed operational taxonomic units (OTUs) and the Faith's Phylogenetic Diversity (PD)[220]. FMT and SAL piglets had similar numbers of OTUs detected pre-treatment, but FMT had significantly more detected in the post and day 3 samples (both $p<0.05$), though the increase from pre to post within FMT piglets was not significant **(Fig. 12)**. The Donor had significantly more OTUs than FMT and SAL piglets at all timepoints (all $p<0.001$). Likewise, the Donor was significantly more diverse than either SAL or FMT as measured by Faith's PD **(Fig. 13)**. SAL was significantly less diverse at all timepoints ($p<0.05$). FMT was less diverse than the Donor pre-treatment, but this was not significant after applying the Bonferroni correction for multiple analyses ($p=0.05$). The comparison between FMT and Donor was significant post-treatment, and on days 3 to 6 (all $p<0.05$). The FMT piglets experienced a non-significant increase in diversity from pre to post, which made them significantly more diverse than the SAL piglets post-treatment ($p<0.01$), and on day 3 ($p<0.05$), but this was not sustained.

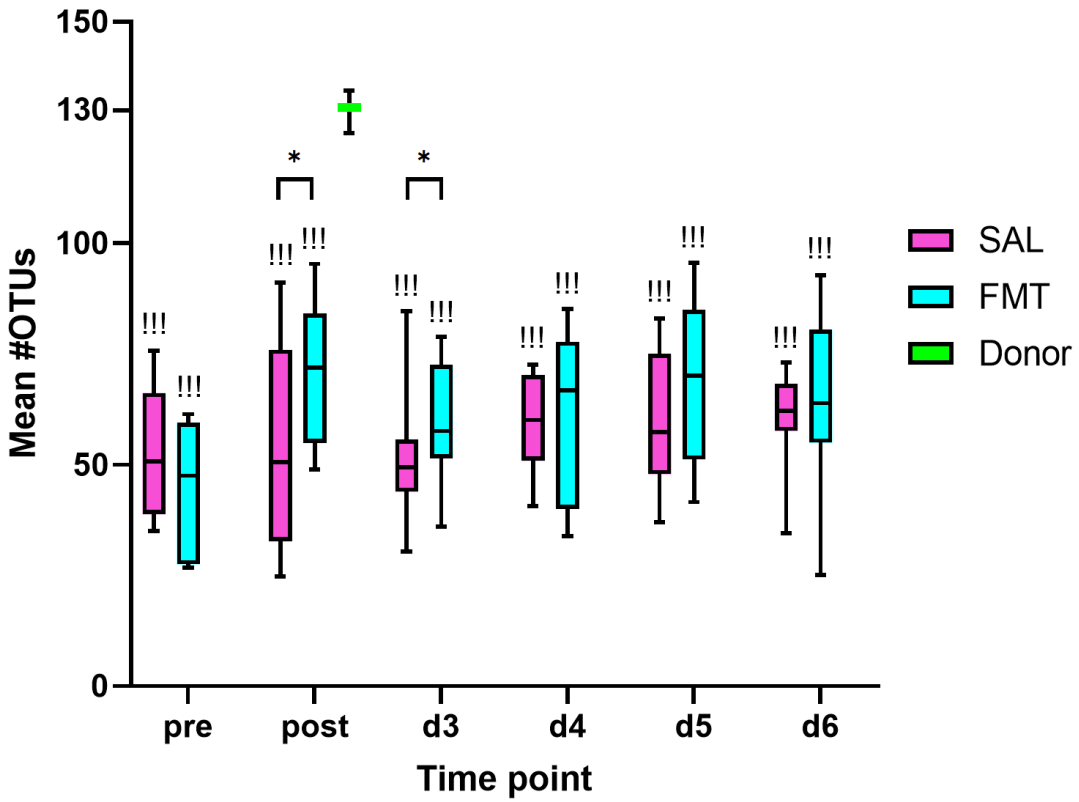


Figure 12 – Observed assigned OTUs in daily stool samples of SAL and FMT compared to Donor. Shown above are the mean OTUs from a read depth of 2000 for SAL and FMT by daily stool collection, and for Donor FMT material that was administered on day 2. Samples were taken day 2 pre-treatment (pre), day 2 post-treatment (post), and days 3 – 6 (d3 – d6). Comparisons between SAL, FMT, and Donor in pre - d6 samples made using independent samples Kruskal-Wallis test reported with Bonferroni correction. Post-hoc differences to Donor shown by !$p<0.05$, !!$p<0.01$, !!!$p<0.001$ and between FMT and SAL by *$p<0.05$, **$p<0.01$, ***$p<0.001$. See Table S8 for full quantification.

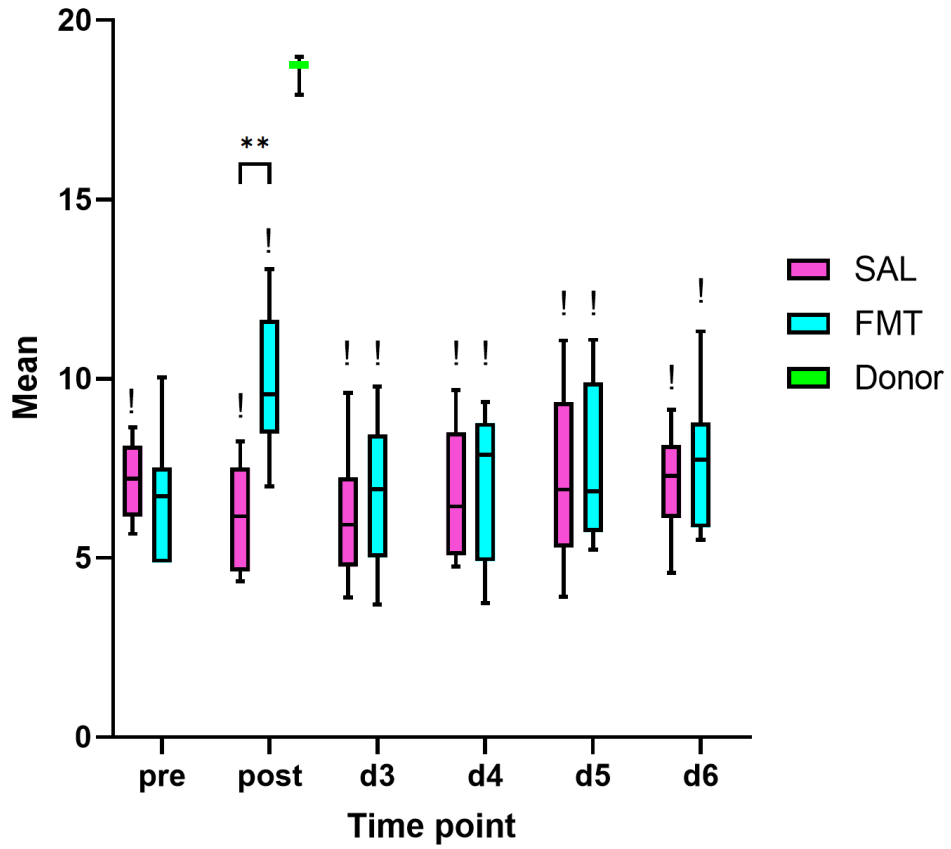


Figure 13 – Faith's Phylogenetic Diversity in daily stool samples of SAL and FMT compared to Donor. Shown above are the mean Faith's Phylogenetic Diversity (PD) measurements for SAL and FMT by daily stool collection, and for Donor FMT material that was administered on day 2. Samples were taken day 2 pre-treatment (pre), day 2 post-treatment (post), and days 3 – 6 (d3 – d6). Comparisons made in pre - d6 samples made using independent samples Kruskal-Wallis test reported with Bonferroni correction. Post-hoc differences shown to Donor by !$p<0.05$, and between FMT and SAL by *$p<0.05$, **$p<0.01$. See Table S8 for full quantification.

Beta diversity between piglet daily stools was measured first by Bray-Curtis Dissimilarity[221] and then unweighted and weighted unique fraction (unifrac). Pre-treatment, the Donor FMT material was significantly different from SAL pigs ($p=0.04$), but SAL and FMT were not different from each other **(Fig. 14)**. Post-treatment, SAL and FMT were significantly different ($p<0.05$), and both were different from Donor ($p<0.05$). These differences increased slightly by day 3 (all $p<0.01$), but by days 4 to 6, FMT and SAL were not significantly dissimilar. Donor was

dissimilar to FMT and SAL on day 4 ($p<0.05$), day 5 ($p<0.05$), and day 6 ($p<0.01$). The unweighted unique fraction (Unifrac) distances **(Fig. 15)** of species present between SAL and FMT piglets were significantly different post-treatment ($p<0.01$) and on day 3 ($p<0.05$) only, while Donor was significantly different at all time points (pre & post, $p<0.05$; day 3, $p<0.01$; day 4, $p<0.05$; days 5 & 6, $p<0.01$). The weighted Unifrac, which considers abundances of species in addition to their presence or absence, was similar to the unweighted analysis **(Fig. 16)**. SAL and FMT were significantly different post-treatment ($p<0.01$), and on day 3 ($p<0.05$), but no longer different on days 4 to 6. Donor was significantly different than both SAL and FMT at all time points (pre & post, $p<0.05$; days 3-5, $p<0.01$; day 6, $p<0.05$).

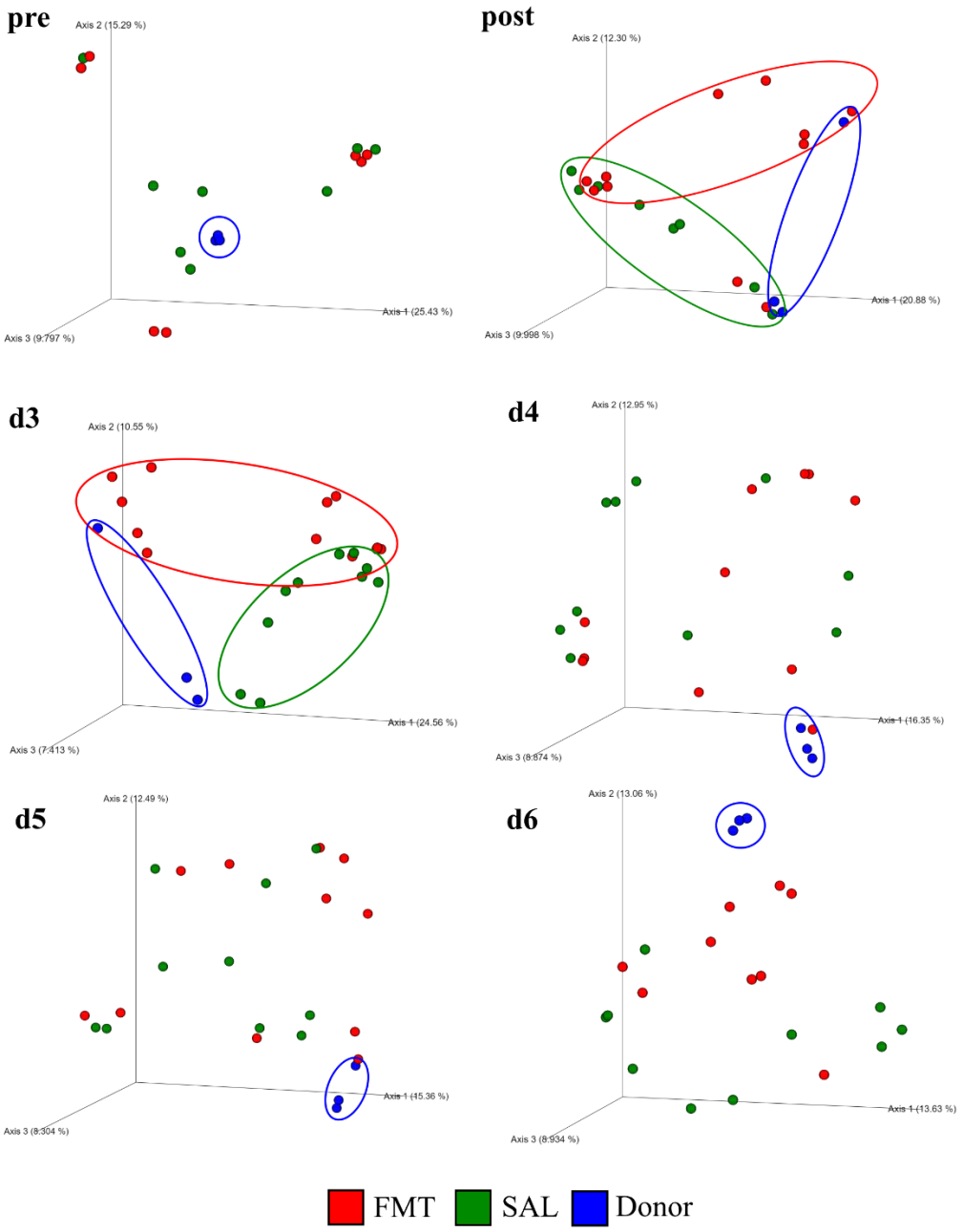


Figure 14 – Bray-Curtis dissimilarity between FMT and SAL daily stools compared to Donor. Shown above are Principal Components Analysis (PCoA) plots of the Bray-Curtis dissimilarity measurements between saline (SAL, green circles) and fecal microbial transplant (FMT, red circles) treated piglets compared also with the Donor FMT material (blue circles) that was administered on day 2. Samples were taken day 2 pre-treatment (pre), day 2 post-treatment (post), and days 3 – 6 (d3 – d6). Statistical analysis was performed using permanova with superimposed ovals indicating groups that were significantly different. Results were considered significant at $p<0.05$.

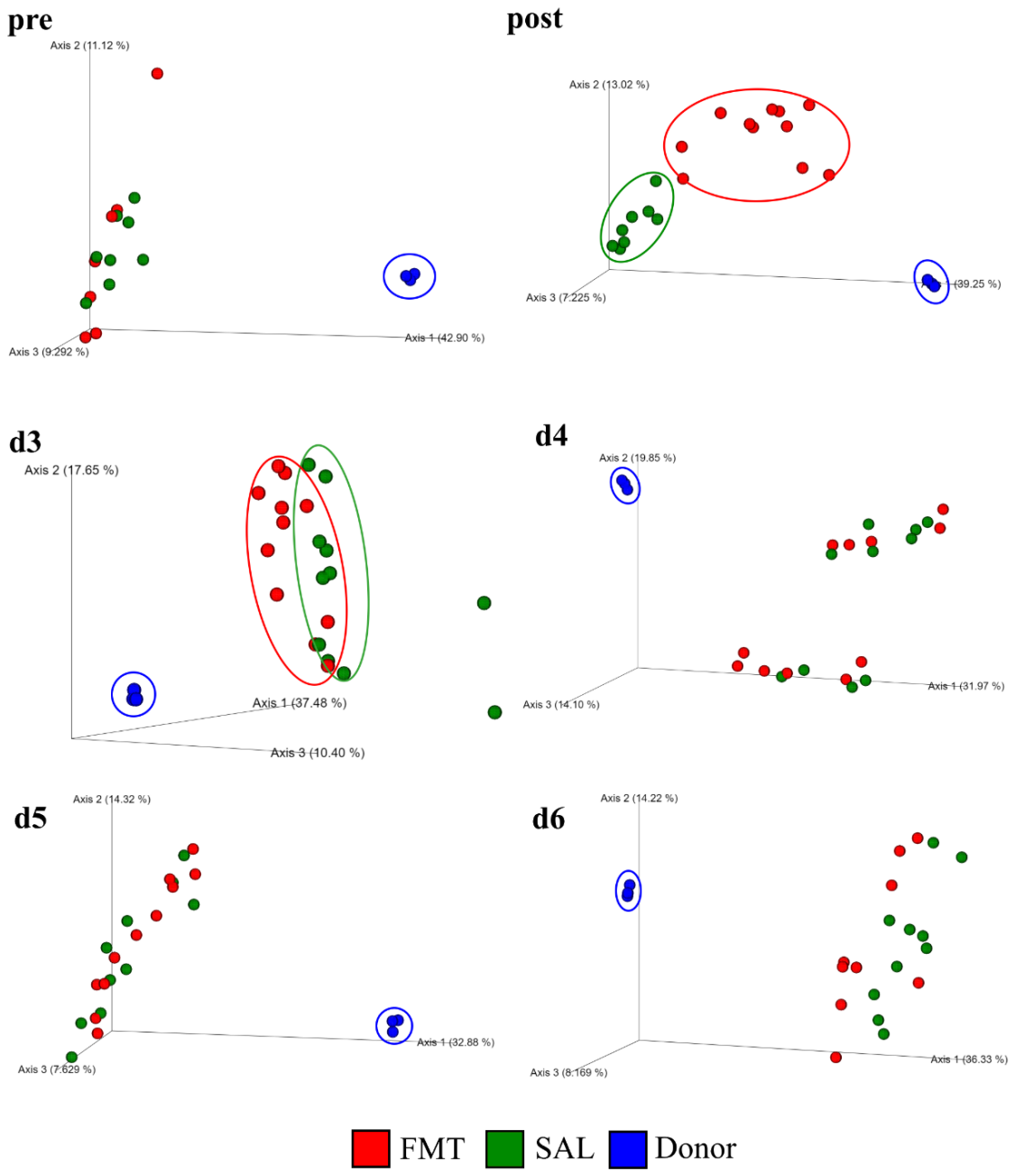


Figure 15 – Unweighted Unifrac distances between FMT and SAL compared with Donor. Shown above are Principal Components Analysis (PCoA) plots of the unweighted unique fraction (Unifrac) measurements between saline (SAL, green circles) and fecal microbial transplant (FMT, red circles) treated piglets compared also with the Donor FMT material (blue circles) that was administered on day 2. Samples were taken day 2 pre-treatment (pre), day 2 post-treatment (post), and days 3 – 6 (d3 – d6). Statistical analysis was performed using permanova with superimposed ovals indicating groups that were significantly different. Results were considered significant at $p<0.05$.

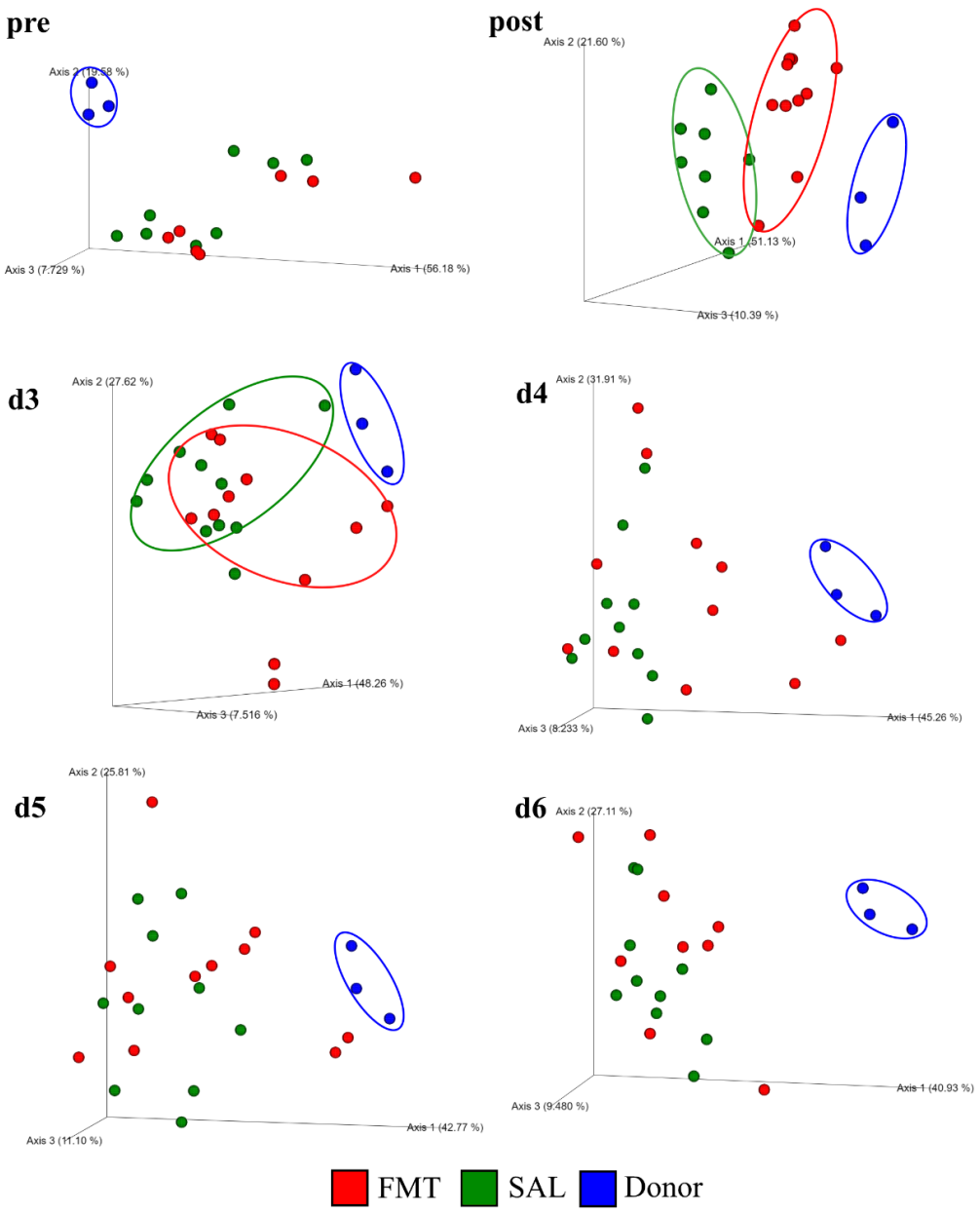


Figure 16 – Weighted Unifrac distances between FMT and SAL compared with Donor. Shown above are Principal Components Analysis (PCoA) plots of the unweighted unique fraction (Unifrac) measurements between saline (SAL, green circles) and fecal microbial transplant (FMT, red circles) treated piglets compared also with the Donor FMT material (blue circles) that was administered on day 2. Samples were taken day 2 pre-treatment (pre), day 2 post-treatment (post), and days 3 – 6 (d3 – d6). Statistical analysis was performed using permanova with superimposed ovals indicating groups that were significantly different. Results were considered significant at $p<0.05$.

3.4.3.8. Diversity of Base and Term tissues

Alpha diversity was measured in the mucosal associated bacteria of the baseline and termination tissue samples for each group. At baseline, the numbers of observed OTUs was not different between SAL and FMT piglets **(Fig. 17)**, nor were there differences within SAL or FMT groups between base and term. At termination, the jejunum of FMT piglets had more OTUs than SAL ($p<0.05$). SOW piglets had significantly more OTUs than SAL at termination in the jejunum ($p<0.001$) and colon ($p<0.001$), and more than FMT in the jejunum ($p<0.05$). The Donor material had significantly more OTUs than SAL, FMT, and SOW piglet tissue samples at all time points ($p<0.001$). SAL, FMT, and SOW piglets all had higher Faith's PD measured in the jejunum than the colon **(Fig. 18)**. SOW piglets had more diverse jejunum samples at termination than SAL ($p<0.05$), and more diverse colons than FMT ($p<0.05$). Donor material was more diverse than FMT colon ($p<0.05$) only.

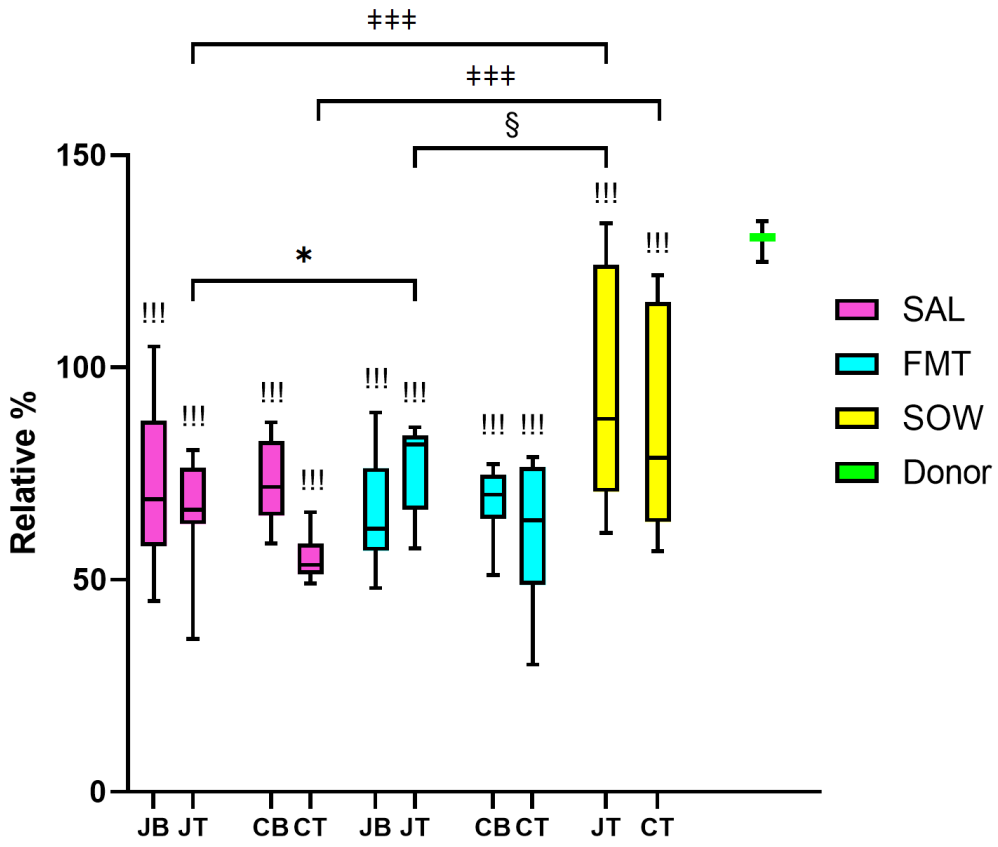


Figure 17 – Observed assigned OTUs in base and term tissue samples of FMT, SAL, SOW, and Donor. Shown above are the observed OTUs from a read depth of 2667 for FMT, SAL, and SOW piglet baseline (B) and termination (T) tissue samples from jejunum (J) and colon (C), and for Donor FMT material that was administered on day 2. Comparisons were made using independent samples Kruskal-Wallis test reported with Bonferroni correction. Post-hoc differences shown for Donor (!$p<0.05$, !!$p<0.01$, !!!$p<0.001$), FMT and SAL (*$p<0.05$), SOW to SAL (ǂ$p<0.05$, ǂǂ$p<0.01$), and SOW to FMT (§$p<0.05$, §§$p<0.01$). See Table S9 for full quantification.

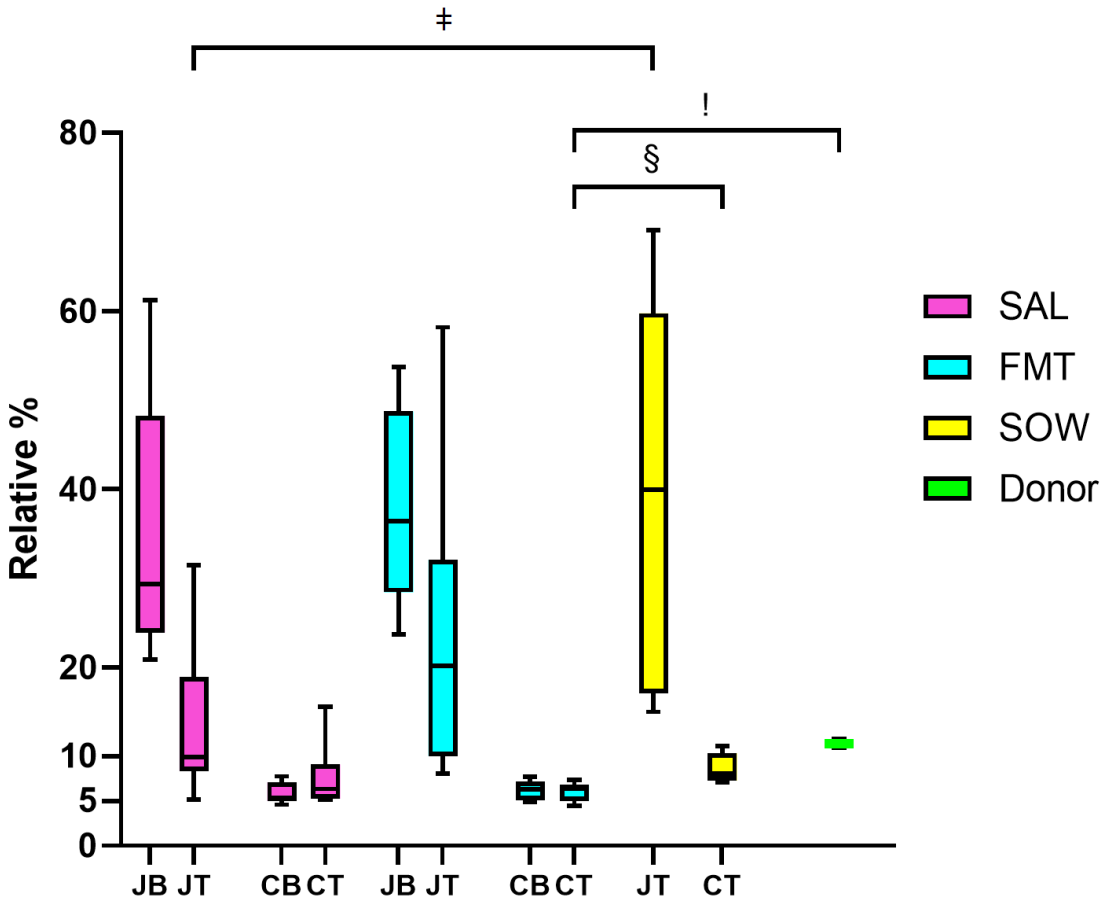


Figure 18 – Faith's Phylogenetic Diversity in base and term tissue samples of FMT, SAL, SOW, and Donor. Shown above is the calculated Faith's Phylogenetic Diversity (PD) for FMT, SAL, and SOW piglet baseline (B) and termination (T) tissue samples from jejunum (J) and colon (C), and for Donor FMT material that was administered on day 2. Comparisons were made using independent samples Kruskal-Wallis test reported with Bonferroni correction. Post-hoc differences shown for Donor (!$p<0.05$, !!$p<0.01$, !!!$p<0.001$), FMT and SAL (*$p<0.05$), SOW to SAL (ǂ$p<0.05$, ǂǂ$p<0.01$), and SOW to FMT (§$p<0.05$, §§$p<0.01$). See Table S9 for full quantification.

At baseline, Bray-Curtis dissimilarity **(Fig. 19)** was not significantly different between SAL and FMT piglets. At termination, SAL, FMT, and SOW piglets were not dissimilar in the jejunum, but were in the colon ($p<0.05$). Donor material was significantly different than SAL and FMT in the jejunum ($p<0.01$) and colon ($p<0.05$). Unweighted Unifrac **(Fig. 20)** was not different between SAL and FMT groups either at baseline or termination in either jejunum or colon. At

termination, SOW was different only compared to SAL in the jejunum ($p<0.05$) but was different than SAL and FMT in the colon ($p<0.01$). Donor was different in jejunum ($p<0.01$) and colon ($p<0.05$) compared to both SAL and FMT. The weighted unifrac **(Fig. 21)** reflects the same trend as the unweighted unifrac, in that SAL and FMT were not different from each other in either tissue at baseline nor termination. SOW piglets were only different compared to SAL and FMT in the colon at term ($p<0.05$), and likewise the Donor was only significantly different in the colon ($p<0.05$).

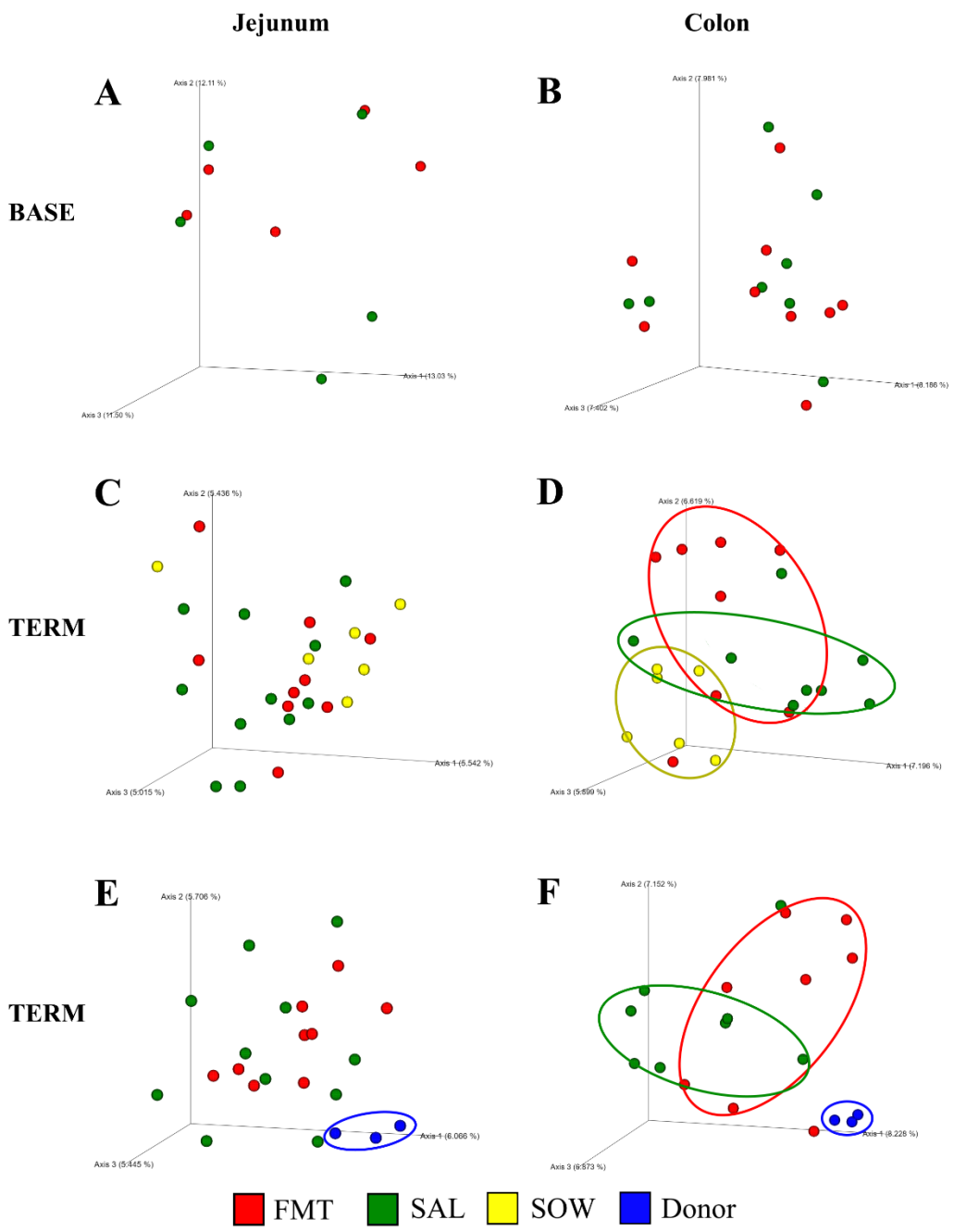


Figure 19 – Bray-Curtis dissimilarity in base and term tissue samples from treatment piglets compared with Donor. Shown above are Principal Components Analysis (PCoA) plots of the Bray-Curtis dissimilarity measurements between saline (SAL, green circles) and fecal microbial transplant (FMT, red circles) treated piglets in baseline (BASE) **A)** jejunum tissue, and **B)** colon tissue. SAL and FMT tissues at termination (TERM) are compared with **C,D)** sow-reared piglets (SOW, yellow circles), and **E,F)** Donor FMT material (blue circles) administered on day 2. Statistical analysis was performed using permanova with superimposed ovals indicating groups that were significantly different. Results were considered significant at $p<0.05$.

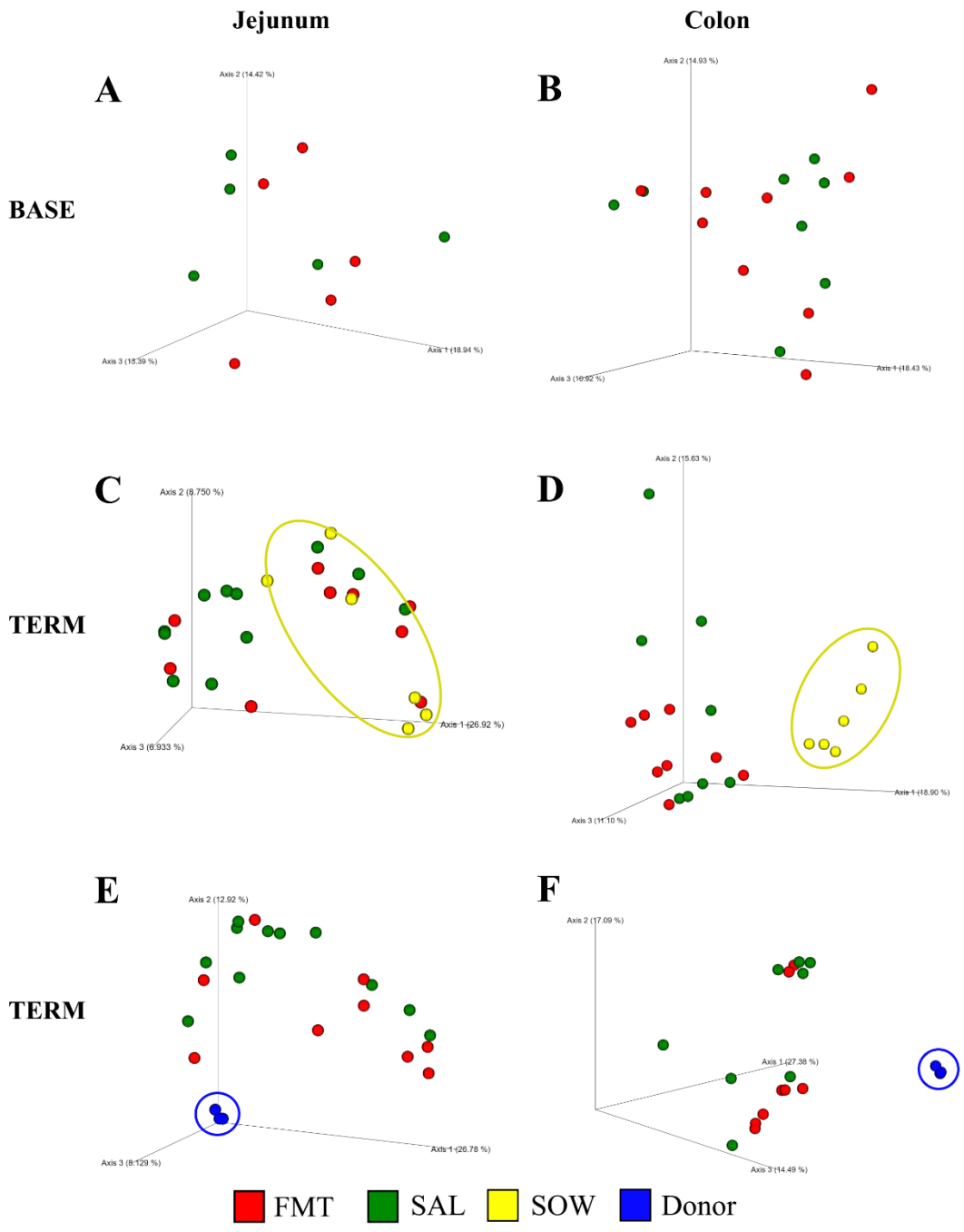


Figure 20 – Unweighted Unifrac distances between FMT and SAL compared with SOW and Donor. Shown above are Principal Components Analysis (PCoA) plots of the unweighted unique fraction (Unifrac) measurements between saline (SAL, green circles) and fecal microbial transplant (FMT, red circles) treated piglets in baseline (BASE) **A)** jejunum tissue, and **B)** colon tissue. SAL and FMT tissues at termination (TERM) are compared with **C,D)** sow-reared piglets (SOW, yellow circles), and **E,F)** Donor FMT material (blue circles) administered on day 2. Statistical analysis was performed using permanova with superimposed ovals indicating groups that were significantly different. Results were considered significant at $p<0.05$.

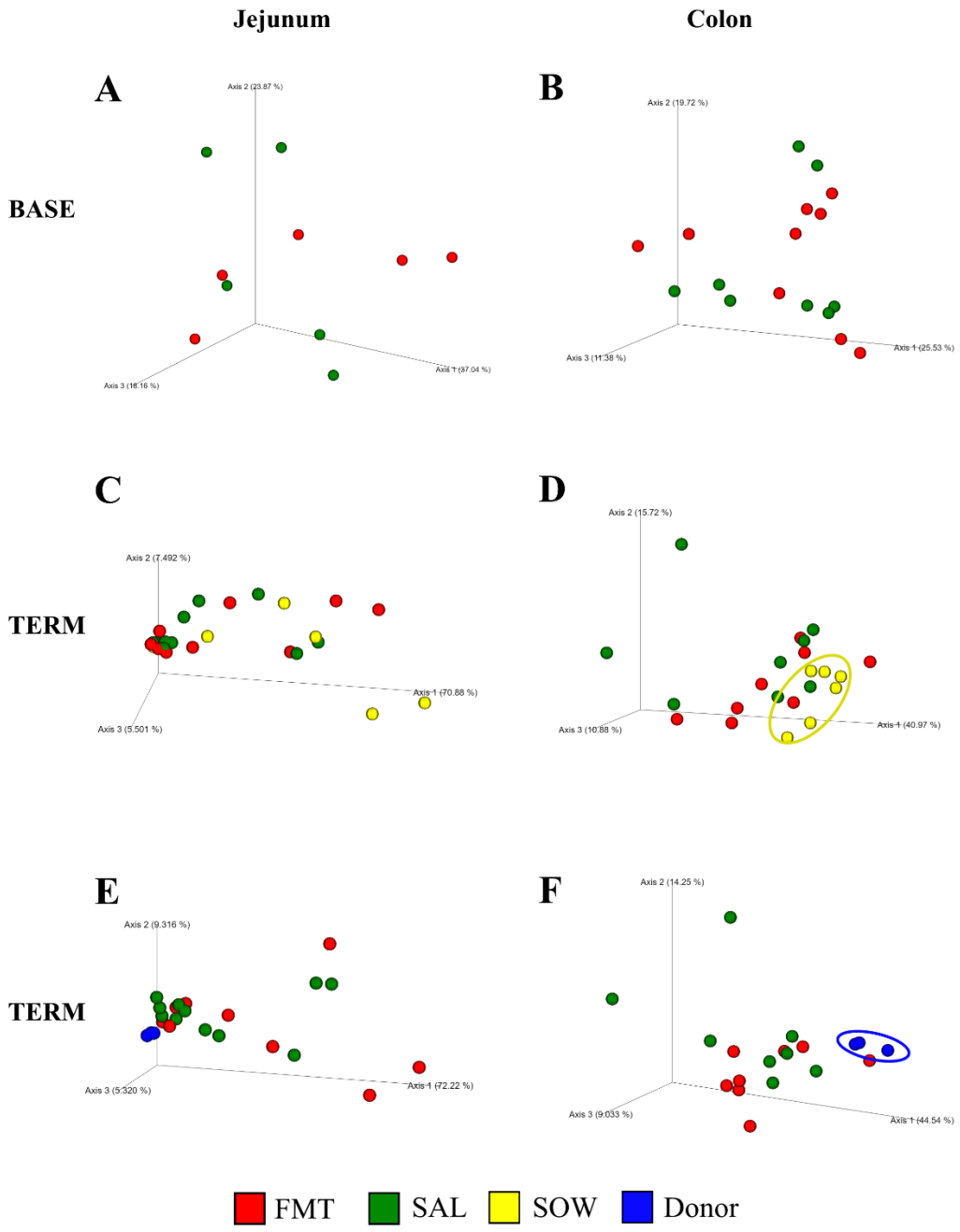


Figure 21 – Weighted Unifrac distances between FMT and SAL compared with SOW and Donor. Shown above are Principal Components Analysis (PCoA) plots of the unweighted unique fraction (Unifrac) measurements between saline (SAL, green circles) and fecal microbial transplant (FMT, red circles) treated piglets in baseline (BASE) **A)** jejunum tissue, and **B)** colon tissue. SAL and FMT tissues at termination (TERM) are compared with **C,D)** sow-reared piglets (SOW, yellow circles), and **E,F)** Donor FMT material (blue circles) administered on day 2. Statistical analysis was performed using permanova with superimposed ovals indicating groups that were significantly different. Results were considered significant at $p<0.05$.

3.5. Discussion

Fecal microbial transplant (FMT) is a promising therapy known to have excellent efficacy in treatment of recurrent *C. difficile* infections[150] and potential to treat colonization with antibiotic resistant organisms[152]. Initial promising results have encouraged clinicians to begin investigating its use in other areas as well, including IBD, IBS, and chronic constipation, as well as non-gastrointestinal-related illnesses such as chronic fatigue syndrome, insulin resistance, and others[146]. FMT has also already seen success in the context of short bowel syndrome, when it was used to treat small intestinal bacterial overgrowth (SIBO)-induced D-lactic acidosis in two patients for which all other treatments had failed[53,153]. The most common cause of SBS in preterm neonates, necrotizing enterocolitis, is a condition initiated by either a disruption of or failure to properly establish the microbiota, and the most common complications of SBS are sepsis with gut-derived organisms and IFALD, which is caused in part due to microbial endotoxin initiating inflammation in the liver. With these factors in mind, we felt the time was right to experimentally evaluate the use of FMT in SBS to restore an ideal microbiota and thus hopefully improve patient outcomes.

In this study, we used our well-established neonatal piglet model of SBS to investigate the effects of delivering an FMT prepared from feces collected from a healthy gilt to piglets that had undergone a 75% distal jejunocolic resection. Our primary objective in doing so was to determine whether or not this practice would lead to an increase in mortality. Neonates who have undergone extensive intestinal resection are an extremely vulnerable population, and any treatment proposed must not cause any increase in mortality or complications that reduce an already impacted quality of life. In our cohort, there was no disease-specific mortality, and all piglets tolerated FMT well. One piglet from the SAL group was removed from the study early due to a breakage in the gastrostomy tube that had been inserted for delivery of enteral nutrition. A second piglet, from the

FMT group, was removed from the study early due to intestinal obstruction. We do not believe that the obstruction was caused by the FMT, but rather likely caused by severe adhesions, which is something that can occur as a consequence of abdominal surgery in this model at random. In this trial, no other piglets developed an obstruction and at termination there was no subjective difference in the severity of adhesions between SAL and FMT groups (data not shown). Careful monitoring and attentive care of future SBS patients that may receive FMTs is prudent, however, to ensure that this is indeed the case.

Our second objective was to evaluate whether or not a dysbiotic microbiota can be restored to that of a healthy, ideal community structure after a single infusion of donor fecal material, and for this we analyzed the daily stool samples. Our results indicate that an immediate alteration of the SBS microbiota can be detected at the Family level of classification within hours after delivery of FMT material, and that many of these changes persist through the following day. As shown in Figure 9, in 5 of 14 families, FMT piglets experienced a significant increase after treatment compared with SAL piglets, 3 of these maintained this increase through the day following treatment (day 3), and a further 2 families experienced increases that achieved significance on the day after treatment. This same trend of a slight increase in percent composition after FMT treatment can be observed even in families which did not reach statistical significance, such as *Ruminococcaceae* and *Lachnospiraceae*. This trend is dramatic enough to be observed even at the higher phylum level of classification, as shown in Actinobacteria (Fig. 10). When compared to the Donor material, Actinobacteria in FMT piglets was significantly lower pre-treatment, but then increased so the post-treatment sample was significantly higher compared to both their own pre-treatment measurements, and the SAL piglets post-treatment, while not being significantly different to the Donor. This trend is seen in the Actinobacteria families *Bifidobacteriaceae* and

Coriobacteriaceae (Fig. 11), as well as similar effects in several Firmicutes families (namely *Clostridiaceae, Lachnospiraceae, Peptostreptococcaceae, Ruminococcaceae,* and *Erysipelotrichaceae*).

We also examined for FMT changes over the course of the experiment in mucosal-associated bacteria, comparing baseline and termination tissue samples taken from the jejunum and colon of each piglet. Mucosal-associated bacteria are important in this model as those are the organisms that most closely interact with the epithelial barrier, and thus are more likely to exert effects upon it. These effects can be beneficial, in terms of SFCA production, or they can be harmful, including inflammation and translocation leading to liver fibrosis, depending on the species present. SAL and FMT piglets experienced modest increases in the mucosal Bacteroidetes in their jejunum so they were not different than sow-fed piglets (SOW) at term, but the same phylum decreased in the colon of both SAL and FMT, so that SOW had significantly more (Fig. 3). Fusobacteria, a phylum that contains many Gram negative pro-inflammatory species, remained constant in SAL and low in SOW piglets, but modestly increased in FMT. Proteobacteria also increased in the colons of SAL and FMT between base and term, suggesting an inflammatory state could have been developing in the colons. This could be due to effects of initial antibiotics and oxygen exposure during the short-bowel surgery, as well as housing in metabolic cages and administration of sterile diet rather than solely due to treatment, as both SAL and FMT experienced this increase. This is supported by analysis of the Family-level of distinction, which shows that while Firmicutes as a phylum made no significant changes in the stool (Fig. 5), families tended to increase in the jejunum between base and term, decrease in the colons, and at term were significantly lower than the sow-fed colonic microbiotas (Fig. 7). Pro-inflammatory *Campylobacteraceae* and *Enterobacteriaceae* also increased in the colons of treatment piglets

(Fig. 7), while decreasing in their sow-fed peers (Table S3). Increases in the jejunum accompanied by decreases in the colon could also indicate retrograde migration of the species due to loss of the ileocecal valve.

Transient increases are reflected further in the measures of alpha and beta diversity. The number of OTUs observed in FMT piglet daily stool samples increases between pre- and post-treatment after exposure to the Donor material (Fig. 12), and is significantly more than SAL piglets post-treatment and on day 3, remaining higher than their pre-treatment levels through to termination. Faith's Phylogenetic Diversity (PD) also experienced a post-treatment bump (Fig. 13), that did not persist and returned to pre-treatment levels. Together with the OTUs, this suggests that while some new OTUs may have been introduced to FMT piglets, these species did not persist in high enough numbers, or they could be closely related, both factors that would not consistently affect diversity on days 3 to 6. Beta diversity, the differences in species detected between individuals, reflects this post-treatment and day 3 change when measured by Bray-Curtis Dissimilarity (Fig. 14), Unweighted Unifrac (Fig. 15), and Weighted Unifrac (Fig. 16). SAL and FMT piglets are significantly different to each other only briefly, and both remain different than the Donor material at all time points.

The reasons for this lack of persistence can be explained by looking at both the source of the donor FMT material, and at the composition of the enteral nutrition provided to the piglets. Microbiotas are made of live organisms that must have acceptable nutritional substrates in order to persist, and if the diet does not contain microbiota-accessible carbohydrates (MAC), then compositional changes will occur that predispose the gut to an inflammatory state[91]. The donor that we used for our FMT material was an adult gilt (unfarrowed female), and as piglets are weaned at 3 weeks of age and thereafter fed a solid chow diet high in complex glycans and protein, this

results in gradual changes to the microbial composition so that adult pigs are distinct from nursing piglets[191,222].

The parenteral and enteral nutrition formulas that we use in our lab are elemental, composed of amino acids, lipids, vitamins, and minerals, with glucose as carbohydrate source in the PN and polycose in the EN, to reduce osmotic diarrhea[18]. Polycose is a modified glucose polymer made from cornstarch[223], and so does not represent a source of complex glycans or SCFAs that are required by many beneficial microorganisms for their survival. While this is nutritionally sufficient to ensure adequate health and growth of our experimental piglets, it does not appear to promote an ideal microbiota with diversity comparable to sow-reared piglets. SAL piglets had significantly fewer OTUs in the jejunum and colon (Fig. 17) and less alpha diversity in the jejunum (Fig. 18) than SOW piglets, while FMT piglets had fewer OTUs in the jejunum and less diverse colons than SOW. Beta diversity measures show that treatment piglet mucosal-associated bacteria were significantly different in the colons, compared to both SOW and Donor (Fig. 19-21), while the jejunums tended to not be significantly different at termination. Adult pig microbiota is composed of fibre-fermenting species (ie. *Bacteroidaceae, Lachnospiraceae, Ruminococcaceae*) that survive on different substrate than either nursing piglets or weaned piglets fed elemental diets, and thus cannot be expected to persist in high numbers under starvation conditions.

In this experiment we did not perform a bowel preparation procedure on the piglets before administering the FMT, which may have prevented the transplanted organisms from establishing growth. In human patients, preparation of the bowel with a polyethylene glycol-based solution (ie. GoLYTELY®) prior to colonic administration of FMT is common in order to remove the dysbiotic community and allow for open niches that the newly transplanted organisms can then occupy[165]. While a bowel preparation procedure is not recommended for upper GI delivery of FMT in

humans, it does appear that colonic delivery may have a slightly higher efficacy than upper delivery (84-93% colonic delivery versus 81-86% upper) in clinical practice[149]. Colonization history affects the growth fitness and ability of later-arriving organisms to survive, as shown in mice[104] and pigs[202]. In the latter experiment, the largest contribution to a pig's mature microbiota came from organisms acquired during the nursery and growing stage, when solid feeds were first introduced, showing that the first and largest changes to community structure are the ones with the greatest longevity. This experiment also investigated the effects of FMT on the progression of the microbiota in healthy pigs and found that while FMT-treated pigs had increased growth performance, there were no significant differences between FMT or Control pig diversity indices. This experiment also did not perform a bowel prep procedure, and used very small amounts of FMT filtrate (3mL, twice), both of which may have negatively affected their results.

Previous FMT experiments in pigs using larger volumes of Donor material[196,199-201] have shown significant changes in the microbiota after treatments, but one[201] also found negative impacts on the intestinal morphology, poor nutrition, and a reduction in growth, while another[198] found good growth, increased Firmicutes and decreases in inflammatory Families, but also a decrease in some beneficial Bacteroidetes groups. One of our FMT piglets did experience severely damaged epithelium that prevented histological measurements, but there were no noticeable significant differences in microbiota between this piglet and the rest of the group. These conflicting results highlight the complicated nature of attempting to alter the microbiota using FMT, with many unresolved controversies. However, overall research in swine shows that it is possible and that beneficial results can be obtained dependent upon the conditions and treatment regimens employed.

Our final objective was to ensure that FMT can be delivered to post-surgical neonatal piglets without an increase in septic episodes. Only one piglet was suspected of being septic, but as this piglet not only survived without medical intervention, but returned to excellent health after single instance of vomiting, it is likely this was not sepsis. Surgical piglets with true sepsis will usually deteriorate and require euthanasia without additional antibiotic treatment, which was deliberately not included in our protocol for this experiment. However, there may have been a transient bacteremia as blood cultures for this piglet did return a result for *C. coli*. Importantly, this organism was not detected in the piglet's microbiota, nor in any other study piglets, nor in the Donor FMT material. Our previously published data show that sepsis occurs in our model between 11% and 14% of piglets[18,209], which are then treated with antibiotics according to our in-house protocols. Piglets in all our studies receive florfenicol at initial surgery, and as this drug has a metabolic half-life of 48 hours, it is not present to a substantial degree after day 2 and we do not believe would have impacted the establishment of FMT or later sepsis outcomes. In short, in this study we did not find that FMT caused an increase in sepsis, and that overall this appears to be a safe treatment to administer to post-surgical neonatal piglets with short bowel syndrome.

Chapter Four: Conclusion and Future Directions

4.1. Conclusion

In this experiment, we tested the hypotheses that fecal microbial transplant would be safe for use in a post-surgical neonatal population for the treatment of dysbiosis associated with short bowel syndrome, and that this method could be used to return the microbiota to a composition more closely resembling that of the ideal sow-reared piglet. Over the course of this one-week trial, we found no disease- or treatment-related mortality and no sepsis developed, in contrast to the sepsis rates of 11-14% seen in our previous trials. This is promising initial data that supports further investigation of this technique's use to improve the health of a vulnerable population at a critical moment in their development.

Disruptions to the microbiota in children with SBS has been linked to poor growth outcomes[126], vitamin B_{12} deficiencies that lead to irreversible neurological effects[127], and liver fibrosis due to bacterial LPS and flagellin from translocated bacteria[50]. Children and neonates with SBS are exposed to multiple rounds of antibiotics early in life as a consequence of their repeated surgeries, and to treat secondary conditions such as SIBO when they arise. We have shown previously that antibiotics and lack of enteral nutrition have a greater impact upon the microbiota than surgery does alone[69], and in this study we showed that our saline-treated surgical piglets had lower alpha diversity in the jejunum than sow-reared piglets, and different beta diversity in the colon than either sow-reared or FMT-treated piglets. For human patients, we must develop ways to correct this dysbiosis before it causes irreversible damage to a child's growth and development.

While we did see some transient changes to the microbiota after FMT, these changes in general did not persist beyond the second day after treatment. We used a mature pig's feces as the

donor material, which is comprised of bacteria that survive on a different diet than neonatal piglets, and we also did not perform a bowel preparation procedure prior to treatment. Together, these factors potentially hindered the ability of our transplanted organisms to persist long term. The initial results are promising, however, as there were noticeable increases to overall alpha diversity, and some individual families of bacteria did persist at levels higher than prior to treatment. The mucosal-associated bacteria in the colon also experienced increased beta diversity that persisted 5 days after treatment.

4.2. Future Directions

There are several ways in which future experiments could address the lack of long-term persistence seen with this experiment. First, supplementation with oligosaccharides found in sow milk[193] could be added to the diet in order to provide nutrition for the transplanted bacteria, in the same way that infant formulas are recommended to be supplemented with complex fiber or oligosaccharaides to mimic breast milk[224]. For human infants, fiber sources such as amylopectin, inulin (fructooligosaccharide, FOS), pectic galactans, arabinoxylan, β-glucan, cellobiose, and dextran, which support the growth of beneficial *Bacteroides spp*, *Roseburia intestinalis, E. rectale*, and *F. prausnitzii*, while also not being accessible by and limiting the growth of potentially harmful species such as *E. coli* or *Akkermansia mucinophila*[205], would be promising avenues of exploration. One systematic review and meta-analysis[225] found mixed results with regard to the effect of fiber supplementation in EN on both the microbiota (mainly *Bifidobacteria*) and production of SCFAs; However, in all cases these studies included adult participants (mean/median ages between 45 – 79) and none of them supplemented the beneficial bacterial species as well as fiber, after long fiber-free periods during which the fermentative species cannot be expected to have survived in large numbers. Combining the reintroduction of a range of healthy bacteria with sufficient levels

of their specific accessible food sources (ie. use of a synbiotic, rather than prebiotic and probiotic separately) is key to effecting long-term changes on the microbiota.

Additionally, rather than using feces from an adult to treat an infant (either human or pig), selection of a donor that is more closely matched developmentally may provide more contemporary fecal composition that has a better chance of establishing. For pediatric patients, if such transplants were to occur multiple times as both donor and recipient aged, it may induce the recipient microbiota to follow a typical succession pattern as seen in healthy children. In piglets, sow-reared littermates may serve in this capacity in an experimental context. A study longer than 7 days with only 5 days of treatment may also provide more time for differences to develop enough to be detectable. Multiple treatments are another way to increase efficacy, and potentially larger sample sizes may increase the power of the study and overcome the high interindividual variation seen. Further, a bowel preparation procedure using a polyethylene glycol solution delivered enterally or via enema should be used to clear the bowel prior to transplantation so that the newly introduced organisms find empty niches to populate. Such a procedure has already been tested in sows and found to be safe and efficacious[201]. This will eliminate or reduce the colonization order complications that often prevent later species from establishing entirely, or from thriving in large numbers.

With regard to investigating safety of FMT in a neonatal population, studies with longer treatment periods than 5 days should be planned, to ensure that the lack of increase in sepsis observed herein is a lasting effect. One main point of caution in this study was the presence of inflammatory species (*Peptrostreptococcaceae, Veillonellaceae, Fusobacteriaceae*) in the Donor material. Such groups contain many opportunistic pathogens which may not cause issues in small numbers in a healthy adult pig, but transplanted into a surgical neonatal piglet with a gut

predisposed to inflammation due to oxygen exposure and lack of fiber have the potential to cause negative impact. In order to eliminate this possibility, a synthetic microbial community that is free of any potential pathogens could be created and used as the Donor material. Such a synthetic microbiota has already been used in one of the first modern references to use of FMT to cure RCDI[161]. Tvede and Rask-Madsen used a mix of bacteria cultured from feces and shown to inhibit growth of *C. difficile* for their inoculum, however as their mixture contained *E. coli*, a different mixture should be used for neonates. The healthy developing infant microbiome is typically comprised of mostly *Bifidobacteriaceae, Lachnospiraceae, Ruminococcaceae*, and *Lactobacillaceae*[102], so these Families should be included, ensuring that vital fiber fermenters and butyrate producers such as *F. prausnitzii, E. rectale,* and *B. thetaiotamicron* are present for optimal effect.

Careful consideration of the timing of FMT used to treat dysbiosis in neonates should be considered also. At this present time, it would not be prudent to recommend using FMT prophylactically to prevent dysbiosis immediately post-surgery. Although dysbiosis in neonates and children with SBS is likely universal, some children adapt on their own and experience minimal complications. Close monitoring of the stool for the development of dysbiosis in terms of species and metabolic pathways may provide insight as to the optimal time to use FMT on an individual case by case basis.

In terms of a deeper look at the effects of an FMT, there are many potential avenues of investigation. As discussed, the benefits of the microbiota are derived not only from the individual species, but from the metabolic pathways present in their genomes. It is possible that changes to the microbiomes of the study piglets were made that were not detected by focusing only on their microbiotas. Metabolomic and metagenomic studies that look into the predicted or actual

functionality of the microbiota could help bring further understanding of common complications like malnutrition, lack of satiety, and vitamin deficiencies seen in children with SBS. This leads into looking at the effects of FMT on liver function as well, in terms of bile flow and bile acid transport, histology and any presence of fibrosis, as well as inflammation and cytokines present in the tissues or serum. The impact of the FMT on gut adaptation should also be investigated. As shown in a case report of using FMT to treat D-lactic acidosis[153], relative abundance of *Lactobacilli* did not change after FMT, but serum D-lactate lowered and fecal D-lactate increased, indicating that D-lactate was still present, but was not being absorbed. A deeper look at tight junction protein expression, mucosal proliferation and quality, apoptotic index, stem cell proliferation, and expression and regulation of trophic hormones such as GLP-2 and IGF-1 may help shed light on the mechanics of increasing barrier function using FMT. Such a study should also employ metagenomic methods in order to detect which exact species are present. This study was hindered in its ability to resolve species-level differences reliably, as it used 16S rRNA, which has limitations in this regard.

In summary, FMT has been shown to be a safe treatment used in surgical neonatal piglets in this context, producing no disease-specific mortality and no increased incidence of sepsis, in contrast to the sepsis rates typically seen in this model. We succeeded in altering the microbiota of FMT-treated piglets, and although most differences did return to pre-treatment levels, there were significant differences in colonic mucosal-associated bacteria that persisted through to termination on day 7, 5 days after treatment. These initial results are encouraging and raise several exciting new questions for future avenues of research into refining this technique for use in children with dysbiosis due to short bowel syndrome.

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Appendix A: Supplementary Tables and Figures

Table S1 - Quantification of Phyla in base and term tissue samples from SAL, FMT, and SOW piglets.

			JB	JT	CB	CT
Group	**Phylum**	**n**	11	11	11	11
SAL	Actinobacteria		1.6 (2.4)	3.5 (4.1)	4.4 (3.5)	6.2 (7.2)
	Bacteroidetes		6.5 (9.1)#	14.8 (8.2)	18.1 (8.1)#	8.9 (10.7)
	Firmicutes		42.2 (26.3)	53.4 (15.3)	65.8 (13.7)	62.8 (18.9)
	Fusobacteria		2.4 (3.5)	3.7 (5.2)	3.4 (3.6)	3.5 (5.1)
	Proteobacteria		4.2 (4.4)	7.6 (4.3)	4.3 (2.8)##	15.4 (8.8)
	Unassigned		43.0 (29.4)#	16.8 (17.8)	3.4 (2.6)	3.2 (5.0)
	Other		0.0	0.2 (0.6)	0.5 (1.6)	0.0
FMT		**n**	11	11	11	11
	Actinobacteria		1.9 (2.7)	1.7 (2.2)	4.4 (2.6)	3.1 (4.1)
	Bacteroidetes		2.7 (3.0)##	15.0 (9.7)	21.3 (11.5)#	8.7 (12.4)
	Firmicutes		34.2 (18.5)	45.1 (12.5)	64.8 (10.9)	57.8 (17.2)
	Fusobacteria		0.7 (1.2)#	4.2 (5.1)	2.2 (3.0)	2.9 (4.9)
	Proteobacteria		6.0 (4.2)	7.1 (5.6)	3.8 (3.2)##	23.7 (17.3)
	Unassigned		54.4 (21.8)#	26.4 (21.6)	3.0 (2.6)	2.9 (3.1)
	Other		0.04 (0.1)	0.4 (0.8)	0.5 (1.5)	0.8 (2.7)
SOW		**n**		6		6
	Actinobacteria		-	2.2 (2.9)	-	1.5 (1.4)
	Bacteroidetes		-	11.5 (10.3)	-	27.5 (6.3)ǂ§
	Firmicutes		-	35.5 (16.1)	-	56.5 (7.8)
	Fusobacteria		-	2.7 (3.2)	-	3.6 (1.0)
	Proteobacteria		-	6.2 (3.2)	-	4.0 (1.5)ǂ§§
	Unassigned		-	41.6 (25.2)	-	5.6 (3.2)
	Other		-	0.4 (0.4)	-	1.5 (1.5)

Relative % compositions are presented as mean (SD). Samples were taken from saline (SAL, n=11), fecal microbial transplant (FMT, n=11), and non-surgical sow-reared (SOW, n=6) piglets. For SAL and FMT base to term, comparisons made using related samples Wilcoxon Signed Rank tests, #p<0.05, ##p<0.01. For SOW to SAL and SOW to FMT, comparisons made using independent samples Kruskal-Wallis tests. Significant differences as indicated: SOW and SAL (ǂp<0.05, ǂǂp<0.01) and SOW and FMT (§p<0.05, §§p<0.01) phyla. JB = jejunum base, JT = jejunum term, CB = colon base, CT = colon term.

Table S2 – Quantification of Phyla in daily stool samples from SAL and FMT piglets.

Group	Phylum	n	pre	post	d3	d4	d5	d6
SAL			8	8	11	10	10	10
	Actinobacteria		1.9 (1.9)	2.3 (2.9)	1.1 (2.4)	2.9 (2.7)	6.6 (8.8)	7.8 (10.4)
	Bacteroidetes		9.0 (8.3)	1.7 (1.9)	5.7 (6.0)	4.5 (3.7)	6.2 (8.5)	4.7 (5.8)
	Firmicutes		52.3 (30.8)	55.1 (28.4)	41.5 (20.5)	49.9 (20.1)	51.5 (19.8)	54.6 (23.8)
	Fusobacteria		0.7 (1.6)	0.7 (2.1)	2.7 (5.8)	0.9 (1.6)	3.4 (5.5)	5.0 (6.1)
	Proteobacteria		35.3 (28.9)	39.6 (30.2)	48.5 (16.7)	41.5 (18.0)	31.7 (17.2)	27.6 (15.6)
	Unassigned		0.4 (0.8)	0.5 (0.7)	0.4 (0.7)	0.2 (0.3)	0.6 (0.9)	0.3 (0.4)
	Other		0.4 (1.1)	0.0	0.0	0.0	0.0	0.0
FMT		**n**	7	11	11	11	11	10
	Actinobacteria		0.6 (0.9)	8.6 (4.0)**	5.5 (6.0)*	5.0 (3.2)	4.9 (4.5)	7.5 (3.5)
	Bacteroidetes		4.4 (4.0)	4.0 (2.4)	3.8 (4.5)	7.8 (11.1)	7.0 (9.5)	5.6 (8.2)
	Firmicutes		59.3 (37.8)	54.3 (17.7)	50.1 (24.2)	59.3 (24.7)	58.3 (19.5)	61.9 (20.6)
	Fusobacteria		0.08 (0.14)	0.30 (1.0)	1.3 (2.4)	4.0 (8.3)	5.5 (8.1)	3.9 (8.9)
	Proteobacteria		34.5 (36.6)	29.7 (20.9)	38.8 (23.8)	23.1 (21.0)	24.0 (19.7)	20.0 (13.8)
	Unassigned		1.0 (1.9)	2.7 (1.5)***	0.4 (0.8)	0.8 (1.2)	0.4 (0.7)	1.1 (1.3)
	Other		0.0	0.4 (0.4)	0.0	0.0	0.0	0.02 (0.07)

Relative % compositions are presented as mean (SD). Samples were taken from saline (SAL, n=11), and fecal microbial transplant (FMT, n=11) piglets. Comparisons made using independent samples Mann-Whitney U test, *p<0.05, **p<0.01.

Table S3 - Quantification of Families in base and term tissue samples.

Group	Family	n	JB	JT	CB	CT
SAL			11	11	11	11
	Bifidobacteriaceae		0.4 (0.8)	0.7 (1.5)	0.5 (1.1)	1.4 (3.1)
	Bacteroidaceae		0.8 (2.8)#	3.0 (3.0)	2.0 (2.8)	2.1 (3.8)
	Lactobacillaceae		17.0 (12.9)	16.5 (9.4)	14.5 (5.2)	23.0 (21.9)
	Streptococcaceae		1.0 (1.4)	0.2 (0.5)	2.2 (2.7)#	0 (0)
	Clostridiaceae		5.6 (7.6)	4.1 (3.1)	6.5 (5.8)	11.1 (23.4)
	Lachnospiraceae		4.0 (5.3)##	13.5 (5.5)	18.7 (9.2)	12.8 (13.2)
	Peptostreptococcaceae		1.8 (4.4)	2.4 (2.3)	3.4 (1.9)#	0.9 (1.6)
	Ruminococcaceae		1.4 (1.2)	3.3 (3.6)	6.6 (4.2)##	1.7 (1.3)
	Veillonellaceae		1.2 (2.3)	0.6 (1.2)	0 (0)	0 (0)
	Erysipelotrichaceae		1.2 (1.9)	2.2 (2.0)	3.6 (3.3)	1.8 (2.3)
	Fusobacteriaceae		2.4 (3.5)	3.7 (5.2)	3.4 (3.6)	3.5 (5.1)
	Campylobacteraceae		1.0 (2.4)	1.1 (1.9)	0.1 (0.4)#	2.9 (5.2)
	Enterobacteriaceae		1.0 (1.1)	2.6 (3.6)	0.3 (0.9)##	10.4 (8.6)
	Coriobacteriaceae		1.1 (1.7)	2.7 (3.6)	2.9 (2.3)	3.3 (2.9)
	Unassigned		57.0 (23.4)	35.3 (18.4)	25.2 (9.5)	19.4 (11.3)
	Other		3.2 (2.4)	7.2 (4.0)	10.1 (4.3)	5.8 (6.1)

	n	11	11	11	11
FMT	Bifidobacteriaceae	0.8 (2.1)	0.4 (1.4)	0.3 (0.8)	1.5 (2.8)
	Bacteroidaceae	0.3 (0.6)	1.1 (1.2)	1.4 (2.2)	0.4 (1.0)
	Lactobacillaceae	18.6 (16.0)	14.2 (7.5)	15.7 (6.7)	21.2 (15.5)
	Streptococcaceae	0.7 (1.7)	1.8 (2.5)	3.3 (6.1)	2.7 (5.0)
	Clostridiaceae	2.8 (3.3)	5.8 (5.4)	6.5 (3.7)	8.3 (14.4)
	Lachnospiraceae	2.2 (2.1)#	8.9 (5.8)	17.2 (10.1)#	5.9 (7.1)
	Peptostreptococcaceae	0.4 (0.9)#	1.8 (1.6)	4.3 (3.1)#	1.3 (3.6)
	Ruminococcaceae	0.7 (1.0)#	3.8 (3.6)	5.5 (5.2)#	2.2 (2.6)
	Veillonellaceae	0.2 (0.5)	0.5 (1.1)	0.1 (0.5)#	2.6 (3.7)**
	Erysipelotrichaceae	0.3 (0.4)	1.2 (1.7)	2.5 (2.2)#	0.9 (1.4)
	Fusobacteriaceae	0.7 (1.2)#	4.2 (5.1)	2.2 (3.0)	2.9 (4.9)
	Campylobacteraceae	1.7 (3.3)	1.1 (2.1)	0.4 (1.1)#	9.7 (15.6)
	Enterobacteriaceae	2.1 (3.1)	1.9 (2.2)	0 (0)##	4.5 (3.3)
	Coriobacteriaceae	0.5 (0.8)	0.9 (1.3)	2.9 (2.4)	1.5 (3.0)
	Unassigned	64.8 (17.4)#	44.7 (14.6)	28.2 (7.8)	22.0 (11.5)
	Other	3.2 (2.9)	7.7 (5.0)	9.4 (4.8)	12.2 (8.8)

	n		6		6
SOW	Bifidobacteriaceae	-	0.6 (1.2)	-	0.3 (0.7)
n=6	Bacteroidaceae	-	0.5 (1.2)	-	0.8 (1.4)
	Lactobacillaceae	-	9.0 (4.0)	-	9.7 (3.6)
	Streptococcaceae	-	1.2 (1.4)	-	0.9 (1.7)
	Clostridiaceae	-	3.4 (3.4)	-	8.4 (3.5)
	Lachnospiraceae	-	8.8 (10.5)	-	8.3 (2.6)
	Peptostreptococcaceae	-	2.1 (1.3)	-	9.0 (3.8)ǂ§§
	Ruminococcaceae	-	3.5 (3.0)	-	11.0 (3.2)ǂ§§
	Veillonellaceae	-	0.01 (0.02)	-	0.1 (0.3)
	Erysipelotrichaceae	-	0.7 (0.8)	-	0.4 (0.7)
	Fusobacteriaceae	-	2.6 (3.2)	-	3.4 (1.1)
	Campylobacteraceae	-	0.5 (1.3)	-	0 (0)
	Enterobacteriaceae	-	2.3 (2.1)	-	0.7 (0.9)ǂ
	Coriobacteriaceae	-	1.5 (2.7)	-	0.8 (0.8)
	Unassigned	-	57.8 (20.1)	-	34.8 (9.3)ǂ
	Other	-	5.4 (2.6)	-	11.5 (3.7)

Relative % compositions are presented as mean (SD). Samples were taken from saline (SAL, n=11), fecal microbial transplant (FMT, n=11), and non-surgical sow-reared (SOW, n=6) piglets. For base to term within SAL and FMT groups, comparisons made using related samples Wilcoxon Signed Rank tests, #p<0.05, ##p<0.01. For SOW to SAL (ǂp<0.05, ǂǂp<0.01) and SOW to FMT (§p<0.05, §§p<0.01), comparisons made using independent samples Kruskal-Wallis tests. For FMT to SAL comparisons made using independent samples Mann-Whitney U test, *p<0.05, **p<0.01. JB = jejunum base, JT = jejunum term, CB = colon base, CT = colon term.

Table S4 – Quantification of Families in daily stool samples.

Group	Family	n	pre 8	post 8	d3 11	d4 10	d5 10	d6 10
SAL	Bifidobacteriaceae		0.4 (1.0)	0.1 (0.3)	0 (0)	0.5 (1.7)	2.3 (7.2)	3.1 (9.5)
	Bacteroidaceae		3.8 (6.2)	0.3 (0.8)	1.1 (1.7)	0.7 (1.2)	0.4 (0.8)	0.8 (1.5)
	Lactobacillaceae		22.5 (19.6)	13.0 (19.2)	11.4 (16.0)	32.3 (24.3)	34.7 (24.5)	33.0 (18.9)
	Streptococcaceae		0.6 (0.8)	0.5 (0.8)	0 (0)	0.1 (0.2)	0.03 (0.09)	0.05 (0.15)
	Clostridiaceae		2.9 (4.0)	1.6 (3.5)	1.0 (2.0)	1.4 (1.6)	2.8 (3.7)	1.2 (1.5)
	Lachnospiraceae		2.0 (2.0)	3.8 (6.3)	1.3 (3.4)	2.8 (5.1)	5.9 (7.0)	5.9 (6.5)
	Peptostreptococcaceae		3.4 (4.5)	1.0 (1.4)	0.3 (0.7)	0.6 (1.4)	0.06 (0.2)	1.1 (1.4)
	Ruminococcaceae		0.3 (0.5)	0.8 (1.4)	0.3 (0.7)	0.4 (0.9)	0.3 (0.6)	0.5 (0.7)
	Veillonellaceae		0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Erysipelotrichaceae		0.5 (0.7)	1.0 (1.7)	0.1 (0.2)	0.5 (0.8)	0.5 (1.0)	0.6 (1.1)
	Fusobacteriaceae		0.7 (1.6)	0.7 (2.1)	2.7 (5.8)	0.9 (1.6)	3.4 (5.5)	5.0 (6.1)
	Campylobacteraceae		0.02 (0.06)	0 (0)	0.2 (0.6)	0 (0)	0.1 (0.4)	0.6 (1.2)
	Enterobacteriaceae		26.3 (32.5)	37.8 (32.1)	47.8 (16.9)	40.7 (17.7)	30.1 (17.0)	25.3 (15.6)
	Coriobacteriaceae		0.7 (1.2)	1.7 (3.1)	1.0 (2.4)	2.3 (2.7)	4.3 (3.3)	4.4 (2.6)
	Unassigned		22.1 (27.2)	33.6 (25.7)	29.7 (19.4)	13.5 (11.3)	11.2 (6.7)	14.8 (14.5)
	Other		13.8 (15.1)	4.0 (5.3)	3.2 (3.4)	3.3 (3.1)	3.8 (3.5)	3.7 (4.4)

Group	Family	n	pre 8	post 8	d3 11	d4 10	d5 10	d6 10
FMT	Bifidobacteriaceae		0.3 (0.7)	1.9 (1.6)**	2.3 (4.4)*	1.3 (1.8)	1.2 (1.3)	2.4 (2.5)
	Bacteroidaceae		1.1 (1.9)	0.2 (0.6)	0.3 (0.7)	1.1 (2.5)	0.3 (0.6)	0.8 (1.5)
	Lactobacillaceae		15.7 (13.2)	29.2 (15.1)*	28.6 (25.4)*	33.0 (29.7)	33.1 (25.4)	33.8 (22.8)
	Streptococcaceae		0.6 (1.1)	1.6 (1.3)	3.2 (4.3)**	2.0 (4.8)	2.7 (5.6)	7.3 (21.8)
	Clostridiaceae		1.5 (3.2)	7.4 (4.7)**	2.4 (2.4)	4.0 (4.1)	3.4 (7.4)	2.9 (2.5)
	Lachnospiraceae		2.2 (4.2)	2.5 (3.4)	2.3 (3.0)	6.4 (8.0)	6.8 (8.0)	5.2 (6.2)
	Peptostreptococcaceae		1.2 (2.4)	3.9 (2.2)**	0.8 (1.1)	1.0 (1.6)	0.2 (0.5)	0.7 (1.9)
	Ruminococcaceae		0.4 (1.0)	1.7 (1.9)	0.5 (1.4)	0.9 (1.5)	1.2 (1.8)	0.8 (1.3)
	Veillonellaceae		0 (0)	0.1 (0.3)	0.4 (1.3)	1.3 (1.9)	1.4 (2.1)	1.4 (2.5)
	Erysipelotrichaceae		0.7 (0.8)	1.6 (1.7)	1.7 (1.8)*	2.5 (2.7)	1.6 (2.3)	1.5 (2.0)
	Fusobacteriaceae		0.1 (0.1)	0.3 (1.0)	1.3 (2.4)	4.0 (8.3)	5.5 (8.1)	3.9 (8.9)
	Campylobacteraceae		0.3 (0.9)	0 (0)	0.2 (0.4)	0.4 (1.1)	0.8 (1.8)	1.5 (3.5)
	Enterobacteriaceae		27.7 (36.0)	30.9 (20.4)	38.1 (23.7)	20.9 (22.0)*	20.9 (20.5)	13.8 (14.8)
	Coriobacteriaceae		1.3 (2.8)	6.3 (3.0)**	3.2 (2.2)*	3.4 (2.9)	3.6 (3.6)	4.9 (4.3)
	Unassigned		42.5 (39.3)	9.1 (5.1)*	12.9 (5.3)*	14.9 (8.3)	13.7 (7.8)	15.6 (9.0)
	Other		4.5 (4.3)	3.2 (2.3)	1.8 (2.4)	2.9 (2.7)	3.6 (5.2)	3.5 (3.6)

Relative % compositions are presented as mean (SD). Samples were taken from saline (SAL, n=11), and fecal microbial transplant (FMT, n=11) piglets. Comparisons of FMT to SAL made using independent samples Mann-Whitney U test, *$p<0.05$, **$p<0.01$.

Table S5 – Comparison of Donor FMT material to FMT piglet daily stools.

			FMT				
	Donor	pre	post	d3	d4	d5	d6
Phylum **n**	3	7	11	11	11	11	10
Actinobacteria	4.5 (0.9)	0.6 (0.9)!	8.6 (4.0)#**	5.5 (6.0)	5.0 (3.2)	4.9 (4.5)	7.5 (3.5)
Bacteroidetes	27.0 (3.7)	4.4 (4.0)!	4.0 (2.4)	3.8 (4.5)!!	7.8 (11.1)	7.0 (9.5)!	5.6 (8.2)!
Firmicutes	48.1 (3.0)	59.3 (37.8)	54.3 (17.7)	50.1 (24.2)	59.3 (24.7)	58.3 (19.5)	61.9 (20.6)
Fusobacteria	0.0	0.08 (0.14)	0.30 (1.0)	1.3 (2.4)	4.0 (8.3)	5.5 (8.1)	3.9 (8.9)
Proteobacteria	4.4 (1.4)	34.5 (36.6)	29.7 (20.9)	38.8 (23.8)	23.1 (21.0)	24.0 (19.7)	20.0 (13.8)
Unassigned	11.9 (3.4)	1.0 (1.9)!	2.7 (1.5)*	0.4 (0.8)!	0.8 (1.2)!	0.4 (0.7)!	1.1 (1.3)
Other	4.1 (0.1)	0.0	0.4 (0.4)	0.0	0.0	0.0	0.02 (0.07)
Family							
Bifidobacteriaceae	1.0 (0.4)	0.3 (0.7)	1.9 (1.6)#**	2.3 (4.4)*	1.3 (1.8)	1.2 (1.3)	2.4 (2.5)
Bacteroidaceae	0.4 (0.3)	1.1 (1.9)	0.2 (0.6)	0.3 (0.7)	1.1 (2.5)	0.3 (0.6)	0.8 (1.5)
Lactobacillaceae	2.4 (1.0)	15.7 (13.2)	29.2 (15.1)	28.6 (25.4)	33.0 (29.7)!	33.1 (25.4)	33.8 (22.8)!
Streptococcaceae	0 (0)	0.6 (1.1)	1.6 (1.3)	3.2 (4.3)!***	2.0 (4.8)	2.7 (5.6)	7.3 (21.8)
Clostridiaceae	5.3 (1.3)	1.5 (3.2)	7.4 (4.7)#*	2.4 (2.4)	4.0 (4.1)	3.4 (7.4)	2.9 (2.5)
Lachnospiraceae	14.9 (3.9)	2.2 (4.2)	2.5 (3.4)!	2.3 (3.0)!	6.4 (8.0)	6.8 (8.0)	5.2 (6.2)
Peptostreptococcaceae	2.0 (1.9)	1.2 (2.4)	3.9 (2.2)#*	0.8 (1.1)	1.0 (1.6)	0.2 (0.5)	0.7 (1.9)
Ruminococcaceae	17.1 (1.3)	0.4 (1.0)	1.7 (1.9)#	0.5 (1.4)!!	0.9 (1.5)!	1.2 (1.8)!	0.8 (1.3)!
Veillonellaceae	0.3 (0.1)	0 (0)	0.1 (0.3)!	0.4 (1.3)!	1.3 (1.9)	1.4 (2.1)	1.4 (2.5)
Erysipelotrichaceae	0.3 (0.1)	0.7 (0.8)	1.6 (1.7)	1.7 (1.8)	2.5 (2.7)	1.6 (2.3)	1.5 (2.0)
Fusobacteriaceae	0 (0)	0.1 (0.1)	0.3 (1.0)	1.3 (2.4)	4.0 (8.3)	5.5 (8.1)	3.9 (8.9)
Campylobacteraceae	0.3 (0.3)	0.3 (0.9)	0 (0)!!	0.2 (0.4)	0.4 (1.1)	0.8 (1.8)	1.5 (3.5)
Enterobacteriaceae	0.9 (0.7)	27.7 (36.0)	30.9 (20.4)	38.1 (23.7)	20.9 (22.0)	20.9 (20.5)	13.8 (14.8)
Coriobacteriaceae	3.5 (0.5)	1.3 (2.8)	6.3 (3.0)#	3.2 (2.2)	3.4 (2.9)	3.6 (3.6)	4.9 (4.3)
Unassigned	38.1 (3.7)	42.5 (39.3)	9.1 (5.1)!#	12.9 (5.3)!	14.9 (8.3)	13.7 (7.8)	15.6 (9.0)
Other	13.3 (0.9)	4.5 (4.3)	3.2 (2.3)	1.8 (2.4)	2.9 (2.7)	3.6 (5.2)	3.5 (3.6)

Relative % compositions are presented as mean (SD). Daily stool samples were taken from fecal microbial transplant (FMT) treated piglets and compared to the processed Donor FMT material, as well as to samples taken from saline (SAL) piglets (see Table S6). Comparisons made using independent samples Kruskal-Wallis test reported with Bonferroni correction, !$p<0.05$, !!$p<0.01$. Comparisons between pre and post within FMT made using related samples Wilcoxon Signed Rank test, #$p<0.05$.

Table S6 – Comparison of Donor FMT material to SAL piglet daily stools.

			SAL					
		Donor	pre	post	d3	d4	d5	d6
Phylum	**n**	3	8	8	11	10	10	10
Actinobacteria		4.5 (0.9)	1.9 (1.9)	2.3 (2.9)**	1.1 (2.4)	2.9 (2.7)	6.6 (8.8)	7.8 (10.4)
Bacteroidetes		27.0 (3.7)	9.0 (8.3)	1.7 (1.9)!!	5.7 (6.0)!	4.5 (3.7)	6.2 (8.5)	4.7 (5.8)!
Firmicutes		48.1 (3.0)	52.3 (30.8)	55.1 (28.4)	41.5 (20.5)	49.9 (20.1)	51.5 (19.8)	54.6 (23.8)
Fusobacteria		0.0	0.7 (1.6)	0.7 (2.1)	2.7 (5.8)	0.9 (1.6)	3.4 (5.5)	5.0 (6.1)
Proteobacteria		4.4 (1.4)	35.3 (28.9)	39.6 (30.2)	48.5 (16.7)!	41.5 (18.0)!	31.7 (17.2)!	27.6 (15.6)!
Unassigned		11.9 (3.4)	0.4 (0.8)	0.5 (0.7)!!*	0.4 (0.7)!	0.2 (0.3)!	0.6 (0.9)!	0.3 (0.4)!!
Other		4.1 (0.1)	0.4 (1.1)	0.0	0.0	0.0	0.0	0.0
Family								
Bifidobacteriaceae		1.0 (0.4)	0.4 (1.0)	0.1 (0.3)**	0 (0)*!!	0.5 (1.7)	2.3 (7.2)	3.1 (9.5)
Bacteroidaceae		0.4 (0.3)	3.8 (6.2)	0.3 (0.8)	1.1 (1.7)	0.7 (1.2)	0.4 (0.8)	0.8 (1.5)
Lactobacillaceae		2.4 (1.0)	22.5 (19.6)	13.0 (19.2)	11.4 (16.0)	32.3 (24.3)!	34.7 (24.5)	33.0 (18.9)!
Streptococcaceae		0 (0)	0.6 (0.8)	0.5 (0.8)	0 (0)***	0.1 (0.2)	0.03 (0.09)	0.05 (0.15)
Clostridiaceae		5.3 (1.3)	2.9 (4.0)	1.6 (3.5)*	1.0 (2.0)!	1.4 (1.6)	2.8 (3.7)	1.2 (1.5)!
Lachnospiraceae		14.9 (3.9)	2.0 (2.0)	3.8 (6.3)	1.3 (3.4)!!	2.8 (5.1)	5.9 (7.0)	5.9 (6.5)
Peptostreptococcaceae		2.0 (1.9)	3.4 (4.5)	1.0 (1.4)*	0.3 (0.7)	0.6 (1.4)	0.06 (0.2)	1.1 (1.4)
Ruminococcaceae		17.1 (1.3)	0.3 (0.5)	0.8 (1.4)!!	0.3 (0.7)!!	0.4 (0.9)!!	0.3 (0.6)!!	0.5 (0.7)!
Veillonellaceae		0.3 (0.1)	0 (0)	0 (0)!!	0 (0)!!	0 (0)!	0 (0)!	0 (0)!
Erysipelotrichaceae		0.3 (0.1)	0.5 (0.7)	1.0 (1.7)	0.1 (0.2)	0.5 (0.8)	0.5 (1.0)	0.6 (1.1)
Fusobacteriaceae		0 (0)	0.7 (1.6)	0.7 (2.1)	2.7 (5.8)	0.9 (1.6)	3.4 (5.5)	5.0 (6.1)
Campylobacteraceae		0.3 (0.3)	0.02 (0.06)	0 (0)!!	0.2 (0.6)	0 (0)!	0.1 (0.4)	0.6 (1.2)
Enterobacteriaceae		0.9 (0.7)	26.3 (32.5)	37.8 (32.1)	47.8 (16.9)!	40.7 (17.7)!	30.1 (17.0)!!	25.3 (15.6)!!
Coriobacteriaceae		3.5 (0.5)	0.7 (1.2)	1.7 (3.1)!	1.0 (2.4)	2.3 (2.7)	4.3 (3.3)	4.4 (2.6)
Unassigned		38.1 (3.7)	22.1 (27.2)	33.6 (25.7)	29.7 (19.4)	13.5 (11.3)!	11.2 (6.7)!	14.8 (14.5)!
Other		13.3 (0.9)	13.8 (15.1)	4.0 (5.3)	3.2 (3.4)	3.3 (3.1)	3.8 (3.5)	3.7 (4.4)

Relative % compositions are presented as mean (SD). Daily stool samples were taken from saline (SAL) treated piglets and compared to the processed Donor FMT material, as well as to samples taken from fecal microbial transplant (FMT) piglets (see Table S5). Comparisons made using independent samples Kruskal-Wallis test reported with Bonferroni correction, !$p<0.05$, !!$p<0.01$.

Table S7 – Observed OTUs and Faith's Phylogenetic Diversity for Daily Stool samples.

			Stool					
			pre	post	d3	d4	d5	d6
Group		**n**	8	8	11	10	10	10
SAL	Observed OTUs		53 (14)	53 (22)!!!	50 (14)!!!	60 (11)!!!	60 (15)!!!	61 (11)!!!
	Faith's PD		7.2 (1.1)	6.1 (1.4)!	6.2 (1.8)!	6.8 (1.9)!	7.2 (2.4)!	7.1 (1.4)!
FMT		**n**	7	11	11	11	11	9
	Observed OTUs		44 (14)	71 (14)*!!!	59 (13)*!!!	62 (18)!!!	68 (18)!!!	64 (19)!!!
	Faith's PD		6.6 (1.9)	9.9 (2.0)**!	6.8 (1.9)!	7.0 (2.0)!	7.8 (2.1)!	7.7 (1.8)!
Donor		**n**		3				
	Observed OTUs			130 (4)				
	Faith's PD			18.4 (0.6)				

Shown above are summarized details on observed operational taxonomic units (OTUs) to a read depth of 2000, and Faith's Phylogenetic Diversity (PD) for saline (SAL) and fecal microbial transplant (FMT) treated piglets, compared to Donor material. Samples were taken day 2 pre-treatment (pre), day 2 post-treatment (post), and days 3 – 6 (d3 – d6). Data are presented as mean (SD). OTU comparisons between FMT and SAL pre-treatment made using independent samples Mann-Whitney U test. OTU comparisons between SAL, FMT, and Donor in post - d6 samples and Faith's PD between SAL, FMT, and Donor made using independent samples Kruskal-Wallis test reported with Bonferroni correction. Differences shown to Donor by !$p<0.05$, !!$p<0.01$, !!!$p<0.001$ and between FMT and SAL by *$p<0.05$, **$p<0.01$. See Table 2 for full quantification.

Table S8 – Observed OTUs and Faith's Phylogenetic Diversity for base and term tissue samples.

			JB	JT	CB	CT
Group		**n**	11	11	11	11
SAL	Observed OTUs		73 (18)!!!	66 (13)§§§!!!	73 (9.1)!!!	55 (5.4)ǂǂ!!!
	Faith's PD		35 (16)	13 (7.8)ǂ	5.9 (1.2)	7.7 (3.5)
FMT		**n**	11	11	11	11
	Observed OTUs		67 (12)!!!	76 (10)*§!!!	68 (7.8)!!!	61 (17)!!!
	Faith's PD		38 (11)	23 (16)	6.2 (1.1)	6.1 (1.0)!§
SOW		**n**		6		6
	Observed OTUs		-	94 (26)!!!	-	86 (24)!!!
	Faith's PD		-	40 (23)	-	8.7 (1.6)
Donor		**n**		3		
	Observed OTUs		-	111 (4.9)		
	Faith's PD		-	11 (0.5)		

Shown above are summarized details on observed operational taxonomic units (OTUs) to a read depth of 2667, and Faith's Phylogenetic Diversity (PD) for saline (SAL), fecal microbial transplant (FMT) treated piglets, sow-reared control piglets (SOW), compared to Donor material. Data are presented as mean (SD). Comparisons made using independent samples Kruskal-Wallis tests reported with Bonferroni correction. Post-hoc differences shown for Donor (!$p<0.05$, !!$p<0.01$, !!!$p<0.001$), FMT and SAL (*$p<0.05$), SOW to SAL (ǂ$p<0.05$, ǂǂ$p<0.01$), and SOW to FMT (§$p<0.05$, §§$p<0.01$).