Designer probiotics as a novel therapeutic against inflammatory bowel disease

by

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Abstract

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a major health burden globally. Current pharmaceutical therapies are risky or ineffective, for long-term use and are associated with severe side effects. Therefore, new alternative therapies for IBD are needed. Probiotic therapy, which is the ingestion of non-pathogenic microorganisms to provide health benefits, is considered a potential treatment option. However, clinical trials using probiotics for IBD treatment have yielded very inconsistent and difficult to interpret data. The gut environment of IBD patients undergoes inflammation and oxidative stress, which may interfere with the growth and therefore beneficial effects of probiotics. Current probiotics on the market are ineffective at survival and colonization in the hostile gut of IBD patients. We hypothesize that novel designer probiotics, which are genetically modified to enhance survival and colonization in an IBD gut, will result in better efficacy of probiotic therapy against IBD. The overall objective was to determine if these genetically modified probiotics had improved survival and growth in an inflamed IBD gut and enhanced ability to colonize the mammalian gut. The probiotics were tested in murine colitis models, DSS-induced colitis and $Muc2^{-/-}$ spontaneous colitis, to determine if the modified probiotics were more efficacious during colitis compared to their parent strains. Both the *E. coli* and *L. reuteri* designer strains, compared to their parent strains, were shown to be more protective in the DSS-induced colitis through lower clinical scores, reduced histopathological scores, and increased protective responses including butyric acid, RegIIIγ, and Muc2. In the $Muc2^{-/-}$ spontaneous colitis model, the *E. coli* designer strain, was shown to be protective through lower clinical scores, reduced frequency of rectal prolapses, lower CFU bacterial counts, lower histopathological scores, and

decreased expression of pro-inflammatory cytokine IFN-γ gene expression. With strain detection, only the *E. coli* designer strain had an established detection assay and was shown to have non-persistent detection in C57BL/6 mice but had semi-persistent detection in the $Muc2^{-/-}$ mice. This research could result in genetically improved probiotics, with enhanced persistence and colonization, leading to better efficacy during IBD therapy and a potential alternative therapeutic option for IBD.

Lay Summary

Inflammatory bowel disease (IBD) is a major health burden in developed countries. Current pharmaceutical therapies are risky or ineffective, cost and health-wise, for long-term use and are associated with severe side effects. Therefore, new alternative therapies for IBD are needed. Probiotic therapy, which is the ingestion of non-pathogenic microorganisms to provide health benefits, is considered a potential treatment option. However, clinical trials using probiotics for IBD treatment have yielded very inconsistent and difficult to interpret data. Specific to IBD, the gut environment is highly inflamed and oxidized; these properties may interfere with the growth and therefore beneficial effects of probiotics. As such, current probiotics are ineffective at colonization and survival in the hostile gut of IBD patients. This study looks at designer probiotics as an alternative to traditional probiotics to enhance bioavailability. This research could result in improved probiotics as a potential alternative therapeutic option for IBD patients.

Preface

Chapters 2 and 3 are based on work completed in the IDEAS Lab at UBC Okanagan under the supervision of Dr. Deanna Gibson. The designer strains were designed by Dr. Deanna Gibson and Dr. Artem Godovannyi and created by Dr. Artem Godovannyi. I was responsible for all the experimental work and data analysis. All the animal experiments were approved by the UBC Animal Care Committee (certificate #: A15-0201). I conducted all of the animal work in the Biosciences Facility at UBC Okanagan with assistance from Jacqueline Barnett. All tissues for histopathology and immunofluorescence microscopy were processed by Wax-It Histology and the Child & Family Research Institute (CFRI) histology groups in Vancouver, BC. Short-chain fatty acid analysis was conducted on the GC machine in collaboration with Dr. Sanjoy Ghosh. Strain detection assay with PCR was established with help from Dr. Anton Callaway. Dr. Anton Callaway also helped with PCR, gel electrophoresis, and gene expression qPCR of the $Muc2^{-/-}$ spontaneous colitis experiment.

Work from this thesis was presented as a poster at the 2017 CAG CDDW conference in Banff, AB and as an oral presentation at the 2017 9^{th} Probiotics, Prebiotics, and New Foods conference in Rome, Italy. Work from this thesis was submitted as a patent filed on February 19, 2018: International Patent: Probiotic compositions and uses thereof. (Held with UBC) Patent No: PCT/CA2018/050188, in which I assisted in drafting. Chapter 1 is being written as a manuscript for a review article on designer probiotics and work from Chapter 3 is being drafted as a manuscript.

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List of Abbreviations

AAD	Antibiotic Associated Diarrhea
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CD	Crohn's Disease
CDI	*Clostridium difficile* Infection
CO_2	Carbon Dioxide
dH_2O	Deionized Water
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DCs	Dendritic Cells
DSS	Dextran Sodium Sulphate
EtOH	Ethyl Alcohol
EEF2	Eukaryotic Elongation Factor 2
EHEC	Enterohaemorrhagic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FID	Flame Ionization Detector
FMT	Fecal Microbial Transplantation
GIT	Gastrointestinal Tract
H&E	Hematoxylin and eosin
H_2O	Water
IFN	Interferon
IBD	Inflammatory Bowel Disease

IgG	Immunoglobulin G
IL	Interleukin
IECs	Intestinal Epithelial Cells
IBS	Irritable Bowel Syndrome
LAB	Lactic Acid Bacteria
LD	Light day
LN2	Liquid Nitrogen
MHC	Major Histocompatibility Complex
MCT	Microcentrifuge Tube
MAPK	Mitogen Activated Protein Kinase
MALT	Mucosa-Associated Lymphoid Tissue
MLN	Mesenteric Lymph Nodes
NBF	Neutral Buffered Formalin
NCBI	National Center for Biotechnology Information
NF	Nuclear Factor
NK	Natural Killer
NOD	Nucleotide-Binding Oligomerization Domain
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PRRs	Pathogen Recognition Receptors
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species

sIgA	Secretory Immunoglobin A
SCFA	Short Chain Fatty Acids
SOD	Superoxide Dismutase
SPF	Specific Pathogen Free
TBP	TATA-Binding Protein
TH	T helper
TER	Trans-Epithelial Electrical Resistance
TFF	Trefoil Factor
TLRs	Toll-like Receptors
TNF	Tumor Necrosis Factor
UC	Ulcerative Colitis
WHO	World Health Organization

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Lastly, I would like to acknowledge that this research was supported through a grant from Crohn’s and Colitis Canada awarded to Dr. Deanna Gibson.

Dedication

To my parents.

Chapter 1: Introduction

1.1 Probiotics

According to the World Health Organization (WHO), probiotics are “live microorganisms which when administered in adequate amounts, confer a health benefit on the host” (World Health Organization & Food and Agriculture Organization of the United Nations, 2002). The theory of probiotics exerting health benefits dates back to Élie Metchnikoff, who was the first to suggest that the consumption of fermented milk containing lactic acid bacteria (LAB) would lead to good health (Metchnikoff & Mitchell, 1908). LAB are commonly used as probiotics but there is a diverse range of bacteria and even yeast that can be considered probiotics. The WHO outlines guidelines for probiotics and states that probiotics must be identified by their strain name, have proof of safe use, and have some evidence of a beneficial effect (World Health Organization & Food and Agriculture Organization of the United Nations, 2002).

Given the increasingly widespread use, probiotic therapy has been used for the treatment of both gastrointestinal (GI) and non-GI associated illnesses. However, the evidence to support the use of probiotics has been inconclusive. Possible beneficial evidence is shown for necrotizing enterocolitis in pre-term infants (AlFaleh & Anabrees, 2014) and acute diarrhea (Allen, Martinez, Gregorio, & Dans, 2010). However, the efficacy of probiotics in many other conditions, like inflammatory bowel disease (IBD), *Clostridium difficile*-associated diarrhea, urinary tract infections, eczema, non-alcoholic fatty liver disease, allergies, food hypersensitivity, and irritable bowel syndrome (IBS) is absent or very minimal. There is still much need for randomized, controlled human trials to document the health benefits and mechanisms for the use of probiotics. Many of the

current claims of probiotics are inflated and further research is needed to test the efficacy of probiotics.

1.1.1 Probiotic market and regulation

Globally, the probiotic market has become a multi-billion dollar market (Grand View Research Inc, 2018). Although probiotic consumption is usually in the form of yogurt, probiotics can be found in other forms such as food supplements. Besides the traditional pills, capsules, or powder, probiotics are available as juices, sugars, sprays, gum, soaps, cleaning products, and even mattresses. Often manufacturers select certain bacterial strains that are easy to mass produce, and not necessarily because they have evidence for beneficial use. This often includes strains from *Lactobacillus* and *Bifidobacterium* genera. Studies have been carried out to test such probiotic products to match their label strain and dose with what is detected in the product. For example, a study by Lewis *et al.* tested 16 probiotic products and found that only 1 out of the 16 products contained its label claims for the presence of Bifidobacterial strains (Lewis et al., 2016). The lack of product quality regulations can interfere with potential beneficial effects of probiotics. This can have adverse effects on certain individuals. For instance, a study looking at celiac patients found that those supplementing with probiotics, reported more symptoms. A test of probiotics products that were labelled gluten-free revealed that 55% of the probiotics were contaminated with gluten (Science Daily, 2015).

The misuse of the word probiotic has been highlighted as a potential cause of misleading concepts for use in certain illnesses and conditions. A misconception arises

from claims made on probiotic labels. Stricter regulations are needed to ensure the quality of products labelled as probiotics. Because of these claims, a common misconception is that probiotics can have an overall beneficial effect on gut health. There is no evidence to suggest probiotics have any benefit in healthy individuals.

Product labels have different claims about potential benefits, which range from general “core benefits” to specific examples of mechanisms. The WHO recommends that if scientific evidence was available to back claims, it should be publicly made available. However, in Canada, there is a lack of rigorous screening processes since probiotics are marketed as food supplements. The Food and Drugs Act (FDA) is the primary regulatory act that regulates quality of food in Canada (Agriculture and Agri-Food Canada, 2010). Depending on whether the product is a drug or food supplement, regulations differ. There are different regulations for whether a product is a functional food or natural health product (NHP). If the products are advertised as dietary supplements or foods, manufacturers are allowed to make vague claims without any scientific evidence. The categorization of products into these groups remains ambiguous and often products can be miscategorized and therefore not regulated correctly.

1.2 Inflammatory bowel disease

Within the spectrum of IBD, there is Crohn’s disease (CD) and ulcerative colitis (UC). UC involves inflammation of the colon, whereas CD can occur anywhere in the GIT (Abraham & Cho, 2009). This is accompanied by mucosal ulceration, increase of immune cells, abscess or granuloma formation, and depletion of goblet cell mucin

(Xavier & Podolsky, 2007). This can lead to symptoms such as abdominal pain, diarrhea, rectal bleeding, weight loss, and fatigue. With respect to the pathogenesis of IBD, both genetic and non-genetic factors can contribute. Environmental factors would include diet (Hou, Abraham, & El-Serag, 2011), antibiotic use (Hviid, Svanstrom, & Frisch, 2011), smoking habits (Seksik, Nion-Larmurier, Sokol, Beaugerie, & Cosnes, 2009), and stress (Bernstein et al., 2010). In the last couple of decades, there has been an increase in incidences of IBD, which likely can be attributed to the environmental factors (Atreya & Siegmund, 2017). IBD is a complex multifactorial disease and it has been shown that often clinical symptoms correlate poorly with underlying mucosal inflammation (Peyrin-Biroulet et al., 2016).

1.2.1 Inflammatory bowel disease and inflammation

The mucosa acts as the primary area where mucosa-associated lymphoid tissue (MALT) is able to interact with the external environment (Turner, 2009). The GIT is the largest mucosal surface in the human body and, therefore, is in a continual interaction with bacteria and antigens. The regions of the GIT are divided into four layers. This includes the mucosa (epithelium, lamina propria, and muscular mucosae), submucosa (with submucosal plexus), muscularis propria (inner circular muscle layer, mesenteric plexus, and outer longitudinal muscle layer), and serosa. The mucosa, innermost layer, is the site at which the majority of absorption occurs. This mucosal layer consists of three sections. The first section facing the intestinal lumen is made up of epithelial cells. This connects to the basement membrane that overlays the second layer called the lamina propria, which consists of sub-epithelial connective tissue and lymph nodes.

Underneath these layers, is the deepest layer called the muscularis mucosa. This entire mucosa sits on the submucosa, with the muscularis propria underneath it. The submucosal region is where a diverse range of inflammatory cells like eosinophils, lymphocytes, plasma cells, and neutrophils are found. When the levels of these cells are elevated, colonic inflammation can occur (Al-Ghadban, Kaissi, Homaidan, Naim, & El-Sabban, 2016).

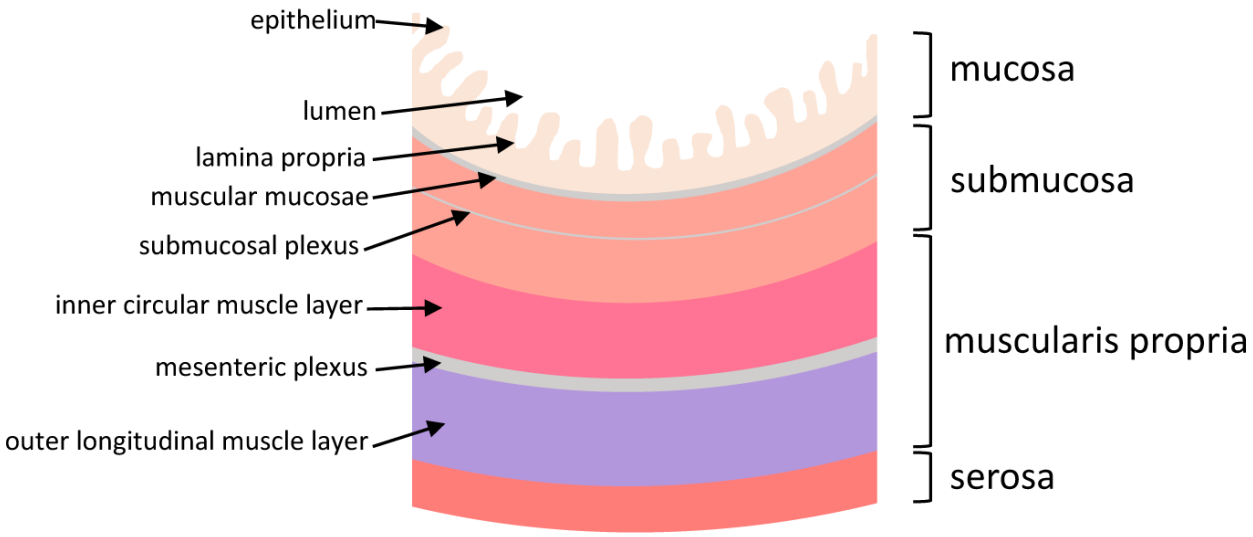

Figure 1. Layers of the tissues found in the large intestine

IBD is characteristic of infiltration of immune cells into the mucosa of the intestines (Al-Ghadban et al., 2016). There is an infiltration of immune cells (neutrophils, macrophages, DCs, and NK cells) into the lamina propria. Increases in the numbers of these cells leads to activation of immune mediator cytokines including TNF-α, IL-1β, and IFN-γ. T helper cells (Th1, Th2, and Th17). Immune cells can facilitate immune response by secreting cytokines, such as IL-17A, IFN-γ, IL-10, and IL-4. The activation

of epithelial cells, macrophages, and dendritic cells stimulates the nuclear factor ϰB (NF-ϰB) and mitogen activated protein kinase (MAPK) signalling pathways, which leads to the production of further cytokines. These cells that become closer to the epithelial layer can play a role in its regulation. Epithelial dysfunction results in intestinal inflammation, which can include dysfunctions in epithelial cell development, barrier, or cell matrix adhesion (Abraham & Cho, 2009). Breaking the epithelial barrier results in inflammation due to epithelial dysfunction. A defective epithelial layer can lead to antigens or bacteria leaking into the surrounding tissues, which can further the inflammation and lead to infection.

1.2.2 Dysbiosis in inflammatory bowel disease

A variety of diseases are associated with a microbial profile with lower species diversity, a decrease in beneficial microbes, and an increase of pathogenic microbes. IBD has been linked to dysbiosis, which is the imbalance of beneficial and pathogenic microbes in the gut (Chan, Estaki, & Gibson, 2013). An altered gut microbiota plays a role in the pathogenesis of IBD (Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gómez-llorente, & Gil, 2012). Although the exact etiology of IBD is yet to be determined, the gut microbiota is central to the disease (Nagao-Kitamoto et al., 2016). It has been shown that germ-free (GF) mice do not develop age-associated inflammation, due to the lack of a gut microbiota and dysbiosis (Thevaranjan et al., 2017).

Overall, IBD patients show shifts in *Faecalibacterium* and increases in Gammaproteobacteria (Frank et al., 2011). Specifically, in CD patients, there are reduced levels of beneficial microbes like *Eubacterium rectale*, *Bacteroides fragilis*, and

Ruminococcus but increased levels of potentially pathogenic Enterococcus, *Clostridium difficile*, and *Shigella flexerni* (Kang et al., 2010). In particular, a study by Sokol *et al*. (Sokol et al., 2008) showed that CD patients had a reduction in *Faecalibacterium prausnitzii*. *F. prausnitzii* has been shown to lower pro-inflammatory IL-12 and IFN-γ production, increase secretion of anti-inflammatory IL-10, and reduce TNBS-induced colitis in mice. Furthermore, adherent-invasive *E. coli* (AIEC) strains have been isolated from biopsies of CD patients (Sepehri et al., 2011) and has shown to be increased in UC patients (Sokol, Lepage, Seksik, Doré, & Marteau, 2006). *Fusobacterium* has also been detected in higher abundances in the colonic mucosa of UC patients (Ohkusa et al., 2002).

Nonbacterial members like fungi and viruses have also been associated with gastrointestinal diseases. Virome shifts have been shown in CD and UC patients compared to healthy controls (Norman et al., 2015). A decrease in overall viral diversity, with an increase in richness of *Caudovirales* phage has been shown in CD and UC patients (Wagner et al., 2013). The virome can participate in intestinal inflammation since the lysis of bacteria would cause the release of proteins, lipids, and nucleic acids that could induce inflammation. Recently, the FDA has cleared a phase 1 and 2 study for the use of bacteriophages designed to treat IBD by targeting AIEC strains in CD patients (Young, 2018). Fungi groups have also been shown to be changed in IBD patients compared to healthy controls (Sokol et al., 2017). There is an increased Basidiomycota/Ascomycota ratio, decreased *Saccharomyces cerevisiae*, and increased *Candida albicans*. This shows that fungi could play a role in IBD pathogenesis.

1.2.3 Pharmaceutical treatments

Current pharmaceutical treatments are intended to maintain remission and control symptoms in patients rather than reversing the pathogenic mechanism. The IBD treatment pyramid shown in Fig 1, has been used as a treatment plan for decades. The treatment options start at the bottom of the pyramid and move up to stronger drugs as the disease severity increases.

5-aminosalicylates (5-ASAs) start at the bottom of the pyramid. Mesalazine is the main 5-ASA currently used for IBD treatment. 5-ASAs are recommended for UC, but for CD they show little or no efficacy in controlling clinical symptoms (Lim, Wang, MacDonald, & Hanauer, 2016). 5-ASAs have been shown to be effective for UC (Wang, Parker, Bhanji, Feagan, & MacDonald, 2016) and have been shown to have reduced risk in developing colorectal tumors (Velayos, Terdiman, & Walsh, 2005).

Antibiotics for IBD can be rationalized since bacteria can play a role in the development of IBD. Certain antibiotics like ciprofloxacin or rifaximin against Gram-negative bacteria have been investigated. These are however, associated with side effects like CDI (Nitzan, Elias, Chazan, Raz, & Saliba, 2013). A study looking at children and development of CD shows that antibiotic use is an associated environmental risk factor that can influence early life changes in the gut microbiota (Hviid et al., 2011). Antibiotic resistance is another major concern of antibiotic use for IBD. A study looking at CD patients found that 2/3 of the Gram-negative isolated strains were resistant to ciprofloxacin (Park et al., 2014). Another study found that 12/48 IBD associated *E. coli* strains were associated with prior rifaximin treatment (Kothary et al., 2013).

Corticosteroids show evidence for inducing remission in UC and CD (Baumgart & Sandborn, 2012; Danese & Fiocchi, 2011). However, corticosteroids are not recommended for maintenance long term therapy. They have adverse effects like hypertension, hyperglycaemia, psycho-neurological disturbances, cataracts, adrenal suppression, growth failure in children, osteonecrosis (Stein & Hanauer, 2000), and steroid dependent disease (Khan, Mehmood, & Khan, 2015).

Immunosuppressants are used to induce remission in steroid-dependent or steroid-refractory disease. Examples of immunosuppressants include 6-mercaptopurine (6-MP) and azathioprine (AZA). Patients taking immunosuppressants, like AZA and 6-MP, have an increased risk of lymphoma (Kandiel, Fraser, Korelitz, Brensinger, & Lewis, 2005) and this has been linked to increases in the Epstein-Barr virus (Dayharsh et al., 2002; Vos et al., 2011).

Many IBD patients are refractory or intolerant to the 5-ASA, corticosteroids, and immunosuppressants. Biological agents for IBD target the TNF since the cytokine TNF plays a key role in intestinal inflammation. Remicade, or infliximab, is the most widely used biological agent. However, biological agents are not suited for long-term use. The main side effects include opportunistic infections like tuberculosis and granulomatous infections (Ford & Peyrin-Biroulet, 2013; Nyboe Andersen, Pasternak, Friis-Møller, Andersson, & Jess, 2015; Shah, Farida, Siegel, Chong, & Melmed, 2017). Anti-TNF for CD shows no efficacy, suggesting differences in the mechanism of drugs on different disease profiles (Sandborn et al., 2001). In addition, CD patients taking anti-TNF agents along with immunosuppressants have an increased risk of non-Hodgkin's lymphoma (Siegel, Marden, Persing, Larson, & Sands, 2009).

The last treatment on the pyramid is surgical removal or rejoining of affected regions in the GIT. This is a treatment option for patients with aggressive disease that is refractory. There are complications associated with surgery including impaired anal function that can cause neurological damage in the rectum, urinary and sexual dysfunction, diarrhea, frequent bowel movements, and an overall decreased quality of life (Ishii et al., 2015). Shown in meta-analyses, 50% of CD patients need a surgical resection, and within 10 years 35% of those need a second operation (Frolkis et al., 2014). It has been shown that endoscopic recurrence occurs in 70-90% of patients, in as little as 1 week after surgery (Olaison, Smedh, & Sjödahl, 1992). In UC patients, “pouchitis” can develop, which is inflammation in the ileal pouch of patients who undergo colectomy (Paiva et al., 2018). This can affect up to 45% of patients that undergo surgery. Surgery itself has many complications and does not provide a long-term treatment option. Since, the current pharmaceutical therapies are either risky or ineffective, cost and health-wise, for long-term use, new therapies for IBD are needed.

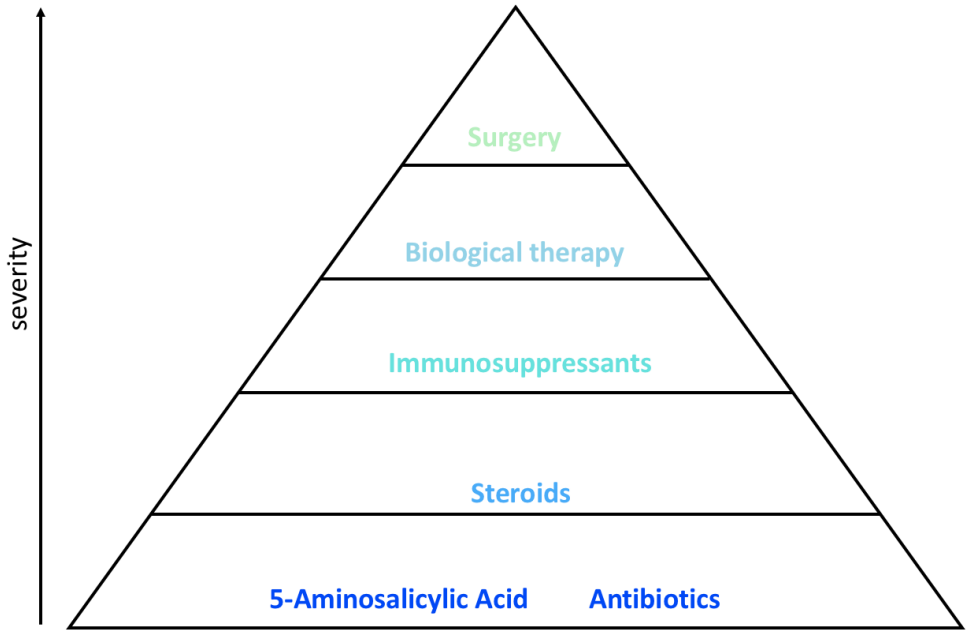

Figure 2. Traditional IBD pharmaceutical treatment approach.

1.2.4 Alternative treatments

There has been a growing interest and need in alternative therapeutic options for IBD. Among them is fecal microbial transplantation (FMT). It consists of administering fecal solution from a donor, that is mixed with saline solution, strained, and then placed into a recipient. (Smits, Bouter, Vos, Borody, & Nieuwdorp, 2013). The first use of FMT was shown with *C. difficile* infection, in which there was an 81% resolution of CDI after just the first infusion (van Nood et al., 2013). Since then, many case studies have reported cure rates of 87-90% (Kelly et al., 2015). In addition, the recipient microbial community has been shown to resemble those of the donor after transplantation (Khoruts, Dicksved, Jansson, & Sadowsky, 2010). Thus, FMT is being considered for use in other conditions that are associated with dysbiosis.

Preliminary studies of FMT in IBD have been shown to be promising, with many patients achieving remission (Paramsothy, Kamm, et al., 2017; H. Thomas, 2017). Several studies for IBD conclude that patients receiving the donor FMT received clinical remission in both UC and CD (He et al., 2017; Narula et al., 2017). A recent meta-analysis shows that 36% of UC and 51% of CD patients received clinical remission with FMT (Paramsothy, Paramsothy, et al., 2017). In another study, patients with UC showed increased fever and C-reactive protein (Angelberger et al., 2013). Overall, FMT appears to be safe and beneficial for the treatment of UC. However, there is a need for more research since many studies thus far have small sampling size, lack standardization, and control groups. In addition, the patients have variation in disease type, severity, and medications. Other factors that vary are the route of transplantation, the number of fusions, and the variability within fecal donors.

1.3 Probiotics and inflammatory bowel disease

There has been much interest in the use of probiotics for IBD. A study from Sonneburg *et al.* (2006) shows that manipulating the microbiota with probiotics can influence the host. Germ free mice were colonized with *B. thetaiotaomicron* (part of the adult gut microbiota) along with a probiotic strain *B. longum*. *B. longum* was able to repress the expression of *B. thetaiotaomicron* antibacterial proteins and promote its own survival and influence the composition and function of the microbial community (Sonnenburg, Chen, & Gordon, 2006). Metabolic studies have shown that probiotics can modulate the gut microbiome through metabolism of SCFA, amino acids, bile acids, and plasma lipoproteins showing the symbiotic metabolic relationship between the gut

microbiota and the host (Martin et al., 2008). Studies with probiotics and animal models have been promising. Studies of probiotics in DSS-induced murine colitis (Atkins et al., 2012; Cui et al., 2016; Talero et al., 2015; Toumi et al., 2013; Zhang et al., 2016) and IL-10$^{-/-}$ mice (McCarthy et al., 2003; O'Mahony et al., 2001; Schultz et al., 2002; Sheil et al., 2004) show that probiotics can improve clinical symptoms or disease severity through various possible mechanisms such as modulation through junction proteins (Bermudez-Brito et al., 2012; Johnson-Henry, Donato, Shen-Tu, Gordanpour, & Sherman, 2008), reduction of pro-inflammatory cytokines (Hacini-Rachinel et al., 2009; Kamada et al., 2008; Madsen et al., 2001), increase in T cells (Roselli et al., 2009), SCFA production (Ahrne, Jeppsson, & Molin, 1998; Yoshida, Tsukahara, & Ushida, 2009), promotion of mucus secretions (Mack, Ahrne, Hyde, Wei, & Hollingsworth, 2003), and Ig production (Fukushima, Kawata, Hara, Terada, & Mitsuoka, 1998; Kaila et al., 1992; Ohashi & Ushida, 2009).

Because of the potential of certain probiotics in influencing the gut microbiota composition, there have been many randomized controlled trials using probiotic supplementation for IBD. However, studies of probiotics in IBD have yielded mixed results with variation in the disease severity and probiotic strain. Importantly, in human trials, the proof of efficacy and safety is still lacking. Many meta-analyses conclude that there is not enough evidence to recommend probiotics for maintenance in UC (Naidoo, Gordon, Fagbemi, Thomas, & Akobeng, 2011), induction in UC (Mallon, McKay, Kirk, & Gardiner, 2007), maintenance in CD (Rolfe, Fortun, Hawkey, & Bath-Hextall, 2006), and induction of remission in CD (Butterworth, Thomas, & Akobeng, 2008).

1.3.1 Current probiotics for Crohn's disease

The evidence to support the use of probiotics for CD in human trials is limited. Table 1 summarizes all of the randomized, controlled human trials in adults. In a few studies, the effects of the non-pathogenic yeast *Saccharomyces boulardii* was examined in CD patients in remission (Bourreille et al., 2013; Garcia Vilela et al., 2008; Guslandi, Mezzi, Sorghi, & Testoni, 2000). In the first study by Guslandi et al, CD patients were given the probiotic in combination with mesalazine for 6 months (Guslandi et al., 2000). Overall, this study showed that those supplementing with the probiotic and mesalazine combined had significantly lower relapses. Garcia et al showed that supplementing with *S. boulardii* with baseline 5-ASAs had improved intestinal barrier function, measured by lactulose/mannitol ratio, compared to the placebo group (Garcia Vilela et al., 2008). However, this improvement was only seen at the end of the third month of supplementing and this study did not report on any relapse rates. In addition, the last study looking at *S. boulardii* in CD patients showed that *S. boulardii* had no beneficial effects on relapses, disease activity index scores, or C-reactive protein compared to the placebo group (Bourreille et al., 2013). In this study, CD patients were given 5-ASAs or steroids to induce remission and then weaned off them before the probiotic treatment. From these 3 studies looking at *S. boulardii*, there are conflicting results. In addition, two other studies looking at *Lactobacillus strains*, *L. rhamnosus* GG and *L. johnsonii* LA1, given in combination with corticosteroids for 6 months failed to significantly induce remission or prevent endoscopic recurrence in probiotic groups compared to the placebo groups (Marteau et al., 2006; Schultz et al., 2004)

Overall, probiotics have been shown to be tolerated in CD but the concluding results from these studies is not positive. Currently, there is no sufficient evidence to recommend probiotics for CD. This may be because there is no clearly defined dose that is ideal for testing probiotics. CD is a complex disease and different patients may have a different disease severity depending on the location of the disease in the GIT. In conclusion, larger scale trials are needed before probiotics can be recommended for CD patients.

Table 1. Probiotic studies for Crohn's disease

Paper	Probiotic	Conclusion
Guslandi et al (2000)	*Saccharomyces boulardii*	may be beneficial
Schultz et al (2004)	*Lactobacillus rhamnosus* GG	no beneficial effects
Marteau et al (2005)	*Lactobacillus johnsonii* LA1	no beneficial effects
Garcia et al (2008)	*Saccharomyces boulardii*	possible improved intestinal health
Bourreille et al (2013)	*Saccharomyces boulardii*	no beneficial effects

1.3.2 Current probiotics for ulcerative colitis

The evidence for randomized, controlled human trials for UC is also conflicting. Studies looking at EcN in UC show that EcN is just as effective as standard pharmaceutical treatments. In some of the first trials looking at EcN in UC patients for inducing remission and for maintaining remission, there is no difference in clinical scores and relapse rates between the EcN probiotic and mesalazine groups (Kruis et al., 1997, 2004a; Rembacken, Snelling, Hawkey, Chalmers, & Axon, 1999). In a study looking at probiotic enemas for EcN, remission rates were dose-dependent on the volume of probiotic enema administered (Matthes, Krummenerl, Giensch, Wolff, &

Schulze, 2010). Although some studies showed possible beneficial effects with *Lactobacillus* strains on disease scores (D'Incà et al., 2011; Hegazy & El-Bedewy, 2010), other studies show that there is no difference between probiotic and mesalazine groups (Zocco et al., 2006). Many of the probiotics used in combination with standard therapy, do not provide any benefit when compared to the standard therapy alone. Another probiotic, VSL#3 cocktail, tested for UC shows mixed results. Some studies show that it does not provide any benefit alone in inducing remission, clinical score, or histological activity (S. C. Ng et al., 2010). Other studies show that it was able to decrease disease activity and induce remission when given in combination with standard therapies vs therapy alone or a placebo group (Sood et al., 2009; Tursi et al., 2004, 2010).

Overall, majority of the clinical studies for UC show no significant differences in effectiveness between the probiotic group and the control/placebo. If there is a difference, it is seen when the probiotic is given in combination with a standard pharmaceutical therapy. It is difficult to generalize results between studies since the strains, dose, patient profile, antibiotic, and pharmaceutical drug use is different across studies. Further studies are needed to investigate the role of probiotics and any beneficial effects in UC.

Table 2. Probiotic studies for ulcerative colitis

Paper	Probiotic	Conclusion
Kruis et al (1997)	*E. coli* Nissle 1917	no difference compared to mesalazine
Rembacken et al (1999)	*E. coli Nissle 1917*	no difference compared to mesalazine
Ishikawa et al (2003)	Yakult	possible preventive effects on relapse rates
Cui et al (2004)	BIFICO	could be effective in preventing flare-ups
Kato et al (2004)	Yakult	possible beneficial effects
Tursi et al (2004)	VSL#3	balsalazide & VSL#3 may be a good choice in the treatment of active mild-to-moderate active UC instead of balsalazide or mesalazine alone
Kruis et al (2004)	*E. coli* Nissle 1917	no difference compared to mesalazine
Zocoo et al (2006)	*Lactobacillus rhamnosus*	no difference compared to mesalazine
Sood et al (2009)	VSL#3	VSL#3 is safe and effective in achieving clinical responses and remissions in patients with mild-to-moderately active UC.
Zwolinska et al (2009)	Lacidofil	probiotics with mesalazine or azathioprine improve the healing process in UC patients with significant fungal colonization of the colon mucosa
Hegazy et al (2010)	*Lactobacillus delbruekii* and *Lactobacillus fermentum*	could be helpful in maintaining flare ups
Matthes et al (2010)	*E. coli* Nissle 1917	possible supplementary treatment option
Ng et al (2010)	VSL#3	no clinical changes alone, only with probiotic + corticosteriods
Tursi et al (2010)	VSL#3	VSL#3 supplementation reduces clinical scores in patients with relapsing mild-to-moderate colitis in combination with 5-ASA and/or immunosuppressant's
D'Inca et al (2011)	*Lactobacillus casei* DG	manipulation of mucosal microbiota needed to mediate beneficial effects
Tomasello et al (2011)	Acronelle Bromatech	patients receiving the combined treatment responded better than those receiving only 5-ASA
Wildt et al (2011)	*Lactobacillus acidophilus* La-5 and *Bifidobacterium* BB-12	no difference between placebo group

Paper	Probiotic	Conclusion
Groeger et al (2013)	*Bifidobacterium longum* subsp infantis 35624	may reduce systemic inflammatory biomarkers
Petersen et al (2014)	*E. coli* Nissle 1917	does not support use of probiotic as an add on to conventional therapy
Shadnoush et al (2015)	*Lactobacillus acidophilus* La-5 and *Bifidobacterium* BB-12	may help improve intestinal health
Yoshimatsu et al (2015)	Bio-Three	may be effective for maintaining clinical remission in patients with inactive ulcerative colitis
Palumbo et al (2016)	Acronelle Bromatech	long term therapy with probiotics + mesalazine could be an alternative to corticosteriods
Tamaki et al (2016)	*Bifidobacterium longum* 536	reduced clinical scores in patients with mild-moderate UC

1.3.3 Challenges in the gut

There are many challenges associated with the delivery of probiotics into the GIT. With a supply of nutrients and environmental protection, the gut is an ideal environment for many bacteria, but it can be a struggle to reach the intestines. Probiotics have to be able to survive and move down to the GIT. There are many host-associated stresses like low pH in the stomach, bile production, and high osmolarity in the intestines (Culligan, Hill, & Sleator, 2009). Probiotics have to be able to face such conditions in order to survive and make it down to the GIT. One of these challenges is the process of peristalsis. In humans, the mean velocity of the movement of luminal contents is 20 µm/s (Cremer et al., 2016). This inflow to the colon has a low bacterial count. A study looking at cecal and fecal samples, found that there were lower bacterial counts in fecal samples compared to cecal (Marteau et al., 2001). For example, facultative anaerobes were lower in the fecal samples with 1% representation of total

bacteria compared to 25% in cecal. It can be expected that the inflow to the colon would have bacterial depletion. Certain bacteria that are motile are able to overcome this, but many of the abundant bacterial groups do not have genes for the flagellin proteins for movement (Cremer et al., 2016).

Microbes face competition when trying to compete and survive. This includes competition for environmental niche and nutrients. More established dominating bacterial strains are able to compete against other strains. Niches in the gut have been shown to only support a limited population, indicating competition amongst microbes (S. M. Lee et al., 2013). For example, Bacteroides species have ccf genes that are upregulated during colonization at the colonic surface in the gut. Specifically, *B. fragilis* has been shown to penetrate the colonic mucus and reside in the crypt channels. Mono-colonization in a gnotobiotic mouse with *B. fragilis* blocks colonization by a second inoculation of the same strain (S. M. Lee et al., 2013). For nutrients, microbes have certain strategies that they use when trying to compete. The bacterial strain *B. thetaiotamicron* has been shown to attach to food particles and mucus in gnotobiotic mice. This would give this strain an advantage in accessing nutrients over bacterial strains that cannot attach.

Pathogen invasion is another challenge and competition for microbes in the gut. Pathogens have complex systems that allow them to defend and successfully establish in the gut. This includes overcoming the host immune system. For example, *Vibrio cholerae* has been shown to avoid secretory IgA (sIgA) by downregulating certain receptors (Hsiao, Liu, Joelsson, & Zhu, 2006). SIgA is part of the first line of defense in protection of the epithelial layer from antigens and pathogenic microorganisms (Mantis,

Rol, & Corthésy, 2011). Strains of *E. coli* are able to express a binding antigen that interacts with sIgA through immunocapturing (Pastorello et al., 2013). This allows the bacteria to inhibit the sIgA-induced neutrophil chemotaxis and avoid clearance by immune cells. *Salmonella* Typhimurium is able to secrete a cytokine, IL-22, which results in production of antimicrobials against *E. coli* and therefore *S.* Typhimurium is able to outcompete for nutrients (Behnsen et al., 2014). In addition, *S.* Typhimurium uses secretory systems that act against strains from the Proteobacteria and Bacteroidetes phyla (Bönemann, Pietrosiuk, Diemand, Zentgraf, & Mogk, 2009; Hood, Peterson, & Mougous, 2017). Many pathogens act opportunistically and are able to invade when the host is undergoing an undesirable state. *C. difficile* is able to expand and cause infection when the host is taking broad-spectrum antibiotics (Loo et al., 2011). This results in niche environments and nutrients being freed up. *C. difficile* and *S.* Typhimurium are able to use liberated mucosal fucose and sialic acid found in the lumen of the gut (K. M. Ng et al., 2013). Such pathogens make it hard for probiotic bacteria to be able to find adequate nutrients and a niche to establish.

Oxygens levels can also factor into bacterial survival. The mucus layer is high in oxygen whereas, the lumen is very low (Albenberg et al., 2014). Oxygen tolerant bacteria are able to grow near the mucosal layer. Given that most bacteria are strict anaerobes, it is difficult for them to grow within the mucosal layer. Certain bacteria like *E. coli* and *E. faecalis* are able to utilize different electron acceptor systems, making this a growth advantage for them (Jones et al., 2007).

1.3.3.1 Probiotics ineffective at colonizing the gut

An important factor in the success of probiotics is the ability to colonize and establish into the microbiota. If colonization occurs, this could provide for the beneficial effects of the probiotic without needing to continually supplement. The ability to be able to colonize depends on the mechanisms the bacteria may have to help it overcome the host defense mechanisms. To avoid being removed, bacteria have to be able to attach to the host cells early on. Studies have looked at the isolation of supplemented probiotic strains from fecal and biopsy samples and have shown that there is no stable colonization of the gut past 2 weeks. Probiotic strains are not able to outcompete and they face strong competition when trying to colonize (Alander et al., 1999). In a study with healthy adults, *E. coli* Nissle 1917 was administered for 17 days and it was shown that only 45% of those individuals had EcN positive stool samples at 2 weeks (Joeres-nguyen-xuan, Boehm, & Joeres, 2010). When the probiotic samples were assessed at 48 weeks, the probiotic was no longer detectable. In another study, a probiotic mixture with *L. rhamnosus* GG was administered for a 2-week period to healthy adults (Saxelin et al., 2010). Only 30% of the participants had probiotic positive samples at 3 weeks. Other strains of *Lactobacillus* found in the same probiotic mixture were undetectable in all of the participants. Similarly, *L. rhamnosus* was undetectable after 2 weeks in adults given a commercial drink (Alander et al., 1999). This demonstrates that even adherent strains can be gradually diluted out of the colon unless they are replenished with a fresh inoculum of the strain. There is a lack of studies to support the colonization and consequent persistence of probiotics in the gut. Even in the absence of any disease, there is a lack of evidence to support colonization of probiotics in healthy individuals.

1.3.3.2 Probiotics ineffective at surviving in the gut

Specifically, for IBD patients, the gut is highly inflamed and oxidized and this may prevent probiotic strains from surviving. The gut is hostile when undergoing chronic inflammation and oxidative stress during an active flare-up of IBD (Abraham & Cho, 2009). Tissue damage has been shown to be a result of the mucosal immune reactions that initiate inflammatory cascades (Keshavarzian et al., 2003). Oxidative stress is caused by reactive oxygen (ROS) and nitrogen (RNS) species like peroxide, superoxide, and nitric oxide (Barboza, Guizzardi, Moine, & Talamoni, 2017). This begins with inflammatory cells and proinflammatory mediators being recruited. ROS and RNS at low amounts are involved as signalling molecules but high amounts can lead to oxidative stress, in which there is an imbalance in production and elimination of reactive molecules (Pereira, Grácio, Teixeira, & Magro, 2015). These molecules are produced by cellular systems on membranes, cystol, and peroxisomes (Di Meo, Reed, Venditti, & Victor, 2016). In the gut, these molecules are produced by gut epithelial cells during inflammatory conditions (Winter et al., 2011). These molecules can damage all forms of cellular macromolecules. They can do so by modifying protein functions and causing lipid peroxidation (Andersen, 2004). ROS and RNS at low amounts are involved as signalling molecules and under physiological conditions, the cell can tolerate some levels of ROS and RNS, but high amounts can cause oxidative stress. (Di Meo et al., 2016). These reactive species have been shown to play a role in diseases like type 2 diabetes, cancer, cardiovascular disease, neurological diseases, and autoimmune diseases like IBD (Sedelnikova et al., 2010). In IBD, these species can cause disturbances in the mucosal barrier and immune cell infiltration due to the body's

defense system. This makes it hard for symbiotic bacteria to survive. Only bacteria that are resilient to these conditions are able to survive and this may hinder the survival and colonization of probiotics. For instance, *S.* Typhimurium uses its virulence factors to be able to use by-products during inflammation and oxidative stress (Diaz-Ochoa et al., 2016). For example, it can utilize tetrathionate, usually found during gut inflammation, as a terminal electron acceptor (Winter et al., 2011). It can outcompete some of the other bacterial strains like certain commensals and thrive in inflammatory and oxidized conditions. The low survivability of probiotics during intestinal inflammation and oxidative stress may contribute to the low efficacy of probiotics in IBD patients.

1.4 Designer probiotics

Despite the potential health benefits of probiotics, there are still limitations. The exact mechanism or therapeutic mode of action is still not fully understood and the evidence to show current probiotics being effective in the treatment of IBD is very limited. However," designer probiotics", "recombinant probiotics, or "probiotics 2.0" provide a means of overcoming the limitations of current probiotics. This involves introducing new genetic factors to strains to improve their efficacy.

For example, modifying a *L. lactis* strain that was engineered to produce interleukin-10 (IL-10), has shown direct modulation of the mucosal immune system (Steidler, Lieven, Fiers, & Remaut, 2000). IL-10 is an anti-inflammatory cytokine that inhibits cytokine production by T cells and natural killer (NK) cells, and thus can down-regulate cytokine cascades and prove to be therapeutic during inflammation (Moore, Malefyt, Robert, & Garra, 2001). This modified probiotic strain was shown to ameliorate

disease in mice with a 50% reduction in colitis with DSS-induced colitis (Steidler et al., 2000) and TNBS colitis (del Carmen et al., 2014). It also prevented disease development in IL-10$^{-/-}$ mice (Steidler et al., 2000). *L. lactis* can also be used to produce other anti-inflammatory cytokines like IL-27, which it reduces colitis in mice by increasing the production of IL-10 (Hanson et al., 2014) or heme oxyegnaze-1 (HO-1). It increased production of the anti-inflammatory cytokine interleukin IL-10 and decreased the expression of pro-inflammatory cytokines (IL-1α and IL-6) (Shigemori et al., 2015). Genetic techniques have also been used to modify *L. lactis* to produce trefoil factor (TFF). TFFs are protective compounds that promote wound healing in the GIT (Sturm & Dignass, 2008). Manipulation of the *L. lactis* strain to enable delivery of TFF resulted in amelioration of DSS-induced colitis in mice and stimulated prostaglandin-endoperoxide synthase 2 expression, improving colitis in IL-10$^{-/-}$ mice (Vandenbroucke et al., 2004).

Another study looked at reducing reactive oxygen species (ROS) through the enzyme superoxide dismutase (SOD). The SOD gene from *S. thermophilus* was inserted into the probiotic strain *L. gasseri*, improving inflammation in IL-10$^{-/-}$ mice (Carroll et al., 2007). Similarly, *Bacteriodes ovatus* was engineered to deliver human keratinocyte growth factor-2 and was able to reduce DSS-induced colitis (Hamady et al., 2010). *Bifidobacterium longum* has been used to deliver the peptide alpha-melanocyte-stimulating hormone (α-MSH) and this shows reduction in pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and an increase in anti-inflammatory IL-10 (Wei et al., 2016).

These studies show the potential of designer probiotics in mice. Engineering strains for therapeutic use has become increasingly popular since the control of certain therapeutic molecules is possible. Some designer probiotics have even advanced to

human clinical trials. Recently, LACTIN-V, a designer probiotic to treat the recurrence of bacterial vaginosis, was registered for a phase II trial (ClinicalTrials.gov, 2016). In order to maintain the potential benefits and to improve the clinical efficacy of probiotics, further optimization of strains need to be achieved through the use of such novel approaches.

1.5 Design of *Lactobacillus reuteri*

Lactobacillus reuteri is a Gram-positive, rod shaped, anaerobic bacteria that is found in the intestines of healthy animals and humans. It is heterofermentative and can produce carbon dioxide, ethanol, acetate, and lactic acid from glucose fermentation (Morita, Toh, & Fukuda, 2008). It can anaerobically metabolize glycerol producing the antimicrobial reuterin (3-hydroxypriopionaldehyde). Reuterin can act against enteropathogens, yeasts, fungi, protozoa and viruses (Cleusix, Lacroix, Vollenweider, Duboux, & Le Blay, 2007). Reuterin is synthesized, *in vitro*, under pH, temperature, and anaerobic conditions similar to what would be found in the GIT (Chung, Axelsson, Lindgren, & Dobrogosz, 1989). *L. reuteri* has been shown to also produce folate and cobalamin, which are vitamin B_{12} nutrients that mammals are able to utilize (Sriramulu et al., 2008; C. M. Thomas et al., 2016).

L. reuteri has been shown to ameliorate clinical signs of DSS-induced colitis. This is through reduction of MPO, IL-1*β*, IL-6, and mKC, increase in mucus thickness, and increased expression of tight junction proteins occludin and ZO-1 (Ahl et al., 2016). Another study in the DSS model showed that *L. reuteri* supplementation suppressed P-selectin, which decreased leukocyte-endothelial cell interactions and platelet-endothelial

cell interactions (Schreiber et al., 2009). A study by Dicksved et al, showed that treatment with *L. reuteri* in the DSS model, decreased bacterial translocation from the intestines to the mesenteric lymph nodes (Dicksved et al., 2012). In the *Citrobacter*-induced model, *L. reuteri* reduced expression of the chemokine CCL2 and attenuated stressor-enhanced infectious colitis (Mackos et al., 2016). Thus, *L. reuteri* has been shown to have beneficial effects in a range of different mouse models.

Many microorganisms use specific proteins called adhesins to attach to specific surfaces to allow them to occupy their niches. The N-acetyl-glucosamine binding protein (GbpA) is an adhesin with a modular multi-domain structure (E. Wong et al., 2012). It has been shown that the first N-terminal domain ($GbpA_{D1}$) is required for binding to the intestinal mucin. GbpA binds to N-acetly-glucosamine (GlcNAc) oligosaccharides. *V. cholerae* is a well-known pathogen that is able to colonize on the intestinal mucin. Mucins contain GlcNAc as part of their network of O-linked glycans and therefore *V. cholerae* is able to colonize (Jensen, Kolarich, & Packer, 2010). GbpA is secreted by *V. cholerae* and this facilitates its growth in the intestinal area. Studies have shown that the survival of *V. cholerae* in the gut is dependent on its ability to be able to colonize successfully (Kirn, Jude, & Taylor, 2005).

We expressed the first domain of the GbpA adhesion protein from *V. cholerae* on the surface of *L. reuteri* DSM 20016 to enhance its ability to colonize in the mucosal surface in the gut. *L. reuteri* has a mucus binding protein (MBP) that has mucus binding domains (Etzold et al., 2014). The $GbpA_{D1}$ was expressed at the end of the MBP at the N-terminal domain. This makes the MBP more accessible and can increase mucosal colonization. A poly-glyine-serine linker was added between the two proteins to allow

independent folding and a strep-tag was added at the N-terminal of GbpA. The GbpA protein acts as a supplementary protein in addition to the MBP of the strain. This can lead to enhanced colonization and can promote more adhesion in the gut.

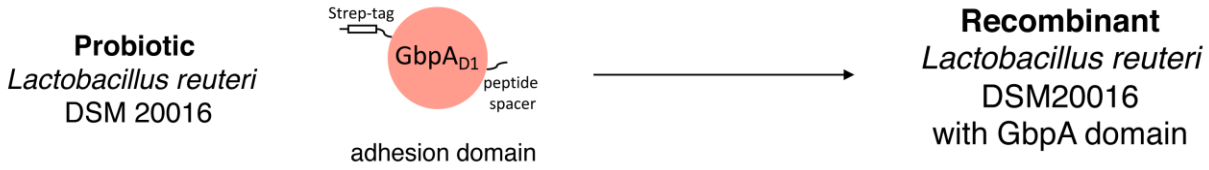

Figure 3. Design of recombinant *L. reuteri* probiotic strain

1.6 Design of *Escherichia coli* Nissle 1917

E. coli Nissle 1917 (EcN), or Mutaflor, is Gram-negative, facultative anaerobe bacteria. It was first isolated from a World War I solider, who did not develop *Shigella* bacterial dysentery like others had (Nzakizwanayo et al., 2015). Since *E. coli* strains are associated with pathogenicity, the biosafety of EcN has been tested. None of the pathogenic properties of enteropathogenic *E. coli* have been found (Sonnenborn & Schulze, 2009). It does not carry pathogenic adhesion factors, does not produce any enterotoxins or cytotoxins, and is not invasive. EcN has been shown to have an indirect antagonistic effect against invasive bacteria. It does so by stimulating non-specific defense mechanisms, like the synthesis of human β-defensin-2 in colonic epithelial cells (Wehkamp et al., 2004) and secretory components called microcins (Altenhoefer et al., 2004). EcN has been shown to inhibit EHEC in animal models (Maltby, Leatham-Jensen, Gibson, Cohen, & Conway, 2013), inhibit growth of and reduce Shiga toxin levels in STEC cultures (Reissbrodt et al., 2009), and prevent EPEC barrier disruption

by restoring trans-epithelial resistance and enhanced ZO-2 expression (Zyrek et al., 2007). In addition, expression of a “wxy” gene, which is required for LPS core biosynthesis, increases the ability to be able to withstand bacterial defense mechanisms (Grozdanov et al., 2002). Since EcN is able to act against pathogenic strains of *E. coli*, it is hypothesized that in an IBD gut, that would have these pathogenic strains present, it can provide some protection.

Studies in DSS-induced colitis show that EcN ameliorates colitis in mice via TLR-2- and TLR-4-dependent pathways (Grabig et al., 2006) and in chronic colitis was able to reduce levels of pro-inflammatory IFN-γ and IL-6 cytokines (Schultz et al., 2004). Human randomized controlled trials with EcN show that the probiotic was well tolerated with no adverse effects (Kruis et al., 1997, 2004b; Rembacken et al., 1999). However, it was not shown to have any differences between the placebo or mesalazine groups when looking at clinical parameters. Overall, EcN is equivalent to the pharmaceutical drug mesalazine in the maintenance of remission in UC.

The enzyme tetrathionate reductase is encoded from a 5-gene operon in *Salmonella* species (Winter et al., 2011). It has been shown that bacteria, specifically Enterobacteriaceae, are able to use tetrathionate as a terminal respiratory electron acceptor (Hensel, Hinsley, Nikolaus, Sawers, & Berks, 1999). This is due to the ttrACBSR operon from *Salmonella enterica* that encodes for tetrathionate reductase. Tetrathionate is a highly oxidized compound that is a by-product of oxidative stress. ROS generated during oxidative stress reacts with luminal sulphur compounds like thiosulphate, to form tetrathionate. It has been shown that *S.* Typhimurium can grow and persist during inflammation due to its ability to utilize tetrathionate (Winter et al.,

2011). These compounds provide little to no growth for bacteria unless they are able to utilize them through special systems.

We have cloned the ttr operon from *Salmonella enterica* and inserted it into *E. coli* Nissle 1917 and enabled it to utilize tetrathionate. The recombinant strain of *E. coli* Nissle 1917 that is able to utilize tetrathionate, will allow it to survive during oxidative stress and inflammation. This could allow the recombinant probiotic to be able to survive and persist during IBD.

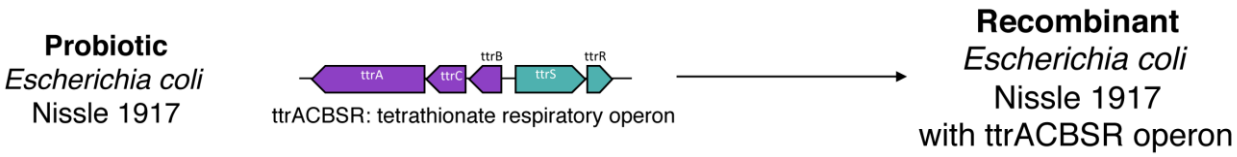

Figure 4. Design of recombinant *E. coli* probiotic strain

1.7 Murine colitis models

Animal models in colitis are used to investigate the pathogenesis and treatments of IBD. There are currently many mouse models for IBD, with the majority of them for UC. These include models with genetic defects, chemically induced, and bacterially induced.

1.7.1 DSS-induced colitis model

One of the most widely used colitis models is DSS-induced colitis. When the DSS polymer is administered to mice in their drinking water, they develop inflammation, which resembles UC (Wirtz, Neufert, Weigmann, & Neurath, 2007). A dosage of 2-5%

over 7-10 days induces a strong acute colitis disease with low mortality rates. This involves colonic inflammation, weight loss, and bloody diarrhea. It leads to NF-κB activation and shows expression of pro-inflammatory cytokines that are seen with clinical symptoms of colitis. Inflammatory markers like TNF-α, IL-1β, IFN-γ, IL-10, and IL-12 are observed. Histological examination of tissues shows mucin depletion, epithelial degeneration, infiltration of immune cells, ulceration, and possibly necrosis (Perše & Cerar, 2012).

The DSS acts against the epithelial cells in the gut and can disrupt the epithelial barrier. It causes defects in the barrier and increased colonic permeability to allow the DSS molecules to pass through (Perše & Cerar, 2012). This also allows the passage of luminal antigens and bacteria into the mucosa and underlying tissue and therefore activation of an inflammatory response (Jeengar, Thummuri, Magnusson, Naidu, & Uppugunduri, 2017). However, the mechanism behind how DSS passes through the membrane is still unknown.

The severity of the colitis depends on the molecular weight of DSS. Studies have shown that mice administered DSS with approximately 40kDa develop the most severe colitis located in the distal colon (Shuji Kitajima, Takuma, & Morimoto, 2000). Mice treated with DSS below 40kDa develop a milder form of DSS that is located in the cecum and proximal colon, while mice treated with DSS above 40kDa fail to develop lesions. The higher molecular weight DSS is not able to pass through the mucosal membrane and therefore is not effective. DSS is able to penetrate in the colon but can be detected in other parts of the body including MLN, macrophages in the large intestine, liver Kupffer cells, and the kidney (S Kitajima, Takuma, & Morimoto, 1999). It

is excreted via urine and feces. DSS is stable in that it is resistant to breakdown by intestinal microbiota, pH changes, and anaerobic conditions (Shuji Kitajima, Morimoto, & Sagara, 2002).

The gut microbiota is also shown to be involved in the development of DSS-induced colitis. Shifts are observed in *Bacteroidales* and *Clostridiales* and increases in *Enterobacteriales*, *Deferribacterales*, *Verrucomicrobiales* and *Erysipelotrichales* (Schwab et al., 2014). Meta-transcriptome sequencing showed that the decrease of *Clostridiales* in DSS-induced colitis is seen with a reduction of transcripts related to butyrate formation and a downregulation of their flagellin-encoding genes. An increase in *Bacteroidales* showed an increase in transcripts related to mucin degradation. This shows that the change in the microbiota can affect the disease progression. Overall, DSS is a simple, inexpensive model for UC. This makes it one of the most commonly used murine IBD models.

1.7.2 Muc2$^{-/-}$ spontaneous colitis model

A newer IBD murine model is the Muc2$^{-/-}$ spontaneous colitis model. The GIT is lined by a layer of mucus that is composed of glycosylated proteins called mucins, and it has been shown that Muc2 is the prominent mucin synthesized in the colon (Tytgat et al., 1994). Muc2 is stored in the granules of the goblet cells and is the most important factor determining their morphology (Velcich et al., 2002). In UC patients, there is a significant decrease in Muc2 synthesis, which can be related to mucosal inflammation (Tytgat, van der Wal, Einerhand, Dekker, & Buller, 1996).

To obtain Muc2$^{-/-}$ mice, there is an inactivation of the Muc2 gene. This missense mutation causes the epithelial barrier to be disrupted, and microbes and luminal antigens to pass through. Goblet cells produce defense factors like trefoil factor 3 (TFF3) and resistin-like molecule-beta (RELM-β) (Morampudi et al., 2016). It has been shown that in the absence of Muc2, there is an upregulation of RELM-β. These mice develop a spontaneous colitis, since a defective mucus barrier is seen in UC. Histological changes are characterized by hyperplasia, crypt abscesses, immune cell infiltration, and sub-mucosal edema. These all represent clinical features of active UC. It has been shown that Muc2$^{-/-}$ mice can develop tumors with age (Velcich et al., 2002). By 1 year of age, 65% of Muc2$^{-/-}$ mice develop tumors in the small and large intestine. Muc2$^{-/-}$ mice are shown to carry an altered gut microbiota (Huang et al., 2015). There is a greater number of Proteobacteria, Clostridiales, and a decrease in Lactobacillaceae.

Muc2$^{-/-}$ mice appear healthy at both birth and after weaning at 1 month of age (Morampudi et al., 2016). They produce smaller litters (3-4) of 4-8 pups. As they age, they develop diarrhea and eventually a rectal prolapse. A rectal prolapse occurs when part of the intestines and rectum protrude out of the anus, indicating humane endpoint. Prior to 2 months of age, 6% of mice develop a rectal prolapse, 17% by 2-4 months, and then a total of 40% by 6 months of age. The Muc2$^{-/-}$ spontaneous colitis represents a defective barrier that results in a chronic colitis.

1.8 Research overview and hypothesis

Evidence to suggest probiotic supplementation for IBD is weak. There are many meta-analyses to show that well-designed randomized controlled trials supporting the

use of probiotics for IBD management are limited. Many of the included studies report no statistically significant differences between the probiotic and placebo or control groups. Even though there is much evidence to show that probiotics are effective in reducing symptoms in animal models or *in vitro*, human clinical trials have reported low efficacy of probiotics. For IBD, current probiotics are ineffective at both colonization and survival in the hostile gut of patients. During IBD, there is an influx of immune cells and signalling molecules while undergoing an inflammatory response. There is the production of ROS with oxidative stress that can hinder the growth of beneficial strains and promote growth of select microbes that can survive these pro-inflammatory conditions. A novel therapeutic approach is to engineer designer probiotics that target these specific limitations. Genetically improved designer probiotics, with enhanced colonization and persistence, can lead to better efficiency during IBD therapy and a potential alternative therapeutic option for IBD patients.

The central hypothesis of this thesis proposes that the novel designer probiotics that are genetically modified to enhance colonization and survival in an IBD gut, will result in better efficacy of probiotic therapy against IBD compared to their parent strains. The objectives of the thesis are:

1) To determine whether the designer strains, as compared to their parent strains, have improved efficacy during DSS-induced colitis.

2) To determine whether the designer strains, as compared to their parent strains, have improved efficacy during the Muc2$^{-/-}$ spontaneous colitis model.

3) To determine whether the designer strains, as compared to their parent strains, have increased colonization/detection.

Chapter 2: Materials and Methods

2.1 Probiotic strains

Both designer and parent *E. coli* strains were cultivated in liquid Luria-Bertain-Miller (LB) media and on 1.8% LB agar. LB media was made in deionized water (dH_2O) according to the recipe in Table 3 adjusted to a pH of 7.5. Bacteria were grown with agitation at 37°C. Media was supplemented with 4 ug/mL tetracycline (Tc) for designer probiotic *E. coli* strain.

Table 3. LB media recipe for 1L volume for *E. coli* strains

Ingredients	Amount
Tryptone	10g
Yeast Extract	5g
Sodium Chloride (NaCl)	10g

Both designer and parent *L. reuteri* strains were cultivated in De Man, Rogosa, and Sharpe (MRS) media and on 1.8% MRS agar according to the recipe in Table 4 adjusted to a pH of 6.5. Bacteria were grown without agitation in anoxic conditions of an anaerobic jar at 37°C. Media was supplemented with 5ug/mL erythromycin (Erm) for designer probiotic *L. reuteri* strain.

Table 4. MRS media recipe for 1L volume for *L. reuteri* strains

Ingredients	Amount
Peptone	10g
Beef extract	10g
Yeast extract	5g
Dextrose	20g
Sodium Acetate	5g
Polysorbate 80 (Tween 80)	1g
Dipotassium Phosphate	2g
Ammonium Citrate	2g
Magnesium Sulfate	0.1g
Manganese Sulfate	0.05g

2.2 Mice

C57BL/6 male and female mice (Charles River; Wilmington, Massachusetts) were maintained in specific pathogen free (SPF) conditions at the Bioscience Facility at the University of British Columbia Okanagan (UBCO; Kelowna, British Columbia). Upon arrival, mice were quarantined and allowed to acclimatize for 1 week prior to the start of experiments. Muc2 knockout ($Muc2^{-/-}$) male and female mice (Bruce Vallance Lab at CFRI, Vancouver, British Columbia) bred on a C57BL/6 background were maintained in SPF conditions at the Bioscience Facility at UBCO. $Muc2^{-/-}$ mice were generated as described (Velcich et al., 2002). All animals were caged on ventilated racks in a temperature controlled (22±2°C) room with 12:12 light day (LD) cycle. All mice cages had filter cage tops and were housed in the same room. C57BL/6 mice were fed Envigo Teklad 7001 Mouse Diet (Envigo; Huntingdon, United Kingdom) or standard chow and autoclaved sterile water ad libitum. $Muc2^{-/-}$ mice were fed PicoLab Rodent 20 (Envigo)

and acidified water at pH of 3. All mice were bred on PicoLab Mouse Diet 20 breeder chow (Envigo). Mice were given autoclaved bedding, mouse huts, and crinkle paper for enrichment. The protocols used were approved by the University of British Columbia Animal Care Committee and in direct accordance with guidelines drafted by the Canadian Council on the Use of Laboratory Animals.

2.3 DSS-induced colitis animal experiment

C57BL/6 mice were weaned at 19-21 days of age and assigned one of 3 groups: no probiotic, modified designer *E. coli* or *L. reuteri* probiotic, and unmodified parent *E. coli* or *L. reuteri* probiotic. Probiotic groups received 3 doses of 100 uL of $3x10^{12}$ CFU/mL for the *E. coli* parent and designer strains. Probiotic groups received a single probiotic dose of 100 µL of $2x10^{9}$ CFU/mL for the *L. reuteri* parent and designer strains. The third treatment group served as the control group and received no oral gavage or probiotic supplementation. Mice were orally gavaged with an animal feeding 22G x 1.5” 1.25 mm straight needle (Cadence Science; Cranston, Rhode Island). Mice were given the single probiotic dose either when they reached a body weight of at least 15 g or when they reached 5 weeks of age. Mice were assessed immediately after gavage, 1 hour after gavage, and then 24 hours after gavage. Mice were immediately euthanized if they showed signs of distress due to gavage such as: lethargy, hunched posture, difficulty breathing, blood emerging from the mouth and/or nose or a loss in total body weight ≥ 15%. Mice were then exposed to 3.5% dextran sodium sulfate (DSS) (MP Biomedicals; Santa Ana, California) at a molecular weight of 35-50 kDa via drinking water and monitored throughout the 7-day exposure for morbidity. DSS treatment

groups were given 3.5% DSS dissolved in sterile water. Non-DSS treatment groups were given sterile water. Water intake for DSS treatment groups was monitored since colitis development is dependent on DSS water intake. Fresh stool collection was carried out every day for the duration of the experiment. Stool pellets were collected in microcentrifuge tubes (MCT) tubes (VWR) and immediately flash frozen in LN2. For the experimental endpoint, mice were sacrificed at day 7 of the DSS exposure. Body weights were measured daily, and data is presented as percent weight change compared to the initial body weight, prior to DSS exposure. A cohort of mice that were assigned to one of 3 treatment groups but not exposed to DSS were used as a set of controls. The set up for the DSS-induced colitis experiment is shown in Figure 1.

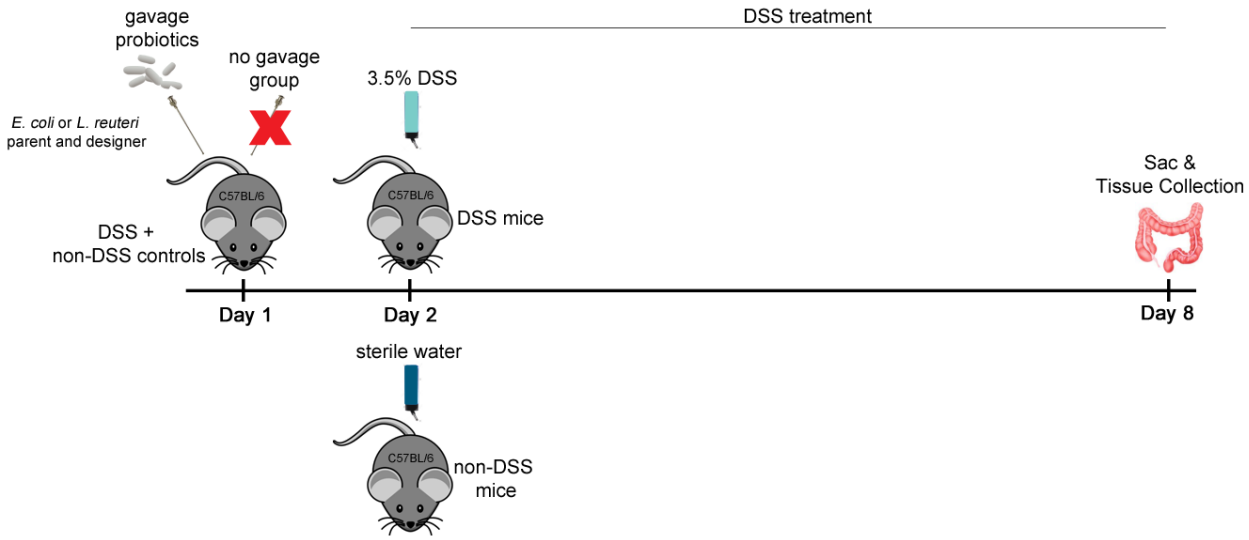

Figure 5. Timeline of DSS-induced colitis experiment

2.4 Muc2$^{-/-}$ spontaneous colitis experiment

Muc2$^{-/-}$ mice were weaned at 28-30 days of age and assigned one of 3 groups: no probiotic saline Muc2$^{-/-}$ control, modified E. coli designer strain, and unmodified E. coli parent strain. Probiotic groups received one dose of 100 uL of 3x10^{12} CFU/mL for the *E. coli* parent and designer strains once weekly for 4 consecutive weeks. Mice were orally gavaged with an animal feeding 22G x 1.5" 1.25mm straight needle (Cadence Science). Mice were given the first probiotic dose either when they reached a body weight of at least 15 g or when they were 5 weeks of age. Mice were assessed immediately after gavage, 1 hour after gavage, and then 24 hours after gavage. Mice were immediately euthanized if they showed signs of distress due to gavage such as: lethargy, hunched posture, difficulty breathing, blood emerging from the mouth and/or nose or a loss in total body weight ≥ 15%. Fresh stool collection was carried out every 2 weeks for the duration of the experiment. Stool pellets were collected in MCT tubes (VWR) and immediately flash frozen in LN2. One cohort of animals was sacrificed at 3 months of age and the second cohort at 4 months of age. Body weights were measured weekly, and data is presented as percent weight change compared to the initial body weight. The set up for the Muc2$^{-/-}$ spontaneous colitis experiment is shown in Figure 2.

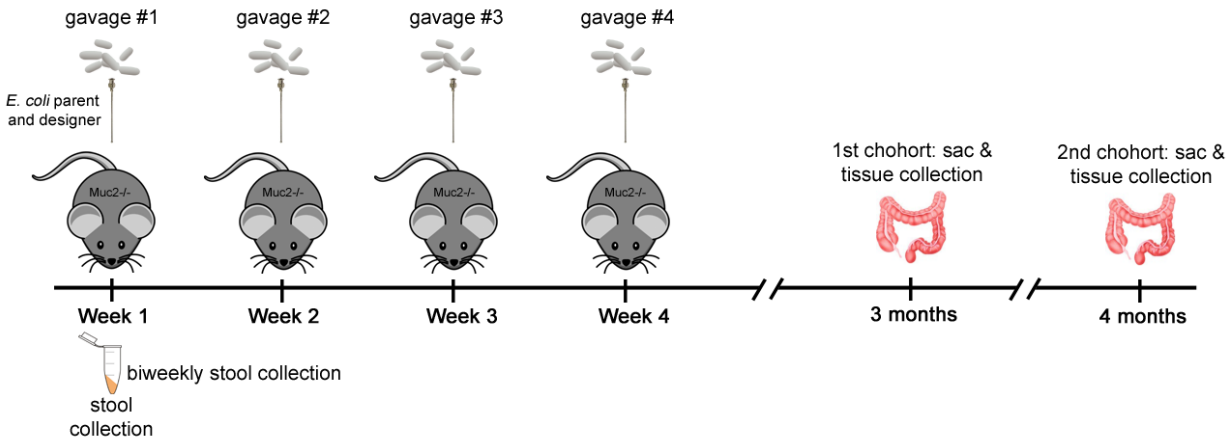

Figure 6. Timeline of Muc2$^{-/-}$ spontaneous colitis experiment

2.5 Strain detection animal experiment

C57BL/6 mice were weaned at 19-21 days of age and assigned one of 4 groups: modified designer *E. coli*, modified *L. reuteri* probiotic, unmodified parent *E. coli*, or unmodified *L. reuteri* probiotic. Probiotic groups received a single probiotic dose of 100 uL of $3x10^{12}$ CFU/mL for the *E. coli* parent and designer strains. Probiotic groups received a single probiotic dose of 100 µL of $2x10^{9}$ CFU/mL for the *L. reuteri* parent and designer strains. Mice were orally gavaged with an animal feeding 22G x 1.5" 1.25 mm straight needle (Cadence Science; Cranston, Rhode Island). Mice were given the single probiotic dose either when they reached a body weight of at least 15 g or when they were 5 weeks of age. Mice were assessed immediately after gavage, 1 hour after gavage, and then 24 hours after gavage. Mice were immediately euthanized if they showed signs of distress due to gavage such as: lethargy, hunched posture, difficulty breathing, blood emerging from the mouth and/or nose or a loss in total body weight ≥ 15%. Fresh stool collection was carried out every 2 weeks for the duration of the

experiment. Stool pellets were collected in MCT tubes (VWR) and immediately flash frozen in LN2. Mice were sacrificed at 2 months of age. Body weights were measured weekly, and data is presented as percent weight change compared to the initial body weight. The set up for the colonization experiment is shown in Figure 2.

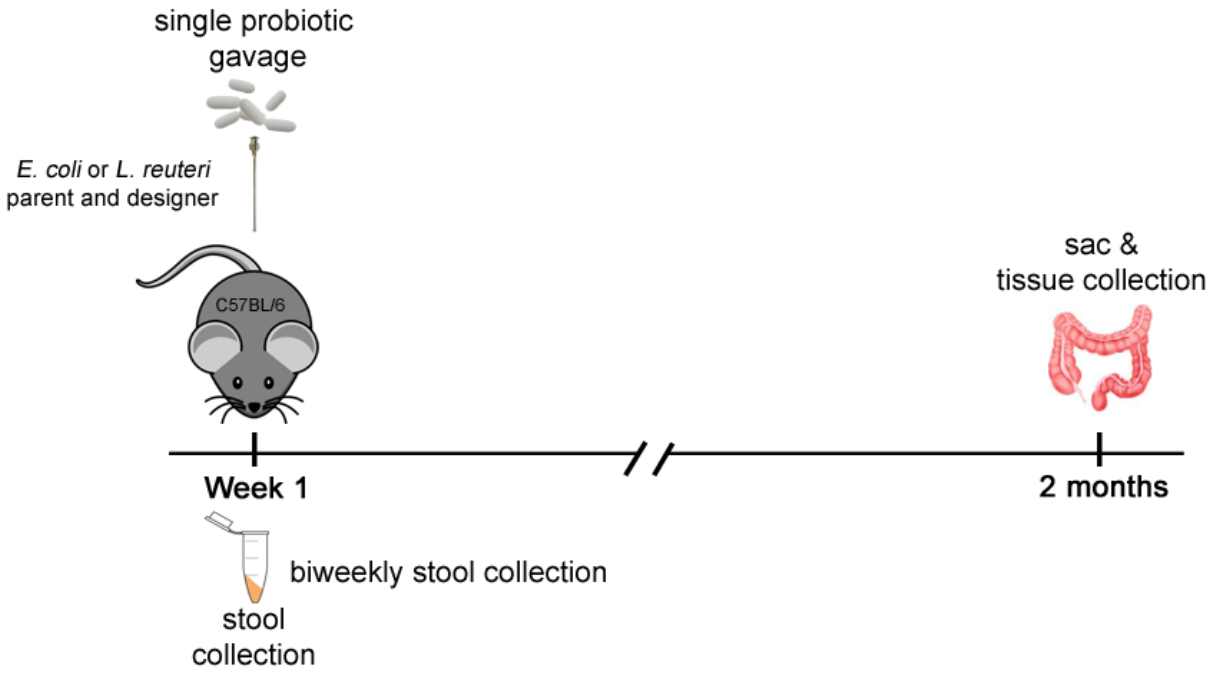

Figure 7. Timeline of strain detection experiments

2.6 Tissue collection

For DSS-induced colitis and colonization experiments, mice were first anesthetized with isoflurane (Fresenius Kabi Canada; Toronto, Ontario), sacrificed by asphyxiation by CO_2 (Praxair Canada; Kelowna, British Columbia), and then followed by cervical dislocation. For $Muc2^{-/-}$ spontaneous colitis, mice were first anesthetized with isoflurane (Fresenius Kabi), sacrificed with cardiac puncture, and then followed by cervical dislocation. The large intestine was cut near the rectum and the distal colon,

ileum, and cecum were removed. Each was cut into 3 sections, with the first section for histology immersed in 1 mL of 10% neutral buffered formalin (NBF) (Ricca Chemical; Arlington, Texas). The second section was used for RNA extractions and quantitative polymerase chain reaction (qPCR) cytokine analysis and immersed in 1 mL of RNA-later (Qiagen). The last section was used for microbiota analysis and flash frozen in LN2 (liquid nitrogen) (Praxair Canada).

2.7 DSS-induced colitis clinical scores

Mice were monitored twice and weighed once daily for the duration of the DSS treatment. Mice were scored based on their body movement, rectal bleeding, stool consistency, weight change, and hydration, summarized in Table 1, with interpretations summarized in Table 2. Animals were scored while blinded by an observer and then recorded by an un-blinded second observer. All scores from each category were added and a final clinical score per day for each mouse was given during the DSS treatment.

Table 5. Clinical scoring system used to score mice for DSS-induced colitis

Clinical Score Categories	Clinical Score				
	0	**1**	**2**	**3**	**4**
Body Movement	Normal	N/A	Piloerection, movement reduced	Hunched	Inactive/ shaking
Rectal Bleeding	None	Positive fecal occult blood test	Blood in stool	Large amount of blood in stool	Extensive amount of blood in stool and visible at anus
Stool Consistency	Normal	Loose stool	Watery stool	Diarrhea	No formed stool
Weight Change	No weight loss, loss up to 5%	Loss of 5-10%	Loss of 10-15%	Loss of more than 15%	N/A
Hydration	Normal	Slightly sunken eyes	N/A	Dehydrated/sunken eyes	Skin tent

Table 6. Interpretation of clinical scores

Score range	Action
1-4	Expected for DSS, monitor as per protocol
5-8	Monitor as per protocol, pay close attention to disease progression (AM vs. PM scores)
9-10	Consider euthanasia, increase monitoring to a minimum of 3 times daily (at least once every 8 hours)
11 or more or body weight loss ≥ 15% for 2 consecutive days	Immediate euthanasia

2.8 Muc2$^{-/-}$ spontaneous colitis clinical scores

Mice were monitored daily and assessed for clinical scores and weighed weekly on a clinical scoring system shown in Table 5, with score interpretation shown in Table 6. Mice were scored based on their body movement, rectal bleeding, stool consistency,

weight change, and hydration. Animals were scored while blinded by an observer and then recorded by a un-blinded second observer. All scores from each category were added and a final clinical score per week for each mouse was given during the Muc2$^{-/-}$ spontaneous colitis. As per UBC policy, mice were also assessed for grade level of colitis. Grade levels were assessed for parameters such as behavior, appearance, prolapses, dehydration, stool consistency, rectal bleeding, respiration, abdomen, body weight, and pain. Grades are summarized in Table 7, with grade interpretations shown in Table 8.

Table 7. Clinical scoring system used to score mice for Muc2$^{-/-}$ spontaneous colitis

Clinical Score Categories	Clinical Score					
	0	**1**	**2**	**3**	**4**	**5**
Body Movement	Normal	Piloerection	Movement reduced	Hunched/Inactive	Shaking	N/A
Rectal Bleeding	None	Rectal swelling	Visible blood in stool	Large amount of blood in stool/or cage	Rectal swelling (immediate euthanize)	N/A
Stool Consistency	Normal	Soft stool	Diarrhea	N/A	N/A	N/A
Weight Change	No weight loss	↓ up to 5%	↓ 5-10%	↓ 10-19%	↓ more than 20%	N/A
Hydration	Normal	Slightly sunken eyes	N/A	Dehydrated/sunken eyes	Skin tent	N/A

Table 8. Interpretation of clinical scores

Score range	Action
0-7	Monitor as per protocol
8-10	Consider euthanasia, increase monitoring to minimum once daily
11 or more, body weight loss ≥ 25% for 2 consecutive days, or rectal prolapse	Immediate euthanasia

Table 9. UBC colitis grading system for Muc2$^{-/-}$ spontaneous colitis

Health Observations	Grade +	Grade ++	Grade +++	Grade ++++
Behaviour: Activity Gait	Slightly slow moving still interested in environment	Less interested in the environment, interacts less with cage mates, disregards observer, when nudged, reluctantly moves away, occasional abnormal gait (limping or "tip-toed" gait)	Isolated from cage mates, minimally active, does not readily move when cage disturbed, when nudged reluctantly moves, frequent limping or "tip-toed" gait	Immobile or hyperactive, not moving when nudged, animal cannot right itself, stereotypic behavior that cannot be stopped, impacting health and welfare (ex. poor body condition, lesions)
Appearance: Grooming/Fur Posture Head shape	Piloerection/ruffled (< 10% of body), runted weanling	Piloerection (25% of body ex. base of neck) and dull fur (not shiny or smooth), slight hunching in back	Piloerection (50% of body), matted and un-groomed, whiskers barbered, severe hunching in back (even when walking)	Piloerection (>75% of body), matted and un-groomed with other severe signs of illness, barbering with > 50% loss of fur and signs of inflammation, hydrocephalus

Health Observations	Grade +	Grade ++	Grade +++	Grade ++++
Prolapses (rectal, vaginal, penile)	Intermittent prolapse but tissue healthy, tissue easily reduced (returned to normal location), animal can urinate and defecat	Moderate amount of tissue exposed which requires treatment to reduce, tissue healthy and animal can urinate and defecate	Fully prolapsed tissue, tissue swollen and red or bleeding, requires treatment to reduce and improve tissue health, unsure if animal can urinate or defecate	Prolapsed tissue severely inflamed, infected, necrotic or dry, animal unable to urinate or defecate, evidence of mutilation, no response to treatment
Dehydration	Mildly sunken eyes (appear >75% open)	Skin tent > 2 seconds (decrease in skin elasticity) represents 10-15% dehydration, sunken eyes (appear half closed)	Skin tent > 5 seconds represents 15- 20% dehydration, completely closed or severely sunken eyes, tail feels square, cool to touch	Animal unresponsive and cold to touch, severe skin tent (> 10 seconds)
Stool Consistency	Loose stool	Watery stool with occasional formed soft feces, small area of fecal staining around anus, hydration normal	Diarrhea, large area of fecal staining on fur or visible fecal smearing on cage wall	No formed stool, unable to maintain hydration
Rectal Bleeding	Guaic test positive	Visible blood in stool	Extensive blood in stool and visible at anus lasting less than 48 hours	Extensive blood in stool and visible at anus lasting more than 24-48h

Health Observations	Grade +	Grade ++	Grade +++	Grade ++++
Respiration: **Rate** **Rhythm** **Effort**	Subtle change in rate or effort with activity but normal at rest, regular rhythm	Obvious change in rate or effort with activity and at rest (see chest expansion), occasional irregular rhythm, small amount of nasal discharge or sneezing	Reduced rate (easy to count) at rest and when active, irregular rhythm, appears to require effort (head bobs or body moves with breathing), noisy breathing, nasal discharge affecting breathing	Reduced rate (≅Mice <95), irregular rhythm, gasping, struggling to breath, open mouth breathing, skin cyanotic (blue)
Abdomen	Mild abdominal distention	Moderate abdominal distention, abnormal increase in body weight	Enlarged and tense abdomen or palpable mass, body weight increases	Ascites/abdominal distension where burden exceeds 10% of body weight (looks full term pregnant) with other clinical signs, abnormal body weight increase, abdominal hernia
Body Weight	↓ weight by 5-9%	↓ weight by 10-14%	↓ weight by 15-19%	↓ weight by ≥ 20%
Pain	Facial Grimace: Narrow or closed eyes, bulge on top of nose, cheek (between eye and whiskers), bulge, ears back or flat, whiskers pointing back or standing out on end Other: Muscle twitching or flinching, staggering, back stretch, abdominal writhing or pressing			Persistent signs of pain that interfere with normal functions or cannot be alleviated

Table 10. Interpretation of UBC colitis grading system for Muc2$^{-/-}$ spontaneous colitis

Grade	Response
+	Maintain monitoring as in protocol
++	Increase monitoring as appropriate. Supportive care as appropriate
+++	Increase monitoring as appropriate., Supportive care as appropriate, Consult clinical veterinarian
++++	Euthanasia

2.9 Bacterial counts

For Muc2$^{-/-}$ spontaneous colitis, the mesenteric lymph nodes (MLN) and spleen were collected and stored in 1 mL of sterile 1x PBS (Lonza). Tissues were homogenized with 5 mm stainless steel beads (Qiagen) using a Retsch Mixer Mill M400 homogenizer (Retsch) set to 30 Hz for 2 x 2 min runs. Homogenates were diluted to a 10-fold serial dilution and plated on 1.8% LB agar. Plates were incubated 16-18 hours and then counted for bacterial colonies. Bacteria colonies were counted on plates by dividing plates into quadrants, counting colonies in one quadrant, and then multiplying by four to get total bacterial colonies on a plate. Dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were plated and counted for bacterial colonies. Only plates with colonies between 30-300 were used in CFU/mL calculations.

2.10 DSS-induced colitis histopathological scoring

Tissue sections were placed in 10% NBF (Ricca Chemical) left overnight at 4°C, and then transferred into 70% ethanol (Anachemia; Richmond, British Columbia) after 2 1 x PBS washes. Tissues were pooled by mouse cage and placed into cassettes. The

cassettes were then paraffin embedded and cut into 5 µm sections onto slides. One slide was stained for Hematoxylin and eosin stain (H&E) staining for histopathological scoring. Slide cutting and staining were performed (Wax-It Histology Services; Vancouver, British Columbia). The H&E stained slides were used to assess damage and scores were measured, summarized in Table 9. The histopathology scores were based on 4 parameters as described (Laroui et al., 2012) with slight modifications. Scores were determined from crypt damage, ulceration, inflammation, and goblet cell depletion. Scores in each category were added up and a total histopathological score was given. The maximum histopathological score was 16. Tissues were scored by 2 blinded observers. Slides were viewed and images were taken with an Olympus IX81 inverted microscope (Olympus Life Sciences Solutions; Toronto, Ontario) equipped with a QImaging EXi aqua Bio-Imaging camera (QImaging; Surrey, British Columbia). Histopathological images were taken using MetaMorph Advanced version 7.7.8.0 software (MetaMorph for Olympus).

Table 11. Histopathological scoring system used to score for DSS-induced colitis

Histopathological Scoring Parameters	Clinical Scores					
	0	**1**	**2**	**3**	**4**	**5**
Crypt damage	Intact	Loss of 1/3 basal	Loss of 2/3 basal	Entire crypt loss (one area)	Change of epithelial surface with erosion (two areas)	Confluent erosion (whole area – no crpyts)
Ulceration	Absence of ulcers	1 or 2 foci of ulcerations	3 or 4 foci of ulcerations	Confluent or extensive ulcerations (more than 4 foci)	N/A	N/A
Inflammation	Normal	Minimal (larger focal area, minimal diffuse, no separation of glands, may be mostly in areas of submucosal edema or mesentery)	Mild (diffuse mild, multifocal affecting 11-25% of mucosa with minor focal or multifocal gland separation, no separation in most areas)	Moderate (26-50% of mucosa affected with minimal to mild focal or multifocal separation of glands by inflammatory cell infiltrate, milder in remaining areas of mucosa with some areas having no gland separation by inflammation)	Marked (51-75% of mucosa affected with mild to moderate separation of glands by inflammatory cell infiltrate, minimal to mild in remaining areas of mucosa but all glands have some separation by infiltrate)	Severe (76-100% of mucosa affected with moderate to marked areas of gland separation by inflammatory cell infiltrate, mild to moderate in remaining areas of mucosa)

2.11 Muc2$^{-/-}$ spontaneous colitis histopathological scoring

Tissue sections were placed in 10% NBF (Ricca Chemical) left overnight at 4°C, and then transferred into 70% ethanol (Anachemia; Richmond, British Columbia) after 2 1 x PBS washes. Tissues were pooled by mouse cage and placed into cassettes. The cassettes were then paraffin embedded and cut into 5 µm sections onto slides. One slide was stained for Hematoxylin and eosin stain (H&E) staining for histopathological

scoring. Slide cutting and staining were performed (Histology & Image Core Lab at BC Children's Hospital Research Institute; Vancouver, British Columbia). The H&E stained slides were used to assess damage and scores were measured, which are summarized in Table 10. The histopathology scores were based on parameters as described (K. S. Bergstrom et al., 2015). Scores were determined from edema, epithelial hyperplasia, epithelial integrity, and cell infiltration. The maximum histopathological score was 13. Tissues were scored by 2 blinded observers. Slides were viewed and images were taken with an Olympus IX81 inverted microscope (Olympus Life Sciences Solutions; Toronto, Ontario) equipped with a QImaging EXi aqua Bio-Imaging camera (QImaging; Surrey, British Columbia). Histopathological images were taken using MetaMorph Advanced version 7.7.8.0 software (MetaMorph for Olympus).

Table 12. Histopathological scoring system used to score mice for Muc2$^{-/-}$ spontaneous colitis

Histopathological Scoring Parameters	Clinical Score				
	0	**1**	**2**	**3**	**4**
Edema	No change	Mild (<10%)	Moderate (10-40%)	Profound (>40%)	N/A
Epithelial hyperplasia	No change	1-50%	51-100%	>100%	N/A
Epithelial Integrity	No change	<10 epithelial cells shedding per lesion	11-20 epithelial cells shedding per lesion	Epithelial ulceration	Epithelial ulceration with severe crypt destruction
Cell Infiltration	None	Mild (2-43)	Moderate (44-86)	Severe (87-217)	N/A

2.12 Immunofluorescence staining

Paraffin-embedded tissue sections were deparaffinized using Xylene (VWR International; Edmonton, Alberta) washes and then rehydrated using an EtOH gradient from 100-70% (Anachemia) with a final wash in distilled water (dH_2O). For antigen retrieval, 1 mg/ml trypsin (Sigma Aldrich; Saint Louis, Missouri) was incubated on tissues for 30 mins at 37°C. Bovine serum albumin (BSA) (Sigma Aldrich) was used as a blocking solution, to block potential non-specific binding sites, incubated at room temperature for 20 mins. Slides were incubated with either rat monoclonal IgG_{2a} antibody raised against F4/80 (Cedarlane; Burlington, Ontario) to examine macrophages or rabbit polyclonal antibody IgG raised against MPO-1 (Thermo Fisher Scientific; Waltham, Massachusetts) to examine polymorphonuclear leukocytes or neutrophils at 1:50 dilution. This was followed by secondary antibodies of goat anti-rabbit IgG AlexaFluor-conjugated 594-red antibody (Invitrogen; Carlsbad, California) or goat anti-rabbit IgG AlexaFluor-conjugated 488-green antibody (Invitrogen) at a dilution of 1:1000. Tissue sections were mounted using fluoroshield with 4',6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich) and viewed on an Olympus IX81 inverted microscope (Olympus Life Sciences Solutions) equipped with a QImaging EXi aqua Bio-Imaging camera (QImaging). A negative control for primary antibody non-specific binding and background signal were performed for each antibody used.

For inflammatory cell counts, positive cells were quantified in the sub-mucosal region of the distal colon and the total number of positive cells in all sub-mucosal regions per mouse tissue were calculated as a count per mouse colon. Cell counts were

made by a blinded observer and verified by another from a stitched image using MetaMorph Advanced version 7.7.8.0 software (MetaMorph for Olympus).

2.13 RNA extraction and cytokine analysis

Total RNA was purified using Qiagen RNAeasy Fibrous Tissue Mini Kit (Qiagen) following the manufacturer's instructions. 200 mg of colon tissue was homogenized using RLT lysis buffer and β-mercaptoethanol (Sigma) to denature RNases. Tissues were homogenized with 5 mm stainless steel beads (Qiagen) using a Retsch Mixer Mill M400 homogenzier (Retsch; Haan, Germany) set to 30 Hz for 2 x 2 min runs. Proteinase K was added to digest proteins followed by ethanol to precipitate nucleic acids. The solution was applied to a spin column and washed with Buffer RW1 and then incubated with DNase I solution to digest contaminating genomic DNA. The column was then washed with Buffer RW1 and Buffer RPE and then the resulting RNA was eluted with nuclease free H_2O.

Extracted RNA was then purified using Oligo (dT) purification of mRNA using Dynabeads mRNA purification kit (Invitrogen). 5-10 µg of DSS-exposed total RNA (estimated to contain 5000 ng of mRNA) was used with 0.25 mg of Dynabeads Oligo $(dT)_{25}$ in a total volume of 200 µl (including buffers). The beads were washed with buffers according to the manufacturer's instructions. This was eluted in 20 µl of Tris-HCl and 7.5 µl of this elute was used for cDNA synthesis. DNA was synthesized with iScript cDNA Synthesis Kit (Bio-rad Laboratories; Hercules, California) according to manufacturer's instructions. cDNA was synthesized by mixing 0.5 µl of iScript reverse transcriptase enzyme, 2 µl of iScript reaction mix, and 7.5 µl of eluted clean mRNA.

cDNA was synthesized on the Bio-rad S1000 Therma Cycler (Bio-rad) using the following protocol: primer annealing at 5 min at 25°C, DNA polymerization at 30 mins at 42°C, enzyme deactivation at 5 mins at 85°C, and then held at 4°C. The final cDNA was diluted 1:10 dilution in nuclease free water (IDT).

Quantitative PCR (qPCR) was performed in duplicates in a volume of 10 µl with SsoFast EvaGreen Supermix (Bio-rad Laboratories) on the Biorad CFX 96 real time PCR detection system (Bio-rad Laboratories). 1 µl of cDNA was used with 5 µl SsoFast, 0.2 µl of each primer (10mM), and 3.6 µl of nuclease free water (IDT). Cycling conditions of denaturation at 95°C for 30 s and 39 cycles of 95°C for 5 s, followed by annealing at 58°C for 5 s, and then dissociation of primers at 95°C for 10 s. This was followed by a melt curve (65°C to 95°C in 0.5°C increments for 5 s). Relative expression of cytokines (TNF-α, IFN-γ, IL-1β, IL-17A, IL-10, RegIIIγ, and Muc2) was quantified by CFX Manager software version 1.6.541.1028 (Bio-rad). Relative normalized gene expression was quantified via the $\Delta\Delta C_t$ method. All primers were synthesized by the Integrated DNA Technology (IDT; Coralville, Iowa), Canada and sequences are shown in Table 8. Primer efficiencies were verified according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. The specificity of the primers was verified by blasting sequences in the NCBI database and efficiencies were determined using standard curves using CFX software (Bio-rad). Expression of 18S, EEF2, and TBP were used as reference genes for gene expression analysis for DSS-induced colitis and 18S, TBP, EEF2, and RPLP0 were used as reference genes for gene expression analysis for $Muc2^{-/-}$ spontaneous colitis.

Table 13. Primers used for mRNA cytokine analysis for qPCR

Primer	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
18S	CGGCTACCACCCAAGGAA	GCTGGAATTACCGCGGCT
TBP	ACCGTGAATCTTGGCTGTAAC	GCAGCAAATCGCTTGGGATTA
EEF2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA
TNF-α	CATCTTCTCAAAATTCGAGTGACA	TGGGAGTAGACAAGGTACAACCC
IFN-γ	TCAAGTGGCATAGATGTGGAAGA	TGGCTCTGCAGGATTTTCATG
IL-1β	AGCTTCCTTGTGCAAGTGTC	CCCTTCATCTTTTGGGGTCC
IL-17A	TCCCTCTGTGATCTGGGAAG	CTCGACCCTGAAAGTGAAGG
IL-10	AGGGCCCTTTGCTATGGTGT	TGGCCACAGTTTTCAGGGAT
RegIIIγ	CCCGTATAACCATCACCATCAT	GGCATCTTTCTTGGCAACTTC
Muc2	GCCAGATCCCGAAACCA	TATAGGAGTCTCGGCAGTCA

2.14 Short chain fatty acid analysis

The amount of short chain fatty acids (SCFAs) were analyzed in cecal samples by gas chromatography (Brown et al., 2015). Cecal tissue samples were homogenized with 700 µl isopropyl alcohol (BDH Chemicals; London, UK), containing 2-ethylbutyric acid (Sigma Aldrich) at 0.01% (volume/volume) v/v as internal standard at 30 Hz for 13 minutes in a Retsch MixerMill MM 400 (Retsch) homogenizer with stainless steel metal beads (Qiagen). Samples were kept at room temperature for 15 mins and then centrifuged in a Megafuge 40R (Thermo Fisher Scientific) at 15,100 x g for 10 mins at 4°C. Resulting supernatant was collected and the procedure was repeated for a second time on the leftover pellet to confirm complete extraction. 0.9 µl of the cleared supernatant was directly injected to a Trace 1300 Gas Chromatograph in splitless mode, equipped with a flame-ionization detector (FID), and an AI/AS 1310 series auto sampler (Thermo Fisher Scientific). A fused silica FAMEWAX column 30 m × 0.32 mm I.D. coated with 0.25 µm film (Restek; Bellefonte, Pennsylvania) was used. Helium (Praxair) was supplied as the carrier gas at a flow rate of 1.8 mL/min. The initial oven temperature was 80°C, maintained for 5 mins, rose to 90°C at 5°C/min, then increased

to 105°C at 0.9°C/min, and finally increased to 240°C at 20°C/min and held for 5 mins. The temperatures of the FID and the injection port were 240 and 230°C, respectively. The flow rates of hydrogen, air and nitrogen as makeup gas were 30, 300 and 20 mL/min, respectively. Data analysis was carried out with Chromeleon software version 7.2 (Thermo Fisher Scientific). Standard volatile acid mix (Sigma Aldrich) was used to determine retention times of acids. Peaks were analyzed on the software and the area under the peaks for acetic, propionic, and butyric acid were represented as weight percentage of the total cecal tissue (g of SCFA / g of cecal tissue x 100).

2.15 Colonization PCR detection

Bacterial DNA was purified using QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. 200 mg of colon tissue was homogenized in Buffer ASL at 30 Hz for 2 x 2 min runs. Tissues were homogenized with 5 mm stainless steel beads (Qiagen) using a Retsch Mixer Mill M400 homogenzier (Retsch). Samples were heated at 95°C for 5 mins to lyse cells and denature nucleases. 1 InhibitEX tablet was added to absorb PCR inhibitors. Proteinase K was added to digest proteins, followed by Buffer AL and then heated at 70°C for 10 mins to activate Proteinase K. Ethanol was added to precipitate out nucleic acids. Wash Buffers AW1 and AW2 were then added, followed by a final elution in Buffer AE.

PCR was performed with EconoTaq DNA polymerase (Lucigen; Middleton, Wisconsin) in a mix of 12.5 uL EconoTaq (Lucigen), 2.5 uL of forward and reverse primers at 10 uM, 5.5 uL of nuclease free water (IDT), and 2 uL of extracted DNA. PCR was performed on the Bio-rad S1000 Therma Cycler (Bio-rad) using the following

protocol: 95°C for 2 mins, 95°C for 30 secs, T_m for 30 secs, 72°C for X, repeated at cycles of 30 or more, and then 4°C for storage. T_m being the melting temperature and X being the annealing time. For ttr detection, Nested PCR was performed with primer pair #1 and then this PCR1 product was used as a template for PCR2 using primer pair #2. The final amplified DNA product was loaded onto a 1% agarose (Mandel Scientific Company; Guelph, Ontario) gel and run at 90-100 V for 30-40 mins. Band separation was compared to either 50 bp DNA Ladder (VWR Canada) or 1 kb DNA Ladder (Bioneer; Alameda, California). Gels were imaged on the Herolab UVT-28 ME gel imager Doc (Herolab; Wiesloch, Germany).

Table 14. Primers used for detection of recombinant strains

Primer Pair	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
E. coli **Detection**		
1	ACAGTGCGGCGCAGGCGGCAATGC	TAACAAACGCTGCGGCTGCGCC
2	ATTCAGACCTCCTGCCAAAG	CCTTACGACGCCCGATTTAT
L. reuteri **Detection**		
1	AATTCACCACTACCAGCAGCA CACCAGCACTACCACCGTCAA ACTTAACGTCAATA	GTGCTGAAGCTGGTAGTAATTCGAGT
2	CGTATCGGCAGTTGAAAACGG	ACGTTGTAGAACGAGGCTGC
3	GTTGGGTCAAGCCTGCTATT	GGGTTCCAGTTTGGCTTAGT
4	TTGGAAATGTTCCACAAGAC	TTGTGAGTTATTGAACC
5	CGTATCAGTTGAAACCG	GCATAGTCAACTTTGGC
6	CTTTGAAATCGGCTCAGGAA	TAATGCCAATGAGCGTTTTG
7	TGATCCCGTTTTCATCCAA	ACTAGGGCAAAAGTAGGTT
8	GGTCGTAGAGCACACGGTTT	TTGGGATAGAGCGTTTTTGG
9	TGCTGATGTGTTTTCATCCAA	CTGGTTGTTGCTCAGGTGTTT

2.16 Statistical analysis

RStudio Version 1.1.442 (RStudio Inc; Boston, Massachusetts) was used to conduct statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) test or with the non-parametric Kruskal-Wallis rank sum test, with Dunn's test for post hoc test and Bonferroni correction for multiple comparisons. One-way ANOVA was tested using the code aov and Tukey HSD test was performed using the code TukeyHSD both from the base R stats package. Kruskal-Wallis test was performed using kruskal.wallis from the base R stats package and Dunn's test with Bonferroni correction was performed using the code dunn.test from the R package "dunn.test". Normality was tested using both the Shapiro-Wilk test and quantile-quantile (Q-Q) plots. The shapiro.test and qqnorm codes were used from the base R stats package. Homogeneity of variance was tested using Levene's test using the LeveneTest code from the package "car". Power analysis was computed using G*Power (University of Dusseldorf; Dusseldorf, Germany).

Line graphs and box-plots were created using the R package "ggplot2". Data are presented as means +/- standard error of the mean (SEM). P-value of less than 0.05 was considered significant with asterisks indicating significance level of * for <0.05, ** for <0.001, and *** for <0.0001. Figure panels were made using Adobe Photoshop Elements 12 (Adobe Systems; San Jose, California). Figures with illustrations were created in Microsoft PowerPoint (Microsoft; Redmond, Washington).

Chapter 3: Results

3.1 Designer probiotics and DSS-induced colitis model

To test the designer probiotics *in vivo*, the DSS-induced colitis model was used since it is a representative epithelial damage model to study murine colitis (Eichele & Kharbanda, 2017). The probiotics were tested during the 7-day DSS treatment following pre-treatment with probiotic strains. Experiments were conducted as described in section 2.1.3. Mice were gavaged once daily for three days for testing of *E. coli* strains and one gavage for testing of *L. reuteri* strains. A DSS control with no probiotic supplementation was used to provide a control for the DSS-induced colitis. The no DSS probiotic groups and control data is shown in Appendix B.

3.1.1 Clinical data with DSS-induced colitis model

After probiotic supplementation, the mice were given 3.5% DSS in drinking water for 7 days. Prior to the probiotic and DSS treatment, all mice had the same starting body weight (Appendix A, Table 20). The body weights of the mice were recorded daily for the duration of the experiment. Weight change was calculated as a percentage from the starting body weight prior to DSS water exposure. For both the *E. coli* and *L. reuteri* probiotic strains, there was no differences in weight change between probiotic strains and the DSS control (Figure 8 A&B). However, comparing day one to day seven, the mice did lose weight over the course of the DSS treatment.

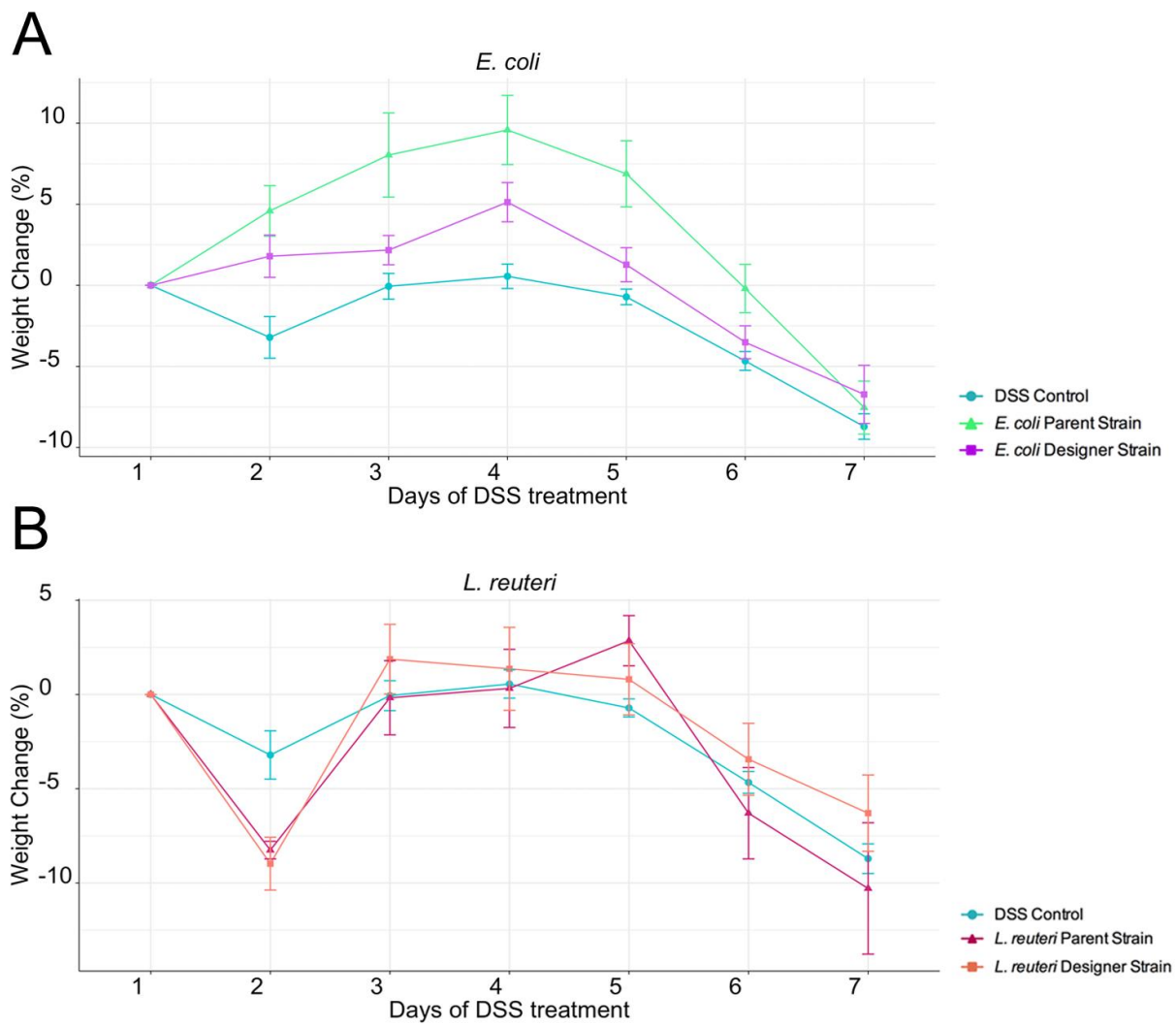

Figure 8. No differences in body weight change between strains during DSS-induced colitis at day 7 of DSS treatment.
Body weight change during the DSS-induced colitis in mice pre-treated with probiotic strains (A) *E. coli* Nissle or B) *L. reuteri*) labelled as *E. coli* or *L. reuteri* parent strains (triangles), designer probiotic strains labelled as *E. coli* or *L. reuteri* designer Strain (squares), and the no probiotic DSS control (circles). Weight loss was calculated as a percentage of the weight loss from the starting body weight prior to DSS exposure. Values are expressed as means +/- SEM (n=10-12). Non-parametric one-way ANOVA (Kruskal-Wallis) was used for statistical analysis.

Intake of 3.5% DSS drinking water was measured to ensure mice in all the groups were exposed to the same amount of DSS water. This was important since the disease severity depends on the amount of DSS water consumed. Water bottles were weighed for every cage and an average value per mouse per day was recorded. All mice across all groups had the same DSS water intake (Appendix A, Table 21).

As DSS-induced colitis was allowed to progress, mice were given daily clinical scores to score and assess the visual clinical symptoms observed. Mice were scored based on the scoring system described in section 2.1.7. The maximum score from this scoring system was a 19. Higher clinical scores indicate greater disease symptoms. For the *E. coli* probiotic strains, the designer strain had significantly lower clinical scores compared to the parent strain (Figure 9 A). This indicates that clinically, the mice supplemented with the designer strain had lower morbidity compared to the parent strain, as indicated by clinical scores. For the *L. reuteri* strain, the designer strain had significantly lower clinical scores compared to the DSS control and a slight trend compared to the parent strain (Figure 9 B).

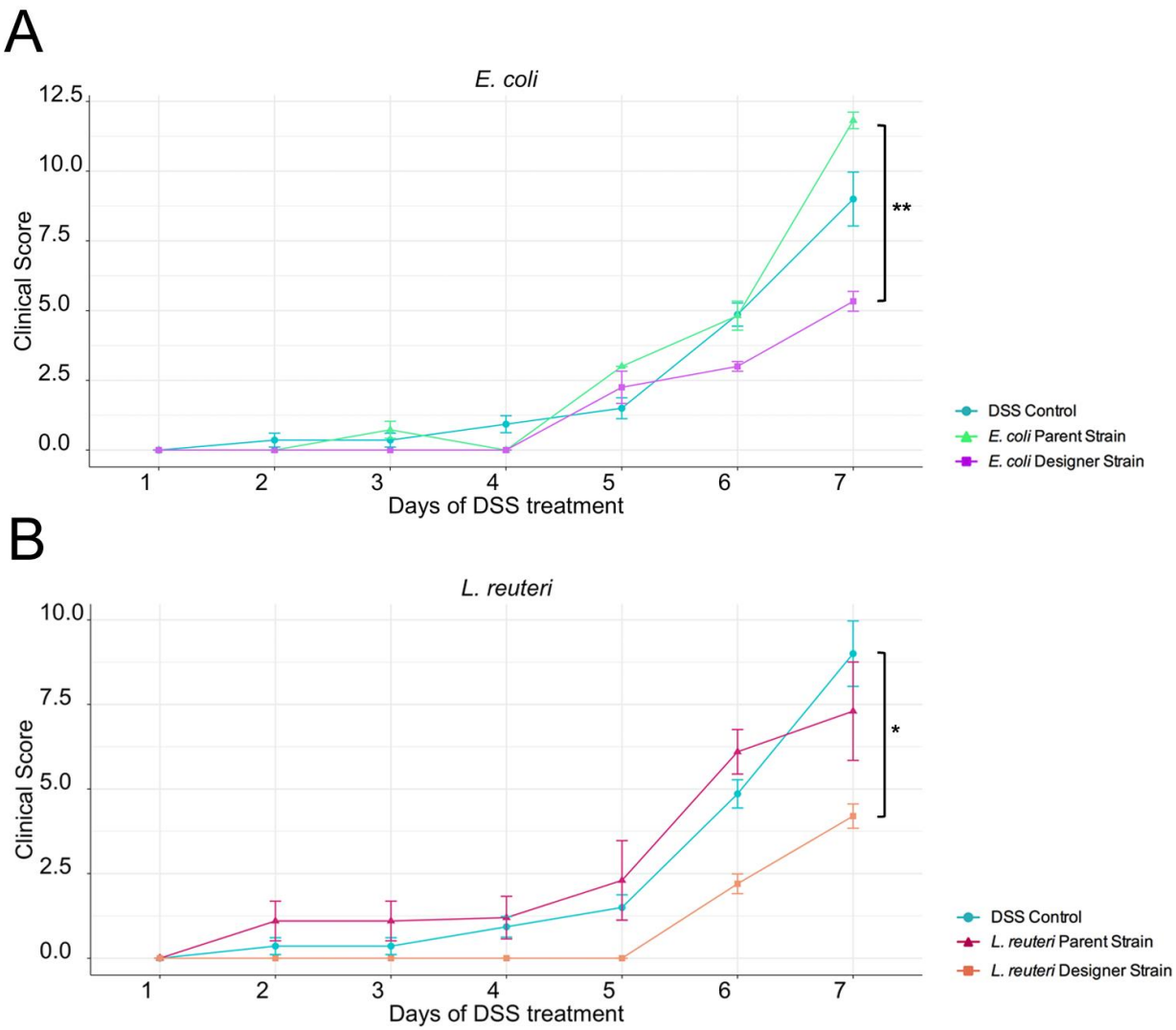

Figure 9. *E. coli* designer probiotic strains show lower clinical scores compared to the parent probiotic and *L. reuteri* designer probiotic strain to DSS control group.

Clinical scores following DSS-induced colitis in mice pre-treated with probiotic strains (A) *E. coli* Nissle or B) *L. reuteri*) labelled as *E. coli* or *L. reuteri* parent strains (triangles), designer probiotic strains labelled as *E. coli* or *L. reuteri* designer Strain (squares), and the no probiotic DSS control (circles). Movement, rectal bleeding, stool consistency, weight loss, and hydration were used to calculate clinical scores. Values expressed as means +/- SEM (n=10-12). Non-parametric one-way ANOVA (Kruskal-Wallis) was used for statistical analysis. Asterisks represent significant p-values of 0.0010 and 0.0106, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

Upon sacrifice on day 7, tissues were collected and examined macroscopically. In both strains, mice who were administered the parent probiotic strain had very dark loose, rather than formed stool, with blood primarily located in the ceca. Additionally, the colons of these mice also had loose bloody diarrhoea in lumps still present within the colon and some ulceration near the distal colon (Figure 10). In contrast, mice who were administered the designer probiotic strains had less bloody diarrhoea in their ceca and distal colons.

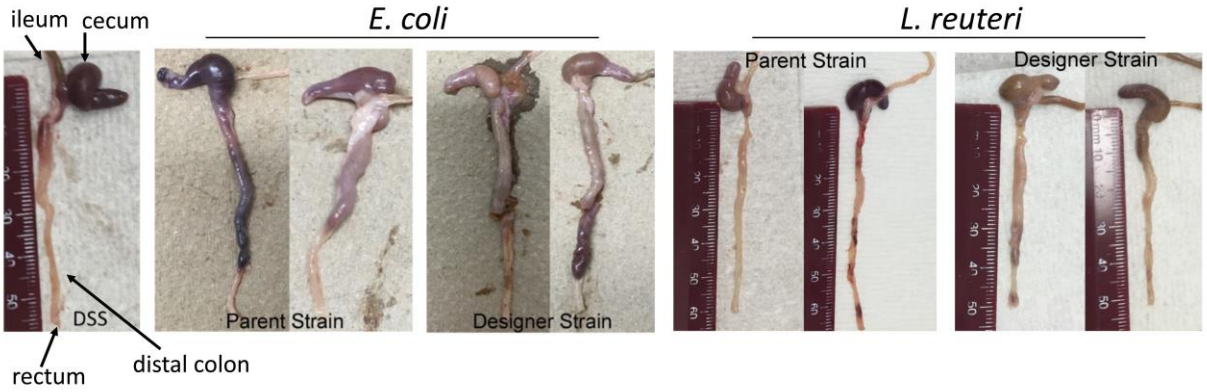

Figure 10. Macroscopic examination of tissues shows that designer probiotic groups have less loose, bloody diarrhea in the distal colon and ceca.
Mice were administered either *E. coli* or *L. reuteri* parent or designer probiotics via oral gavage for 1-3 days and then exposed to 3.5% DSS water for 7 days. Tissues were collected at day 7 of DSS treatment, following sacrifice. Images were taken to show the large intestine of the mice.

3.1.2 Histology and immunofluorescence with DSS-induced colitis

Histopathological scores were examined to assess underlying tissue damage in the mouse tissues on a cellular level. To assess histopathological damage, tissue sections were scored based on the scoring system described in section 2.1.10. A higher

histopathological score indicates more inflammation and thus more damage as a result from the DSS-induced colitis. The maximum histopathological score from this scoring system was a 16. Both designer strains had significantly lower histopathological scores compared to the corresponding parent strains (Figure 11 A). This indicates that there is less tissue damage seen in the distal colons of these mice, as shown by the representative images (Figure 11 B).

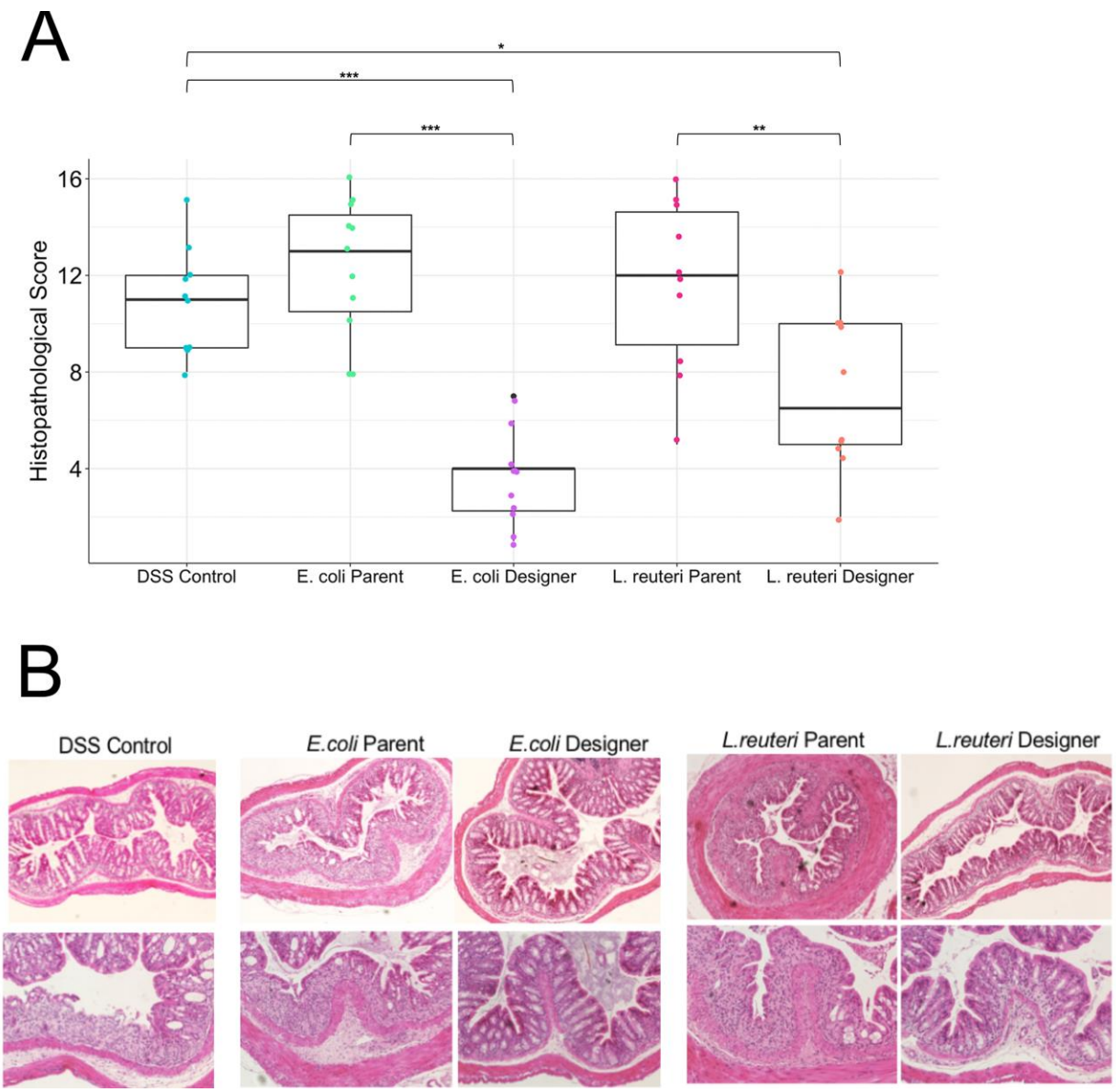

Figure 11. Designer probiotic *E. coli* and *L. reuteri* treated groups show lower histopathological scores compared to the parent probiotic treated groups.
A) H&E stained slides of cross sections of the distal colon were used to calculate histopathological scores. Epithelial integrity, immune cell infiltration, ulceration, and goblet cell depletion were used to calculate histopathological scores. B) Images were taken to show damage representing histopathological scores. Both rows of images represent corresponding treatment group. Images were selected based on the mean histopathological score. Values are expressed as means +/- SEM (n=10-12). One-way ANOVA test was used. Asterisks represent significant p-values of <0.0001, 0.033, <0.0001, and 0.0072, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

During inflammation, immune cells can become activated by molecules such as signaling molecules and play a role in eliciting an immune response. To assess the role of immune cells, sections of cut distal colon tissue on slides was stained for surface markers using immunofluorescence. F4/80 antigen marker was used to stain for F4/80+ macrophages and MPO antigen for MPO+ neutrophils. Cells were co-stained with DAPI to confirm nucleated cells. Both the designer strains showed a reduction in the macrophage colonic infiltration (Figure 12 A&B). The DSS control and parent strains both show high counts of macrophage cells in the sub-mucosal region. Further, looking at MPO marker for neutrophils, a similar pattern is observed, in that the *E. coli* designer strain shows a lower neutrophil infiltration compared to the DSS control and *E. coli* parent strain (Figure 13 A&B). This indicates that the designer strains could be protecting against the DSS-induced colitis.

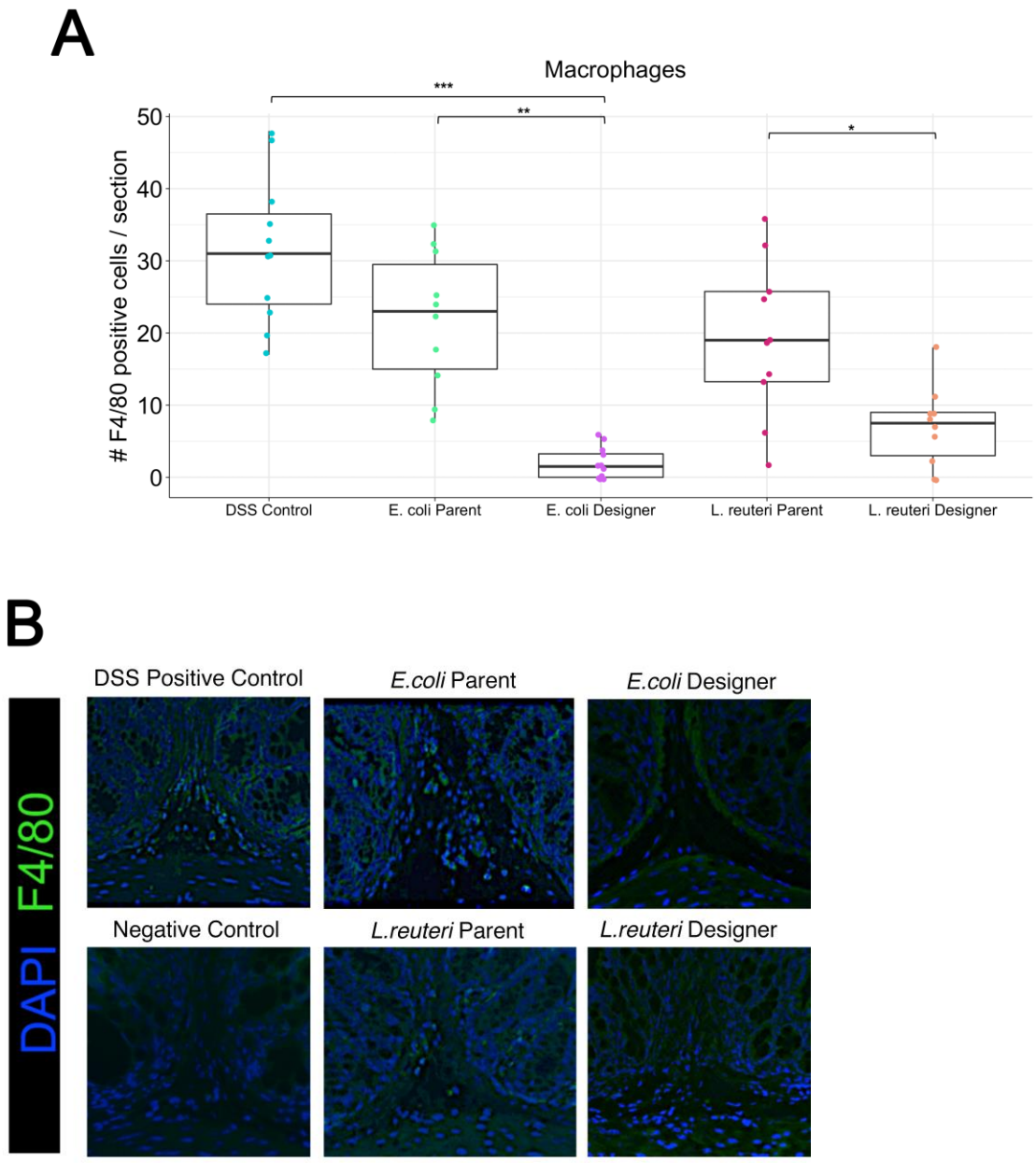

Figure 12. Designer *E. coli* and *L. reuteri* DSS-induced colitis groups show lower macrophage colonic cell infiltration compared to parent probiotic groups.
A) Positive cells were quantified in the sub-mucosal lamina propria region on stained tissues via immunofluorescence. B) Images were taken to show colonic mucosal regions representing colonic cell counts. Blue fluorescence represents DAPI and green FITC fluorescence represents F4/80. Values are expressed as means +/- SEM (n=10-12). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-values of <0.0001, 0.0004, and 0.014, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

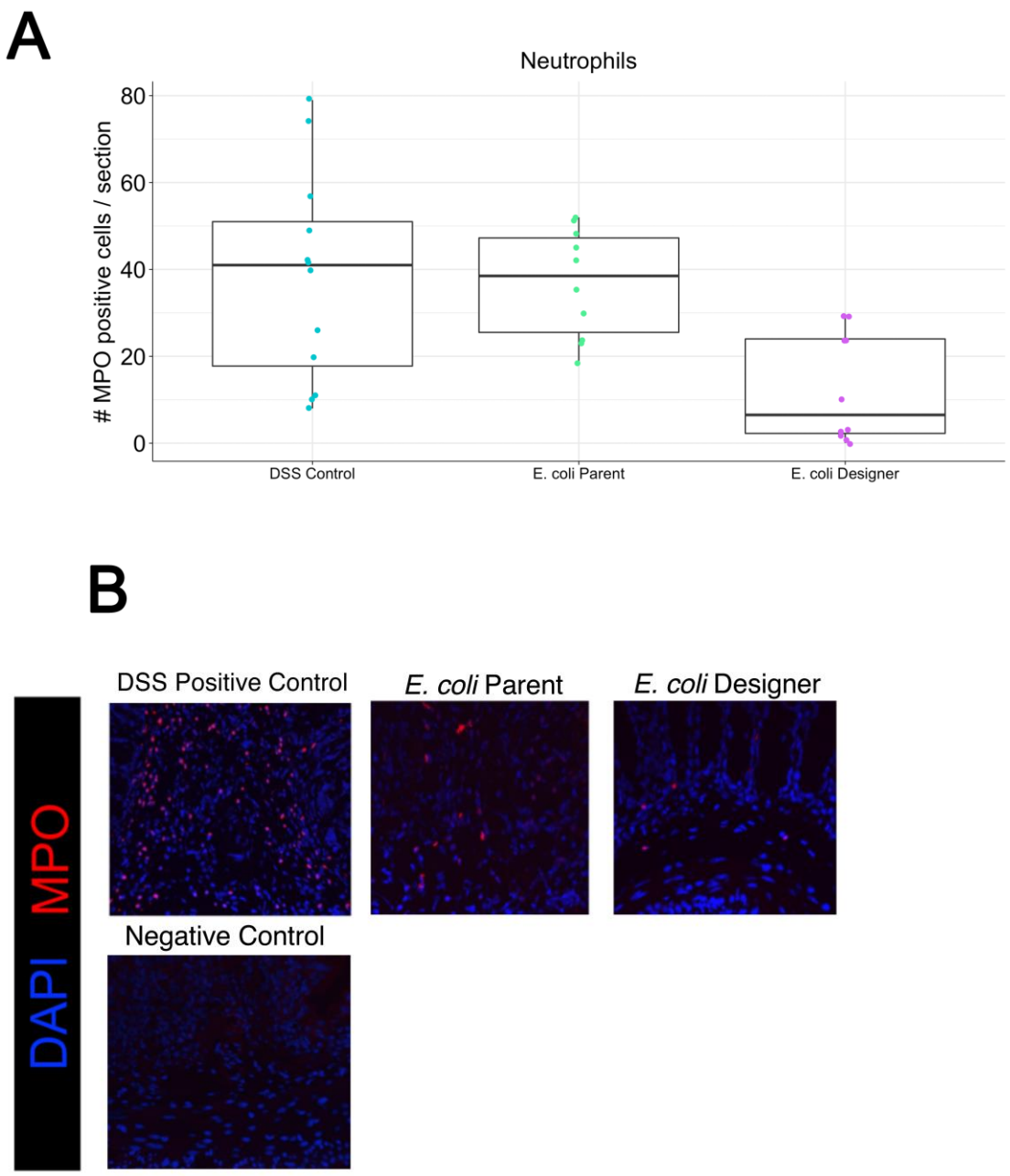

Figure 13. Designer probiotic *E. coli* DSS-induced colitis group shows lower neutrophil colonic cell infiltration compared to probiotic parent group.
A) Positive cells were quantified in the sub-mucosal lamina propria region on stained tissues via immunofluorescence. B) Images were taken to show colonic mucosal regions representing colonic cell counts. Blue fluorescence represents DAPI and red Texas Red fluorescence represents MPO. Values are expressed as means +/- SEM (n=10-12). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-values of 0.0081 and 0.0061, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

3.1.3 Cytokine analysis with DSS-induced colitis

To further explore immune responses and to examine if there were any cytokines that were modulated during DSS-induced colitis, pro-inflammatory cytokines were examined as described in section 2.1.13. Cytokines like TNF-α, IFN-γ, IL-1β, and IL-17A. TNF-α and IFN-γ are cytokines that are usually upregulated during DSS-induced colitis. TNF-α is a signaling molecule that plays a role in the activation of further inflammatory responses. IFN-γ is another typical pro-inflammatory cytokine that further stimulates more immune cells, such as natural killer (NK) and T cells. IL-1β is a mediator of inflammatory responses that are involved in cell proliferation, differentiation, and apoptosis. IL-17A is a signaling molecule secreted by T-helper cells and may be a mediator of inflammatory responses. Such pro-inflammatory cytokines are elevated during conditions like IBD and lead to tissue damage from increased inflammation. In contrast, IL-10, is an anti-inflammatory cytokine that inhibit the development of immune cells and enhance the regulatory T cells (Lyer & Cheng, 2013). Therefore, it would be beneficial in tissues undergoing chronic inflammation to have a suppression of pro-inflammatory cytokines and increase in the anti-inflammatory mediators. Although there was a general trend in which the designer strains showed lowered expression in some of these cytokines compared to the parent strain and DSS control, there were no significant differences in TNF-α, IFN-γ, IL-1β, IL-17A, and IL-10 between the *E. coli* and *L. reuteri* designer, parent strains, and control (Figure 14 A-E).

To further look at markers and possible protective responses, the gene expression of RegIIIγ and Mucin2 was examined. RegIIIγ is an anti-microbial peptide that targets Gram-positive bacteria by binding to the peptidoglycan layer

(Ratsimandresey, Indramohan, Dorfleutner, & Stehlik, 2017). The higher expression of this peptide can help in controlling some of the opportunistic bacteria that can populate as a result of the damaged epithelial layer. Muc2 is a colonic secretory mucin that is synthesized by goblet cells (K. S. B. Bergstrom et al., 2010). It makes up the mucus layer found in the gut epithelial. Increased expression of this would be beneficial in a tissue undergoing inflammation. In both *E. coli* and *L. reuteri* designer strains, the gene expression of RegIIIγ was up-regulated compared to the parent strains and the DSS control groups (Figure 15 A). Similarly, for Muc2 gene expression, there was higher expression seen in the *E. coli* designer strain compared to the parent strain (Figure 15 B). This indicates that there could be some protection offered by the designer strains through these pathways.

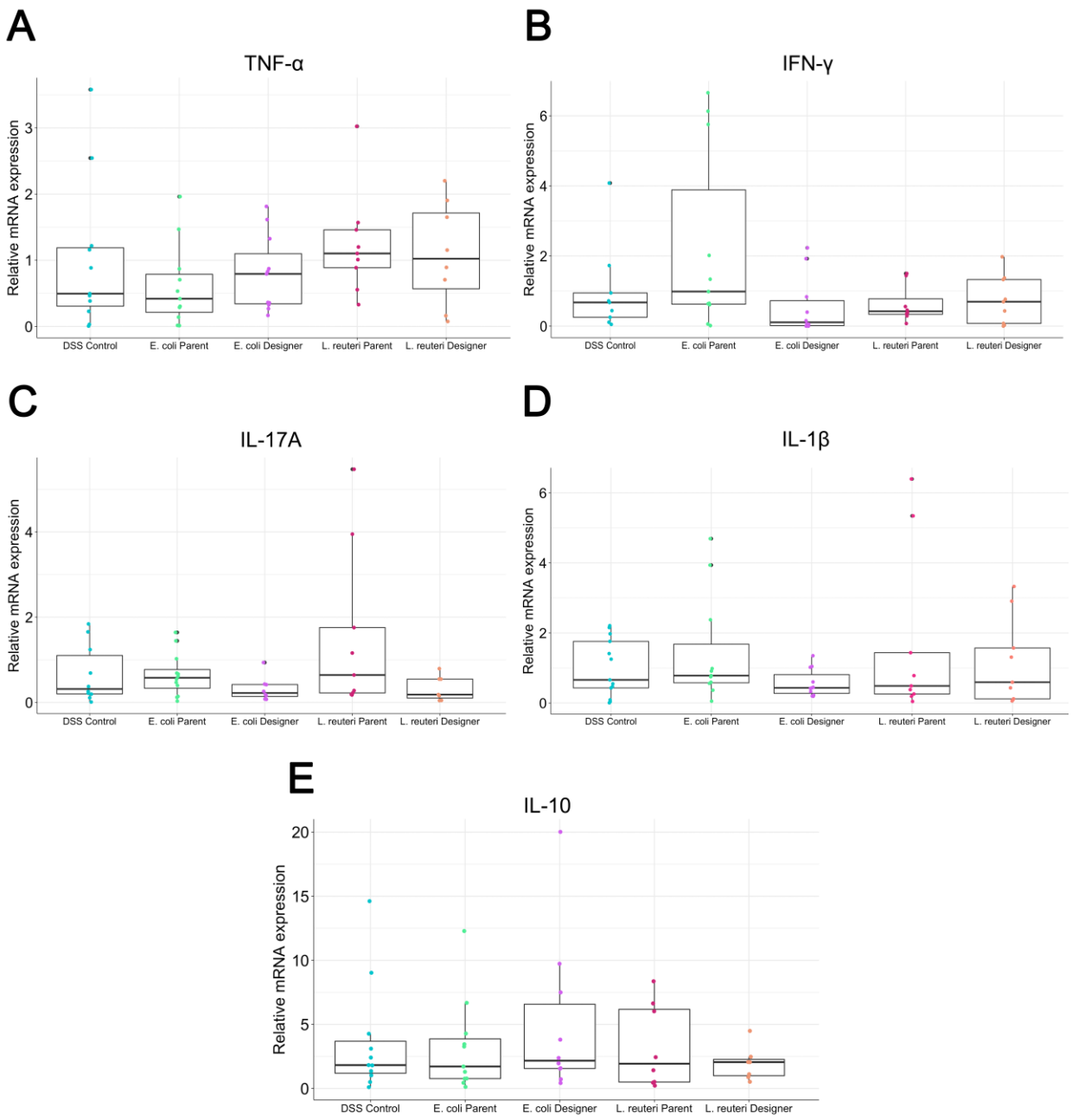

Figure 14. No differences between probiotic groups in pro and anti-inflammatory cytokines.
mRNA gene expression of inflammatory cytokines in the colonic tissue of DSS treated probiotic groups performed via qPCR. Pro-inflammatory cytokines A) TNF-α, B) IFN- γ, C) IL-17A, D) IL-1β, and anti-inflammatory cytokine E) IL-10 were examined. Values are expressed as means +/- SEM (n=10-12).

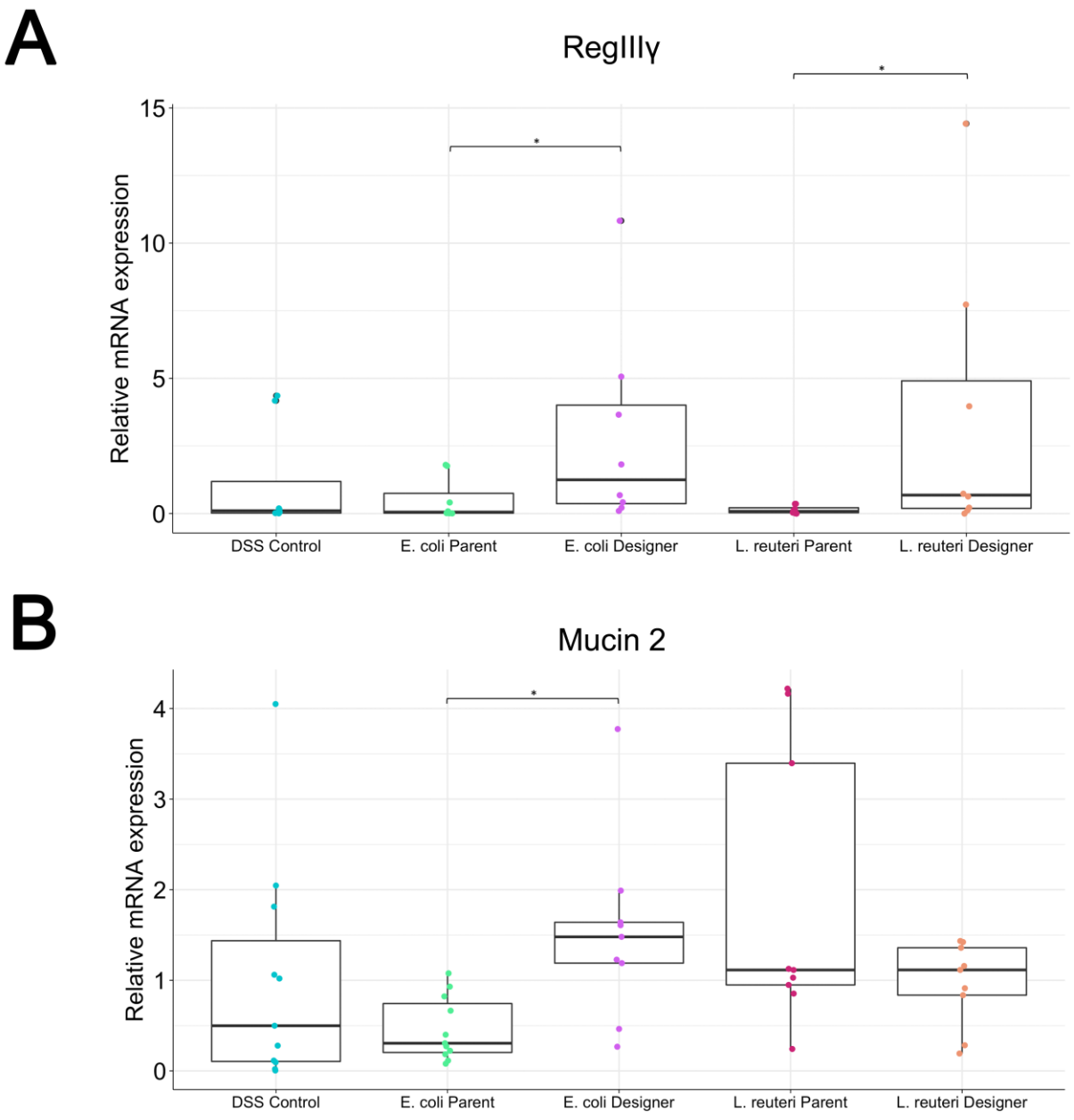

Figure 15. Designer *E. coli* and *L. reuteri* probiotic groups show upregulation of protective Reg3γ and *E. coli* probiotic group shows upregulation of Muc2. Gene expression of protective markers A) RegIIIγ and B) Muc2 in the colonic tissue performed via qPCR. Values are expressed as means +/- SEM (n=10-12). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-values of 0.0124, 0.0211, and 0.0117, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

3.1.4 SCFA and DSS-induced colitis

To further explore protective responses, the production of short chain fatty acids (SCFAs) was examined. SCFAs have many roles such as nutrients for colonic epithelium, mediating intercellular pH, cell volume, ion transport, and regulation of proliferation, differentiation, and gene expression. Specifically, butyric acid is the primary fuel for colonic epithelial cells, but it also regulates cell proliferation and differentiation. Butyric acid is preferred over propionate and acetate in colonocyte metabolism, where butyrate oxidation makes up 70% of the oxygen consumed by colonic tissue (Morrison & Preston, 2016).

SCFAs like acetic acid, propionic acid, and butyric acid were examined using gas chromatography as described in section 2.1.14. Butyric acid was found to be more abundant in mice that received the *E. coli* and *L. reuteri* designer stains compared to the parent stains and DSS control (Figure 16 C). Acetic acid and propionic acid showed no significance differences between probiotic groups and the DSS Control group (Figure 16 A&B). This shows that the designer probiotics could be showing protection through the increased production of bacterial metabolites like SCFAs.

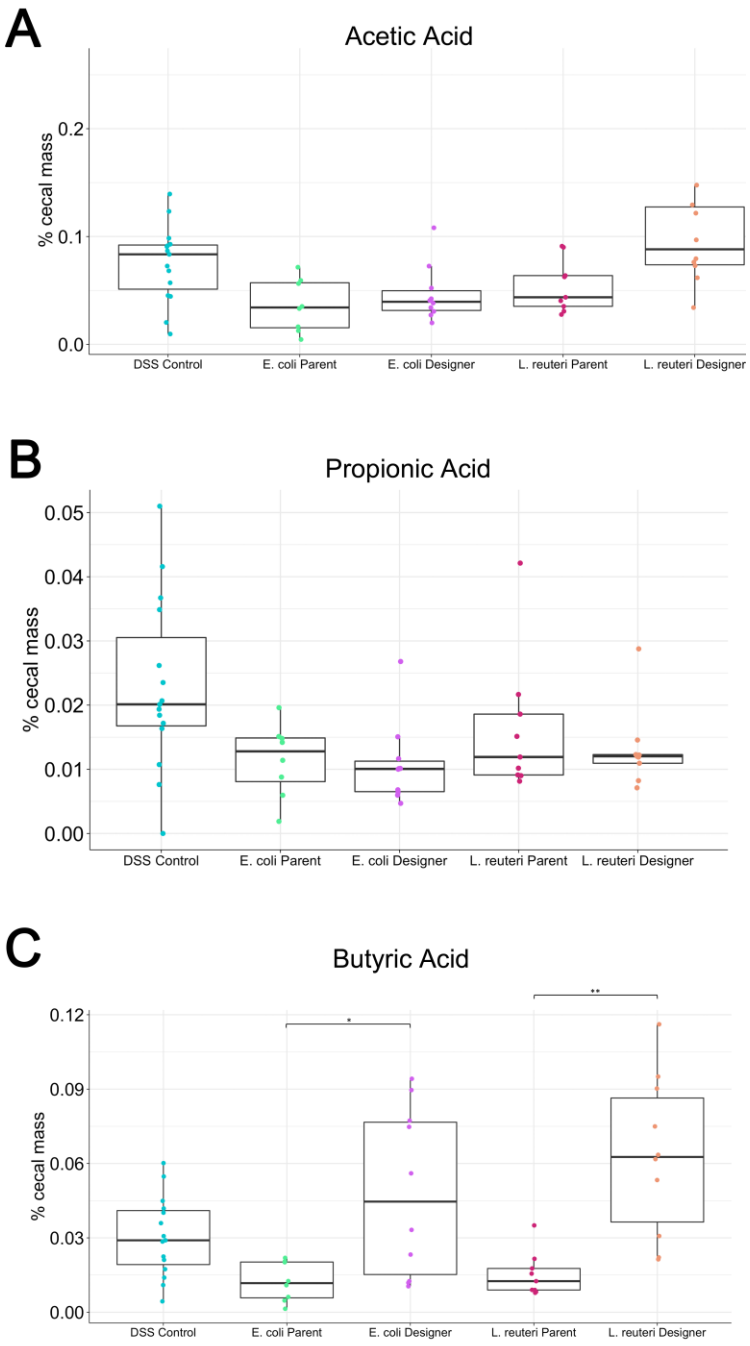

Figure 16. Designer *E. coli* and *L. reuteri* groups show increased amounts of butyric acid.
Short chain fatty acid analysis performed via gas chromatography on cecal samples of mice. Short chain fatty acids A) acetic acid, B) propionic acid, and C) butyric acid were analyzed. Values are expressed as weight percentage shown as means +/- SEM (n=10-12). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-values of 0.0245 and 0.0012, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

3.2 Designer probiotics and Muc2$^{-/-}$ spontaneous colitis model

The designer strains were shown to provide protection during DSS-induced colitis; therefore, we examined another model of murine colitis, Muc2$^{-/-}$ spontaneous colitis. Since the *E. coli* designer strain showed the most protection, the *E. coli* parent and designer strains were tested in the Muc2$^{-/-}$ spontaneous colitis model. Muc2$^{-/-}$ mice develop spontaneous colitis, which is characterized by hyperplasia, crypt abscesses, immune cell infiltration, and sub-mucosal edema. These all represent clinical features of active ulcerative colitis. Mucin 2 is the prominent mucin synthesized in the colon and therefore a defective mucus barrier in animal models allows bacterial contact with the intestinal epithelium (Morampudi et al., 2016). This results in spontaneous colitis since a defective mucus barrier is seen in ulcerative colitis. Muc2$^{-/-}$ mice can develop rectal prolapse and this would indicate severe inflammation and humane endpoint for the mice. Experiments were conducted according to section 2.1.4. Muc2$^{-/-}$ mice were administered either *E. coli* parent stain or designer strain and split into two cohorts at 3 months of age and at 4 months of age. A Muc2 control was used to assess disease progression as a control and also as a gavage control to factor in any stress responses as a result of the gavages. This group was given a media gavage once weekly for 4 consecutive weeks. Additional clinical data is shown in Appendix C.

3.2.1 Clinical data and Muc2$^{-/-}$ spontaneous colitis model

A rectal prolapse indicates a very severe state, in which the animal would need to be euthanized. The rate of rectal prolapse is summarized in Table 15. The parent

strain had the highest number of rectal prolapses at 20%, Muc2 control had 5%, and the designer strain did not have any rectal prolapses.

Table 15. Frequency of rectal prolapses in Muc2$^{-/-}$ colitic mice.

Treatment Groups	Muc2$^{-/-}$ Control	*E. coli* Parent	*E. coli* Designer
Number of Rectal Prolapses	1/19 (5%)	3/15 (20%)	0/20 (0%)

Weekly measurements were taken for food and water intake. There were no differences in food and water intake between different probiotic groups (Table 24, Appendix B). The body weights of the Muc2$^{-/-}$ mice were recorded weekly. Prior to probiotic supplementation, the starting body weights of the mice were the same with no significant differences (Table 23, Appendix C).

The body weight change of these animals was monitored weekly throughout the entire Muc2$^{-/-}$ spontaneous colitis. Weight change was calculated as a percentage from the starting body weight prior to probiotic gavage. For both the *E. coli* parent and designer strains, there was no differences in weight change at 3 months of age (Figure 17 A), 4 months of age (Figure 17 B), and combined data with all mice at 3 months of age (Figure 17 C).

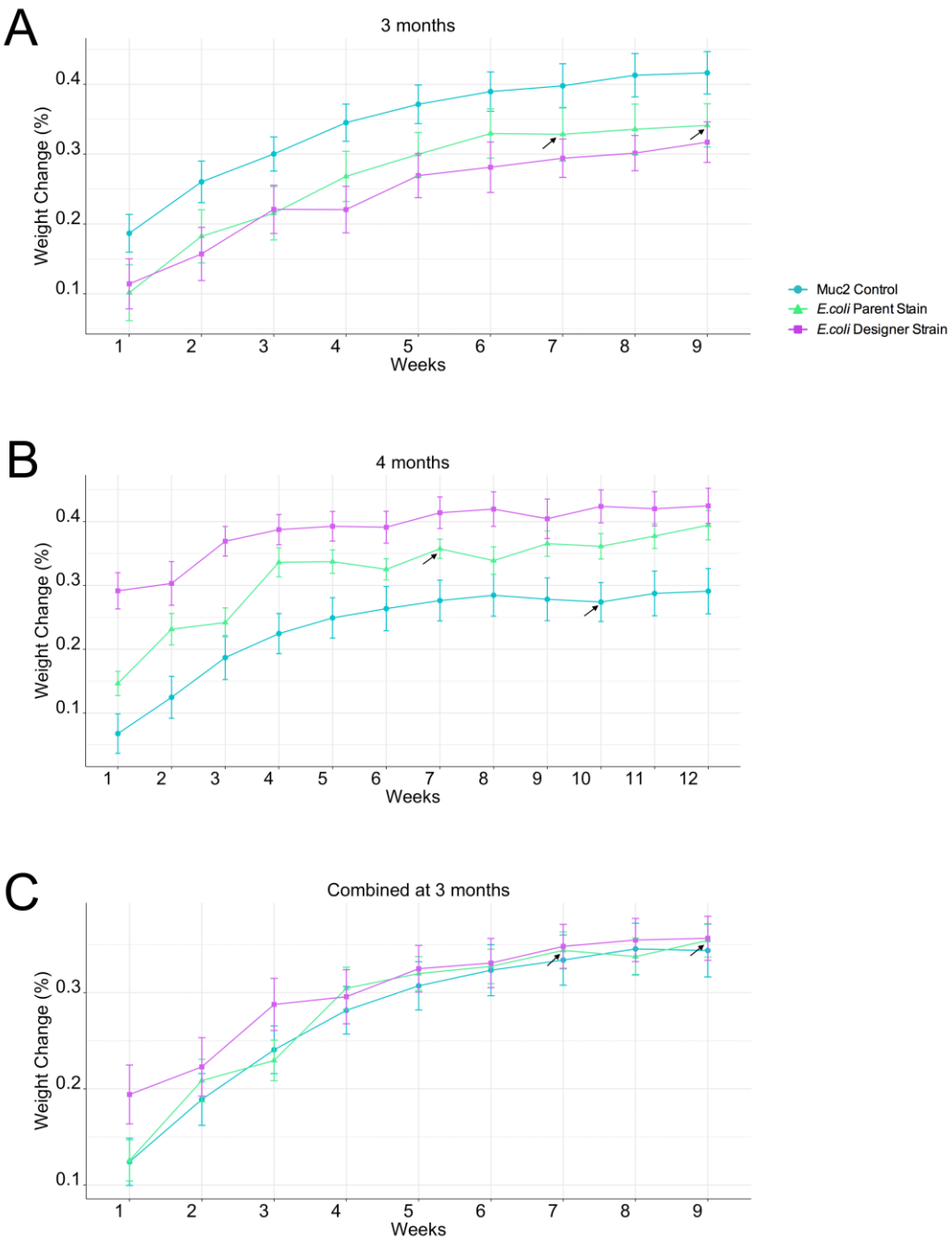

Figure 17. No differences in body weight change between strains during Muc2$^{-/-}$ spontaneous colitis.
Body weight change during the Muc2$^{-/-}$ spontaneous colitis in mice pre-treated with *E. coli* probiotic strains labelled as *E. coli* parent strains (triangles), designer probiotic strains labelled as *E. coli* designer Strain (squares), and the media only Muc2 control (circles). Weight loss was calculated as a percentage of the weight loss from the starting body weight at Week 0. Weight change is shown at A) 3 months of age, B) 4 months of age, and C) combined at 3 months of age. Arrows represent rectal prolapses. Values are expressed as means +/- SEM. Sample size of n=8-20.

As the spontaneous colitis progressed, mice were given daily clinical scores to score and assess the visual clinical symptoms observed. Mice were scored based on the scoring system described in section 2.1.8. The maximum score from this scoring system was an 18. Higher clinical scores indicate greater disease symptoms. At 3 months of age, there was a significant difference in clinical scores at week 9 between the *E. coli* designer strain and Muc2 control (Figure 18 A). Similarly, this difference was also observed at 4 months of age at 12 weeks (Figure 18 B). When the clinical scores were combined together from both cohorts at 3 months of age, this same difference was observed at 9 weeks where both the E. coli parent and designer strains were significantly different from the Muc2 control (Figure 18 C). This shows that supplementation of probiotics lowered clinical scores. As expected, when looking at the black arrows (rectal prolapses), it can be seen that the clinical scores increased when a mouse developed a rectal prolapse (Figure 18 A-C).

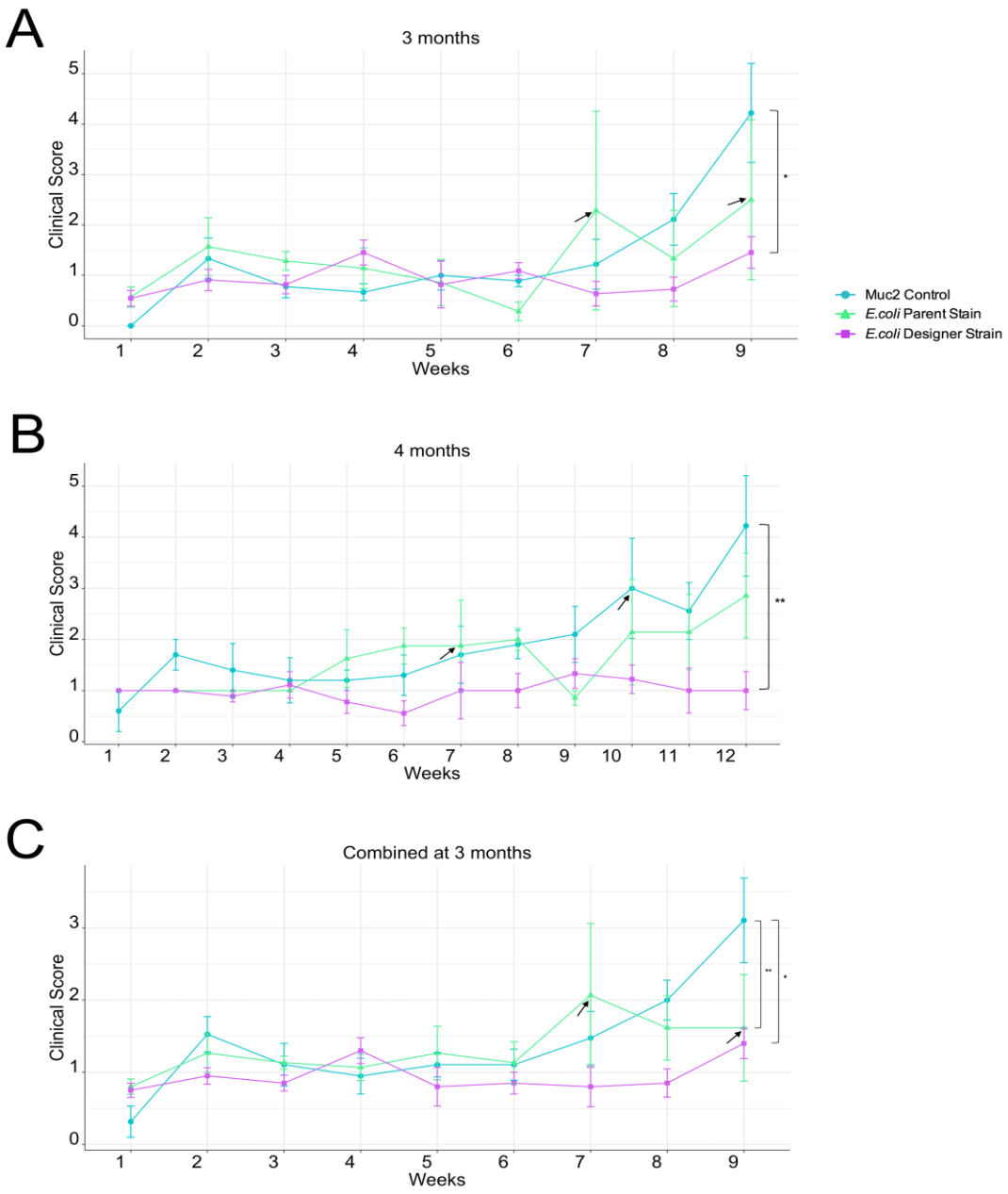

Figure 18. Probiotic supplementation results in lowered clinical scores during Muc2$^{-/-}$ spontaneous colitis.
Clinical scores during the Muc2$^{-/-}$ spontaneous colitis in mice pre-treated with *E. coli* probiotic strains labelled as *E. coli* parent strains (triangles), designer probiotic strains labelled as *E. coli* designer Strain (squares), and the media only Muc2 control (circles). Clinical scores are shown at A) 3 months of age, B) 4 months of age, and C) combined at 3 months of age. Arrows represent rectal prolapses. Values are expressed as means +/- SEM. Sample size of n=8-20. Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-values of 0.0245 and 0.0012, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

To examine if there was a systemic infection, the MLN and spleen were homogenized and then plated on 1.8% LB agar plates to obtain colony counts as described in section 2.1.9. At 3 months of age in the MLN, there was no difference in CFU counts between groups (Figure 19 A). However, at 4 months of age there is a significant reduction in CFU counts in the *E. coli* designer strain compared to the parent strain (Figure 19 B). This is also observed with the spleen, in which there is no difference at 3 months of age (Figure 20 A), but at 4 months of age the *E. coli* designer strain showed a significant decrease in CFU counts compared to the parent stain (Figure 20 B). This indicates that there could be some dysfunction in the epithelial layer leading to passage of bacteria into extra-intestinal sites in mice supplemented with the parent stain. This not seen at 4 months of age in the designer strain, maybe due to some protective epithelial or mucosal pathways.

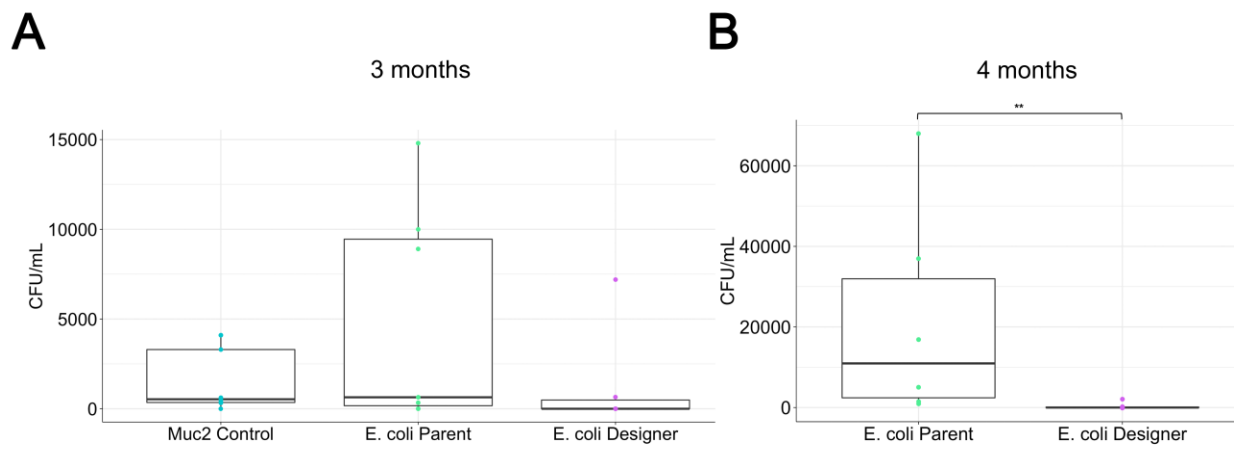

Figure 19. ***E. coli*** **designer probiotic group shows significantly lower MLN CFU counts at 4 months of age.**
CFU/ml calculated from homogenates of MLN grown on 1.8% LB agar. Values are expressed as means +/- SEM (n=8-11). Non-parametric t-test (Mann-Whitney U test) was used. Asterisk represents significant p-value of 0.0016. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

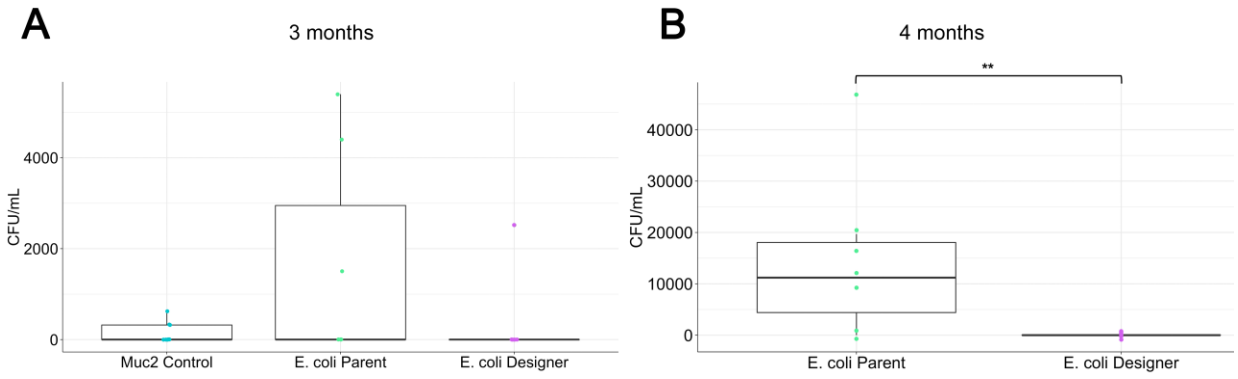

Figure 20. *E. coli* designer probiotic group shows significantly lower spleen CFU counts at 4 months of age.
CFU/ml calculated from homogenates of spleen grown on 1.8% LB agar. Values are expressed as means +/- SEM (n=8-11). Non-parametric t-test (Mann-Whitney U test) was used. Asterisk represents significant p-value of 0.0029. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

Upon sacrifice at 3 or 4 months of age, macroscopic images of the distal colon and ceca were taken. At 3 and 4 months of age, Muc2$^{-/-}$ control and *E. coli* parent strain groups showed more swollen distal colons and ceca compared to the designer strain groups (Figure 21).

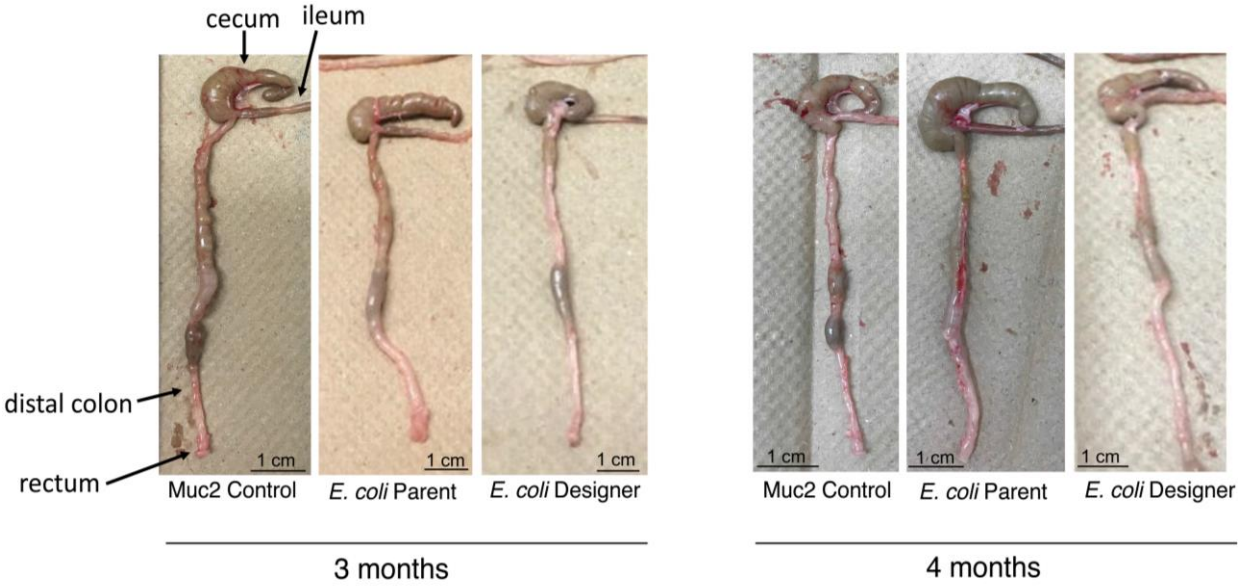

Figure 21. Macroscopic examination shows that the designer probiotic groups appear to have slightly less swollen tissues compared to the other treatment groups.
Mice were administered either parent or designer probiotics via oral gavage weekly for 4 weeks. Tissues were collected at either 3 or 4 months of age. Images were taken to show the large intestine of the mice. Scale measurement shows distance of 1 cm.

3.2.2 Histology and Muc2$^{-/-}$ spontaneous colitis model

Histopathological scores were examined in the Muc2$^{-/-}$ spontaneous colitis model, similar to the DSS model. To assess histopathological damage, tissue sections were scored based on the scoring system as described in section 2.1.11. A higher

histopathological score indicates more inflammation and thus more damage as a result from the Muc2$^{-/-}$ spontaneous colitis. The maximum histopathological score from this scoring system was a 13. At 3 months, there was a significant difference between the *E. coli* designer strain and *E. coli* parent strain. The *E. coli* designer probiotic strain supplementation resulted in significantly lower histopathological scores (Figure 22 A&B). At 4 months, supplementation with the *E. coli* designer strain resulted in significantly lower scores compared to only the Muc2 control group (Figure 22 C&D). When these scores at both 3 and 4 months of ager, were analyzed according to gender, there was a significant difference only at 3 months. At 3 months, the male mice supplemented with *E. coli* designer strain had significantly lower histopathological scores compared to the *E. coli* parent strain (Fig 23 A). The female mice at 3 months had no significant differences, but a trend towards lower sores with the *E. coli* designer strain (Fig 23 B). At 4 months, there were no significant differences, but a similar pattern in lower scores with the *E. coli* designer strain (Fig 24 A&B). When these histopathological scores were broken down by gender, due to the small sample size, some of the significant differences were not seen, compared to all genders grouped together, as in Fig 22.

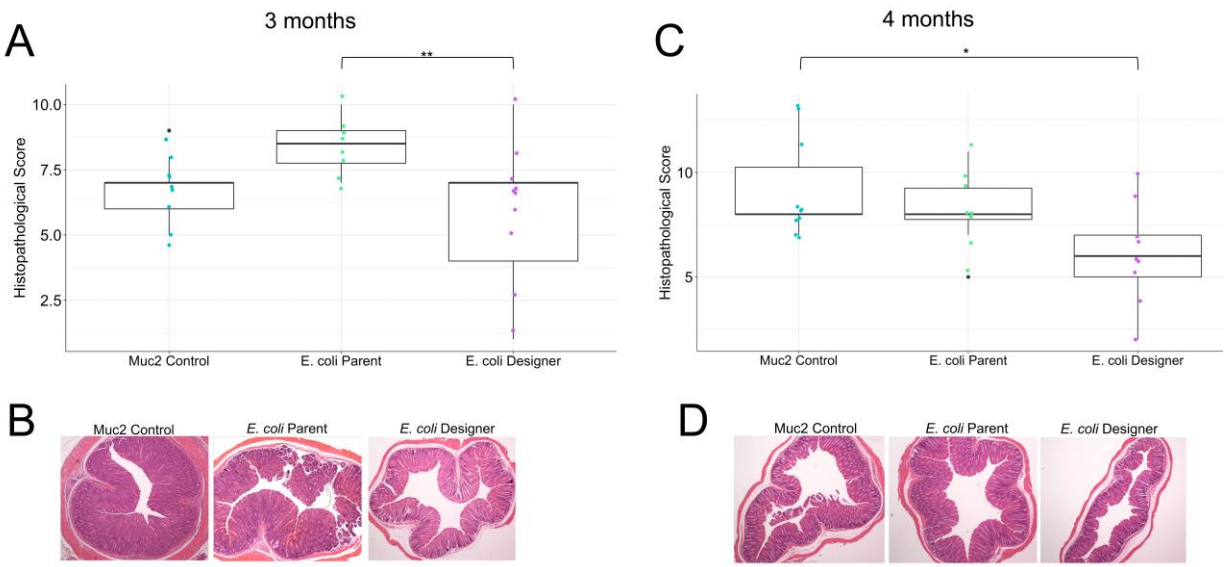

Figure 22. Supplementation with *E. coli* designer strain results in significantly lower histopathological scores compared to *E. coli* parent strain at 3 months and Muc2 control at 4 months of age.

H&E stained slides of cross sections of the distal colon were used to calculate histopathological scores at A) 3 months of age and C) 4 months of age. Epithelial integrity, immune cell infiltration, hyperplasia, and edema were used to calculate histopathological scores. Images were taken to show damage representing histopathological scores at B) 3 months of age and D) 4 months of age. Images were selected based on the mean histopathological score. Values are expressed as means +/- SEM (n=8-11). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-values of 0.0094 and 0.0251, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

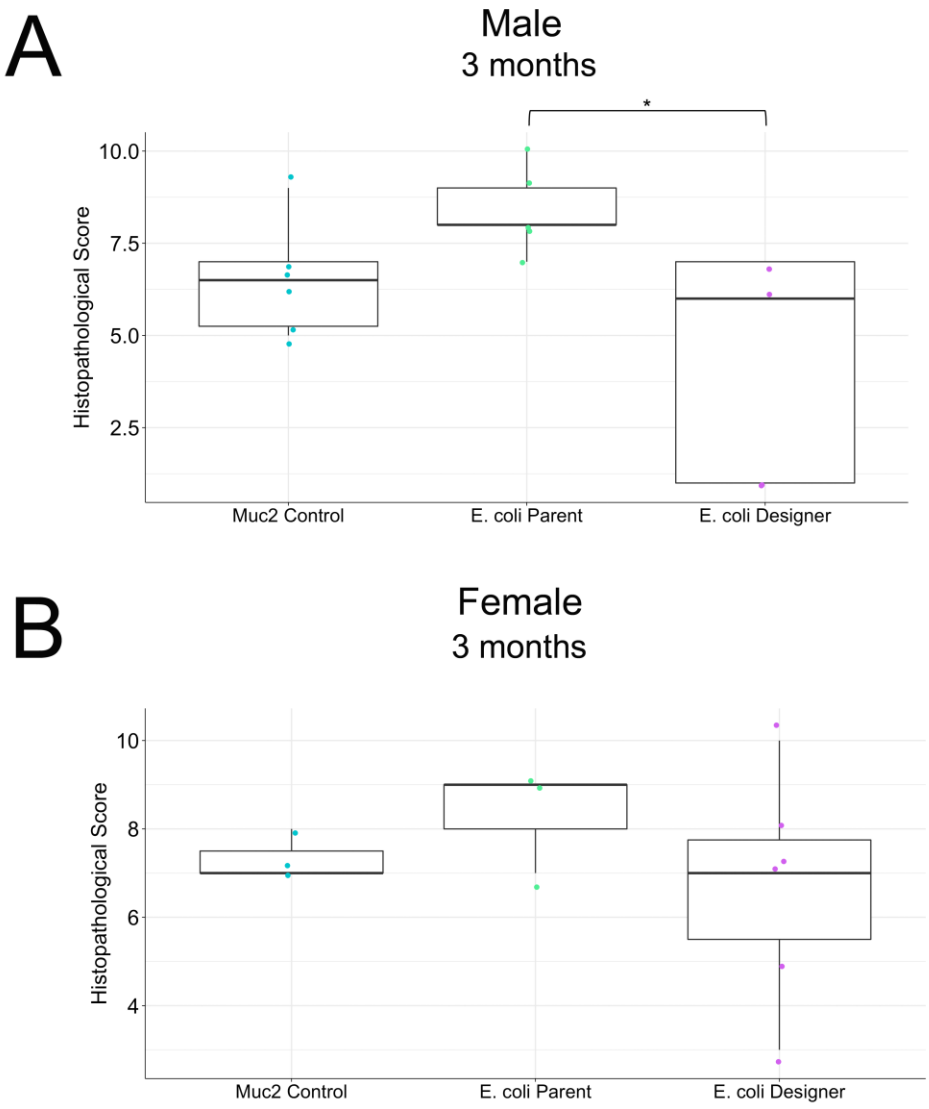

Figure 23. Supplementation with *E. coli* designer strain results in significantly lower histopathological scores in male mice at 3 months of age.
H&E stained slides of cross sections of the distal colon were used to calculate histopathological scores at A) males 3 months of age and B) females 3 months of age. Epithelial integrity, immune cell infiltration, hyperplasia, and edema were used to calculate histopathological scores. Values are expressed as means +/- SEM (n=3-6). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisk represents significant p-value of 0.0152. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

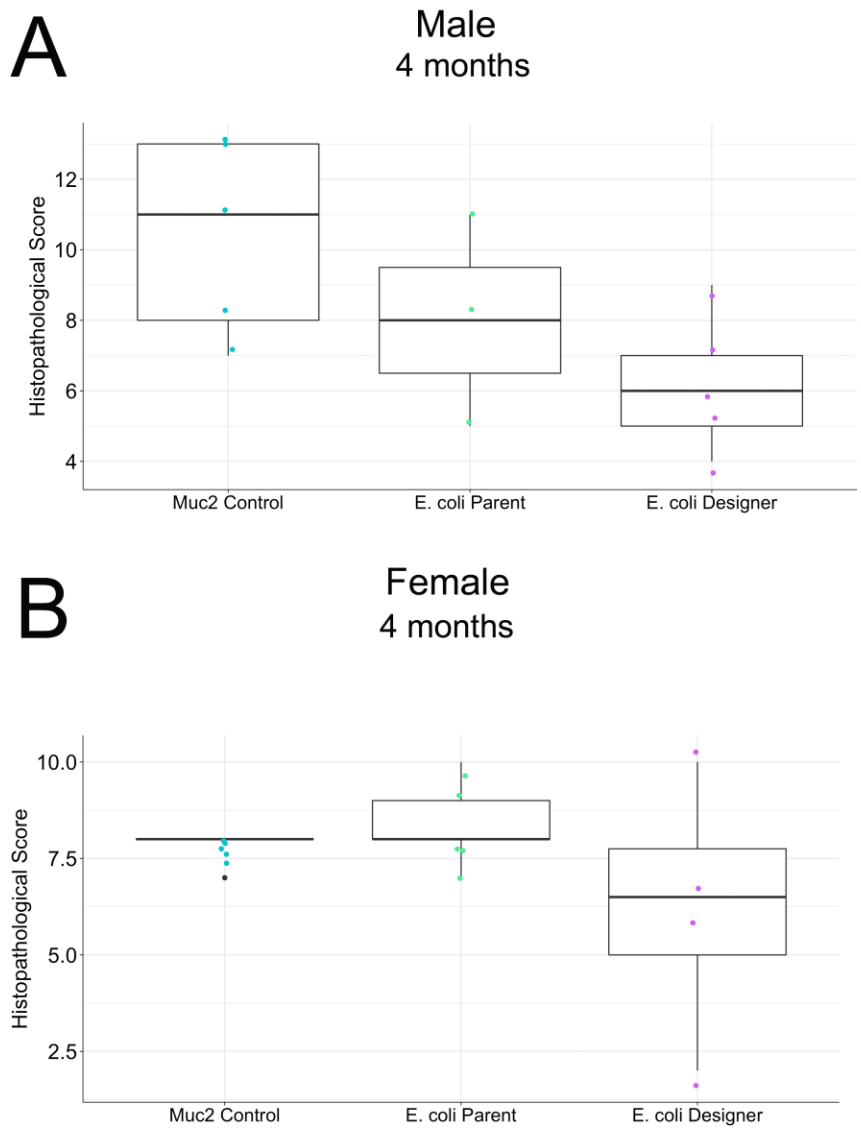

Figure 24. Histopathological scores show trend towards lower histopathological scores with *E. coli* designer strain supplementation at 4 months of age.
H&E stained slides of cross sections of the distal colon were used to calculate histopathological scores at A) males 4 months of age and B) females 4 months of age. Epithelial integrity, immune cell infiltration, hyperplasia, and edema were used to calculate histopathological scores. Values are expressed as means +/- SEM (n=3-5). Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

3.2.3 Cytokines and Muc2$^{-/-}$ spontaneous colitis model

To further explore immune responses and to examine if there were any cytokines that were modulated during Muc2$^{-/-}$ spontaneous, pro-inflammatory cytokines were examined as described in section 2.1.13. Pro-inflammatory cytokines like TNF-α and IFN-γ and markers like RegIIIγ and RELM-β were examined, as described earlier with the DSS model. RELM-β is a defense factor that is upregulated in the absence of Muc2 and has been shown that it drives colitis in the Muc2$^{-/-}$ model by depleting protective commensal microbes (Morampudi et al., 2016). In addition, CXCL-9 and Claudin-10 were also analyzed. CXCL-9 is a chemokine that is a T-cell chemoattractant, induced by IFN-γ, that is shown to be up-regulated in the Muc2 model (Yang et al., 2008). Claudin-10 is a tight-junction protein that has been shown to have increased expression in the Muc2 model (Lu et al., 2011).

There were no differences in TNF-α at 3 (Figure 25 A) and 4 months of age (Figure 25 B) between probiotic groups. For IFN-γ, there were no differences at 3 months of age (Figure 25 C) but at 4 months of age there was significant differences between the groups (Figure 25 D). The *E. coli* designer strain had significantly lower IFN-γ expression at 4 months of age compared to the parent *E. coli* strain and the Muc2 control. For RegIIIγ and RELM-β, at 3 months of age, there were no differences between groups (Figure 26 A&C), but at 4 months there was lower expression of RegIIIγ between the designer and parent *E. coli* strain and the Muc2 control (Figure 26 D). For RELM-β, there was a significant difference between the designer *E. coli* strain and Muc2 control (Figure 26 B). For CXCL-9 and Claudin-10, there were no

differences at 3 and 4 months of age (Figure 27 A-D). This indicates that there could be some protection the designer strains through these pathways.

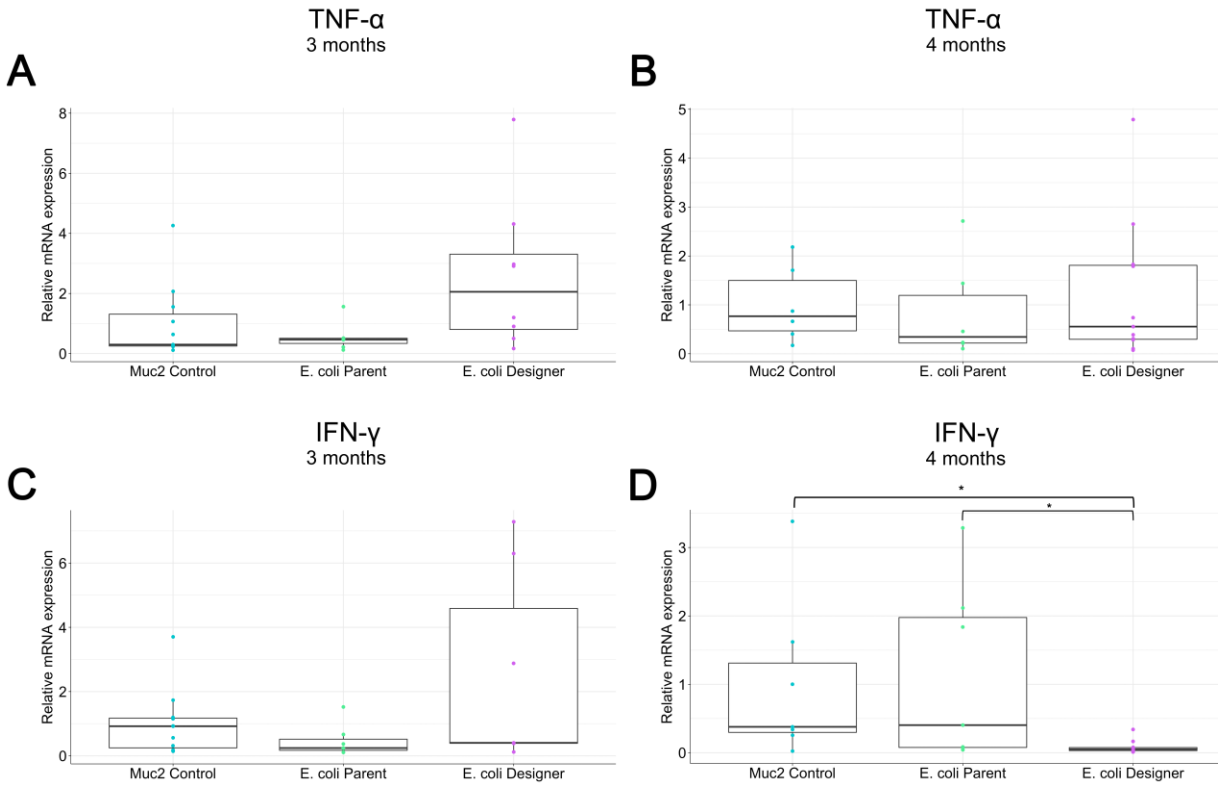

Figure 25. Designer probiotic *E. coli* group shows significantly lower mRNA expression of IFN-γ at 4 months of age compared to parent probiotic group. mRNA gene expression of inflammatory cytokines in the colonic tissue of mice performed via qPCR. Pro-inflammatory cytokines TNF-α were analyzed at A) 3 months of age and B) 4 months of age and IFN-γ at A) 3 months of age and B) 4 months of age. Values are expressed as means +/- SEM (n=8-11). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-values of 0.0221 and 0.0197, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

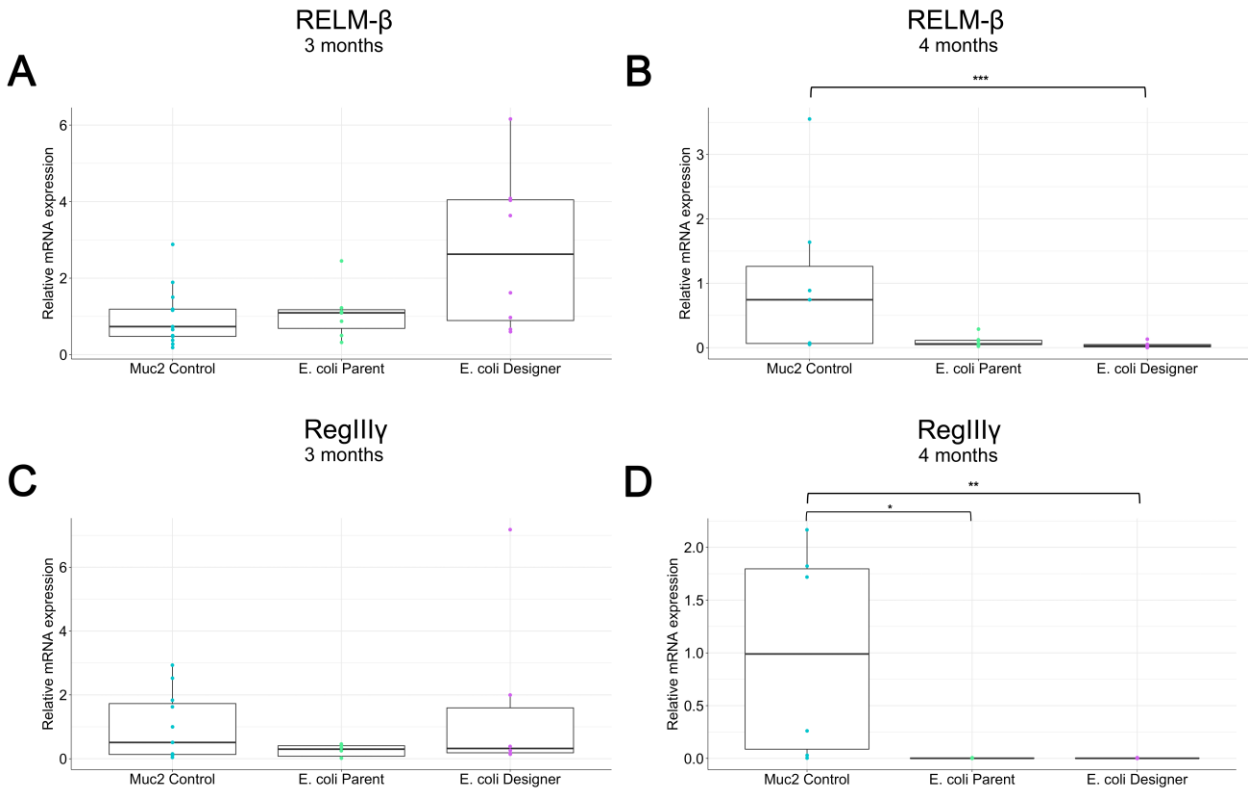

Figure 26. Designer probiotic *E. coli* group shows significantly lower RELM-β and RegIIIγ expression at 4 months of age compared to Muc2 control group.
mRNA gene expression of markers in the colonic tissue of mice performed via qPCR. RELM-β was analyzed at A) 3 months of age and B) 4 months of age and RegIIIγ at A) 3 months of age and B) 4 months of age. Values are expressed as means +/- SEM (n=8-11). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-values of 0.0007 and 0.0026, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

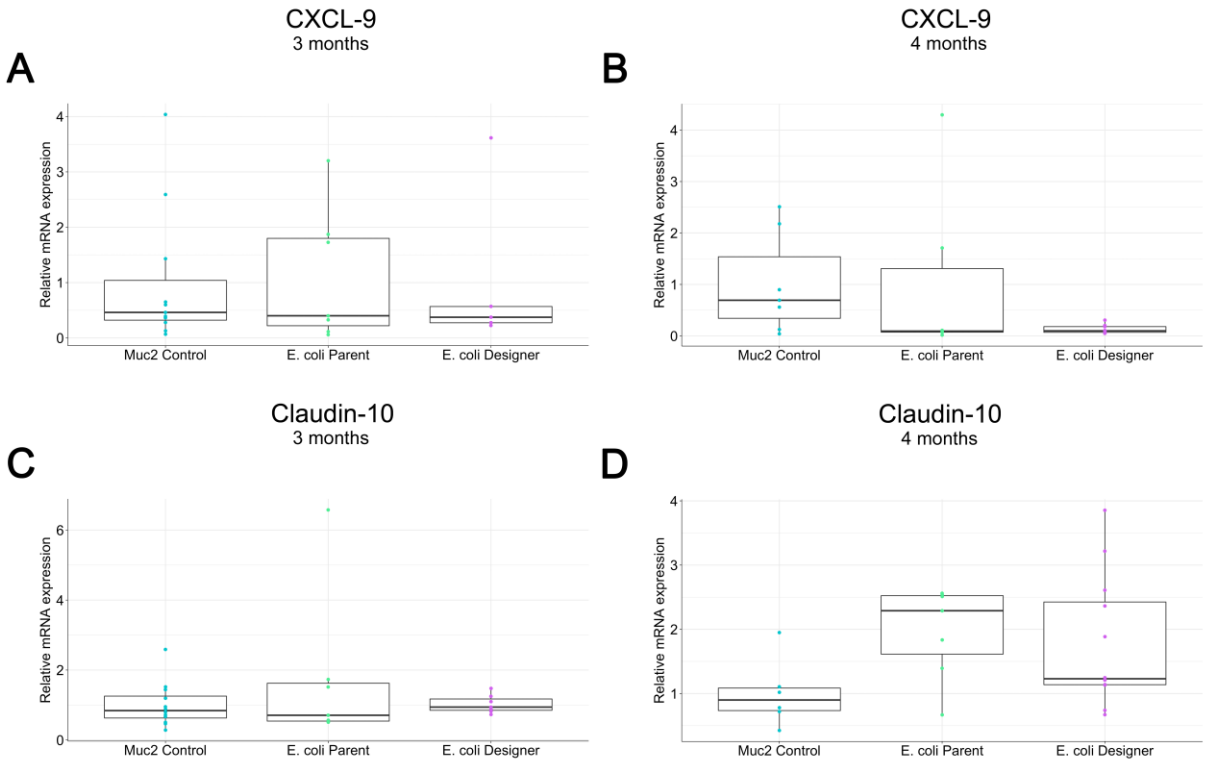

Figure 27. *E. coli* probiotic groups shows no significant difference in CXCL-9 and Claudin-10 expression.
mRNA gene expression of markers in the colonic tissue of mice performed via qPCR. CXCL-9 was analyzed at A) 3 months of age and B) 4 months of age and Claudin-10 at A) 3 months of age and B) 4 months of age. Values are expressed as means +/- SEM (n=8-11).

3.2.4 SCFA and Muc2$^{-/-}$ spontaneous colitis model

To further explore protective responses, the production of short chain fatty acids (SCFAs) was examined as previously explained with the DSS model. SCFAs like acetic acid, propionic acid, and butyric acid were examined as described in section 2.1.14. There were no significant differences between E. coli probiotic strain at 3 and 4 months of age between all SCFAs (Figure 28 A-F). The only differences seen were between the *E. coli* designer strain and Muc2 control at 3 months of age for acetic acid (Figure 28 A) and propionic acid (Figure 28 C).

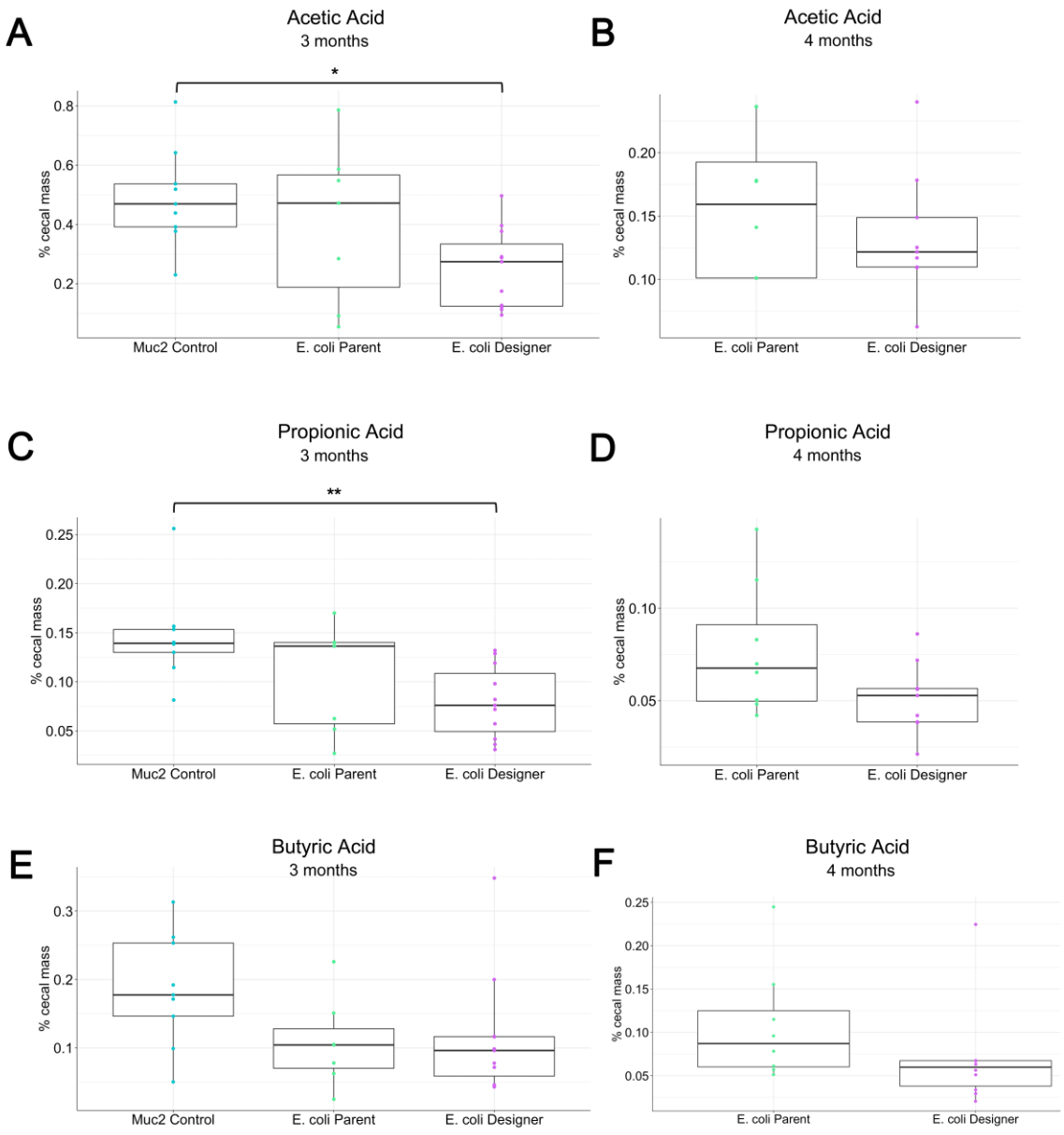

Figure 28. No differences in short chain fatty acids between designer and parent probiotic groups.
Short chain fatty acid analysis performed via gas chromatography on cecal samples of mice. Short chain fatty acids A) acetic acid, B) propionic acid, and C) butyric acid were analyzed. Values are expressed as mass percent of cecal tissue shown as means +/- SEM (n=8-11). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-values of 0.0186 and 0.0055, respectively. Corresponding asterisks for significance level * for <0.05, ** for <0.001, and *** for <0.0001.

3.3 Designer probiotics and strain detection

3.3.1 *E. coli* strain and strain detection experiment

C57BL/6 mice were given a single oral gavage of the *E. coli* parent and designer probiotic strains as described in section 2.1.5. Clinical data for the C57BL/6 detection experiments is shown in Appendix D. *E. coli* designer strain was detected as described in section 2.1.15. Gel electrophoresis images are shown in Appendix E. Primers specific to the ttr operon were used for detection of the *E. coli* designer probiotic strain in C57BL/6 mice. Five different collection points were analyzed (Table 16).

Table 16. Sample key for ttr detection with *E. coli* designer strain in C57BL/6 mice

Collection #	Sample Type	Time Point	Weeks after Gavage
1	Stool	1 week	1
2	Stool	2 weeks	2
3	Stool	1 month	4
4 (euthanization)	Stool	2 months	8
5 (euthanization)	Colon	2 months	8

Stool samples were used to detect ttr in mice supplemented with *E. coli* designer strain. Table 17 shows the results of ttr detection in C57BL/6 mice. In the starting timepoints, detection levels start at 29-36% of the mice but then this drops down to around 12% by the end of the experiment.

Table 17. Summary of ttr detection with *E. coli* designer strain in C57BL/6 mice

Collection #	Strain	Sample Type	Timepoint	# Positive	Total (N)	% Positive
1	Designer	Stool	1 week	4	14	29%
2	Designer	Stool	2 weeks	5	14	36%
3	Designer	Stool	1 month	2	14	12%
4 (euth)	Designer	Stool	2 months	1	14	7%
5 (euth)	Designer	Colon	2 months	2	14	12%

Summarizing the ttr detection in C57BL/6 mice by individual mouse shows non-persistent colonization (Figure 29). Looking at each individual mouse, only mouse #13 seems to have a semi persistent ttr detection pattern. However, at the end of this experiment at 2 months, ttr is detected in stool samples but not colon tissue.

Mouse #	Stool Collection #				
	1	2	3	4*	5*
1	-	-	-	-	-
2	-	-	-	-	-
3	+	-	-	-	+
4	-	+	-	-	-
5	-	+	-	-	-
6	-	+	-	-	-
7	-	-	-	-	-
8	-	-	-	-	-
9	-	-	-	-	+
10	+	+	-	-	-
11	-	-	+	-	-
12	+	+	-	-	-
13	+	-	+	+	-
14	-	-	-	-	-

* = euthanization timepoint

Figure 29. C57BL/6 mice treated with *E. coli* designer probiotic strain show non-persistent detection in stool and colon samples.
Ttr detection with C57BL/6 mice supplemented with a single oral gavage of *E. coli* designer strain. Mouse # refers to different individual mice supplemented with *E. coli* designer probiotic strain. Stool collection # refers to different timepoints of stool or colon collection. Asterisks represent collection at euthanization. A positive and negative result for detection is shown by + or – symbol.

3.3.2 *E. coli* strain and strain detection in Muc2$^{-/-}$ spontaneous colitis model

Muc2$^{-/-}$ mice were given oral gavages of the *E. coli* designer probiotic once weekly for four weeks as described in section 2.1.4. *E. coli* designer strain was detected

as described in section 2.1.15. Gel electrophoresis images are shown in Appendix F. Primers specific to the ttr operon were used for detection of the *E. coli* designer probiotic strain in Muc2$^{-/-}$ mice. Eleven different collection points were analyzed (Table 18).

Table 18. Sample key for ttr detection with *E. coli* designer strain in Muc2$^{-/-}$ mice

Collection #	Sample Type	Total # Gavages	Weeks after Gavage #1	Weeks after Gavage #2	Weeks after Gavage #3	Weeks after Gavage #4
1 (before gav)	Stool	0	0	0	0	0
2	Stool	1	1	0	0	0
3	Stool	3	3	2	1	0
4	Stool	4	5	4	3	2
5	Stool	4	7	6	5	4
6 (euthanization)	Stool	4	9	8	7	6
7 (euthanization)	Colon	4	9	8	7	6
8	Stool	4	9	8	7	6
9	Stool	4	11	10	9	8
10 (euthanization)	Stool	4	12	11	10	9
11 (euthanization)	Colon	4	12	11	10	9

Stool samples were used to detect ttr in mice supplemented with *E. coli* designer strain. Table 19 shows the results of ttr detection in Muc2$^{-/-}$ mice. Detection levels start off at 60-70% and then stay until the last time-points, with 67% for stool and 22% for colon. This shows that there are differences in detection between the stool and colon samples.

Table 19. Summary of ttr detection with *E. coli* designer strain in Muc2$^{-/-}$ mice

Strain	Sample Type	Collection #	# Positive	Total (N)	% Positive
Designer	Stool	1	0	20	0%
Designer	Stool	2	12	20	60%
Designer	Stool	3	14	20	70%
Designer	Stool	4	12	20	60%
Designer	Stool	5	8	20	40%
Designer	Stool (euth)	6	4	11	36%
Designer	Colon (euth)	7	3	11	27%
Designer	Stool	8	3	9	33%
Designer	Stool	9	1	9	11%
Designer	Stool (euth)	10	6	9	67%
Designer	Colon (euth)	11	2	9	22%

Summarizing the ttr detection in Muc2$^{-/-}$ mice by individual mouse shows some persistent colonization (Figure 30). Looking at each individual mouse, mice # 30, 32, 46, and 24 show consistent detection patterns throughout all time points.

	Mouse #	Stool Collection #										
		1	2	3	4	5	6*	7*	8	9	10*	11*
3 month	17	-	+	+	-	-	-	-	N/A	N/A	N/A	N/A
	18	-	-	+	-	-	-	-	N/A	N/A	N/A	N/A
	19	-	-	-	-	-	-	-	N/A	N/A	N/A	N/A
	30	-	-	+	+	+	+	+	N/A	N/A	N/A	N/A
	31	-	-	-	+	+	+	-	N/A	N/A	N/A	N/A
	32	-	+	+	+	+	+	+	N/A	N/A	N/A	N/A
	43	-	+	+	+	+	-	-	N/A	N/A	N/A	N/A
	44	-	+	-	+	-	-	-	N/A	N/A	N/A	N/A
	45	-	-	+	-	-	-	-	N/A	N/A	N/A	N/A
	46	-	+	+	+	+	+	-	N/A	N/A	N/A	N/A
	47	-	-	+	+	+	-	+	N/A	N/A	N/A	N/A
4 month	21	-	+	+	-	-	N/A	N/A	-	-	+	-
	22	-	+	-	-	-	N/A	N/A	-	-	-	-
	23	-	+	-	-	+	N/A	N/A	-	-	+	+
	24	-	+	+	+	+	N/A	N/A	+	+	+	+
	25	-	+	+	+	-	N/A	N/A	+	-	-	-
	26	-	-	+	+	-	N/A	N/A	+	-	+	-
	27	-	-	-	-	-	N/A	N/A	-	-	+	-
	28	-	+	+	+	-	N/A	N/A	-	-	+	-
	29	-	+	+	+	-	N/A	N/A	-	-	-	-

* = euthanization timepoint

Figure 30. Muc2$^{-/-}$ mice treated with *E. coli* designer probiotic strain show semi persistent detection in stool and colon samples.
Ttr detection with Muc2$^{-/-}$ mice supplemented four oral gavages of *E. coli* designer strain. Mouse # refers to different individual mice supplemented with *E. coli* designer probiotic strain. Stool collection # refers to different timepoints of stool or colon collection. Asterisks represent collection at euthanization. A positive and negative result for detection is shown by + or – symbol.

3.3.3 *L. reuteri* and detection of GbpA in C57BL/6 mice

C57BL/6 mice were given a single oral gavage of the *L. reuteri* parent and designer probiotic strains as described in section 2.1.5. Clinical data for the C57BL/6 detection experiments is shown in Appendix D. *L. reuteri* designer strain was detected as described in section 2.1.15. Gel electrophoresis images are shown in Appendix G. Five different collection points were analyzed (Table 16). Gel images were taken to show different primer pairs (Appendix G). Nine different primer pairs were used for detection of GbpA primer. Unfortunately, a primer pair that showed bands in appropriate positive controls and no bands in the negative controls was not established. Therefore, there were no concluding strain detection results for the *L. reuteri* designer probiotic.

Chapter 4: Discussion

4.1 Designer probiotics during murine DSS-induced colitis

Both *E. coli* and *L. reuteri* designer strains were tested in the DSS-induced colitis model. This model is used to study UC since it can be used to study intestinal inflammation due to epithelial damage (Eichele & Kharbanda, 2017).

4.1.1 Designer probiotics significantly lowered clinical scores but not body weight during DSS-induced colitis

Administering mice 3.5% DSS for 7 days induces an acute colitis that can be characterized by clinical symptoms like weight loss, diarrhea, blood in stool, and piloerection (Perše & Cerar, 2012). In our study, for body weight, there were no differences between the DSS control and the probiotic strains. When comparing day one to day seven of the DSS treatment, there was an overall loss in body weight, indicating that in the mice, DSS resulted in weight loss. In literature, during the course of DSS-induced colitis, mice have been shown to lose weight after the third day of DSS treatment, as bleeding starts to initiate (Chassaing et al., 2014). This was similar to what was seen in with the probiotic treatment in the DSS model. However, for clinical scores, the *E. coli* designer strain had significantly lower scores compared to the parent strain. This indicates that the mice supplemented with the designer strain had lower morbidity compared to the parent strain. Whereas, the *L. reuteri* designer strain only differed compared to the DSS control but not the parent strain. In addition, the macroscopic images of the tissues in both designer probiotics showed less bloody, loose diarrhea. Clinically, the designer probiotic supplemented mice appeared to be healthier than the

corresponding parent strain supplemented mice. Often clinical symptoms do not reflect the severity of inflammation and hence it is crucial to examine histopathological damage and not depend solely on clinical scores.

4.1.2 Designer probiotics resulted in significantly lower damage and immune cell infiltration upon histopathological examination

Since histology can show the inflammatory damage in the tissues, histopathological scores were examined. During DSS-induced colitis, both the *E. coli* and *L. reuteri* designer strains had significantly lower histopathological scores. Both the parent and DSS controls showed higher histopathological scores, with some mice even reaching a score of 15, indicating severe inflammatory conditions. Based on these histopathological scores, the parent strains resembled the DSS control with more of an inflamed damage tissue. In contrast, the designer strains showed lower histopathological scores, indicating less tissue damage. These results are similar to what is previously reported as these mice showed active inflammation and mucosal damage. Literature shows histopathological damage in active IBD patients is characterized by inflammation in the colonic mucosa. It is expected that a damaged colitic colon would have inflamed tissue which involves infiltration of immune cells (macrophages, neutrophils etc.) into the sub-mucosal region, destruction or loss of colonic crypts, ulceration present in the crypts, and depletion of mucosal goblet cells (Geboes et al., 2000). This recurrent epithelial damage results in the disruption of the intestinal barrier. Mucosal inflammation has been shown to be associated with

increased relapse rates (Azad, Sood, & Sood, 2011; Riley, Mani, Goodman, Dutt, & Herd, 1991).

Since immune cells play a role in the pathogenesis and excessive inflammation seen during IBD, immunofluorescence was used to examine immune cell infiltration. To assess the role of immune cells, F4/80 antigen marker was used to stain for F4/80+ macrophages and MPO antigen for MPO+ neutrophils. Since IBD involves impairment of the intestinal layer, there can be an infiltration of inflammatory cells into the lamina propria (Al-Ghadban et al., 2016). These immune cells are close to epithelial cell layer and can regulate its function. The innate immune system, which is the first line of defense, includes cells like macrophages and neutrophils (Prame Kumar, Nicholls, & Wong, 2018). These cells act as phagocytes against invading pathogens and are able to digest antigens and present to them to other immune cells. These immune cells are able to move through intestinal mucosa and therefore elicit an immune response (Miura, Hokari, & Tsuzuki, 2012). Although these immune cells are beneficial by acting as the host's defense; excessive recruitment of these cells is seen in inflammatory states. They work in further recruiting more immune cells and signaling molecules like cytokines to the area of inflammation. In a tissue that is undergoing severe inflammation, further recruitment of cells and molecules may be detrimental. It has been shown that in IBD, these immune cells and molecules can result in uncontrolled activation of the immune system and lead to chronic inflammation (Markus F. Neurath, 2014). For example, neutrophils can contribute to IBD pathogenesis through impairment of epithelial barrier, tissue destruction, and release of inflammatory mediators (de Souza & Fiocchi, 2016). Macrophages have been shown to increase in number with increases

in luminal contents that enter the mucosa (Kühl, Erben, Kredel, & Siegmund, 2015). In a normal state, these phagocytic cells produce anti-inflammatory mediators but as inflammation alters the differentiation of cells, it changes their function to promote more inflammation (Maloy & Powrie, 2011; Rivollier, He, Kole, Valatas, & Kelsall, 2012; Tamoutounour et al., 2012). Defects in the epithelial barrier, innate, and immune responses can aggravate inflammatory response in IBD.

Both the *E. coli* and *L. reuteri* designer strains showed lower counts of macrophage cell infiltration and *E. coli* designer strain showed lower neutrophil cell counts compared to the parent strains and DSS only control. Based on the previous histopathological scores that looked at immune cell infiltration as a parameter, this confirms the previous finding that both the DSS control and parent strains showed increased immune cell infiltration. This suggests that the designer strains could be exerting beneficial effects through the suppression of macrophage and neutrophil activation or signaling. Previous literature shows that in a normal state with no inflammation, the gut maintains homeostasis by suppressing the excess immune responses (S. H. Lee, Kwon, & Cho, 2018).Uncontrolled immune cell activation can further increase inflammation and be detrimental in instances like IBD.

4.1.3 Designer probiotics provided protection during DSS-induced colitis through mucosal protective responses but not with reduction of pro-inflammatory cytokines

To further explore immune responses and to examine if there were any cytokines that were modulated during DSS-induced colitis, pro-inflammatory cytokines were

examined. Cytokines play a role in the progression of IBD, in that they can control the inflammatory responses. Some cytokines are even used as therapies as potential targets for inflammation. For example, the blockade of the cytokine TNF is used as a therapy (Danese & Fiocchi, 2011). It is known that excessive amounts of cytokine responses can further increase and worsen intestinal inflammation. During IBD, when there is chronic inflammation, it is usually induced by the uncontrolled activation of mucosal immune cells (Markus F. Neurath, 2014). Several studies in mouse models have shown that the neutralization of pro-inflammatory cytokines can be used to prevent inflammation (M F Neurath, Fuss, Kelsall, Stüber, & Strober, 1995; Powrie et al., 1994; Strober, Fuss, & Blumberg, 2002). Macrophages and dendritic cells are the main antigen-presenting cells in the mucosa and are able to produce pro-inflammatory cytokines like IL-1B, IL-8, and TNF-α through TLR signaling (Markus F. Neurath, 2014).

Cytokines like TNF-α, IFN-γ, IL-1β, and IL-17A. TNF-α and IFN-γ are cytokines that are usually upregulated during DSS-induced colitis. These cytokines are primarily part of the Th1/Th17-mediated inflammation in the acute DSS-colitis model (Alex et al., 2009). DSS is a chemical that is able to disrupt the epithelial layer, which causes antigens to activate mucosal immune cells that in response produce pro-inflammatory cytokines. In particular, TNF-α is able to induce expression of adhesion molecules, fibroblast proliferation, procoagulant factors, and further initiate acute phase responses (Műzes, Molnár, Tulassay, & Sipos, 2012). In IBD, the source of TNF-α comes from activated macrophages or monocytes. IL-17a acts in the delayed-type immune reaction, in which it increases chemokine production and the number of monocytes and neutrophils to the inflamed area. IFN-γ has been shown to augment MHC expression

and expression of T and natural killer cells (Farrar & Schreiber, 1993). Previous studies have shown that IFN-γ plays a major role in the initiation of DSS-induced colitis and further produces chemokines in a IFN-γ dependent manner (Ito et al., 2006). IL-1B promotes Th17 responses in the early phases of inflammation (Muro & Mrowiec, 2015). On the other hand, IL-10, is an anti-inflammatory cytokine that can enhance the protective regulatory T cell responses and inhibit other immune cells that would result in pro-inflammatory responses (Lyer & Cheng, 2013). Studies have shown that mutations in genes that encode IL-10 are associated with early-onset IBD (Kotlarz et al., 2012). Therefore, the lowered expression of the pro-inflammatory responses and higher expression of anti-inflammatory responses, especially during colitis, help to reduce the inflammation and this would be beneficial in controlling symptoms.

To examine local cytokines in the colonic tissues, the main pro-inflammatory cytokines during IBD were analyzed. Although there were general trends in the pro-inflammatory cytokines (TNF-α, IFN-γ, IL-17a, and IL-1β), there were no differences in gene expression of these cytokines between the DSS control and the parent/designer probiotic strains in both the *E. coli* and *L. reuteri* probiotic strains. This could indicate that either the protection from the *E. coli* and *L. retueri* designer strains is not through these inflammatory cytokine pathways or that there was not adequate time for some of the adaptive immune responses to kick in overcome the inflammation. After the initial innate responses kick in, the adaptive immune responses initiate after several days (Chaplin, 2010). The adaptive system is composed of cells that have more specificity for certain antigens. These cells need more sufficient time to elicit an effective response. Perhaps, there are certain adaptive cells or anti-inflammatory cytokines part of the Th2

responses that have not developed yet. These responses would induce changes to overcome some of the inflammation and reduce the pro-inflammatory responses. Previous research in the DSS-induced colitis model shows that both B and T cells were significantly higher after day 8, peaking at day 12 onwards in more chronic models of DSS (Hall et al., 2011). Importantly, many of the current pharmaceutical therapies work through suppression of the T cell responses (Atreya et al., 2011). DSS treatment resulted in significantly increased mRNA expression of pro-inflammatory markers compared to normal C57BL/6. This confirmed that DSS treatment resulted in a pro-inflammatory cytokine response, as expected (Appendix B, Fig 33). Thus, the designer strains may not have had adequate time to develop immune responses to reduce the pro-inflammatory responses that were not shown to be significantly different.

To examine protective responses, RegIIIγ and Mucin2 were analyzed. RegIIIγ is a soluble lectin that can be classified as an antimicrobial peptide (AMP) (Arijs et al., 2009). They are a part of the innate immune response and can help protect the epithelium. RegIIIγ targets Gram-positive bacteria by binding to the peptidoglycan layer (Ratsimandresey et al., 2017). There is evidence in literature to show that AMPs are altered in IBD (Arijs et al., 2009). Decreased secretion of AMPs could further attenuate inflammation, especially when there is uncontrolled epithelial dysregulation, allowing bacteria to invade the mucosa. It has been hypothesized that antimicrobial mucosal barrier defects could be responsible for susceptibility to IBD. The gene expression of RegIIIγ was up-regulated in mice administered the designer probiotic strains compared to the parent strains and the DSS control for both *E. coli* and *L. reuteri* strains. This indicates that the designer strains may be able to exert beneficial effects by pathways or

mediators that reduce bacterial evasion of the mucosa through AMPs. In addition, the expression of Muc2 was examined. This is a secretory mucin that makes up the mucus layer in the epithelial (Morampudi et al., 2016). This mucus layer ensures the protection of the epithelial surfaces from luminal contents and pathogens. In IBD patients, the mucus layer is shown to be altered, specifically in the distal colon. In the DSS-model, there has been shown to be a down-regulation in mucin genes and subsequent depletion of goblet cells (Dharmani, Leung, & Chadee, 2011). Therefore, since the disruption of the epithelial barrier is the primary outcome seen in the DSS-induced colitis, the expression of the Muc2 mucin was analyzed. There was higher expression seen in the *E. coli* designer strain compared to the parent strain but not the *L. reuteri* strain. This further indicates that the strains may be protective due to some epithelial barrier protective responses. RegIIIγ is secreted by IECs and Muc2 is produced by goblet cells, that are associated with the intestinal epithelial layer (Gonçalves, Araújo, & Di Santo, 2018). These protective responses could provide protection of the disrupted barrier function, which is observed in DSS-induced colitis.

4.1.4 Supplementation with designer probiotics but not parent probiotics resulted in increased amounts of butyric acid

SCFAs are by-products of fermentation by intestinal microbiota of dietary residues (J. M. W. Wong, de Souza, Kendall, Emam, & Jenkins, 2006). These SCFAs are beneficent in energy metabolism. The most abundant SCFAs, comprised together of more than 95%, are acetic acid, propionic acid, and butyric acid. These SCFAs are mainly found in the cecum and large intestine, where 95% of the produced SCFAs are

absorbed by colonocytes and 5% secreted in feces (Dawson, Holdsworth, & Webb, 1964; Rechkemmer, Rönnau, & von Engelhardt, 1988; Ruppin, Bar-Meir, Soergel, Wood, & Schmitt, 1980). SCFAs can affect lipid, glucose, and cholesterol metabolism (den Besten et al., 2013). SCFAs from the intestinal lumen can be taken up by organs where they act as substrates or signalling molecules. Specifically, in the intestines, butyrate is taken up by colonocytes as an energy supply. Colonocytes prefer butyrate over acetate or propionate since it can oxidize butyrate to ketone bodies and CO_2. Current research focuses on butyrate and its protective effect in the intestines. It has been shown that butyrate increases the production of total mucins and antimicrobial peptides (Finnie, Dwarakanath, Taylor, & Rhodes, 1995; Raqib et al., 2006; Xiong et al., 2016). Butyrate is able to promote intestinal barrier function since it is able to supress intestinal stem cell proliferation, promote IECs, and modulate tight junctions (Gonçalves et al., 2018). Butyrate has also been shown to inhibit macrophage-mediated (Chang, Hao, Offermanns, & Medzhitov, 2014) and pro-inflammatory cytokines in neutrophils (Vinolo et al., 2011) and thus can decrease inflammatory responses.

The production of SCFAs in cecal tissue was examined. Butyric acid was found to be more abundant in mice supplemented with both *E. coli* and *L. reuteri* designer strains compared to the parent stains and DSS control. This indicates that the designer strains may have formed a synergistic relationship with the established gut microbiota. More specifically, they may have allowed butyrate-producing bacteria to flourish and therefore lead to the increased production of butyrate. Since butyrate is an important regulator of colonic health, increased amounts are beneficial during inflammatory conditions. In IBD patients, there is a reduction of SCFAs-producing bacteria,

Bacteroidetes and *Firmicutes* and therefore a reduction in butyrate production (Arpaia et al., 2013). Therefore, it would be ideal to look at butyrate-producing bacteria and their relative levels between different probiotic treatment groups.

4.2 Designer probiotics during murine Muc2$^{-/-}$ spontaneous colitis

The designer strains were shown to provide protection during DSS-induced colitis; therefore, we examined another model of murine colitis, Muc2$^{-/-}$ spontaneous colitis. Muc2$^{-/-}$ develop spontaneous colitis since they are deficient in Muc2. This results in the eventual loss of epithelial barrier function and causes inflammation (Wenzel et al., 2014). Muc2 is essential to the protection of the epithelial layer (Van der Sluis et al., 2006). Only the *E. coli* probiotic strains were tested, since the *E. coli* designer strain showed more of a protective response.

4.2.1 Clinical scores were lowered at 3 months of age but not at 4 months between parent and designer probiotic strains

To assess the health status of the mice, body weights and clinical scores were measured weekly. It has been reported that Muc2$^{-/-}$ mice weigh significantly less compared to C57BL/6 mice. The body weight change showed a pattern where the early weeks, up until week 5, the mice are increasingly gaining weight as they acclimatize to the environment, food, and water. After this time and after weaning, the weights start to stabilize and plateau as the mice age. Similar to the DSS-induced colitis model, there were no differences in body weight change between treatment groups between the *E.*

coli probiotic groups and the Muc2 control. One thing to note about the body weights and clinical scores is that since this is spontaneous colitis, the mice can develop colitis at different timepoints. This would mean that there may not be a specific time period that would be representative of this decrease in body weight and health. Body weight seems to change the most drastically with the occurrence of a rectal prolapse. Similarly, clinical scores would increase with a rectal prolapse since it would be given a high clinical score. Therefore, it may be difficult to see a change at a specific time-point since mice develop prolapses at different ages. Regarding clinical scores, at 3 and 4 months of age there was a difference between the Muc2 Control and *E. coli* parent strain. Clinical symptoms have also been shown to not be a good predictor of intestinal damage (Peyrin-Biroulet et al., 2016). Therefore, clinical symptoms can be analyzed as a starting parameter, but histopathological damage should be examined for severity of disease.

4.2.2 Frequency of rectal prolapses increased in both Muc2$^{-/-}$ control and parent probiotic strain but not the designer probiotic strain

As Muc2$^{-/-}$ mice age, they start to develop progressively worse symptoms and then eventually can develop a rectal prolapse. The colons start thicken as the mice start to show softer stool (Morampudi et al., 2016). These mice develop a unique phenotype, in which they develop severe rectal inflammation, a rectal prolapse. In the clinical scoring system, a rectal prolapse is an endpoint for the mouse and usually would be associated with a significant loss of body weight. The animal has difficulty defecating and eventually the rectum protrudes out of the anus. This condition is extremely painful,

in which the mouse would not recover. Of all the Muc2$^{-/-}$ mice, the *E. coli* designer strain had no rectal prolapses over a 4-month period, the *E. coli* parent strain showed 20% of the mice develop a prolapse, and the Muc2$^{-/-}$ control showed 5% of the mice develop a rectal prolapse. This indicates that the probiotic supplementation with the *E. coli* designer strain provided resulted in a lower frequency of rectal prolapses.

4.2.3 Designer probiotic lowered CFU counts in both MLN and spleen at 4 months of age

Bacterial CFU counts in the spleen and MLN were examined to see if there was a systemic effect. Bacterial translocation would result in the passage of viable bacteria from the digestive tract into other body sites that normally would not have bacteria present. Such sites like the MLN and spleen can be used as indicators of bacterial translocation and high amounts of this translocation could result in a systemic infection. As a preliminary investigation, these bacterial CFU counts were analyzed. We hypothesized that since there is mucin depletion, the epithelial barrier is not fully functional, and this could lead to bacterial translocation. The *E. coli* designer strain showed significantly reduced bacterial counts compared to the *E. coli* parent strain at 4 months but not 3 months of age. Since the tissues were plated on LB agar, the bacteria could belong to the Enterobacteriaceae group. However, this is unknown what these bacterial colonies are and whether they are pathogens. In IBD patients, bacterial DNA in blood has been reported (Gutiérrez et al., 2009). It has been proposed that this bacterial translocation can occur through possible mechanisms of intestinal bacterial overgrowth, deficiencies in host immune defenses, and increased permeability or damaged mucosal barrier (Berg, 1995). Since the Muc2$^{-/-}$ model has a defective mucin production, this

would could allow the movement of bacteria into other body locations. To confirm the bacterial translocation, other tests like assessing the flow of markers like radioisotopes or sugars, florescent labelled dextrans, looking at tight junctions, or mucus analysis would need to be performed (Bischoff et al., 2014).

4.2.4 Supplementation with designer probiotic resulted in lower histopathological scores but not short chain fatty acid production

Likewise, with the DSS-induced colitis model, both histopathological scores and SCFA production were analyzed. Supplementation with the *E. coli* designer strain resulted in lower histopathological scoring at 3 and 4 months. With gender, males showed lower histopathological scores at 3 months. This shows that the *E. coli* designer strain resulted in less tissue damage and could be protective in this model of colitis. There are gender differences in this model, and therefore for some cohorts it could be possible that the power to detect differences in gender may be too low. This could be due to the low sample size of genders between 3 and 4 months. Future studies would need to be done to increase sample size to further assess gender effect.

There were no significant differences in SCFA production between the *E. coli* designer and parent strains. Since there was protection of SCFAs and lower histological scores in the DSS-induced colitis model, this suggest that there are differences between the two models. The $Muc2^{-/-}$ mice were supplemented with the probiotics once weekly for the first four weeks. Probiotic supplementation in the $Muc2^{-/-}$ mice was stopped at 2 months of age. Perhaps this supplementation was not at the optimal dose, age at supplementation, or number of doses. In addition, the *E. coli* designer strain is

engineered to have the ttr operon. This operon encodes for enzymes essential for tetrathionate respiration (Winter et al., 2011). This allows the strain to utilize alternative metabolites like tetrathionate to outcompete other microbes. Thus, this would give the strain a growth advantage during conditions, in which these alternative metabolites would need to be used. In order to survive the aerobic environment and oxidative stress, the strains have to cope with the reactive oxygen species. Such conditions can be found during IBD. Studies have shown that *S.* Typhimurium infection makes colitic mice susceptible to intestinal inflammation, since it is able to grow during IBD. However, if there is not oxidative stress environment present, maybe the strain will not outcompete since its growth advantage is likely most effective during oxidative and inflammatory conditions. This could explain why protection was not seen in the $Muc2^{-/-}$ mice since the probiotic was supplemented early on when inflammatory conditions may not have been severe enough to allow establishment of the strain. Likewise, these inflammatory conditions may have been too severe for the SCFA producing bacteria to be able to flourish. Previous studies looking at probiotics in $Muc2^{-/-}$ mice, saw protection of the probiotics when they were given at 3 months of age for a 4 week treatment (Morampudi et al., 2016).

4.2.5 Designer probiotic showed significantly lower mRNA expression of IFN-γ only at 4 months of age compared to parent probiotic group but with no differences in other pro-inflammatory markers

To explore immune responses pro-inflammatory cytokines were examined. Similar to the DSS-induced colitis, TNF-α, IFN-γ, and RegIIIγ were analyzed. Further,

RELM-β, CXCL-9, and Claudin-10 were analyzed. RELM-β is a defense factor that is secreted by goblet cells (Morampudi et al., 2016). It has been shown to increase production of Th2 cytokines (Artis et al., 2004). It has been shown to be upregulated during DSS-induced colitis, where it can increase macrophage infiltration (McVay et al., 2006). In the Muc2$^{-/-}$ model, it has also been shown to be upregulated and shown to worsen the colitis (Morampudi et al., 2016). The chemokine CXCL-9, induced by IFN-γ, and the tight junction protein Claudin-10 have been shown to be upregulated in the Muc2$^{-/-}$ model (Lu et al., 2011; Yang et al., 2008).

The pro-inflammatory cytokine IFN-γ was shown to be downregulated at 4 months of age between the *E. coli* designer strain compared to the Muc2 control and *E. coli* parent strain. Similarly, at 4 months of age, RELM-β and RegIIIγ were significantly reduced. RELM-β was reduced in *E. coli* designer strain compared to the Muc2 control and RegIIIγ was reduced in both *E. coli* designer and parent strain compared to the Muc2 control. This is different from the DSS-induced colitis model, where RegIIIγ was considered protective. Both defensins, RegIIIγ and RegIIIβ, were shown to be upregulated in Muc2$^{-/-}$ mice, with RegIIIβ most drastically upregulated. This study showed that RELM-β induced RegIIIβ mediated the anti-microbial effect of *Lactobacilli* depletion (Morampudi et al., 2016). Since *Lactobacilli* bacteria are shown to adhere to mucus (Van Tassell & Miller, 2011), it is expected in Muc2$^{-/-}$ mice there be a decrease in *Lactobacilli* (Morampudi et al., 2016). This leads to the hypothesis that these *Lactobacilli* bacteria do not have a proper niche to grow in. This would lead to a disrupted microbiota and certain strains that would provide protection are not able to optimally grow. In the DSS-induced model, there is a disrupted epithelial but not a

complete absence of mucin. Therefore, some beneficial strains like *Lactobacilli* would still have an environment to grow in. It would be expected that the bacteria that are invading into the mucosa and causing an inflammatory response are Gram-positive pathogens, which would be targeted by the RegIIIγ. Whereas in the Muc2$^{-/-}$ model, since there is not an ideal mucin environment, it could be possible that some non-pathogenic bacteria could be infiltrating and causing an inflammatory response due to differences of the microbiota found near the epithelial. This leads to altered interactions between the bacterial species and the epithelial layer with the absence of the mucus layer. This could lead to increased commensal bacterial-epithelial interactions that normally would not result with a mucus layer (Burger-van Paassen et al., 2012). To confirm this, bacterial DNA sequencing of the mucosa would need to be done to confirm which bacterial species are present. This shows that there could be differences in the models since their mechanism of colitis induction, inflammatory responses, and the microbiota could be different.

None of the inflammatory markers were different at 3 months of age. This coincides with the bacterial counts, in which there was differences only at 4 months of age. This could imply that the responses need a longer time to develop and exert effects or that as the colitis progresses, the *E. coli* strain with the ttr operon is able to grow and flourish at these conditions and show some of the protective responses. In addition, gender differences were not detected with pro-inflammatory markers. However, gender differences here could be too low to detect since the sample size is small and therefore could be contributing to some of the variation see. Future studies would need to be done to increase sample size to further assess gender effect.

4.3 Detection of designer probiotics

In order to see if the probiotic supplementation had any effect on the mice, the *E. coli* designer strain was detected in samples to see if it was still present at different timepoints. This could be done so by the probiotic's continual growth and colonization in the gut. Many studies do not report stable detection of probiotics from stool or tissue samples. This is a challenge that has been identified and may be the reason why many probiotics have limited long-term positive results since the probiotic strains cannot successfully grow and colonize.

4.3.1 Designer probiotics did not show colonization and persistence in healthy C57BL/6 mice but showed semi- persistent detection in the Muc2$^{-/-}$ spontaneous colitis model

Primers designed specific to the ttr operon were used to detect the *E. coli* designer strain. An experiment with C57BL/6 mice given a single probiotic dose of *E. coli* was conducted. At one-week post gavage, the 29% of the animals had detectable ttr in their stool samples. At 2 weeks post gavage, this increased to 36%. This is could be due to the animals being co-housed and being exposed to their own and one another's stool samples. Mice are known to be coprophagic and therefore this could be why the ttr detection increased with age (Kulecka et al., 2016). The ttr detection decreased to 12 and 7% at 3 and 4 weeks post gavage. At euthanization, this signal was 12% detected from colon tissues. Since the ttr detection only was persistent in most time-points in one or two animals, the *E. coli* designer strain did not have persistent survival in the C57BL/6 mice. In some of the mice, the detection of the strain

appeared at later time-points periodically. This may be due to probiotic stain detection below the limits of the assay or because the strain could be present in the stool samples in the cage and be ingested by other mice in the same cage. Since these animals do not have high inflammatory conditions, tetrathionate is not expected to be present at high amounts. Therefore, the *E. coli* designer strain is not expected to utilize the ttr and have optimal growth since inflammatory conditions are absent.

Strain detection was also analyzed in the $Muc2^{-/-}$ spontaneous colitis experiments. For the first four weeks, while the mice were still supplemented with the *E. coli* designer strain, detection levels were around 60-70%. For the animals that were euthanized at 3 months of age, the detection dropped down to around 40%. For the animals euthanized at 4 months of age, the detection dropped down to 33 and then 11%. However, at the very last collection dates at euthanization, the stool detection increased to 67% in stool and 22% in colon tissue. A trend can be seen in which the colon tissue shows lower detection than stool samples at the very last collection timepoint. A lower percentage of mice had detection in the colon possibly because the strain was not able to attach and colonize in the colon tissue. In the stool, it would be expected that if the strain is surviving and present, it wold be shed out. Considering the design of the strain, it was designed to survive and utilize ttr, not necessarily have improved colonization.

Since the *E. coli* designer strain shows more persistent detection during inflammation than in a healthy environment, this is important when looking at regulatory control. Since designer probiotics contain additional genetic components, their regulation will be different, and safety will be a concern. Proper characterization of the

probiotic with full genome sequencing, antibiotic resistance, and toxicology testing needs to be completed. A major environmental concern is the problem with contamination of the designer probiotic. To overcome this problem, it has been suggested that biological containment systems be used to prevent designer probiotics from flourishing in the external environment (Sola-Oladokun, Culligan, & Sleator, 2017). This can be done by auxotrophic systems, in which the survival of the strain depends on certain compounds which may not be found in the external environment. Another example is a suicide system, in which a lethal function is added based on physical or chemical signals in the environment. In this case, since it is shown that the *E. coli* designer is much more persistent under inflammation, this in itself may act as a contamination control. The *E. coli* designer strain would only grow optimally under conditions that favor ttr growth, like those found during inflammation. Therefore, if ttr is absent, this strain would not optimally grow.

4.3.2 Inconclusive detection of GbpA of *L. reuteri* designer probiotic strain and parent strains

For the *L. reuteri* designer strain detection, a reproducible assay was not established. As shown in Appendix G, nine different primer pairs to the GbpA protein were designed and tested. Either the primers were not specific enough or the conditions were not optimal, but an appropriate primer set was not identified.

For the comparisons between the designer strains to the parent strains, no conclusive assay was determined. Many primer sets for strain specific bacteria have

been shown in previous studies, but when tested they are not specific to the bacterial strain and pick up multiple types of strains in a species. There are very limited studies that look at probiotic detection and even those still show primer sets that are designed to the genus or species but not strain level (Alander et al., 1999; Haarman & Knol, 2006; Matsuki et al., 2004; Requena et al., 2002; Rinttila, Kassinen, Malinen, Krogius, & Palva, 2004). Therefore, no comparisons for probiotic detection between the designer and parent strains could be made. For this reason, objective 3 of this thesis was partially completed since the comparison between parent and designer strains could not be made.

4.4 Comparing colitis models

Two different colitis models were used in this thesis; the DSS-induced colitis and $Muc2^{-/-}$ spontaneous colitis. DSS-induced colitis involves induction of colitis with the chemical DSS. Although the exact mechanism by which colitis is induced is not fully understood, it is hypothesized that the disruption of the intestinal epithelial layer which leads to luminal bacteria and antigens into the mucosa initiates an inflammatory response in the tissue (Eichele & Kharbanda, 2017). This model can be considered part of a tissue/epithelial injury and repair mechanism (Cominelli, Arseneau, Rodriguez-Palacios, & Pizarro, 2017). However, since this is an acute model, immune cells like neutrophils dominate over cells like T cells. The acute DSS model is characterized by more Th1 responses, whereas chronic DSS models can include both Th1 and Th2 responses. The $Muc2^{-/-}$ spontaneous colitis model is a genetically engineered model

(Cominelli et al., 2017). It is epithelial-driven colitis, in which the Muc2 gene has been knocked out. With the specific knockout, it is easier to determine the role of specific genes and specific target cell types. Compared to the DSS-induced colitis model, the $Muc2^{-/-}$ spontaneous colitis model is more chronic and therefore would represent both Th1 and Th2 responses. However, the DSS model has a defined onset of disease whereas, the $Mu2^{-/-}$ is spontaneous and mice develop symptoms at different rates. Overall, the two models both are characterized by epithelial disruption, have clinical and histological similarities to IBD, and can provide insights into immune responses looking at immune cells and inflammatory markers.

Comparing both models to each other, there is no direct comparison that can be made at a specific time point. The DSS model is acute and the $Muc2^{-/-}$ model could be considered chronic. For the DSS model, the most clinically severe timepoint is day 7 of the DSS treatment. At this timepoint the mice have severe colitis with rectal bleeding, diarrhea, weight loss, and dehydration. In the $Muc2^{-/-}$ model, a similar timepoint would be at or after 6 months of age. At this timepoint, it has been shown in literature that 40% of mice develop a rectal prolapse (Morampudi et al., 2016). Similarly, the mice develop diarrhea, rectal bleeding, swelling, dehydration, and weight loss. Although there are some similarities in these models of symptoms, these models have different mechanisms of initiating colitis. Therefore, they do have slightly different dominant immune responses. The DSS-induced colitis model may only include Th1 responses but the $Muc2^{-/-}$ model can include Th1 and Th2 responses, more notably the T or B cell responses. Taken together, these results demonstrate that the designer probiotics are

shown to have improved efficacy. These designer probiotics could be an alternative therapeutic option for the treatment of IBD.

4.5 Using probiotics with caution

The theory of probiotics depends on the idea that they are a non-invasive alternative therapeutic. Although, probiotics are considered a safe approach, some cases have raised questions on the safety of the use of probiotics. There have been a few documented cases of infections arising from probiotic consumption. This has been reported in immunocompromised individuals like the elderly or young infants. For example, an elderly patient developed liver abscess infected with *L. rhamnosus* after supplementation (Rautio et al., 1999) and another developed endocarditis after *L. rhamnosus* supplements (Mackay, Taylor, Kibbler, & Hamilton-Miller, 1999). Similarly in another case study, both a 6-week old infant and 6-year-old child developed sepsis after *Lactobacilli* supplementation (Land et al., 2005). More specifically for IBD, a case study was reported in which a 44-year old patient with UC showed *L. rhamnosus* cultured in blood samples (Claudio, Arosio, Mangia, & Moioli, 2001).

VSL#3 administered to Azoxymethane (AOM) IL-$10^{-/-}$ mice resulted in enhanced tumorigenesis (Arthur et al., 2013). This model of colitis-associated colorectal cancer (CRC) shows how certain populations who are at risk or already immunocompromised can result in adverse effects from probiotic supplementation. There was no significant effect of VSL#3 on tumorigenesis, but there was a greater depth of tumor invasion seen in the probiotic-supplemented group. Visible tumors were seen in 91% of the mice in the probiotic group as opposed to the 38% in the control group. This paper concluded that

the increased tumorigenesis could have been due to the depletion of *Clostridium* bacteria. Recently, another study looking at the link between brain fogginess and severe bloating, found that the patients were consuming probiotics (Rao, Rehman, Yu, & Andino, 2018). When probiotic supplementation was stopped, the brain fogginess and gastrointestinal symptoms improved. This study proposed that the production of toxic metabolites, like D-lactic acid, were due to the colonization of probiotic bacteria in the gut. Studies like these highlight that probiotic supplementation should be advised with caution. This is especially important in IBD patients and raises questions over the use of certain probiotic strains for IBD. With complex diseases, each individual can have slightly different responses to treatments. Proper studies looking at potential adverse effects and toxicology should be conducted before probiotics can be routinely recommended as a therapeutic.

Chapter 5: Conclusion

5.1 Conclusion

Despite the potential of probiotics for health benefits, there is a lack of evidence to suggest that current probiotics do work, specifically for IBD. Well-designed randomized controlled trials supporting the use of probiotics for IBD management are limited (Jonkers, Penders, Masclee, & Pierik, 2012). Even though there is much evidence to show that probiotics are effective in reducing symptoms and bio-markers in animal models or *in vitro*, human clinical trials have reported low efficiency of probiotics. As such, current probiotics for IBD are ineffective at colonization and survival in the hostile gut of IBD patients. A novel therapeutic approach is to engineer designer probiotics that target these specific limitations. In order to maintain the potential benefits and to further improve the clinical efficacy of probiotics, further optimization of strains needs to take place to use such approaches.

Through this research, we designed probiotics that targeted survival and colonization during IBD. These probiotics, *E. coli* and *L. reuteri* designer strains, were shown to be protective during murine models of IBD, DSS-induced colitis and $Muc2^{-/-}$ spontaneous colitis. These strains had improved efficacy compared to the parent strains. In the DSS-induced colitis model, efficacy was shown with lower clinical scores, reduced histopathological scores, and increased protective responses including butyric acid, RegIIIγ, and Muc2. In the $Muc2^{-/-}$ spontaneous colitis model, efficacy was shown with lower clinical scores, lower frequency of rectal prolapses, lower CFU bacterial counts, and decreased expression of pro-inflammatory cytokine IFN-γ.

5.2 Limitations

Several limitations exist due to the nature of using murine colitis models. The induction of colitis in the DSS-induced colitis model is dependent upon the amount of DSS water that is consumed by the mice. This is hard to control for since the DSS is dissolved into the drinking water and the water needs to be freely available to consume. This could have contributed to some of the variation seen since different mice in a cage could have consumed different amounts of the DSS water. In the $Muc2^{-/-}$ spontaneous colitis model, mice developed colitic symptoms at different timepoints, making it difficult to assess a certain bio-marker at a defined timepoint during the experiment. Therefore, assessing the rate of colitis in the colony can be helpful when trying to decide what age or timepoint would be most informative.

Additionally, cage effects could have had an effect on the mice. In this thesis, all the experiments were conducted with mice that were bred in house or purchased from the same vendors to minimize environmental factors. For example, mice were kept in the same room, same rack, and same cage throughout the studies. However, cage effects still could have come into play since it has been shown that litters that are split into different cages start to have a divergence in microbiota profiles (Laukens, Brinkman, Raes, De Vos, & Vandenabeele, 2016). This can be diminished by maximizing the number of cages, mixing treatment groups within cages, and distributing littermates before the experiment. However, in the probiotic experiments, this may be difficult to achieve. Mixing treatment groups cannot be done in a study where mice are given a probiotic gavage. Mice are coprophagic, making it difficult as the treatment groups would become mixed. Housing mice individually can be done but would require

extensive resources and money. Mixing littermates prior to the start of a study can be done more easily with female mice but with male mice this can result in fighting, leading to fight wounds and added stress.

Regarding the experimental design, this was the first animal study to test the designer probiotics. Therefore, the optimal dose, amount, and age is not yet determined. The probiotic dose was chosen based on previous studies. The number of doses was given based on both the design theory and preliminary studies. The age of supplementation was chosen since supplementation was meant to be given early on. Performing experiments to optimize these variables would be beneficial.

5.3 Future directions

In this thesis, the patented designer probiotics were tested in two different murine colitis models. Overall, these designer strains showed some protection over the parent strains. For some parameters, trends were observed but no significant differences reported. A power analysis could be performed to determine if an increase in sample size could be helpful.

Future studies are needed to look into the mechanisms by which these designer probiotics show protection. Based on the present study's preliminary insights, investigating mucosal protection and the intestinal barrier function would be useful. In addition, analysis of the microbiome of the mice in the different colitis models could be valuable in determining the effect of the probiotics on the gut microbiota community. To optimize probiotic conditions, performing dose dependent or toxicology studies would be

appropriate. After murine models, these designer probiotic strains could move into larger animals to assess safety and efficacy. In addition, to test for synergistic effect, the probiotics could be tested in a mixed culture to determine if these strains are more protective.

Moving forward, commercialization of these designer strains will be explored. Since these designer strains are genetically engineered, they are not eligible to be regulated as a food supplement or an NHP. They can be regulated as a genetically modified (GM) product and fall under the class of “novel foods” (Government of Canada, 2012). Majority of the labelling rules for GM foods is unclear. While they require that any intentionally modified changes must be stated, it is not stated that the word GM needs to be clearly indicated on the label (Government of Canada, 2018). Worldwide, there is a discrepancy among GM regulations. Some of the regulations have been set up for economic and political reasons. For example, Europe and Japan lean more towards rigorous regulations, whereas the U.S.A is more lenient with these regulations. However, many of these laws are unclear since regulations have not been able to keep up with the new discovery of GM organisms. Public perception is one of the issues for GM organisms. Many consumers have concerns about designer probiotics being unethical and possible dangers to health. For commercialization, regulating these designer probiotics under a drug may be more suitable since these designer strains are targeted for IBD patients.

5.4 Significance of findings

The prevalence of IBD in Western countries has been increasing over the last couple of decades. It is estimated that 1 in 150 Canadians suffer from IBD (Crohn's and Colitis Foundation of Canada, 2012). Among the few available therapies, many of them have severe associated side effects and are not a long-term solution. Thus, current therapies are either risky or ineffective for long-term use and new therapies for IBD are needed for reducing disease burden. There is a lack of evidence to support the use of probiotic supplementation in IBD management in the field. Probiotics are deemed to be beneficial and do show beneficial effects in many other conditions. However, for IBD, they simply do not work. The novel approach of designer probiotics, proposed here, could target these specific limitations. This research could result in genetically improved probiotics, with enhanced colonization and persistence, leading to better efficiency during IBD therapy and a potential alternative therapeutic option for IBD patients. These strains may help control inflammation in IBD patients and be a cost-effective therapy without any significant adverse effects, which are seen in current pharmaceutical treatments. In addition, these designer probiotics could provide new insights and an innovative perspective for the probiotic field.

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Appendices

Appendix A: Additional clinical data for DSS-induced colitis model

Table 20. Starting body weights of DSS-induced colitis mice.

Treatment Groups	DSS Control	*E. coli* Parent	*E. coli* Designer	*L. reuteri* Parent	*L. reuteri* Designer
Starting Body Weight (g)	20.19 ± 1.36	17.12 ± 0.25	17.77 ± 0.19	21.29 ± 1.24	19.38 ± 0.92

Table 21. DSS water intake of DSS-induced colitis mice.

Treatment Groups	DSS Control	*E. coli* Parent	*E. coli* Designer	*L. reuteri* Parent	*L. reuteri* Designer
Water Intake (mL/mouse per day)	4.83 ± 0.85	5.22 ± 1.09	8.48 ± 3.55	5.74 ± 0.48	6.11 ± 1.77

Appendix B: C57BL/6 control data for DSS-induced colitis experiments

Table 22. Starting body weights of C57BL/6 control mice during DSS-induced colitis.

Treatment Groups	Control	*E. coli* Parent	*E. coli* Designer	*L. reuteri* Parent	*L. reuteri* Designer
Starting Body Weight (g)	18.27 ± 0.32	17.80 ± 0.35	18.86 ± 1.16	18.31 ± 0.45	18.83 ± 0.30

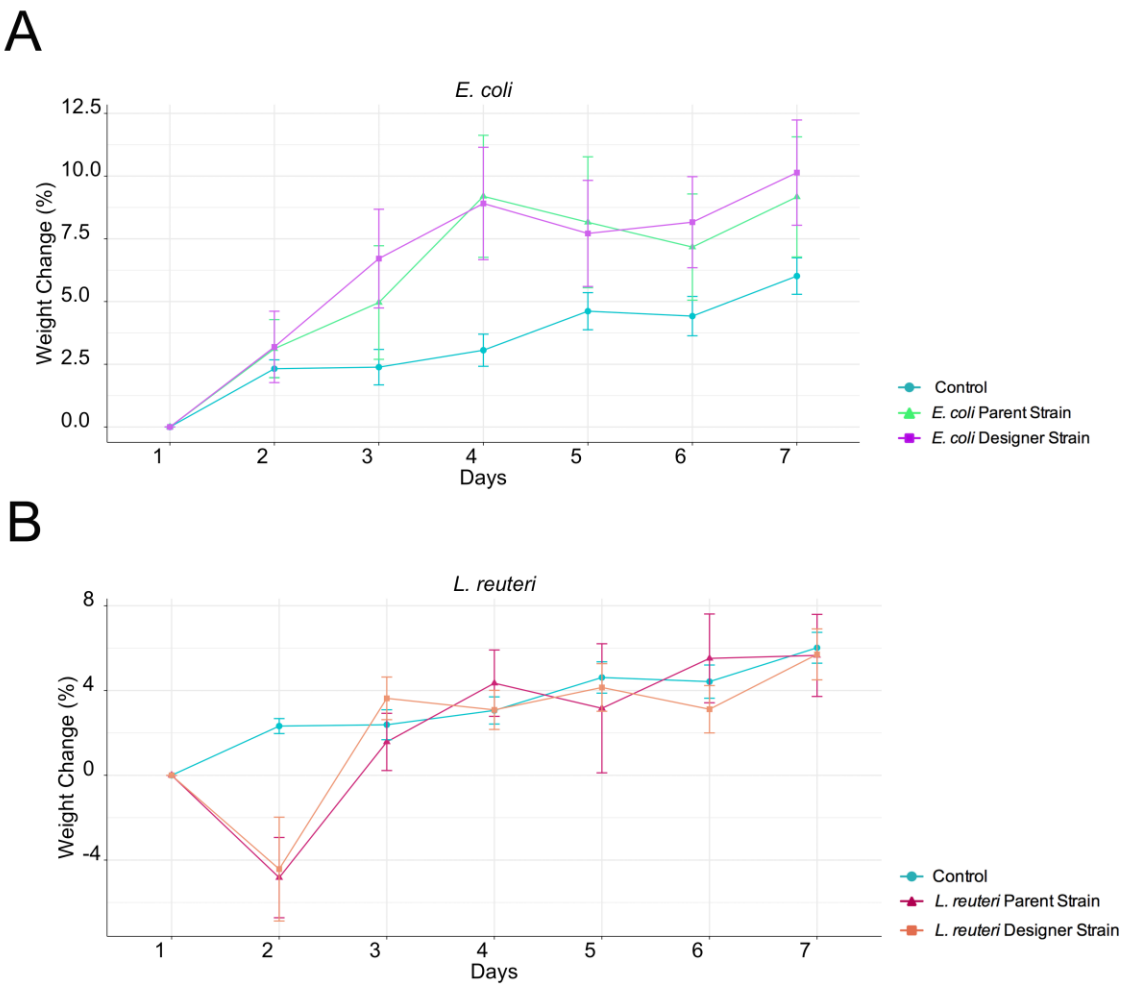


Figure 31. No differences in body weight change between strains.
Body weight change of mice pre-treated with probiotic strains labelled as *E. coli* and *L. reuteri* parent strains (triangles), and designer probiotic strains labelled as *E. coli* and *L. reuteri* designer strain (squares). Control (no probiotic gavage) shown in circles. Weight loss was calculated as a percentage of the weight loss from the starting body weight at Week 0. Weight change is shown for A) *E. coli* strains and B) *L. reuteri* strains. Values are expressed as means +/- SEM (n=7-10)

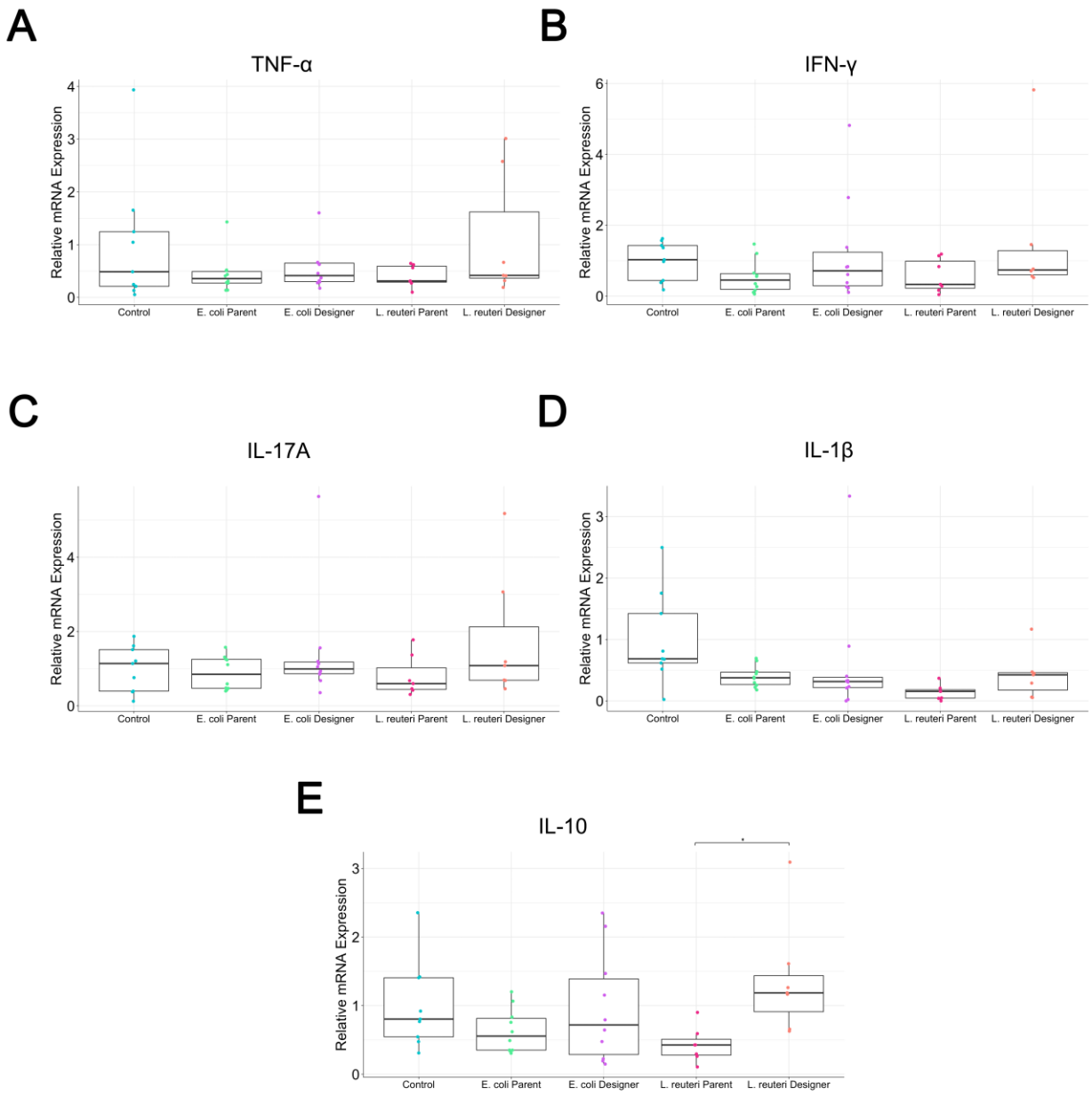


Figure 32. No differences between probiotic groups of anti-inflammatory cytokines in control wild-type mice.
mRNA gene expression of inflammatory cytokines in the colonic tissue of mice pre-treated with probiotics performed via qPCR. Pro-inflammatory cytokines A) TNF-α, B) IFN- γ, C) IL-17A, D) IL-1β, and anti-inflammatory cytokine E) IL-10 were examined. Values are expressed as means +/- SEM (n=7-10).

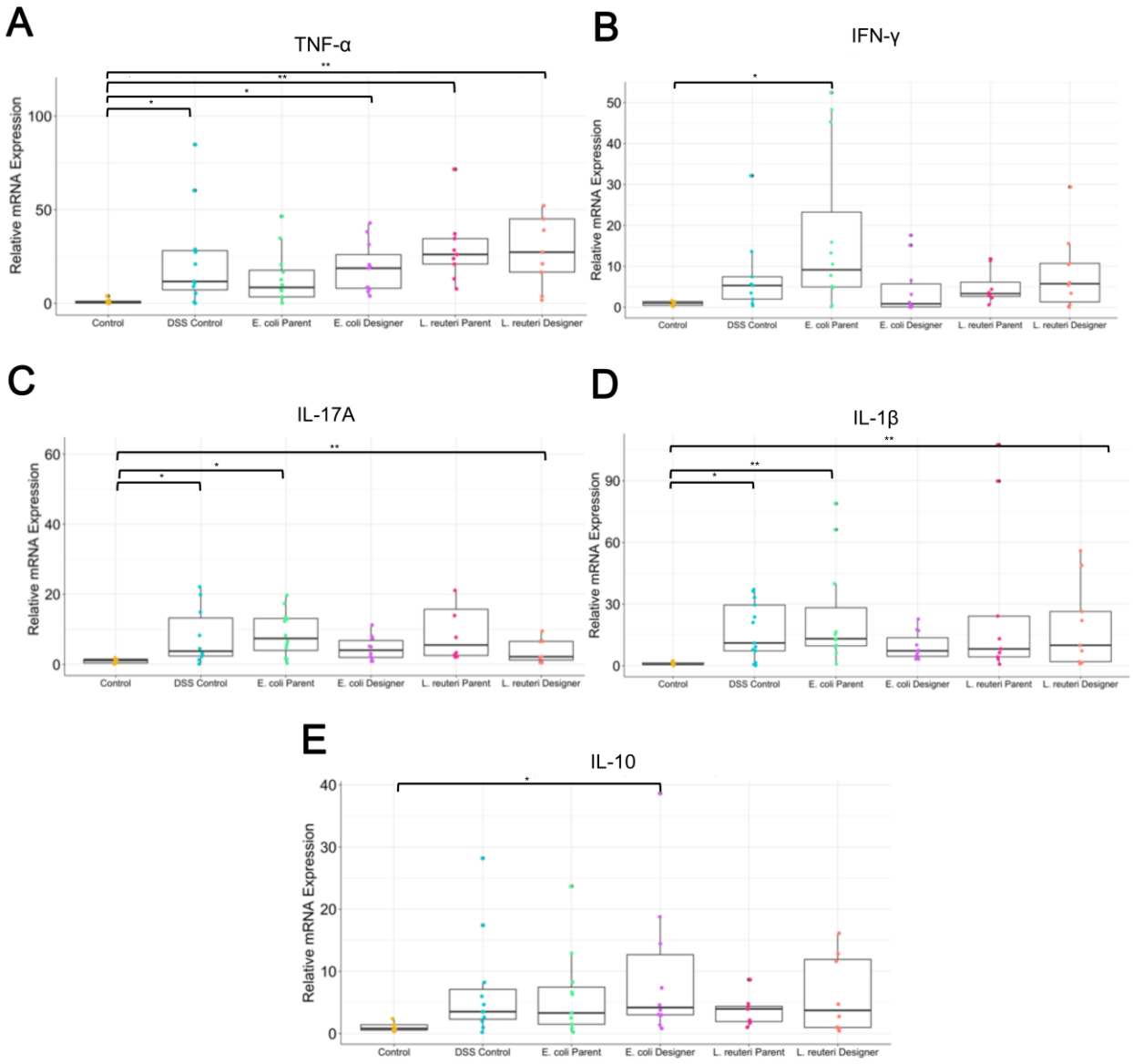


Figure 33. DSS treatment results in significantly higher expression of pro-inflammatory markers compared to control wild-type mice.
mRNA gene expression of inflammatory cytokines in the colonic tissue of mice pre-treated with probiotics performed via qPCR. Pro-inflammatory cytokines A) TNF-α, B) IFN- γ, C) IL-17A, D) IL-1β, and anti-inflammatory cytokine E) IL-10 were examined. Values are expressed as means +/- SEM (n=7-10).

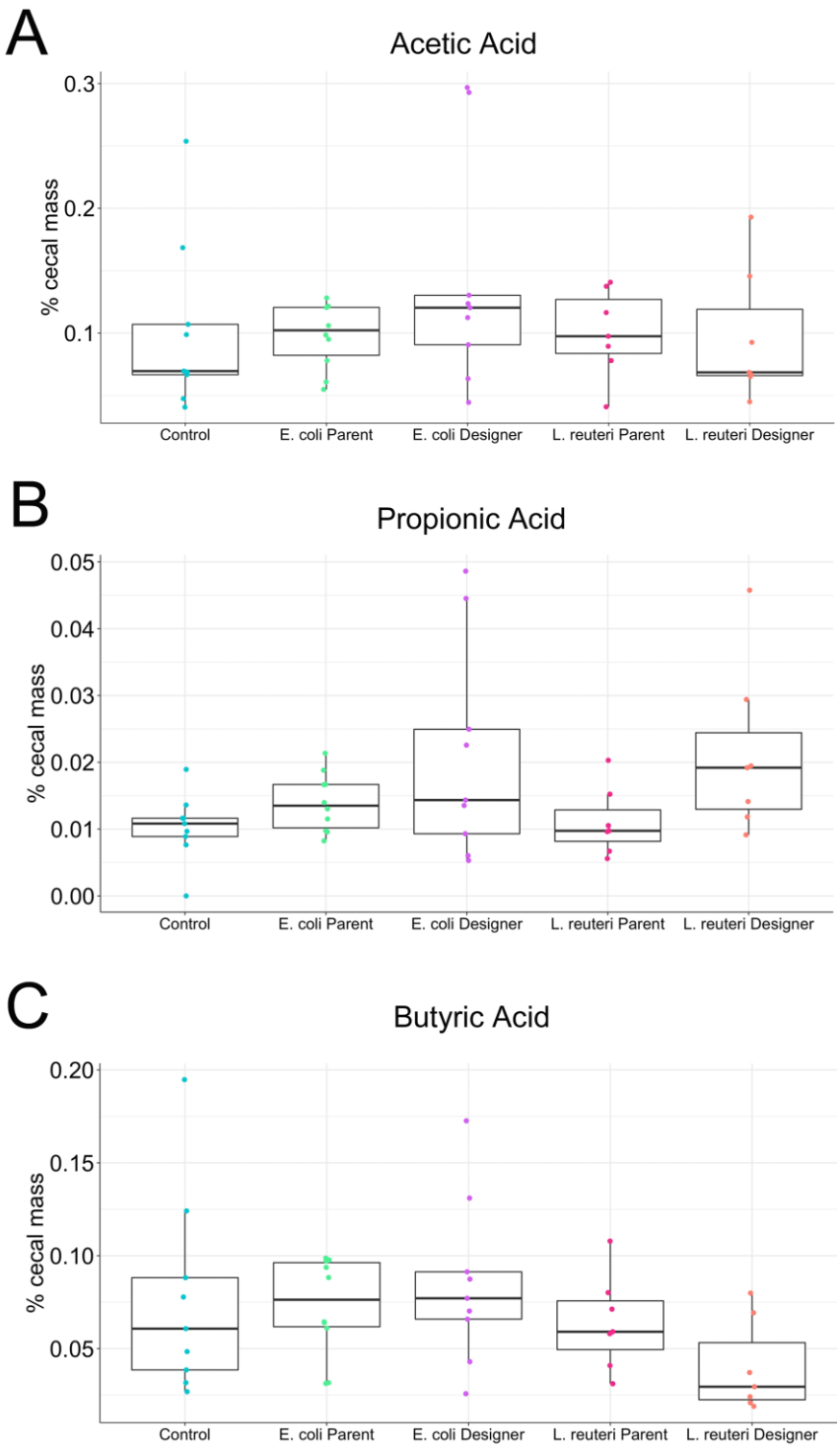


Figure 34. No differences in short chain fatty acids between designer and parent probiotic groups.
Short chain fatty acid analysis performed via gas chromatography on cecal samples of mice. Short chain fatty acids A) acetic acid, B) propionic acid, and C) butyric acid were analyzed. Values are expressed as mass percent of cecal tissue shown as means +/- SEM (n=7-10).

Appendix C: Additional clinical data for Muc2$^{-/-}$ spontaneous colitis model

Table 23. Starting body weights of Muc2$^{-/-}$ spontaneous colitis mice.

Treatment Groups	Muc2$^{-/-}$ Control	*E. coli* Parent	*E. coli* Designer
Starting Body Weights (g)	16.32 ± 0.63	16.07 ± 0.54	15.35 ± 0.42

Table 24. Water and food intake in Muc2$^{-/-}$ spontaneous colitis mice.

Treatment Groups	Muc2$^{-/-}$ Control	*E. coli* Parent	*E. coli* Designer
Water Intake (mL/mouse per day)	6.39 ± 1.56	5.97 ± 0.65	5.35 ± 0.66
Food Intake (g/mouse per day)	4.07 ± 0.35	3.46 ± 0.25	3.56 ± 0.18

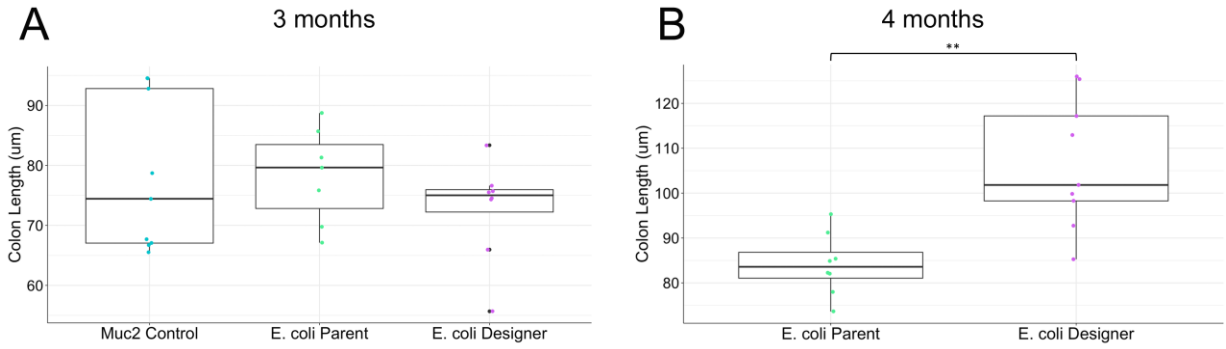


Figure 35. Designer probiotic *E. coli* mice have longer colons than the parent probiotic *E. coli* mice at 4 months of age.
Mice were administered either parent or designer probiotics via oral gavage weekly for 4 weeks. Tissues were collected at either 3 or 4 months of age. Analysis was done on images taken at tissue collection at sacrifice. Values are expressed as means +/- SEM (n=8-11). Non-parametric t-test (Mann-Whitney U test) was used. Asterisks represent significant p-value of 0.0009872.

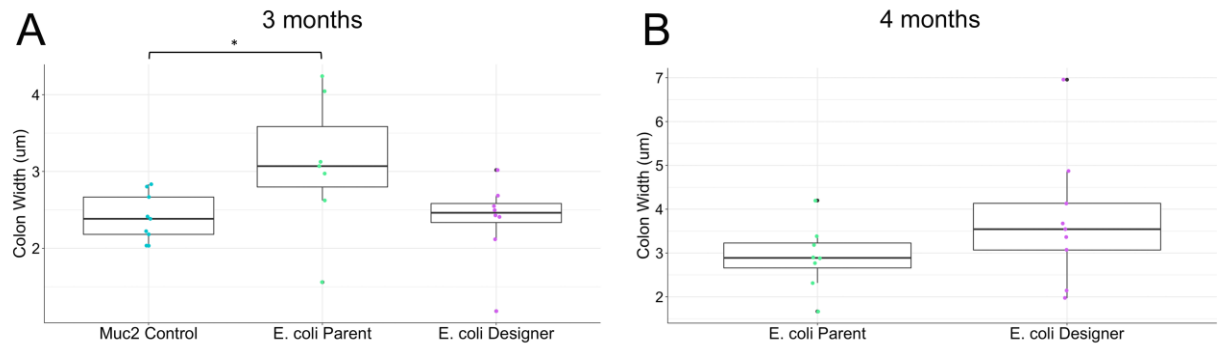


Figure 36. Parent probiotic *E. coli* mice have wider colons than the Muc2 control mice at 3 months of age.
Mice were administered either parent or designer probiotics via oral gavage weekly for 4 weeks. Tissues were collected at either 3 or 4 months of age. Analysis was done on images taken at tissue collection at sacrifice. Values are expressed as means +/- SEM (n=8-11). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-value of 0.0351.

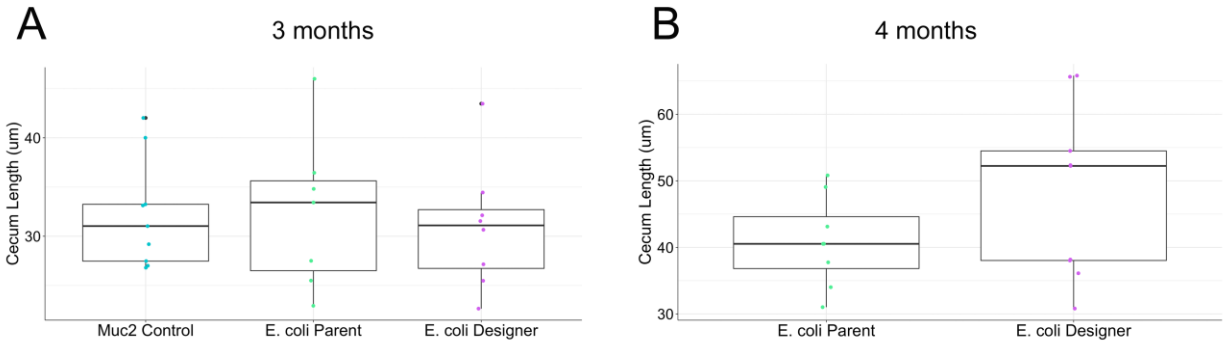


Figure 37. No differences in cecum length between probiotic groups at 3 and 4 months of age.
Mice were administered either parent or designer probiotics via oral gavage weekly for 4 weeks. Tissues were collected at either 3 or 4 months of age. Analysis was done on images taken at tissue collection at sacrifice. Values are expressed as means +/- SEM (n=8-11).

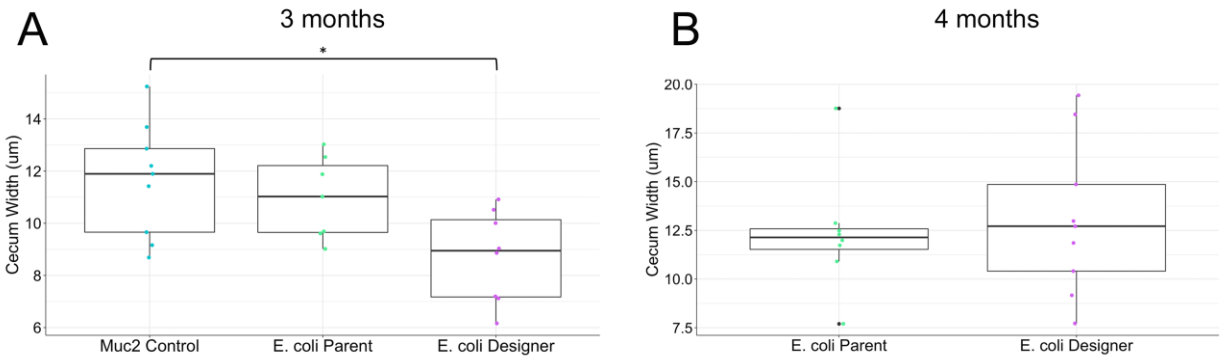


Figure 38. **Designer probiotic *E. coli* mice have smaller ceca than the Muc2 control mice at 3 months of age.**
Mice were administered either parent or designer probiotics via oral gavage weekly for 4 weeks. Tissues were collected at either 3 or 4 months of age. Analysis was done on images taken at tissue collection at sacrifice. Values are expressed as means +/- SEM (n=8-11). Non-parametric one-way ANOVA (Kruskal-Wallis) test was used. Asterisks represent significant p-value of 0.0161.

Appendix D: Additional clinical data for strain detection experiments

Table 25. Starting body weights of C57BL/6 mice during colonization experiments

Treatment Groups	*E. coli* Parent	*E. coli* Designer	*L. reuteri* Parent	*L. reuteri* Designer
Starting Body Weight (g)	21.32 ± 1.32	23.54 ± 0.57	25.91 ± 1.36	26.26 ± 0.88

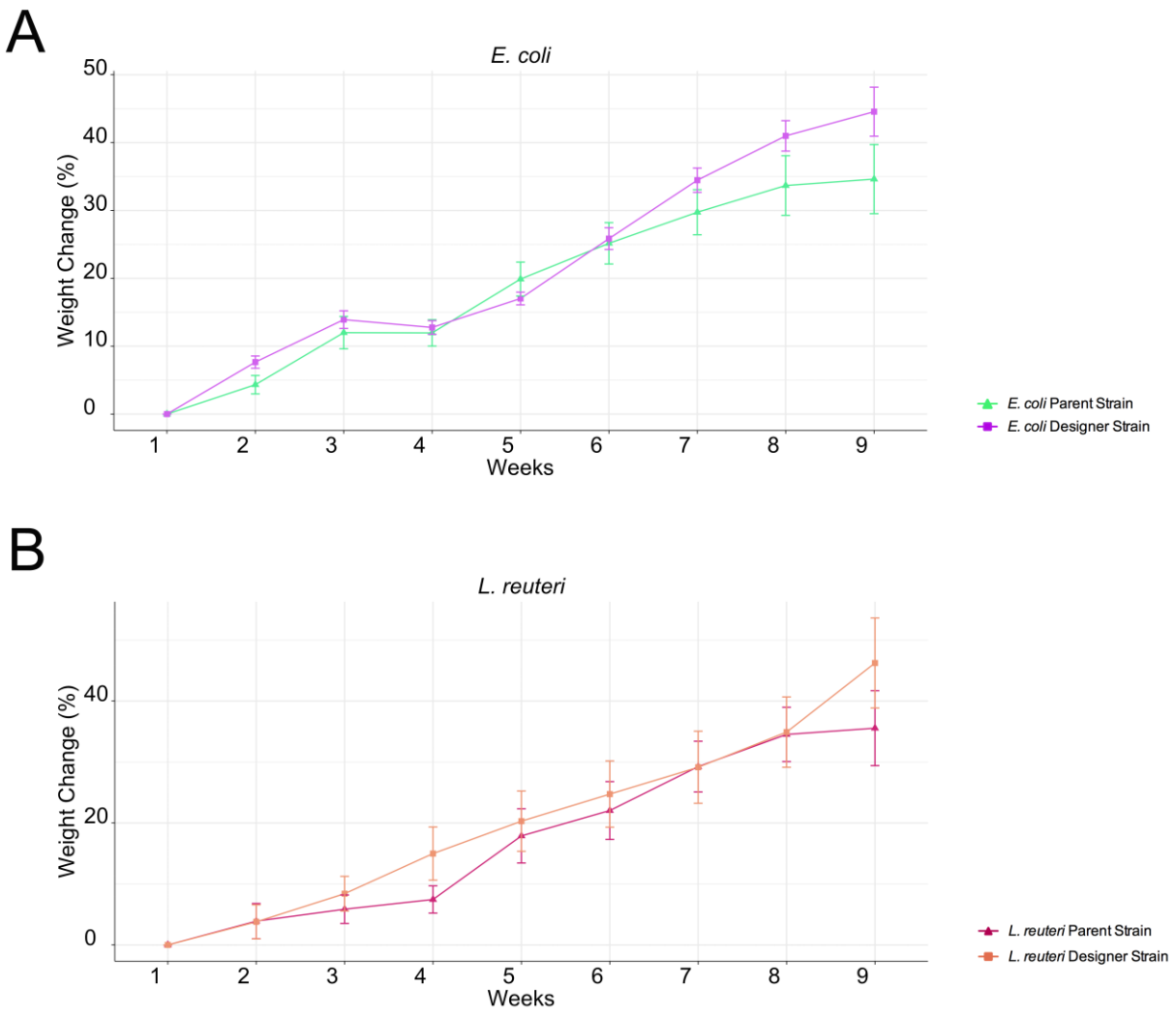


Figure 39. No differences in body weight change between strains during colonization experiments.
Body weight change during the colonization experiment of mice pre-treated with probiotic strains labelled as *E. coli* and *L. reuteri* parent strains (triangles), and designer probiotic strains labelled as *E. coli* and *L. reuteri* designer strain (squares). Weight loss was calculated as a percentage of the weight loss from the starting body weight at Week 0. Weight change is shown for A) *E. coli* strains and B) *L. reuteri* strains. Values are expressed as means +/- SEM (n=8-14).

Appendix E: Gel electrophoresis for ttr strain detection in C57BL/6 mice

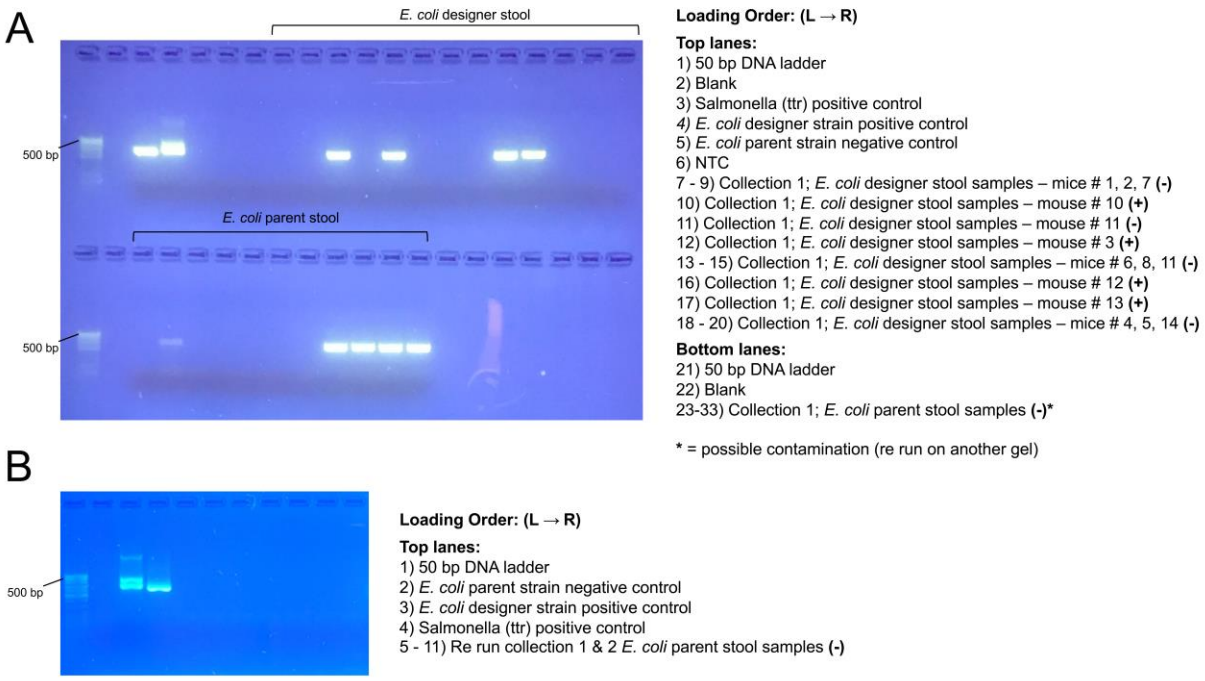


Figure 40. Collection #1 of C57BL/6 stool samples for detection of ttr operon. Ttr detection via PCR from stool samples of C57BL/6 mice given a single oral gavage of *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection # 1 (1 week post gavage). Gel images show A) *E. coli* designer and parent stool samples and B) *E. coli* parent stool samples re-run.

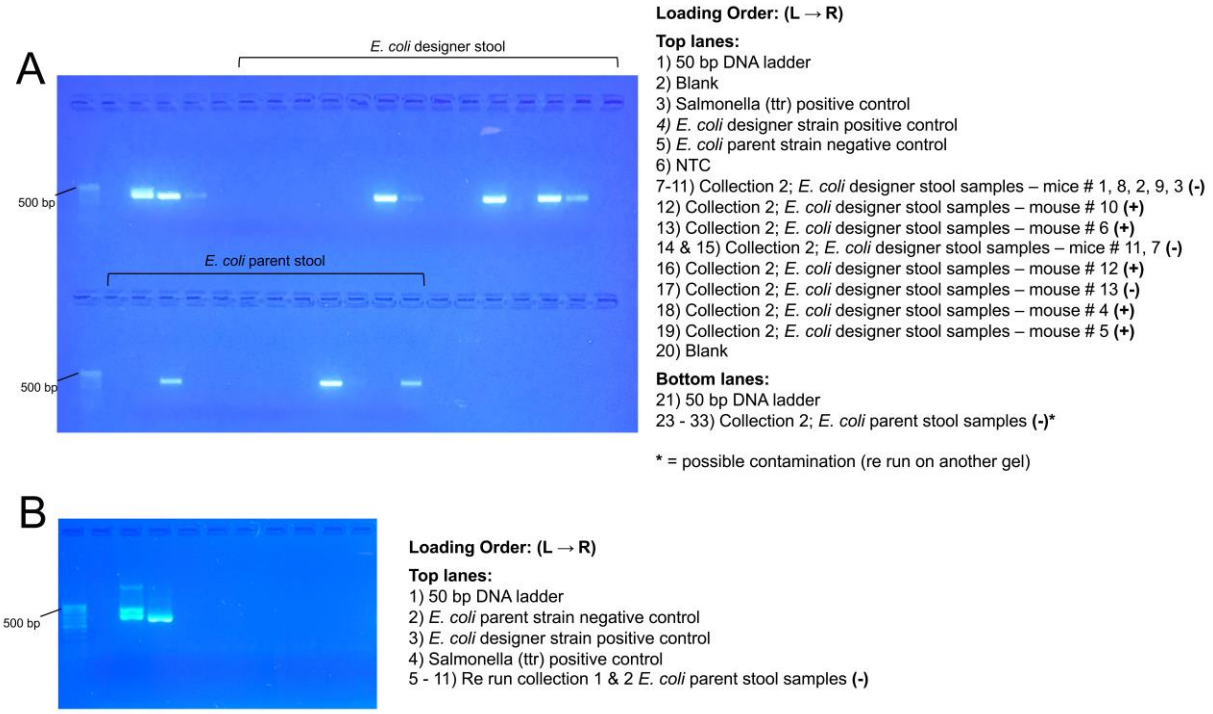


Figure 41. Collection #2 of C57BL/6 stool samples for detection of ttr operon. Ttr detection via PCR from stool samples of C57BL/6 mice given a single oral gavage of *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection # 2 (2 weeks post gavage). Gel images show A) *E. coli* designer and parent stool samples and B) *E. coli* parent stool samples re-run.

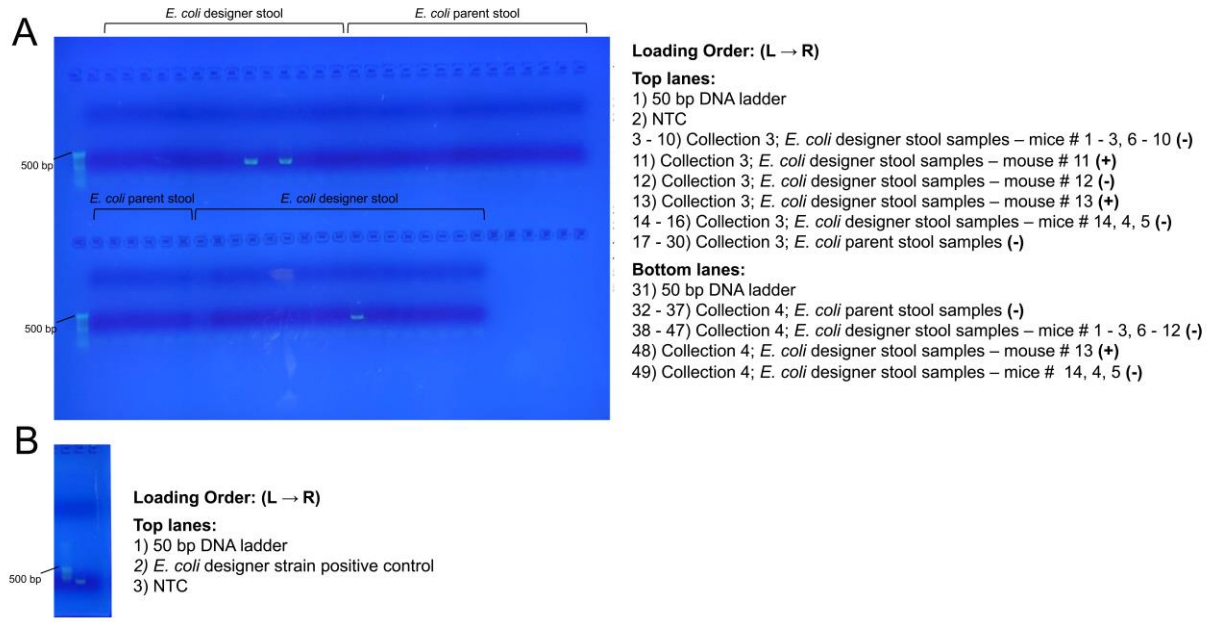


Figure 42. Collection #3 and #4 of C57BL/6 stool samples for detection of ttr operon.
Ttr detection via PCR from stool samples of C57BL/6 mice given a single oral gavage of *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #3 and #4 (1 month and 2 months post gavage). Gel images show A) and B) *E. coli* designer and parent stool samples.

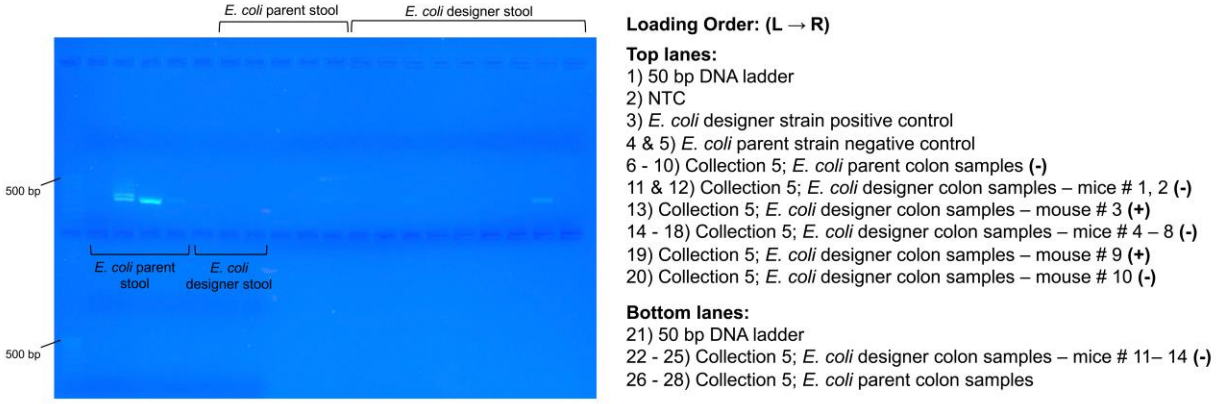


Figure 43. Collection #5 of C57BL/6 colon samples for detection of ttr operon.
Ttr detection via PCR from colon samples of C57BL/6 mice given a single oral gavage of *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #5 (2 months post gavage) at euthanization. Gel image shows *E. coli* designer and parent stool samples.

Appendix F: Gel electrophoresis for ttr strain detection in Muc2$^{-/-}$

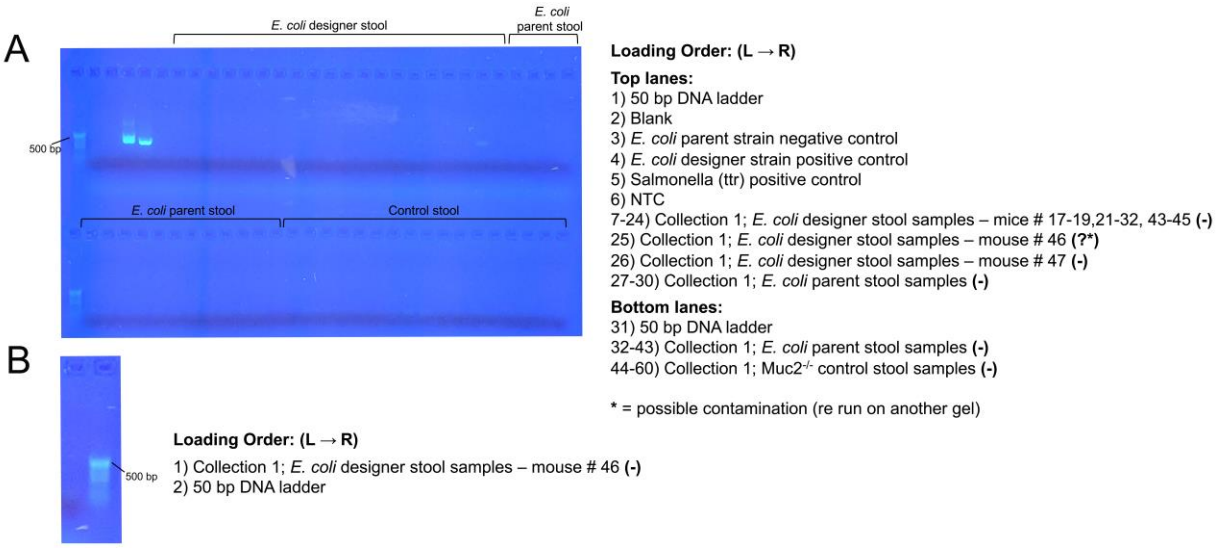


Figure 44. Collection #1 of Muc2$^{-/-}$ stool samples for detection of ttr operon. Ttr detection via PCR from stool samples of Muc2$^{-/-}$ mice supplemented with *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection # 1 (pre-gavage). Gel images show A) and B) *E. coli* designer, *E. coli* parent, and Muc2$^{-/-}$ control stool samples.

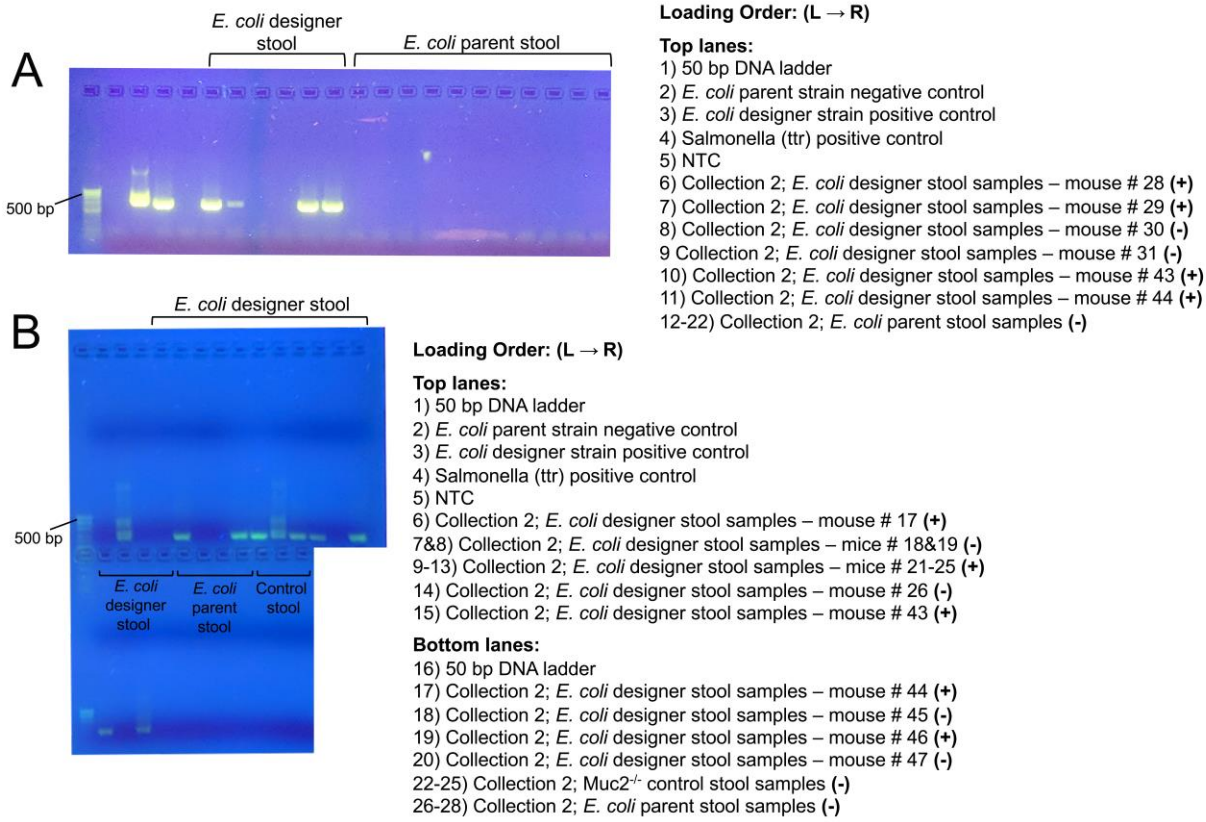
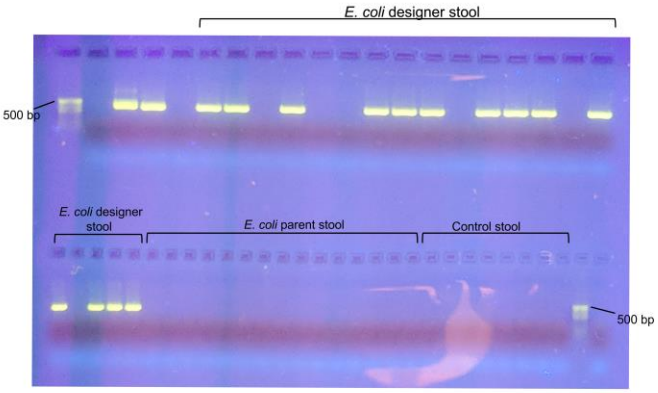


Figure 45. Collection #2 of Muc2$^{-/-}$ stool samples for detection of ttr operon. Ttr detection via PCR from stool samples of Muc2$^{-/-}$ mice supplemented with *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #2. Gel images show A) and B) *E. coli* designer, *E. coli* parent, and Muc2$^{-/-}$ control stool samples.

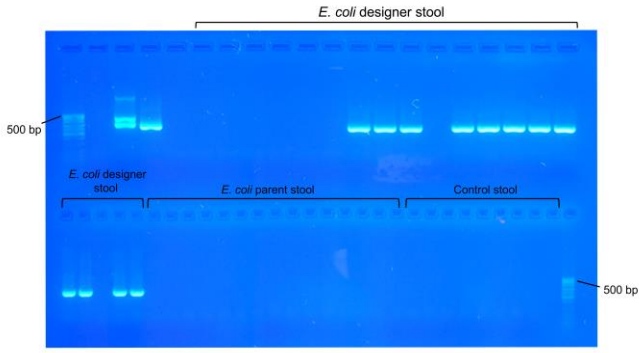


Loading Order: (L → R)

Top lanes:
1) 50 bp DNA ladder
2) *E. coli* parent strain negative control
3) *E. coli* designer strain positive control
4) Salmonella positive control
5) NTC
6) Collection 3; *E. coli* designer stool samples – mouse # 17 **(+)**
7) Collection 3; *E. coli* designer stool samples – mouse # 18 **(+)**
8) Collection 3; *E. coli* designer stool samples – mouse # 19 **(-)**
9) Collection 3; *E. coli* designer stool samples – mouse # 21 **(+)**
10) Collection 3; *E. coli* designer stool samples – mouse # 22 **(-)**
11) Collection 3; *E. coli* designer stool samples – mouse # 23 **(-)**
12) Collection 3; *E. coli* designer stool samples – mouse # 24 **(+)**
13) Collection 3; *E. coli* designer stool samples – mouse # 25 **(+)**
14) Collection 3; *E. coli* designer stool samples – mouse # 26 **(+)**
15) Collection 3; *E. coli* designer stool samples – mouse # 27 **(-)**
16-18) Collection 3; *E. coli* designer stool samples – mouse # 28-30 **(+)**
19) Collection 3; *E. coli* designer stool samples – mouse # 31 **(-)**
20) Collection 3; *E. coli* designer stool samples – mouse # 32 **(+)**

Bottom lanes:
21) Collection 3; *E. coli* designer stool samples – mouse # 43 **(+)**
22) Collection 3; *E. coli* designer stool samples – mouse # 44 **(-)**
23) Collection 3; *E. coli* designer stool samples – mouse # 45 **(+)**
24) Collection 3; *E. coli* designer stool samples – mouse # 46 **(+)**
25) Collection 3; *E. coli* designer stool samples – mouse # 47 **(+)**
26-40) Collection 3; *E. coli* parent stool samples **(-)**
41-48) Collection 3; Muc2$^{-/-}$ control stool samples **(-)**
49) 50 bp DNA ladder

Figure 46. Collection #3 of Muc2$^{-/-}$ stool samples for detection of ttr operon.
Ttr detection via PCR from stool samples of Muc2$^{-/-}$ mice supplemented with *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #3. Gel image shows *E. coli* designer, *E. coli* parent, and Muc2$^{-/-}$ control stool samples.

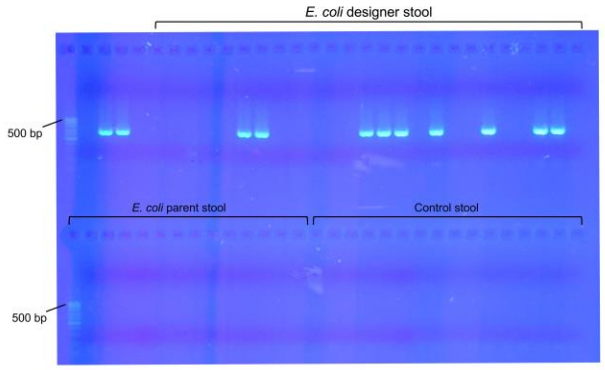


Loading Order: (L → R)

Top lanes:
1) 50 bp DNA ladder
2) *E. coli* parent strain negative control
3) *E. coli* designer strain positive control
4) Salmonella positive control
5) NTC
6-11 Collection 4; *E. coli* designer stool samples – mice # 17-19, 21 & 23 **(-)**
12) Collection 4; *E. coli* designer stool samples – mouse # 24 **(+)**
13) Collection 4; *E. coli* designer stool samples – mouse # 25 **(+)**
14) Collection 4; *E. coli* designer stool samples – mouse # 26 **(+)**
15) Collection 4; *E. coli* designer stool samples – mouse # 27 **(-)**
16-20) Collection 4; *E. coli* designer stool samples – mice # 28-32 **(+)**

Bottom lanes:
21) Collection 4; *E. coli* designer stool samples – mouse # 43 **(+)**
22) Collection 4; *E. coli* designer stool samples – mouse # 44 **(+)**
23) Collection 4; *E. coli* designer stool samples – mouse # 45 **(-)**
24) Collection 4; *E. coli* designer stool samples – mouse # 46 **(+)**
25) Collection 4; *E. coli* designer stool samples – mouse # 47 **(+)**
26-40) Collection 4; *E. coli* parent stool samples **(-)**
41-49) Collection 4; Muc2$^{-/-}$ control stool samples **(-)**
50) 50 bp DNA ladder

Figure 47. Collection #4 of Muc2$^{-/-}$ stool samples for detection of ttr operon.
Ttr detection via PCR from stool samples of Muc2$^{-/-}$ mice supplemented with *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #4. Gel image shows *E. coli* designer, *E. coli* parent, and Muc2$^{-/-}$ control stool samples.

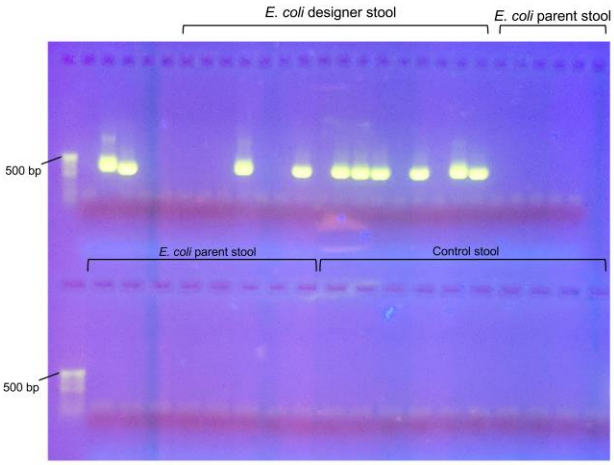


Loading Order: (L → R)

Top lanes:
1) 50 bp DNA ladder
2) *E. coli* parent strain negative control
3) *E. coli* designer strain positive control
4) Salmonella positive control
5) NTC
6-10) Collection 5; *E. coli* designer stool samples – mice # 17-19, 21 & 22 **(-)**
11) Collection 5; *E. coli* designer stool samples – mouse # 23 **(+)**
12) Collection 5; *E. coli* designer stool samples – mouse # 24 **(+)**
13-17) Collection 5; *E. coli* designer stool samples – mice # 25-29 **(-)**
18) Collection 5; *E. coli* designer stool samples – mouse # 30 **(+)**
19) Collection 5; *E. coli* designer stool samples – mouse # 31 **(+)**
20) Collection 5; *E. coli* designer stool samples – mouse # 32 **(+)**
21) Collection 5; *E. coli* designer stool samples – mouse # 22, repeat **(-)**
22) Collection 5; *E. coli* designer stool samples – mouse # 24, repeat **(+)**
23 & 24) Collection 5; *E. coli* designer stool samples – mice # 25 & 26, repeat **(-)**
25) Collection 5; *E. coli* designer stool samples – mouse # 43 **(+)**
26) Collection 5; *E. coli* designer stool samples – mouse # 44 **(-)**
27) Collection 5; *E. coli* designer stool samples – mouse # 45 **(-)**
28) Collection 5; *E. coli* designer stool samples – mouse # 46 **(+)**
29) Collection 5; *E. coli* designer stool samples – mouse # 47 **(+)**

Bottom lanes:
31) 50 bp ladder
32-45) Collection 5; *E. coli* parent stool samples **(-)**
46-60) Collection 5; Muc2$^{-/-}$ control stool samples **(-)**

Figure 48. Collection #5 of Muc2$^{-/-}$ stool samples for detection of ttr operon.
Ttr detection via PCR from stool samples of Muc2$^{-/-}$ mice supplemented with *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #5. Gel image shows *E. coli* designer, *E. coli* parent, and Muc2$^{-/-}$ control stool samples.

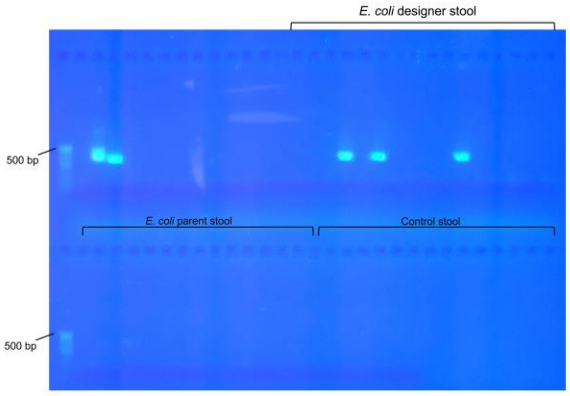


Loading Order: (L → R)

Top lanes:
1) 50 bp DNA ladder
2) *E. coli* parent strain negative control
3) *E. coli* designer strain positive control
4) Salmonella positive control
5) NTC
6-9) Collection 6 & 10; *E. coli* designer stool samples – mice # 17-19, 21 **(-)**
10) Collection 6; *E. coli* designer stool samples – mouse # 24 **(+)**
11-12) Collection 6; *E. coli* designer stool samples – mice # 25 & 26 **(-)**
13) Collection 6; *E. coli* designer stool samples – mouse # 27 **(+)**
14) Collection 6; *E. coli* designer stool samples – mouse # 29 **(-)**
15-17) Collection 10; *E. coli* designer stool samples – mice # 30-32 **(+)**
18) Collection 10; *E. coli* designer stool samples – mouse # 44 **(-)**
19) Collection 10; *E. coli* designer stool samples – mouse # 46 **(+)**
20) Collection 6; *E. coli* designer stool samples – mouse # 22 **(-)**
21 & 22) Collection 6; *E. coli* designer stool samples – mice # 23 & 28 **(+)**
23-27) Collection 6 & 10; *E. coli* parent stool samples **(-)**

Bottom lanes:
24) 50 bp DNA ladder
25-32) Collection 6 & 10; *E. coli* parent stool samples **(-)**
33-42) Collection 6 & 10; Muc2$^{-/-}$ control stool samples **(-)**

Figure 49. Collection #6 and #10 of Muc2$^{-/-}$ stool samples for detection of ttr operon.
Ttr detection via PCR from stool samples of Muc2$^{-/-}$ mice supplemented with *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #6 and #10 at euthanization. Gel image shows *E. coli* designer, *E. coli* parent, and Muc2$^{-/-}$ control stool samples.

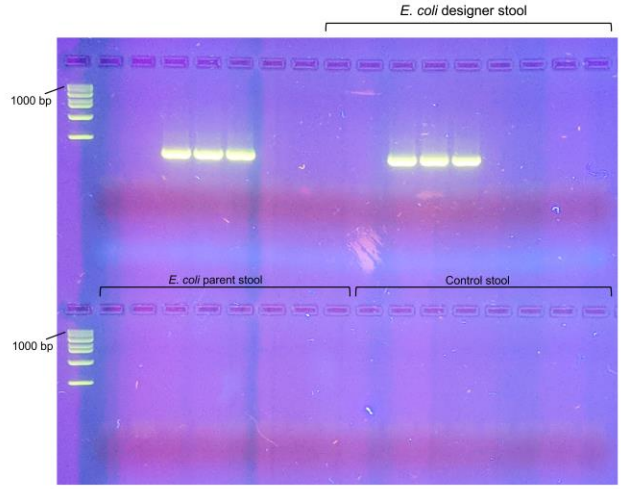


Loading Order: (L → R)

Top lanes:
1) 50 bp DNA ladder
2) *E. coli* parent strain negative control
3) *E. coli* designer strain positive control
4) Salmonella positive control
5) NTC
6-17) Collection 7 & 11; *E. coli* designer colon samples – mice # 17 - 19, 21-29 **(-)**
18) Collection 7; *E. coli* designer colon samples – mouse # 30 **(+)**
19) Collection 7; *E. coli* designer colon samples – mouse # 31 **(-)**
20) Collection 7; *E. coli* designer colon samples – mouse # 32 **(+)**
21-24) Collection 7; *E. coli* designer colon samples – mice # 43-46 **(-)**
25) Collection 7; *E. coli* designer colon samples – mouse # 47 **(+)**
26-30) Collection 7 & 11; *E. coli* parent colon samples **(-)**

Bottom lanes:
31) 50 bp ladder
32-42) Collection 7 & 11; *E. coli* parent colon samples **(-)**
43-51) Collection 7 & 11; Muc2$^{-/-}$ control colon samples **(-)**

Figure 50. Collection #7 and #11 of Muc2$^{-/-}$ colon samples for detection of ttr operon.
Ttr detection via PCR from stool samples of Muc2$^{-/-}$ mice supplemented with *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #7 and #11 at euthanization. Gel image shows *E. coli* designer, *E. coli* parent, and Muc2$^{-/-}$ control stool samples.



Loading Order: (L → R)

Top lanes:
1) 1 kb DNA ladder
2 & 3) *E. coli* parent strain negative control
4 & 5) *E. coli* designer strain positive control
6) Salmonella positive control
7 & 8) NTC
9 -11) Collection 8; *E. coli* designer stool samples – mice # 21-23 **(-)**
12) Collection 8; *E. coli* designer stool samples – mouse # 24 **(+)**
13) Collection 8; *E. coli* designer stool samples – mouse # 25 **(+)**
14) Collection 8; *E. coli* designer stool samples – mouse # 26 **(+)**
15 -17) Collection 8; *E. coli* designer stool samples – mice # 27-29 **(-)**

Bottom lanes:
18 - 25) Collection 8; *E. coli* parent stool samples **(-)**
26 - 32) Collection 8; Muc2$^{-/-}$ control stool samples **(-)**

Figure 51. Collection #8 of Muc2$^{-/-}$ stool samples for detection of ttr operon.
Ttr detection via PCR from stool samples of Muc2$^{-/-}$ mice supplemented with *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #8. Gel image shows *E. coli* designer, *E. coli* parent, and Muc2$^{-/-}$ control stool samples.

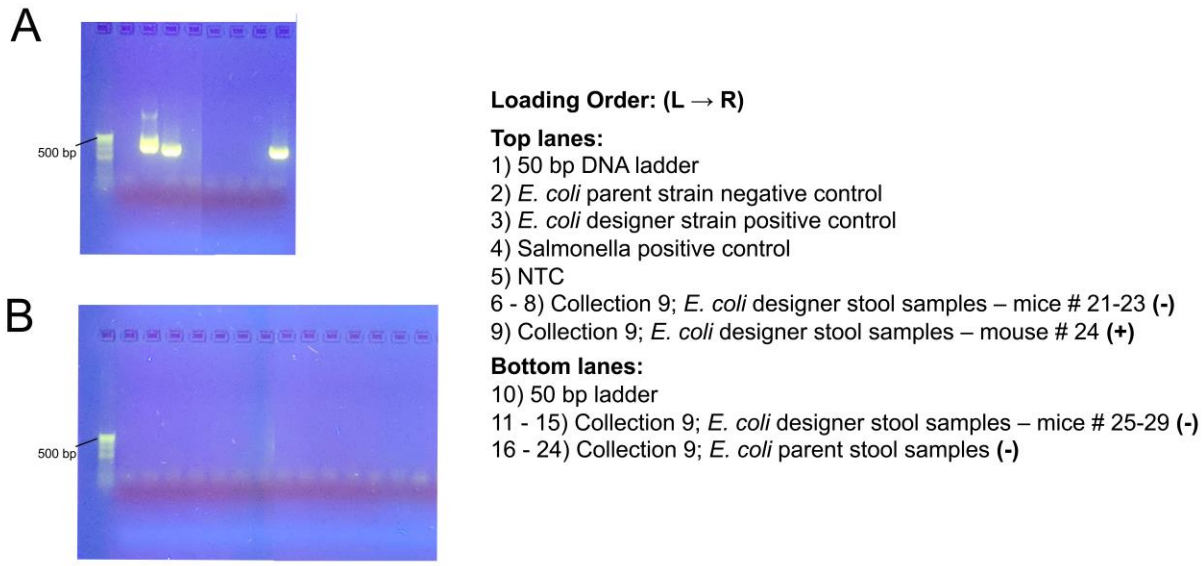


Figure 52. Collection #9 of Muc2$^{-/-}$ stool samples for detection of ttr operon. Ttr detection via PCR from stool samples of Muc2$^{-/-}$ mice supplemented with *E. coli* designer probiotic. Gel electrophoresis image shown of stool samples from collection #9. Gel image shows *E. coli* designer, *E. coli* parent, and Muc2$^{-/-}$ control stool samples.

Appendix G: Gel electrophoresis for GbpA strain detection in C57BL/6 mice

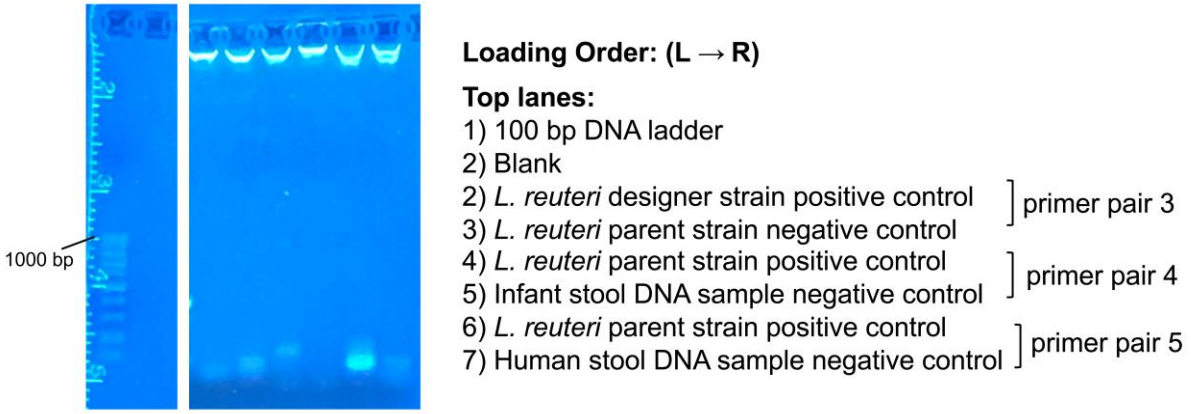


Figure 53. Gel electrophoresis of GbpA primer detection using primer pairs #3, #4, and #5
GbpA detection via PCR for *L. reuteri* designer and parent probiotic samples. Primer pair #3 for *L. reuteri* designer probiotic detection. Primer pairs #4 and #5 for *L. reuteri* parent probiotic detection.

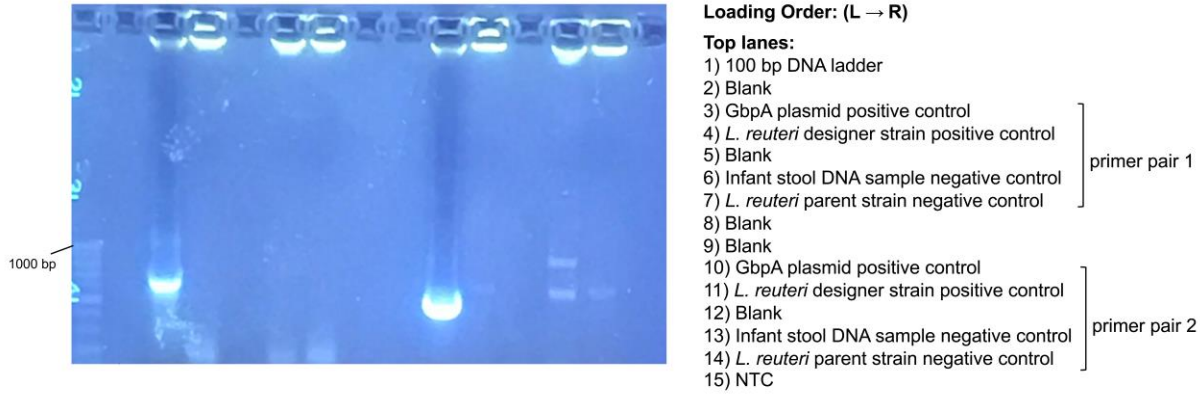
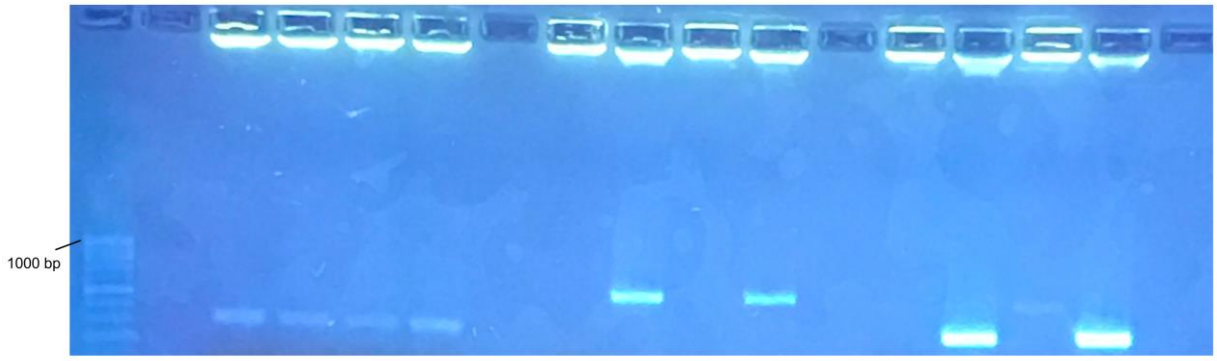


Figure 54. Gel electrophoresis of GbpA primer detection using primer pairs #1, and #2
GbpA detection via PCR for *L. reuteri* designer and parent probiotic samples. Primer pairs #1 and #2 for *L. reuteri* designer probiotic detection.



Loading Order: (L → R)

Top lanes:

1) 100 bp DNA ladder
2) Blank
3) GbpA plasmid positive control
4) *L. reuteri* designer strain positive control
5) Infant stool DNA sample negative control
6) *L. reuteri* parent strain negative control
7) Blank

primer pair 6

8) GbpA plasmid positive control
9) *L. reuteri* designer strain positive control
10) Infant stool DNA sample negative control
11) *L. reuteri* parent strain negative control
12) Blank

primer pair 7

13) GbpA plasmid positive control
14) *L. reuteri* designer strain positive control
15) Infant stool DNA sample negative control
16) *L. reuteri* parent strain negative control

primer pair 8

Figure 55. Gel electrophoresis of GbpA primer detection using primer pairs #6, #7, and #8

GbpA detection via PCR for *L. reuteri* designer and parent probiotic samples. Primer pairs #6, #7, and #8 for *L. reuteri* designer probiotic detection.

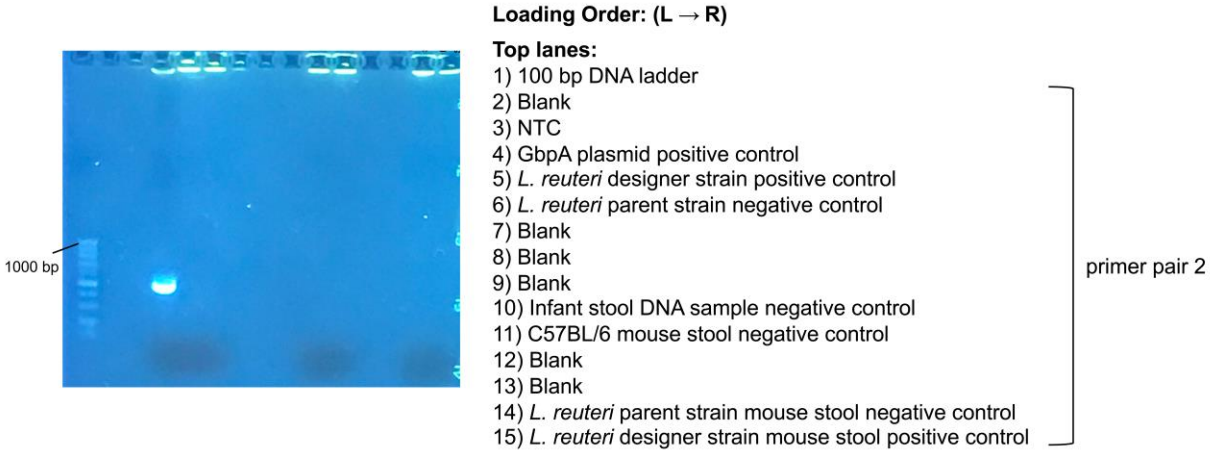


Figure 56. Gel electrophoresis of GbpA primer detection using primer pair #2
GbpA detection via PCR for *L. reuteri* designer and parent probiotic samples. Primer pair #2 for *L. reuteri* designer probiotic detection.

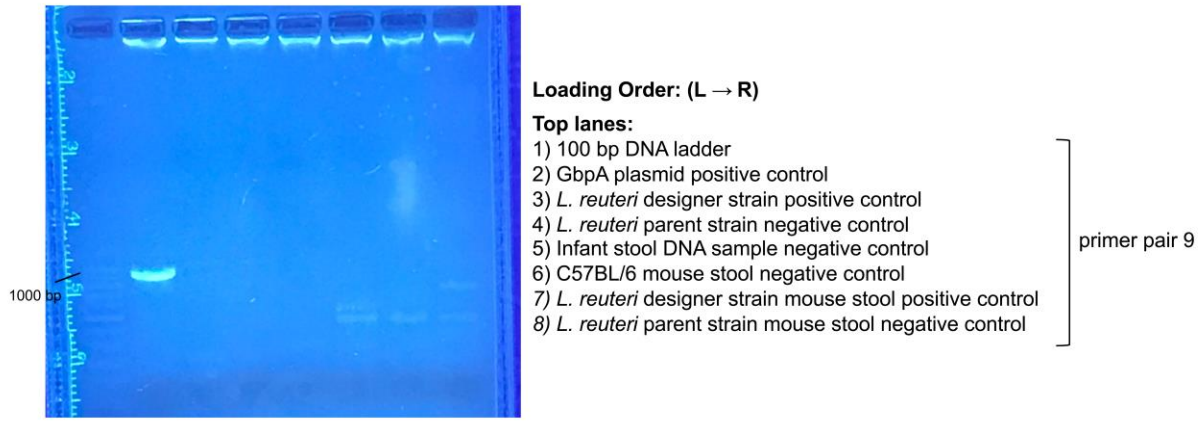


Figure 57. Gel electrophoresis of GbpA primer detection using primer pair #9
GbpA detection via PCR for *L. reuteri* designer and parent probiotic samples. Primer pair #9 for *L. reuteri* designer probiotic detection.