Antibiotic Reduction of Bacterial β-Glucuronidase Activity in the Murine Gut Prevents and Reverses Mycophenolate Mofetil-Induced Gastrointestinal Toxicity

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Antibiotic Reduction of Bacterial β-Glucuronidase Activity in the Murine Gut Prevents and Reverses Mycophenolate Mofetil-Induced Gastrointestinal Toxicity

by

Michael Robert Taylor

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN MEDICAL SCIENCE

CALGARY, ALBERTA

AUGUST, 2018

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Abstract

Mycophenolate mofetil (MMF) is an important immunosuppressive drug widely used post-transplantation, but whose use is often limited by gastrointestinal (GI) side effects including diarrhea, weight loss and colitis. The etiology of this GI toxicity has not been fully explained although we have shown in the mouse that an intact microbiome is required. This thesis developed and tested a potential mechanism, hypothesizing that bacterial conversion of the MMF metabolite mycophenolic acid glucuronide (MPAG) to mycophenolic acid (MPA) in the colon is responsible for the GI toxicity caused by MMF. Vancomycin both prevented and reversed MMF-induced GI toxicity and we characterized its effect on the intestinal microbiota in MMF-treated mice. Vancomycin eliminated many β-glucuronidase-producing bacteria, resulting in decreased hydrolysis of MPAG to MPA that was also associated with decreased weight loss and reduced colonic inflammation and injury. These findings outline a mechanism permitting interventions to improve MMF tolerance.

Keywords: Transplantation, mycophenolate mofetil (MMF), gastrointestinal toxicity, microbiome, bacterial β-glucuronidase
Preface

This thesis is original, unpublished, independent work by the author, Michael R. Taylor.

The methods contained within this thesis were performed solely by the author, Michael R. Taylor with assistance from the following individuals:

Ms. Amina Mohammed, under my supervision, assisted with mouse handling and sample collection during the individual antibiotic administration experiments.

Ms. Hannah Rahim, under my supervision, assisted with the mouse handling, colonic tissue cytokine extraction and *in fimo* bacterial genomic DNA isolation, amplification, and sequencing during the MMF + Vancomycin experiments.

Ms. Laurie Alston performed the MPO assay.

Dr. Bjoern Petri performed the fluorescence imaging for the *in vivo* GUS activity assay.

The Redinbo Laboratory performed the GUS activity screen against MPAG.

Dr. Aadra Bhatt coined the brilliant term ‘*in fimo*’, and has graciously let us adopt it.

The experiments reported in Chapters 2-4 were approved by the University of Calgary Health Sciences Animal Care Committee (ethics certificate number AC14-0068) for the project “The effect of immunosuppressive drugs on the intestinal microbiota – insight into transplantation-associated gastrointestinal complications”.

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Acknowledgements

Firstly, I would like to thank my supervisor Dr. Steven Greenway for providing me with an amazing research opportunity and unrelenting support over the last two years. You not only provided me with the freedom to explore this project in whatever direction I thought may be successful, but also provided me many opportunities for expanding my skills and knowledge in multiple cities across the country. Your teachings and guidance taught me how to be thoughtful and patient with science. Thank you for everything.

To Dr. Simon Hirota and Dr. Ian Lewis, I could not have dreamed for better committee members. You were both involved in every step of my project from day one, never hesitating to help me work through a problem when I dropped by unannounced, helping me direct my project to exactly where it needed to go, or granting me full access to your own laboratories and knowledgeable lab members. I spent nearly as much time working and learning in your laboratories as I did in my own, and I thank you for that.

To my friends, Kara Vanden Broek and Taylor Woo who courteously began their journey of graduate school before me and passed on their immense knowledge of not only science, but how to survive as a graduate student, I cannot thank you enough. This extends to all my friends I have met along the way, and the members of the Greenway, Hirota, and Lewis laboratories, I truly would have been lost without you. In particular, Dr. Kyle Flannigan. You never hesitated to help me with grunt work on a second’s notice, or answer my pleas for help at all hours of the night. It was a pleasure working so closely with you and I thank you for everything.

To my family, despite usually not understanding exactly what I was doing or what I was talking about, you were always excited to speak with me about my research, and encouraged me
to persist through all the challenges I have experienced over my academic years. You pushed me always to persevere and it has always paid off. I would not be here if it wasn’t for you.

To my partner in adventure, the wonderful, caring, and supportive Krystin. Your infinite patience in dealing with the long hours and frustrations of graduate work, and the innumerable nights spent sleeping by the light of my computer, there are not enough thanks in the world. You kept me motivated through the lows and were the first to congratulate me on the highs. There is no one else I would have wanted to experience this roller-coaster of an adventure with.

Thank you.
Dedication

This thesis is dedicated to those individuals who may one day benefit from scientific progress stemming from its content, and to the humble laboratory mice who have given the ultimate sacrifice to progress the knowledge of human health, biology, and science.
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Epigraph

…but if you look a little further, if you dig a little deeper, if you spend the time, you discover an endless supply of good and interesting stuff.

- The late Anthony Bourdain
CHAPTER ONE: INTRODUCTION

1.1 Solid Organ Transplantation

For those individuals suffering from end-stage organ failure, transplantation is an effective and life-saving therapy. Solid organ transplantation (SOT) from a living or deceased donor has now been successfully applied to retina, kidneys, heart, lungs, intestine, pancreas, and liver with thousands of transplant procedures performed worldwide each year. Hematopoietic stem cell transplantation (HSCT) involves transplantation of bone marrow from a healthy donor to a recipient with a life-threatening hematological disease. Another example of cellular transplantation is the donation of healthy hepatocytes to an infant with an inborn error of metabolism to enable survival to liver transplantation. The success of SOT and cellular transplantation, as measured by median survival, has improved substantially since the first kidney transplant in 1954 (between identical twins), due in large part to advances in surgical technique, HLA-antigen matching, and, perhaps most importantly, advances in immunosuppression.\(^1\)\(^{-}\)\(^4\)

Immunosuppression is required after transplantation to prevent rejection of the donated tissue, cells, or organs (allograft). Rejection most commonly occurs when the recipient’s immune system recognizes antigens (major histocompatibility complex, MHC) on the allograft as foreign, and proceeds to mount an immune response involving adaptive immune cells against the allograft.\(^5\) Immunosuppressive drugs (ISDs, commonly referred to as anti-rejection drugs) are used to suppress the activity of the recipient immune system, through a variety of cellular mechanisms and thereby lower the rate of rejection.\(^6\) Without effective immunosuppression, transplantation is rarely successful.\(^7\)
Steroids and then azathioprine were the first ISDs widely used and enabled SOT to expand as a therapeutic option. Cyclosporine was introduced in 1983, followed by MMF in 1995, and then tacrolimus. Today, most patients are maintained on 1-3 immunosuppressive drugs with the most common combination being tacrolimus and MMF although with important differences in the drugs used depending on the transplanted organ.\textsuperscript{8,9}

1.2 The Mammalian Microbiome and Immunosuppressive Drugs

In mammals, the microbiome is composed of all microorganisms, including bacteria, archaea, fungi, and viruses, and their associated genetic content, that exist in and on nonsterile sites of the body, including the skin, respiratory tract, and gastrointestinal (GI) tract.\textsuperscript{10,11} The term microbiome refers to a collection of microbes along with their associated genetic and proteinaceous content, whereas the term microbiota refers only to the microbes themselves. This convention will be followed in the present study. In the human host, microbial cells outnumber human cells by an estimated 1.3–2.3:1 with the ratio of microbial to human genetic content estimated to be an astounding 150:1.\textsuperscript{12,13} Microbial composition varies greatly between individuals as it is influenced by a multitude of factors including age, diet, medication exposures, lifestyle, illness, and stress.\textsuperscript{11} However, functionality of the microbiome is found to be quite uniform among individuals, indicating that assessment of microbial functionality, and not necessarily composition, may be a more important indicator of a perturbed microbiome.\textsuperscript{14,15} Disruption of microbial homeostasis has been implicated in numerous human diseases, many of which affect patients after transplantation.\textsuperscript{16–18}

The mammalian microbiome has only recently been explored as a potential environmental variable affecting health outcomes following transplantation as the capability for
studying these microorganisms on a large scale has emerged only over the last decade.\textsuperscript{19} The development of inexpensive and widely accessible technologies, including next-generation DNA sequencing, highly sensitive instruments for metabolomics, and powerful computing resources for bioinformatics, has led to a rapid expansion of the field of microbiome research.\textsuperscript{20,21} With it has come an explosion of discoveries documenting the significance of the microbiome in nearly all aspects of human health, including xenobiotic metabolism, biosynthesis of nutrients, and development and modification of the immune system, all of which are factors affecting transplant recipient health.\textsuperscript{16,22} Many ISDs in particular have been found to alter gut composition resulting in the flourishing of pathogens\textsuperscript{23,24} or even modifications to host immune cell populations.\textsuperscript{25} Until recently, the impact of mycophenolate mofetil (MMF) on the microbiome was not well known, however, work from the Greenway and Hirota laboratories found not only does the gut microbiome interact with MMF metabolites, but treatment with MMF results in broad compositional changes in the gut microbial populations.\textsuperscript{26} Additionally, they concluded that the presence of an intact microbiome was required for MMF-induced GI toxicity to occur, in a murine model of MMF immunosuppression.\textsuperscript{26}

1.3 Mycophenolate Mofetil

The ISD mycophenolate mofetil, commercially known as CellCept, is used extensively for immunosuppression after both SOT and HSCT and has been associated with more successful SOT outcomes compared to other ISDs.\textsuperscript{2,3,27,28} Unfortunately, MMF is also frequently associated with adverse effects involving the GI tract.\textsuperscript{29}

MMF is the prodrug of mycophenolic acid (MPA). MPA suppresses the adaptive immune system by preventing \textit{de novo} synthesis of guanosine-5’-monophosphate (GMP) through
inhibition of inosine monophosphate dehydrogenase (IMPDH) in a reversible and non-competitive manner. Most cell types are able to utilize a salvage pathway for GMP acquisition, however, lymphocytes rely completely on de novo synthesis of GMP. Additionally, MPA inhibits the IMPDH2 isoform (expressed in lymphocytes) 4-5 fold more effectively than the IMPDH1 isoform (expressed in other cell types). Therefore, MPA prevents the replication of T and B cells by reducing the intracellular availability of guanosine nucleotides for DNA replication and RNA synthesis, effectively suppressing the adaptive immune system.

While it is a highly effective ISD, MMF frequently causes adverse GI side effects ranging from mild to severe, including diarrhea, abdominal pain, and weight loss, and has been reported to induce colitis and an inflammatory bowel disease-like phenotype, with symptoms occurring in up to 79% of patients prescribed MMF. These side effects often necessitate dose reduction or discontinuation which is correlated with increased incidence of rejection and decreased survival, in heart and kidney transplant recipients. Currently, the mechanism of toxicity is not well understood and no effective intervention exists.

The xenobiotic metabolism of MMF within humans has been well characterized (Figure 1.1). Orally-administered MMF is hydrolyzed to MPA in the upper GI tract which then enters the systemic circulation and can act on circulating lymphocytes as well as lymphocytes found within the thymus and spleen. Inactivation of MPA occurs via phase II drug metabolism, specifically the glucuronidation pathway, occurring predominately in the liver where glucuronic acid (GlcA) is conjugated to MPA by UDP-glucuronosyl transferases (UGTs). UGT isoforms 1A8 and 1A9 are primarily responsible for production of the major metabolite mycophenolic acid glucuronide (MPAG) with several other UGT isoforms also contributing to the production of MPAG (e.g.
UGT1A1, UGT1A7, and UGT1A10) but to a lesser extent.\textsuperscript{40} The minor metabolite mycophenolic acid acyl-glucuronide (AcMPAG) is produced by UGT2B7.\textsuperscript{39}

While the majority of both metabolic products are excreted in the urine (~90\%) the remainder (~10\%) is transported into the biliary tract by the multidrug resistance-associated protein 2 (MRP2) for biliary excretion.\textsuperscript{41,42} MPAG and AcMPAG, once excreted through biliary secretion interact with commensal gut bacteria in the lower GI tract where bacterial enzymes known as β-glucuronidases (GUSs) hydrolyze MPAG and AcMPAG to free MPA and GlcA in the GI lumen.\textsuperscript{43} The resulting GlcA is then available as a carbon source for bacterial metabolism via the Entner-Doudoroff pathway.\textsuperscript{44} The freed MPA interacts with the colonic epithelium and undergoes enterohepatic recirculation which is estimated to contribute 10 – 61\% of serum MPA concentration in humans.\textsuperscript{45} Intravenous MMF bypasses the upper GI tract but is hydrolyzed to MPA in the blood, where it follows the same metabolic pathway as oral MMF. Importantly, the frequency of adverse effects is not found to be different between either route of MMF administration.\textsuperscript{46}
Figure 1.1. The known metabolic pathway for mycophenolate mofetil (MMF) in mammals. Oral delivery of the pro-drug (MMF) is followed by hydrolysis in the upper gastrointestinal (GI) tract to the active compound mycophenolic acid (MPA) which then enters the systemic circulation. MPA undergoes phase II glucuronidation by hepatic UDP-glucuronosyltransferases (UGTs) producing MPAG and AcMPAG. MPAG and AcMPAG are primarily (~90%) excreted in the urine but (~10%) are transported by the organic anion transporter multidrug resistance-associated protein 2 (MPR2) into the biliary and GI tracts. In the colon, most MPAG and AcMPAG undergoes deconjugation by bacterial GUS-expressing bacteria to reactivate MPA which re-enters the systemic circulation via enterohepatic circulation. Intravenous MMF enters directly into systemic circulation and enteric-coated MMF bypasses the gastric esterases but thereafter both compounds follow the same metabolic pathway.
1.4 An Intact Microbiome is Required for MMF-Induced Toxicity

Previously, the Hirota and Greenway laboratories observed that MMF produced a phenotype of severe weight loss, GI toxicity, and systemic tissue alterations, in a murine model of immunosuppression, recapitulating the MMF-induced GI toxicity seen in human patients. In this model, mice consistently lost between 20-25% body weight over an 8-day treatment period, in combination with shortened colon length, a reduction in cecum and spleen weight, fat mass and lean mass, and hematocrit. Interestingly, gross colonic tissue damage did not occur, but an innate immune response was observed as measured by increases in granulocyte infiltration and upregulation of many innate inflammatory mediators.²⁶

Furthermore, MMF was found to induce broad scale shifts in the composition of the gut microbiota resulting in decreased species diversity (Observed species index) along with increases in potentially pathogenic bacterial taxa such as *Escherichia/Shigella*. Removal of MMF resulted in loss of toxicity (improved weight gain, decreased gut inflammation) and restoration of many physiological parameters within 8 days, however, the composition of the gut microbiota remained altered.²⁶

To assess the involvement of the gut microbiota in mediating MMF-induced GI toxicity, a broad-spectrum antibiotic cocktail was used to purge the gut microbiota in the MMF-treated murine model. Pretreatment with antibiotics was found to be protective against toxicity. Additionally, supplying antibiotics to mice already consuming MMF was found to be effective in reversing the toxicity phenotype after 8 days of MMF-alone treatment, similar to removal of the ISD. Furthermore, germ-free (GF) mice were found to be unaffected by MMF treatment as they did not experience body weight loss, colonic shortening, or tissue weight loss, indicating that an intact microbiota plays a pivotal role in the development of MMF-induced GI toxicity.²⁶
1.5 Bacterial β-Glucuronidase Implicated in non-ISD Drug Toxicity

Phase II drug metabolism via glucuronidation is one of the most common drug detoxification pathways in the human body, used for approximately 10% of the 200 most commonly prescribed drugs.\(^{47}\) This also includes carboxylic-acid containing nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac and the chemotherapeutic drug irinotecan (prodrug of SN-38) used for the treatment of colon cancer.\(^{48,49}\) Glucuronidation functions by conjugating a GlcA moiety to a xenobiotic, which increases its hydrophilicity and allows the xenobiotic compound to be excreted via the urine or bile.\(^{50}\) These inactivated compounds, referred to as glucuronides, can interact with bacterial β-glucuronidase (GUS) enzymes in the GI tract, possibly re-activating the xenobiotic in a potentially undesirable location.\(^{51}\)

Both SN-38 and NSAIDs are frequently dose limited by GI injury, manifesting as inflammation, bleeding, and perforation of the stomach and small intestine (NSAIDs)\(^{52}\) or severe diarrhea leading to dehydration (irinotecan).\(^{53}\) Similar to MMF, both SN-38 and NSAIDs undergo glucuronidation in the liver and are transported into the small intestine via the biliary tract.\(^{54,55}\) Once in the GI tract, deconjugation of the inactive glucuronides by bacterial GUSs results in reactivation of the active aglycone in close proximity to the gut epithelium, followed by enterohepatic recirculation. The subsequent interaction between the re-activated xenobiotic and the intestinal epithelium is thought to be the cause for the observed GI injuries, validated by an observed reduction in adverse effects after selective inhibition of GUS.\(^{48,49,56}\) Given the similarities in both the host and bacterial metabolism of active SN-38, NSAIDs, and MPA, and their inactive glucuronides, we postulated that a similar mechanism may be occurring in our murine model of MMF-induced GI toxicity.
1.6 Proposed Mechanisms for MMF-Induced GI Toxicity

Despite the frequent and potentially significant adverse effects of MMF, the molecular mechanism of toxicity is not well understood. As stated, GI toxicity is not found to differ between oral or intravenous route of administration, suggesting its etiology is unrelated to the initial circulation of MPA and thus, likely occurs post-inactivation by phase II metabolism occurring in the liver. Of the two main MMF derivatives produced by glucuronidation, MPAG is thought to be physiologically inert, leading to the dominant hypothesis that the minor metabolite AcMPAG may be the toxic entity. Indeed, AcMPAG has been found to bind cytosolic and cytoskeletal proteins in the kidney, liver, and colon, potentially leading to cellular dysfunction, and has also been observed in vivo to induce the release of inflammatory cytokines (i.e. TNFα and IL-6). However, recent in vitro studies have shown that MPA may have the ability to alter colonic epithelial cell metabolism resulting in impaired protein, fatty acid, and lipid metabolism, as well as disrupted epithelial barrier function. This suggests that MPA itself, released into the colonic lumen by bacterial GUS activity, may be directly toxic to the gut epithelium and potentially be a significant contributor to MMF-related adverse events in the GI tract. Despite the acknowledged role of gut bacteria in the enterohepatic recirculation of MPA, the involvement of gut bacteria in MMF-induced GI toxicity has not been thoroughly investigated. Fortunately, recent advances in human gut microbial research have allowed for the study of ISD-human microbiome interactions and are beginning to demonstrate that the microbiome may be an important, and often overlooked, factor in post-transplant health. We investigated the role of gut bacteria in MMF-induced GI toxicity at a mechanistic level.
1.7 Research Hypothesis and Objectives

Studying the interactions between MMF and the gut microbiota in human patients directly is problematic. The most significant obstacle is that treatment protocols after SOT never involve the use of MMF in isolation. Instead, patients are treated with multiple ISDs post-transplant and are often given antibiotics, both prophylactically to prevent infections acquired during transplant surgery, as well as to treat infections occurring due to the immune suppression. Furthermore, many common medications interact with and modulate the microbiome and, therefore elucidating MMF-dependent effects alone can be difficult, if not impossible. As well, recruiting sufficient numbers of human transplant patients for study is problematic. Finally, experimental modulation of the microbiome in human subjects risks unintended consequences, and thus poses ethical concerns. To circumvent these issues the present study utilized the murine model of MMF immunosuppression previously developed by the Greenway and Hirota laboratories. Given that an intact microbial community is required for MMF toxicity, and the precedent of bacterial involvement in the GI toxicity of other glucuronidated xenobiotics, we sought to understand the specific role that bacteria play in MMF-induced GI toxicity and to investigate the mechanistic interactions between MMF and the microbiome. Based on the knowledge that gut bacterial GUSs have an established role in downstream MMF metabolism, and the recent evidence that MPA may be disruptive to enterocyte function, we tailored our investigations to focus on the bacterial catabolism of MPAG to MPA as a driver for MMF-induced toxicity.

We hypothesized that reducing GUS hydrolysis of MPAG to active MPA, specifically by eliminating GUS-expressing bacteria in the colon, would result in reduced inflammation and reduced GI toxicity in our mouse model.
1.8 Specific Aims

1. Characterize the ability of individual antibiotics to relieve MMF-induced GI toxicity in a murine model of immunosuppression.

2. Determine which bacterial populations are eliminated by antibiotic treatment and assess their association with bacterial GUS metabolism as quantified using metabolomics.

3. Explore the impact of both MMF and concomitant antibiotic therapy on bacterial GUS activity and subsequent catabolism of MPAG in the GI tract.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Mice

All animal procedures were approved by the University of Calgary Animal Care Committee (protocol AC14-0068) and were in accordance with the Canadian Council on Animal Care policies.

All mice used in the present study were female C57BL/6 mice, aged 7-10 weeks, acquired from Charles River Laboratories (Sherbrooke, QC), with one exception; the Inferred Functional Analysis Using PICRUSt assay of Control, MMF, and Recovery treated mice (Section 2.6.4, Figure 3.16), used 7-10 week old, female, C57BL/6J mice acquired from Jackson Laboratories (Bar Harbor, ME). Upon arrival to the University of Calgary Health Sciences Animal Resource Centre, mice were randomly divided into treatment groups and placed into sterilized, filter-top cages (Sealsa NExt 1145T, Techniplast) under specific pathogen-free (SPF) conditions. Animals were fed experimental control chow (2016, Teklad Global 16 % Protein Rodent Diet, TD.00217) and allowed to acclimate to their new environment for a minimum of 1 week before commencing any experiments. Soiled cages were changed as needed, with a small portion of old bedding being transferred to the new cage.

In the model used for the present study, no differences were observed between female and male mice (unpublished), nor between C57BL/6(J) or Balb/C mice in previous work.\textsuperscript{26} We therefore chose to use female C57BL/6 mice for the remainder of the experiments as female mice are typically easier to handle and the C57BL/6 strain was used originally for the development of the murine model of MMF immunosuppression.
2.1.1 Fecal Pellet Collection

Fresh fecal pellets were collected for amplicon sequencing of gut microbiota, quantification of colonic lumen metabolites, and bacterial GUS activity assays. Mice were individually placed in clean, ethanol-sterilized cages and monitored closely. Immediately upon defecation each fecal pellet was collected using ethanol-sterilized tweezers, placed in an appropriate clean (and sterile, if required) collection tube, and snap-frozen on dry ice. Pellets were stored at -80°C until further use. Hereinafter any protocol performed using fecal pellets or intestinal luminal contents will be referred to as in fimo from the Latin ‘fimo’ meaning excrement.

2.1.1.1 Fecal Metabolite Extraction

Metabolites (end products of host and bacterial metabolism, including products of MMF metabolism) were extracted from fecal pellets using a water-methanol extraction method (50 % methanol:water, 50 % MeOH), to obtain the largest variety of metabolites possible. Fecal pellets were collected and stored as previously described (Section 2.1.1) in screw-top centrifuge tubes. While on ice, 500 µL of 50 % MeOH was added to 50 mg of fecal pellet (10 µL of 50 % MeOH per mg of fecal pellet), along with 5 × 2.8 mm ceramic beads. Each sample was homogenized using a BeadRupter 4 (Omni, Marietta, GA) on setting 4 for 2 cycles of 60 s with 60 s on ice in between, followed by centrifugation for 15 min at 10,000 RCF at 4°C. Then, 300 µL of supernatant was transferred to a fresh Eppendorf LoBind centrifuge tube. The extraction was repeated by adding 300 µL of 50 % MeOH to the original sample tubes, homogenized again for 60 s, then centrifuged for 10 min at 10,000 RCF at 4°C. Again, 300 µL of supernatant was transferred to the supernatant collection tube. The supernatant collection tube was centrifuged at
max speed for 5 min, to pellet any insoluble matter that was transferred previously, and 200 μL of supernatant was transferred to a 96-well mass spectrometry (MS) sampling plate (1mL, MASTERBLOCK, Greiner Bio-One).

### 2.1.2 Whole Blood Collection and Serum Isolation

Under isoflurane-induced anesthesia, whole blood was collected by cardiac puncture, followed immediately by euthanasia via cervical dislocation. Cardiac puncture was performed using a 26-gauge needle and a 1 mL heparin-coated syringe. The needle was inserted through the chest wall into the heart and a gentle vacuum was created by drawing on the syringe, allowing blood to flow into the syringe. Whole blood was then deposited from the syringe, gently to prevent cell lysis, into 1 mL microcentrifuge tubes containing 10 μL of heparin, to prevent coagulation, and placed on ice.

To obtain serum, whole blood was centrifuged at 1,500 RCF for 15 min followed by transfer of the supernatant fraction (serum) to a fresh 1.5 mL microcentrifuge tube, taking care to avoid transfer of cellular debris. Serum and the remaining cellular fraction were stored at -80°C until further use.

#### 2.1.2.1 Serum Metabolite Extraction

Metabolites were extracted from serum using a water-methanol extraction method (50 % MeOH). Serum was obtained as previously described (Section 2.1.2). Each sample was thawed on ice and set on a shaker at 250 rpm for 10 min then vortexed briefly. Metabolites were extracted by adding 200 μL of 50 % MeOH to 50 μL of serum, vortexed for 1 min, then
centrifuged at maximum speed (14,600 RCF) for 5 min. The supernatant (200 µL) was then transferred to a 96-well MS sampling plate.

2.1.2.2 Hematocrit Measurement

Hematocrit, the ratio of erythrocytes to total blood volume expressed as a percentage, was determined from fresh whole blood. Pre-calibrated Micro-Hematocrit capillary tubes with heparin (60 mm, VWR, Edmonton, AB) were filled with whole blood and centrifuged at 10,000 RCF for 5 min to separate erythrocytes, leukocytes, and plasma. The ratio of erythrocytes to total blood volume was determined using a micro-hematocrit capillary tube reader.

2.1.3 Tissue Collection

Immediately following euthanasia, the liver, spleen, small intestine, cecum, and colon were removed from each mouse. Each tissue was weighed and the length of the colon was measured, before being immediately placed in microcentrifuge tubes and snap-frozen on dry ice. Tissues were stored at -80°C until further use.

2.1.4 Colonic Tissue Cytokine Extraction and Quantification

While on ice, whole colonic tissue, collected as described previously (Section 2.1.3) was thawed and transferred to a 14 mL polystyrene tube containing 500 µL of cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100) per 50 mg of tissue. Cell lysis buffer was prepared by dissolving 1 cOmplete ULTRA Mini Protease Inhibitor tablet and 1 PhosSTOP Phosphatase Inhibitor tablet (Roche, Mississauga, ON) per 10 mL of lysis buffer immediately before use. Tissues were then homogenized using a Polytron
homogenizer (Polytron PT 10-35, 50-60Hz, Kinematica). Cell lysate was returned to the original Eppendorf tube and centrifuged at 4°C at maximum speed for 5 min. The resulting supernatant was transferred to a fresh microcentrifuge tube.

Lysate protein concentration was determined using the Precision Red Protein Assay (Cytoskeleton, Denver, CO). Advanced Protein Assay Reagent (ADV02, 300 µL) was pipetted into wells of a 96 well microplate, then 10 µL of lysate was added and mixed by pipetting until a colour change was observed. After incubation for 1 min at room temperature absorbance at 600 nm was measured using a SpectraMax Plus 384 microplate reader (VWR, Edmonton, AB). Protein concentration was calculated based on an optical density (OD) of 1.00 at 600 nm equating to 125 µg of protein per mL of ADV02 and a dilution factor of 30 (Protein Concentration = OD * 125 µg/ml * 0.3 mL_{ADV02} * 30_{Dilution Factor}). Each sample was measured in duplicate.

All samples were normalized by protein concentration through dilution with lysis buffer. Aliquots (100 µL) of each sample were frozen at -80°C and sent to Eve Technologies at the University of Calgary for analysis of a panel of 31 cytokines and chemokines, performed using a multiplex immunoassay analyzed with a BioPlex 200 Mouse Cytokine Array/Chemokine Array 32-Plex platform (Eve Technologies – Mouse Cytokine Array/Chemokine Array 31-Plex (MD31)).

2.2 Metabolomics for Untargeted and Targeted Metabolite Detection

Metabolites were extracted from fecal pellets and serum as previously described (Section 2.1.1.1 and 2.1.2.1). Chromatographic separation of compounds was conducted on a Thermo Scientific Vanquish Ultra-high performance liquid chromatography (UHPLC) platform (Thermo
Scientific, Waltham, MA) using either a hydrophilic interaction liquid chromatography (HILIC)-based method (all untargeted and fecal pellet targeted runs) or a reverse phase octadecyl carbon chain bonded silica (C18)-based method (serum targeted run). The HILIC runs used a binary solvent mixture of 20 mM ammonium formate (pH 3.0) in LC-MS grade water (Solvent A) and 0.1 % formic acid (% v/v) in LC-MS grade acetonitrile (Solvent B) in conjunction with a Synchronis HILIC LC column (Thermo Scientific, Waltham, MA). For these runs the following gradient was used at a flow rate of 600 µL/min: 0-2 min, 100 % B; 2-7 min, 100-80 % B; 7-10 min, 80-5 % B; 10-12 min, 5 % B; 12-13 min, 5-100 % B; 13-15 min, 100 % B. For the C18-based run, a mixture of 0.1 % (% v/v) formic acid in LC-MS grade water (Solvent A) and 0.1 % formic acid (% v/v) in LC-MS grade acetonitrile (Solvent B) were used in conjunction with an Accucore Vanquish C18+ UHPLC column (Thermo Scientific, Waltham, MA). The gradient for the C18 method was as follows at a flow rate of 200 µL/min: 0-0.5 min, 20 % B; 0.5-14.5 min, 20-100 % B; 14.5-15.5 min, 100 % B; 15.5-16 min, 100-20 % B; 16-18 min, 20 % B. For all runs the sample injection volume was 2 µL.

Mass spectrometry was conducted using a Thermo Scientific Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA) in negative ion full scan mode (50-750 m/z) at 240,000 resolution with an automatic gain control target of 3x10⁶ and a maximum injection time of 200 ms. A Thermo Scientific Ion Max-S API source outfitted with a HESI-II probe (Thermo Scientific, Waltham, MA) was used to couple the mass spectrometer to the UHPLC platform. Heated electrospray source parameters for negative mode were as follows: spray voltage -2000 V, sheath gas 35 (arbitrary units), auxiliary gas 15 (arbitrary units), sweep gas 2 (arbitrary units), capillary temperature 275°C, auxiliary gas temperature 300°C. Raw data
acquisition was carried out using Thermo Xcalibur 4.0.27.19 software (Thermo Scientific, Waltham, MA).

Peak annotation was performed using the open source software MAVEN\textsuperscript{63,64} and ElMaven\textsuperscript{65} (for Windows and Macintosh operating systems, respectively). For untargeted peak detection, metabolite peaks were compared against an in-house library of 412 compound standards based on UHPLC retention time and MS m/z. High quality peaks that aligned closely to a reference compound were annotated. Ion intensity peak area, retention time, and m/z were recorded. All samples of a given experiment were run and analyzed concurrently to minimize technical variation between runs. For targeted detection, each targeted metabolite was aligned with a corresponding standard, run concurrently with the samples, to both validate identification and allow for determination of concentration using a standard curve.

2.2.1 UHPLC-MS Untargeted Metabolite Analysis

Fecal pellet metabolites were separated and identified using UHPLC-MS (Section 2.2). Using the open source statistical computing software environment R (v3.4.0),\textsuperscript{66} individual metabolites were compared between treatment groups by plotting mean ion intensity peak area against time. Metabolites that were differentially affected between treatment groups were selected for further investigation and statistical analysis.

2.2.2 UHPLC-MS Targeted Metabolite Analysis

Targeted metabolites were identified via alignment of retention time and m/z with concurrently run purified standard compounds. The concentration of targeted MMF derivatives in fecal pellets and serum were determined from ion intensity peak area using standard curves
prepared following a protocol modified from Zegarska et al.\textsuperscript{67} Briefly, stock solutions of purified MPA, MPAG, AcMPAG and GlcA were made using 100 % methanol (MPA, MPAG, and AcMPAG) or 100 % ddH\textsubscript{2}O (GlcA) in concentrations of 400 µM, 200 µM, 100 µM, 50 µM, 10 µM, 5 µM, 1 µM, 0.5 µM, and 0.1 µM. To match the dilution factor of the extracted fecal pellet and serum samples, 50 µL of each standard was diluted with 200 µL of 50 % MeOH and mixed. Each standard was then centrifuge at max speed (14,800 RCF) for 2 min and 200 µL of supernatant was transferred to a 96-well MS sampling plate for measurement by UHPLC-MS, run concurrently with the experimental samples. Ion intensity peak area of each purified standard was plotted against its known concentration and a standard curve was calculated using linear regression. Target metabolite concentration was then calculated using the appropriate curve, using measured ion intensity peak area.

\subsection*{2.3 MMF Administration}

MMF was administered \textit{ad libitum} using chow containing MMF at a concentration of 0.563 % w/w, a concentration previously shown to inhibit lymphocyte proliferation in mice.\textsuperscript{68} MMF-containing chow was produced by grinding whole commercial MMF tablets (CellCept, Roche, Mississauga, ON) to a powder, mixing with powered chow (2016, Teklad Global 16 % Protein Rodent Diet, TD.00217) and forming into pellets (performed by Envigo Teklad Diets, Madison, WI). The Teklad Global 16 % Protein Rodent Diet without MMF was used for control chow. Consumption of both chow and water was monitored daily to ensure there were no significant differences between treatment groups.
2.4 Antibiotic Administration

To assess the ability of various antibiotics to prevent MMF-induced weight loss, either ampicillin (1000 mg/L), metronidazole (1000 mg/L), neomycin (1000 mg/L), or vancomycin (500 mg/L) was administered in the drinking water, consumed *ad libitum*, concurrently with MMF-medicated chow for 8 days. MMF-only and Control groups, receiving either MMF-medicated chow only or control chow only, were also included. Mouse body weight was recorded every 24 h. Daily chow and water consumption were also monitored to ensure no differences between groups were present.

2.4.1 Co-Administration of MMF and Vancomycin

To assess the ability of vancomycin to prevent and reverse MMF-induced weight loss, we administered vancomycin in the drinking water (500 mg/L) *ad libitum*, either concurrently with MMF chow for 8 days (concurrent MMF + Vancomycin, cMV) or after 8 days of MMF treatment, with continuation of MMF treatment (MMF + Vancomycin, MV). Control groups consumed either normal chow (Control) or MMF-medicated chow only (MMF-only). Body weight was recorded every second day, along with chow and water consumption. At the cessation of each trial whole blood and tissues were collected as previously described (Sections 2.1.2 and 2.1.3). Hematocrit was determined as described (Section 2.1.2.2).

2.5 Bacterial Community Analysis

We employed 16S rRNA amplicon sequencing to understand how vancomycin affected the composition of the MMF-treated murine gut microbiome by examining the microbiota of both MMF-only and MV treatment groups. This allowed for both intra- and inter-group
comparisons of each treatment over time. Bacterial DNA for amplicon sequencing was obtained from fecal pellets collected on Days 0, 8, 10, 12, 14, and 16, as previously described (Section 2.1.1). These time points allowed for verification of consistency in the MMF-induced alterations of the gut microbial community between the two treatment groups before the addition of vancomycin (Days 0–8), as well as to examine how vancomycin modifies the MMF altered microbiota of the MV treatment group in comparison to the non-antibiotic treated, MMF-only group (Days 10–16).

2.6 Bacterial Genomic DNA Isolation

Bacterial genomic DNA was isolated from mouse fecal pellets (in fimo) using an adapted version of a protocol developed by the Surette Laboratory.69,70 Pellets previously collected in 2 mL screw-top centrifuge tubes (sterile, DNase and RNase free) and stored at -80°C were placed on ice to thaw. Ceramic beads (3 × 2.8mm) were added to each sample tube, along with 800 µL of monobasic sodium phosphate (monobasic NaPO\textsubscript{4}, 200 mM, pH 8) and 100 µL of guanidium extraction solution (GES). Samples were mechanically lysed using a Mini Beadbeater (Biospec Products, Bartlesville, OK) on the “homogenize” setting for 2 cycles of 3 min with 45 s on ice in between. After, 200 mg of 0.1 mm glass beads were added to each sample, followed by a 3 min cycle of homogenization. Next, enzymatic lysis was performed in two stages. First, 50 µL of lysozyme (100 mg/mL, Sigma-Aldrich, Oakville, ON), 50 µL of mutanolysin (10 U/µL, Sigma-Aldrich, Oakville, ON), and 10 µL of RNase A (10 mg/mL in H\textsubscript{2}O, Qiagen, Toronto, ON) were added to each sample, vortexed, and incubated at 37°C for 1.5 h in a dry bath. Second, 25 µL of sodium dodecyl sulfate (SDS, 25 % in ddH\textsubscript{2}O), 25 µL of Proteinase K (Sigma-Aldrich, Oakville,
ON), and 62.5 μL of aqueous sodium chloride (NaCl, 5 M) were added to each sample, vortexed, and incubated at 65°C for 1.5 h in a dry bath.

A phenol-chloroform extraction, in conjunction with the DNA Clean and Concentrator-25 purification kit (Cedarlane Laboratories, Burlington, ON) purification kit was used to isolate bacterial DNA released during mechanical and enzymatic lysis. First, each screw-top tube was centrifuged at 13,500 RCF for 5 min to separate the cellular debris and beads from the soluble content. Then 900 μL of supernatant was transferred to new 2 mL Eppendorf tubes containing 900 μL of phenol-chloroform-isoamyl alcohol (25:24:1, Sigma-Aldrich, Oakville, ON) and vortexed. These tubes were centrifuged at 13,000 RCF for 10 min to separate the top aqueous layer (containing DNA) from the bottom organic layer (containing proteins and lipids). The aqueous layer was transferred to new 1.5 mL Eppendorf tubes containing 200 μL of DNA binding buffer, ensuring that the organic layer and interface were not transferred, and mixed. This was then transferred 600 μL at a time to a DNA column. The solution was moved through each column by brief centrifugation at 12,000 RCF, discarding the flow-through. Next 200 μL of wash buffer was added to the column and moved through again by brief centrifugation at 12,000 RCF, discarding the flow-through. This wash step was repeated once more before the DNA columns were placed in new, sterile, 1.5 mL Eppendorf tubes. Sterile DNase and RNase free ddH₂O (50 μL, preheated to 65°C) was added to the center of the column and incubated at room temperature for 5 min. Centrifugation of these columns at 12,000g for 1 min eluted the DNA into the Eppendorf tubes for storage. The concentration of DNA in each sample was determined using a Qubit 2.0 Fluorometer (Invitrogen, Waltham, MA) and the DNA product was stored at -20°C.
2.6.1 16S rRNA Variable Region 3-4 (V3-V4) Amplification and Sequencing

To amplify the variable 3 and variable 4 (V3-V4) regions of the 16S rRNA gene using next-generation sequencing (NGS) we utilized a protocol developed by the Surette Laboratory, originally adapted from Bartram et al.\textsuperscript{71} This protocol utilized polymerase chain reaction (PCR)-based amplification using barcode-tagged primers, unique to each sample, allowing for multiplex sequencing on the Illumina MiSeq platform (Table S1.1).

In a sterile PCR workstation, a master mix reaction mixture containing 5 µL PCR buffer (10x, Invitrogen, Waltham, MA), 2 µL bovine serum albumin (BSA, 10mg/mL), 1.5 µL magnesium chloride (MgCl\textsubscript{2}, 50 mM), 1 µL dinucleotide triphosphates (dNTPs, 10 mM, Invitrogen, Waltham, MA), 0.25 µL of Taq polymerase (5 U/µL, Invitrogen, Waltham, MA), 10 µL variable region 4 modified reverse primer (v4Rm2) per sample was prepared. Mastermix (19.75 µL) was distributed to 0.2 mL thin-walled PCR tubes, 1 tube per sample. Sample template DNA (50 ng) was then added to each tube along with 5 µL of V3F barcoded primer (1 µM), using a unique barcoded primer for each sample. The total volume of each reaction mixture was made up to 50 µl total volume using UltraPure distilled water (DNAse and RNAse free, Invitrogen, Waltham, MA), and vortexed to mix.

Each reaction mixture underwent PCR in a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) using the following PCR protocol: an initial denaturation step at 94°C for 30 s was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 47°C for 30 s, and elongation at 72°C for 40 s, followed by a final annealing step at 72°C for 5 min.

Successful amplification of the V3-V4 region was assessed using gel electrophoresis. The presence of a single band of approximately 550 bp, without evidence of contamination or primer-dimer formation indicated successful amplification. If required, PCR was repeated. PCR
products were stored at 4°C and sent to the Nicole Perkins Microbial Communities Core Laboratory at the University of Calgary for library construction and sequencing on the Illumina MiSeq platform as done previously.\textsuperscript{72}

\section*{2.6.2 16S rRNA Amplicon Sequence Processing}

Analysis of 16S rRNA V3-V4 amplicon sequencing was performed using a modification of the Microbiome Helper DADA2 16S Chimerin Tutorial\textsuperscript{73} and the DADA2 Pipeline Tutorial (v1.8).\textsuperscript{74} First, primers were removed from each sequence contained in the demultiplexed sample FASTQ files using Cutadapt (v1.16).\textsuperscript{75} Cutadapt first paired forward and reverse sequences then removed the forward and reverse primers from the appropriate read. Any reads that did not pair, contained insertions or deletions (indels), or were not trimmed due to lacking a primer sequence were removed at this stage. Next, the primer trimmed FASTQ files were imported into the statistical computing software environment R\textsuperscript{66} where the remainder of processing was completed using the R package DADA2 (v1.6.0).\textsuperscript{76}

First, reads were trimmed and filtered by truncating the forward and reverse reads at a length of 260 bp and 240 bp respectively, or at the first instance of a quality score of \( \leq 2 \), followed by removal of any reads that contained ambiguous bases, had more than 2 or 5 expected errors (in the forward or reverse read respectively), or if the read matched the phiX genome. Truncation length was determined by approximating when the average base read quality score dropped below Q20. Next, a parametric error model was developed by alternating between estimating error rates and inference of sample composition until convergence was reached. This was followed by de-replication where identical sequences were combined into unique sequences while the corresponding abundance and quality information was retained. Next, true amplicon
sequence variants (ASVs) were inferred from these unique sequences by comparing the abundance and quality information of the unique sequences against the previously produced error model to determine which sequences are true sequences and which were produced due to sequencing error. Next, paired forward and reverse reads were merged and a sequence table was produced using reads with a length of 400-450 bp (length of the V3-V4 region of the bacterial 16S rRNA gene), followed by removal of chimeric sequences. Finally, taxonomy was assigned using the Silva Taxonomy Training Set (v128)\textsuperscript{77} and the Silva Species Assignment Set (v128).\textsuperscript{77} The resultant composition dataset was passed to the R package Phyloseq (v1.23.1)\textsuperscript{78} where further processing and community analysis was performed.

### 2.6.3 Compositional Analysis

Visual and statistical analysis of amplicon sequencing data was performed using the R packages Phyloseq and Vegan (v2.5-2)\textsuperscript{79}, with support from the R packages dplyr (0.7.5)\textsuperscript{80} and ggplot2 (v3.0.0).\textsuperscript{81} Before commencing analysis or statistical testing, the dataset was cleaned and filtered by removing any non-bacterial reads mapping to the kingdom Archea or Eukaryote, family Mitochondria, or class Chloroplast. Next, any samples with a low read depth (<10,000 reads) were excluded from further analysis to prevent statistical errors from insufficient read depth.

Alpha-diversity (within sample diversity) was measured using the metrics Observed species, Shannon’s Diversity Index, and Simpson Index. Multiple indices were used to ensure a potential bias did not arise from unequal numbers of reads between samples.\textsuperscript{82} Beta-diversity (diversity between samples) was assessed using a non-metric multidimensional scaling (NMDS) plot of Bray-Curtis dissimilarity.
The R package DESeq2 (v1.18.1),\(^\text{83}\) along with wrapper functions from the package microbiomeSeq (v0.1)\(^\text{84}\), was utilized to compute significantly differentially abundant taxa in the vancomycin treated group over time. Deseq2 models taxa abundance using a negative binomial distribution and finds differentially abundant taxa using log2 fold change and an alpha value cut off of 0.05.

Prior to further analysis and visualization of taxa specific differences between samples or treatment groups, reads in each sample were normalized to relative abundance (the ratio of reads mapped to a specific taxon to total number of reads in the sample).

### 2.6.4 Inferred Functional Analysis Using PICRUSt

Currently, PICRUSt (v1.1.3)\(^\text{85}\) (Phylogenetic Investigation of Communities using Reconstruction of Unobserved States) requires amplicon sequencing reads be clustered into operational taxonomic units (OTUs) and not ASVs as were previously produced using DADA2. Therefore, the 16S rRNA V3-V4 sequencing reads were reprocessed following the Microbiome Helper 16S pipeline (v1).\(^\text{73}\) First, read quality was assessed using FastQC (v0.11.5),\(^\text{86}\) then paired-end reads were merged using PEAR (v0.9.10).\(^\text{87}\) Successfully merged reads were filtered by ensuring the sequence contained both the forward and reverse primer, had a quality score of ≥30 over 90 % of the bases, and a maximum length of 400 bp. Any remaining sequences that contained ambiguous bases were removed, then chimeric sequences were removed using VSEARCH (v1.11.).\(^\text{88}\) Sequences were clustered into OTUs using a closed reference OTU picking pipeline that utilizes QIIME (v1.91)\(^\text{89}\) This pipeline used a 97 % similarity cut-off and mapped the OTUs against the Greengenes database (v13_8),\(^\text{90}\) producing an OTU table with associated abundances.
Within PICRUSt, the OTU table was first normalized based on the 16S rRNA copy number of each specific bacterial taxa and then functional predictions of KEGG Orthologes (KOs) were predicted using PICRUSt’s Hidden State Prediction (HSP) algorithm\textsuperscript{85}.

2.7 In Fimo Assay for GUS Enzymatic Activity

To validate our \emph{in silico} predictions of GUS enzymatic activity, we used an assay adapted and modified from the protocol described by Asano et al.\textsuperscript{91} to measure GUS activity \emph{in fimo}. Fecal pellets (collected as previously described in Section 2.1.1 in 2 mL screw-top centrifuge tubes) were thawed on ice. Phosphate buffered saline (PBS, 750µL, 0.01M, pH 7.0) and 3 × 2.8 mm ceramic beads were added to each sample, followed by homogenization with a BeadRuptor 4 (Omni, Marietta, GA) at speed setting 2 for two cycles of 90 s, pausing for ≥1 min on ice in between cycles. Next, the samples were sonicated using a Branson 3510 Water-Bath Ultrasonicator (Branson, Danbury, CT) at 4°C for 30 min. Homogenates were centrifuged at 10,000 RCF for 30 min at 4°C to separate the cellular debris and liquid protein containing fraction. The supernatant was transferred to a fresh 650 µL microcentrifuge tube.

Protein concentration for each sample was determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Briefly, 5 µL of supernatant was added to a 96-well plate, followed by 25 µL of reagent A’ (composed of 1µl Reagent S per 50 µL Reagent A) and 200 µL of Reagent B. Each sample was mixed thoroughly and incubated at room temperature for 15 min before reading absorbance at 705 nm using a SpectraMax Plus 384 microplate reader. Protein concentration was determined using a standard curve of protein standards in concentrations of 1.5 mg/ml, 1.0 mg/ml, 0.8 mg/ml, 0.4 mg/ml, and 0.2 mg/ml, ran in conjunction with the experimental samples.
Activity of extracted bacterial GUS was measured by hydrolysis of phenolphthalein glucuronide (Sigma-Aldrich, Oakville, ON) to free phenolphthalein, an indicator compound that can be quantified by spectrophotometry. A reaction mixture (pH 7.0) containing 100 μL of supernatant, 10 μL of 0.01 M phenolphthalein glucuronide and 50 μL of PBS was incubated in a water bath at 37°C for 1 hour. The reaction was terminated by placing the samples on ice for 5 min followed by the addition of 8 μL of alkaline glycine (1 M, pH 10.2) and 3 μL of 5 % trichloroacetic acid. To measure background absorbance of the reaction mixture, sample blanks were prepared identical to the reaction mixture, except the blanks remained on ice and the alkaline glycine (8 μL, 1 M, pH 10.2) and trichloroacetic acid (3 μL, 5 %) was added immediately to prevent action of the enzyme. Each incubation reaction was done in duplicate.

To calculate concentration from absorbance, phenolphthalein (Phe) standard solutions in concentrations of 10 μM, 7.5 μM, 5 μM, 2.5 μM, 1 μM, 0.5 μM, and 0.1 μM were prepared using 95 % ethanol for a standard curve. For each standard, 10 μL of the appropriate Phe stock solution, or 95 % ethanol for the standard blank, was combined with 140 μL of PBS, 10 μL phenolphthalein glucuronide (0.01 M), 8 μL alkaline glycine (1 M, pH 10.2), and 3 μL trichloroacetic acid (5 %).

A final volume of 50 μL for each reaction mixture, blank, and standard was transferred to a clear, flat bottom 96 well microplate. Absorbance was measured at 550 nm using a SpectraMax Plus 384 microplate reader. GUS activity was calculated as the mass of free phenolphthalein (mg) liberated during incubation per mg of protein in the sample supernatant, per hour of incubation (GUS Activity = mg_Phe/mg_Protein/h).
2.8 *In Vivo* Imaging of GUS Activity

Using a modification of the protocol developed by Chen et al., we performed *ex vivo* tissue imaging of intestinal bacterial GUS activity through quantification of fluorescence following hydrolysis of fluorescein di-β-D-glucuronide (FDGlcU, Sigma-Aldrich, Oakville, ON) to fluorescein. FDGlcU is a non-fluorescent compound, however, removal of the glucuronic acid moieties by GUS produces the fluorescent aglycone fluorescein. Three hours prior to imaging, each animal was administered 100 µL of FDGlcU (7.3 µmol/kg). Immediately before imaging, mice were anaesthetized with isoflurane and all thoracic and abdominal organs were excised and imaged using an In-Vivo Xtreme 4MP imaging platform (Bruker, Billerica, MA) Imaging occurred in three steps: reflectance imaging (2 second exposure time), fluorescent imaging (5 second exposure time, 470 nm excitation and 535 nm emission filters) and X-ray imaging (10 second exposure time). Pixel binning was kept constant at 4×4. Images were acquired and analyzed using Bruker molecular imaging software MI SE (v7.1.3.20550, Bruker, Billerica, MA). GUS activity in Control and MMF-only treated animals was quantified by measuring the mean fluorescence (after background subtraction) in a given region of interest (ROI) which was kept constant over the time period of imaging.

2.9 GUS Activity Screen Against MPAG

A total of 279 unique microbial GUS enzymes, organized into six categories based on the active site-adjacent loop structure, have been identified *in silico*. The differing enzyme structure affects enzyme efficiency based on the three-dimensional structure and chemistry of the substrate. To understand which GUS isoforms are active against MPAG, an *in vitro* screen was performed using 23 purified recombinant GUS enzymes from 20 different species of bacteria.
In vitro assays of GUS activity with MPAG as a substrate were carried out in CoStar UV half area 96-well microplates with UV transparent bottom plates. MPAG (5 µL, 4.0 mM) was added to the 96-well plate, along with 10 µL of 5x reaction buffer (250 mM HEPES, 250 mM NaCl, pH 7.0), and 30 µL of water, followed by mixing and pre-incubation at 37°C for 10 min. The reaction was initiated by adding 5 µL of GUS (300 nM). Reaction was monitored continuously by absorbance at 310 nm for 1 hour using an Infinite M1000 PRO microplate reader (Tecan, San Jose, CA). Resultant progress curves were fit by a custom linear regression analysis program in MATLAB. Initial velocities were normalized by protein concentration (30 nM) to determine the apparent catalytic turnover ($k_{cat}^{app}$).

2.10 Intrarectal (IR) Administration of MPA

To assess the effect of free MPA and GlcA on the colonic epithelium in vivo, we administered each metabolite intrarectally, bypassing the digestive and circulatory systems to avoid conversion to MPAG by hepatic UGTs. Experimental groups consisted of four independent groups: 10 % MPA (17.5 mM), 1 % MPA (1.75 mM), 10 % GlcA (17.5 mM), and Vehicle (50 % DMSO). IR dose was calculated as a percentage of the estimated total metabolite to pass through the colon each day, based on the estimated mass of MMF consumed daily.

Stock solutions of MPA were prepared in 100 % DMSO, then diluted with PBS to treatment concentrations of 17.5 mM and 1.75 mM (10 % MPA and 1 % MPA, respectively) in 50 % DMSO. A stock GlcA solution was prepared in PBS, then diluted with 100 % DMSO to a final concentration of 17.5 mM in 50 % DMSO. DMSO (50 %) in PBS was used as a vehicle control.
IR injections were performed daily, for 8 days, using a 1 cc syringe and a polyethylene catheter (PE-50). With the mouse immobilized, the catheter was lubricated with petroleum jelly and inserted 3-4 cm intrarectally. Then 200 µL of the appropriate treatment solution was slowly injected into the colonic lumen. After removal of the catheter the rectum was held closed for a minimum of 2 min to prevent loss of solution. Body weight of each animal was recorded daily. On Day 9, mice were euthanized and whole blood, spleen, cecum, and colon tissues were collected and colon length measured as previously described (Sections 2.1.2, 2.1.3).

Myeloperoxidase (MPO) activity of the colonic tissue was measured to assess the induction of an innate immune response in each treatment group.

2.11 Myeloperoxidase (MPO) Activity

To assess colonic tissue neutrophil infiltration in mice treated intrarectally with 17.5 mM MPA, 1.75 mM MPA, 17.5 mM GlcA, and 50 % DMSO we measured the activity of MPO in whole colonic tissue. Colonic tissue was collected as described previously (Section 2.1.3) in pre-weighed tubes. While on ice, the tissue was transferred to a 14 mL polystyrene tube (VWR, Edmonton, AB) and 40 µL of hexadecyltrimethylammonium buffer (HTAB, 5 g of hexadecyltrimethylammonium (Sigma-Aldrich, Oakville, ON) in 1 L of 50 mM potassium phosphate buffer, pH 6.0) was added per mg of tissue. Each sample was homogenized using a Polytron homogenizer. The homogenate was transferred to original collection tube and centrifuged at 14,000 RCF for 5 min at 4°C then 14 µL of supernatant was transferred to a 96 well microplate. MPO (from human polymophonuclear leukocytes, Calbiochem, Etobicoke, ON) standard solutions (14 µL of 0.8 U/mL, 0.4 U/mL, 0.2 U/mL, 0.1 U/mL, and 0.05 U/mL in ddH2O) were added to the microplate. The MPO substrate, O-dianisidine (Sigma-Aldrich,
Oakville, ON), was prepared in potassium phosphate buffer (50 mM, pH 6.0) at a concentration of 1.67 mg/mL, then diluted with ddH₂O to a final concentration of 0.67 mg/mL. Immediately before use, 1% H₂O₂ was added to the O-dianisidine buffer (0.5 µL/mL). Lastly, 200 µL of the O-dianisidine buffer was added to each sample and MPO standard and absorbance was measured immediately at 450 nm using a SpectraMax Plus 384 microplate reader. Activity was calculated from absorbance based on the standard curve produced from the MPO standard solutions. Each measurement was completed in replicates of 5.

2.12 Statistical Analysis

All statistical analysis was conducted using the statistical computing software R⁶⁶ in conjunction with a number of open-source R packages.⁷⁸–⁸¹,⁸³,⁸⁴,⁹⁴–⁹⁹ Data were expressed as either mean ± standard error of the mean (SEM) or as median with first and third quartiles (boxplot). Where possible, individual data points were included in plots expressing mean ± SEM to allow for visualization of variance. For each set of data, normality was determined using the Shapiro-Wilk Normality Test. Means of parametric data with > 2 treatment variables compared at a single time point were compared using a one-way ANOVA. Significant ANOVA results were followed by the Tukey Honest Significant Differences post hoc test. Means of non-parametric data with > 2 treatment variables compared at a single time were compared using either pairwise Wilcoxon Rank Sum Tests with p-value correction for multiple testing using the Benjamini-Hochberg procedure, or the Kruskal-Wallis Rank Sum Test, followed by Dunn’s Kruskal-Wallis Multiple Comparisons post hoc test, if significant. To compare means of two treatment groups over time, a two-way ANOVA was used, followed by the Tukey Honest Significant Differences post hoc test, if significant. If the two-way ANOVA residuals were non-
parametric the significance level was lowered from 0.05 to 0.01. Intergroup significance in beta-diversity was computed using the Adonis test, a permutational multivariate analysis of variance (PERMANOVA) test, applied pairwise, using the Benjamini-Hochberg procedure for p-value correction. Significant results were verified by ensuring the dispersions of each group were not statistically different. For data with more than 2 treatment groups, each experimental group mean was compared pairwise against the control group mean using either Welch’s t-test (parametric data) or Wilcoxon Rank Sum test (non-parametric data) with multiple test correction using the Benjamini-Hochberg procedure. A p-value < 0.05 was considered significant except for the assessment of colonic tissue cytokines and chemokines in MMF-only and MV treated animals, and the pairwise group comparisons of Observed species alpha diversity and beta-diversity between MMF-only and MV treated animals, where a p value < 0.1 was considered significant.
CHAPTER THREE: RESULTS

3.1 Comparative Efficacy of Individual Antibiotics in Preventing MMF Toxicity

We assessed the preventative capabilities of individual antibiotics (vancomycin, metronidazole, ampicillin, and neomycin) from the previously used broad-spectrum cocktail, using body weight loss (relative to Day 0) as a marker of toxicity. The Vancomycin + MMF treated group was most similar to the control animals with respect to mean percent body weight, after 8 days of concurrent MMF and antibiotic treatment (103.2 % ± 0.68 % vs 98.84 % ± 0.82 %, p = 0.41). As well, the vancomycin-treated mice appeared to maintain their body weight most consistently.

Animals treated with metronidazole, ampicillin, or neomycin lost more weight, were less similar to the control animals, and were observed to have a steady decline in body weight over the 8-day period. In order of effectiveness (lowest incurred weight lost) were neomycin- (95.36 % ± 0.83 %, p = 0.19), ampicillin- (87.98 % ± 1.1 %, p = 0.062), and metronidazole- (74.89 % ± 1.3 %, p = 0.0012) treated groups. The MMF-only group had a mean percent body weight of 80.36 % ± 1.9 % after 8 days of exposure (Figure 3.1).
Figure 3.1. Changes in percent body weight relative to Day 0 of C57BL/6 mice treated concurrently with MMF and one of either ampicillin (1 g/L), metronidazole (1 g/L), neomycin (1 g/L), or vancomycin (500 mg/L) in the drinking water for 8 days. Controls were fed either MMF-containing chow (MMF-only) or control chow (Control) only. Data represent mean ± SEM with n = 4 for all groups except vancomycin (n = 5). Intergroup comparisons were performed for Day 8 using the Kruskal-Wallis multiple comparisons test, followed by Dunn’s post hoc test, with Benjamini-Hochberg multiple test correction. Weights on Day 8 were most similar to Controls for those animals treated with vancomycin (p = 0.41) and neomycin (p = 0.19).
3.2 Vancomycin is Sufficient to Prevent and Reverse MMF-Induced Weight Loss

Based on these data, we determined vancomycin to be the most effective individual antibiotic in preventing MMF-induced weight loss in our mouse model. Therefore, we continued to assess the physiological parameters associated with MMF and vancomycin treatment, as compared to MMF-only treated animals.

3.2.1 Reversal of MMF-Associated GI Toxicity by Vancomycin

Given that vancomycin can prevent much of the weight loss associated with MMF treatment, we sought to determine if vancomycin is also able to reverse MMF-induced weight loss. After 8 days of MMF exposure, vancomycin restored mouse body weight from 75.02 % ± 1.5 % of initial weight to 93.82 % ± 1.2 % within 8 days, despite continuation of MMF treatment. While these mice were still significantly smaller than control treated animals at 16 days (105.7 % ± 1.5 %, p = 0.0030), it represented a significant improvement over the MMF-only group (69.26 % ± 0.93 %, p = 0.014) (Figure 3.2).
Figure 3.2. Changes in percent body weight relative to Day 0 for C57BL/6 mice treated with either control chow (Control, n = 5), MMF (MMF-only, n = 4) or MMF and vancomycin (MV, n = 9). Vancomycin treatment began after 8 days of MMF-only treatment. Data represent two combined separate experiments (MV vs. Control and MV vs. MMF-only) and are shown as mean ± SEM. Intergroup comparisons were performed for Day 16 using multiple Wilcoxon Sum Rank Tests with Benjamini-Hochberg multiple test correction. On day 16, the MV animal group was significantly different from both the MMF-only and control groups. P < 0.05 denoted by ‘*’, p < 0.005 denoted by ‘**’.
3.2.2 Physiological Parameters

We collected cecum, colon, liver, and spleen tissues from Control (n = 5), MMF + Vancomycin (MV, n = 9), and MMF-only (n = 4) treatment groups to assess the effect of vancomycin on individual tissues. In MV mice, cecum (p = 0.99), liver (p = 0.80), and spleen (p = 0.85) weights were not found to be statistically different from Control mice. This contrasted with the MMF-only animals, where all three tissues were found to be smaller than in MV animals (cecum, p = 0.015; liver, p = 0.0026; spleen, p = 0.0030) (Figure 3.3). Additionally, colon length improved significantly with the addition of vancomycin, as compared to MMF-only treated (p = 0.0033) and was not different in length compared to control animals (p = 0.19) (Figure 3.4A). Hematocrit did not improve with the addition of vancomycin as compared to MMF-only animals (p = 0.60), and remained significantly lower than controls (p = 0.015) (Figure 3.4B).

These data are representative of two pooled experiments; one experiment composed of Control animals (n = 5) and MV animals (n = 5), and the second composed of MMF-only animals (n = 4) and MV animals (n = 4). Table 3.1 contains measurement statistics for each physiological parameter.
Figure 3.3 Weights of cecum, liver, and spleen tissues from C57BL/6 mice treated with either control chow (Control, \( n = 5 \)), MMF (MMF-only, \( n = 4 \)), or MMF and vancomycin (introduced after 8 days of MMF treatment, MV, \( n = 9 \)). Data have been combined from two separate experiments (MV vs. Control and MV vs. MMF-only) and are shown as mean ± SEM. Intergroup comparisons were performed using a one-way ANOVA followed by Tukey’s Honest Significance Differences post hoc test with \( p < 0.05 = \text{‘*’} \) and \( p < 0.005 \) denoted by ‘**’. ns = p > 0.05.
Figure 3.4. Colon length (A) and hematocrit (B) of C57BL/6 mice treated with either control chow (Control, n = 5), MMF (MMF-only, n = 4) or MMF and vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 9). Data represent mean ± SEM and are combined data from two separate experiments (MV vs. Control and MV vs. MMF-only). Intergroup comparisons were performed using a one-way ANOVA followed by Tukey’s Honest Significance Differences post hoc test (A) or Kruskal-Wallis multiple comparison test, followed by Dunn’s post hoc test, with Benjamini-Hochberg multiple test correction (B). p < 0.05 denoted by ‘*’ and ns = p > 0.05.
Table 3.1. Physiological parameters of mice treated with either control chow (Control, n = 5), MMF-only (n = 4) or MMF and vancomycin (introduced following 8 days of MMF-only treatment, MMF + Vancomycin, n = 9). Data are a combination of two separate experiments (MV vs. Control and MV vs. MMF-only).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 5)</th>
<th>MMF-only (n = 4)</th>
<th>MMF + Vancomycin (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecum Weight (mg)</td>
<td>350.7 (16.72)</td>
<td>245.9 (32.75)</td>
<td>353.8 (19.73)</td>
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<td>Liver Weight (mg)</td>
<td>905.4 (37.21)</td>
<td>553.6 (99.11)</td>
<td>965.9 (61.73)</td>
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<tr>
<td>Spleen Weight (mg)</td>
<td>71.5 (3.935)</td>
<td>30.33 (4.470)</td>
<td>66.87 (6.309)</td>
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<tr>
<td>Colon Length (cm)</td>
<td>7.75 (0.0645)</td>
<td>8.40 (0.100)</td>
<td>8.90 (0.212)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47 (0.37)</td>
<td>23 (2.5)</td>
<td>24 (1.9)</td>
</tr>
</tbody>
</table>
3.2.3 Changes in Colonic Cytokines and Chemokines

Using a 31-plex array, we measured the concentration of cytokines and chemokine in whole colonic tissue homogenates from MMF-only (n = 4) and MMF + Vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 4) treatment groups. Vancomycin was found to have a large effect on the overall cytokine/chemokine profile of the MMF-treated mice (Figure 3.5). Specifically, it was found to have significantly reduced the concentration of 7 cytokines, G-CSF, IL-6, KC, LIF, M-CSF, MIP-2, and TNFα (p < 0.1) as compared to MMF-only animals (Figure 3.6). Of these, 4 cytokines (KC, MIP-2, G-CSF and IL-6) were previously found to be significantly upregulated due to MMF treatment when compared to Control animals. In addition, four cytokines (IL-2, IP-10, MIG, and RANTES) were upregulated in MV animals when compared to MMF-only animals (p < 0.1) (Figure 3.7).
Figure 3.5. Heatmap of cytokines and chemokines from whole colonic tissue homogenates of mice treated with either MMF (MMF, n = 4) or MMF + Vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 4). Tile colour represents Z-transformed cytokine/chemokine concentration, applied prior to clustering. Cytokines with missing measurements were removed prior to clustering. Cytokines/chemokines significantly differentially affected by vancomycin are denoted by ↑ (upregulated) or ↓ (downregulated) (p < 0.1). Pairwise comparisons between groups for each cytokine/chemokine performed using multiple Wilcoxon Rank Sum Tests, with Benjamini-Hochberg multiple test correction.
Figure 3.6. Data from Figure 3.5 showing those colonic tissue cytokines that were significantly decreased (p < 0.1) in mice treated with MMF and Vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 4) as compared to mice treated only with MMF (MMF-only, n = 4). Pairwise comparisons between groups for each cytokine performed using multiple Wilcoxon Rank Sum Tests, with Benjamini-Hochberg multiple test correction.
Figure 3.7. Data from Figure 3.5 showing those colonic tissue cytokines that were significantly increased (p < 0.1) in mice treated with MMF and Vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 4) as compared to mice treated only with MMF (MMF-only, n = 4). Pairwise comparisons between groups for each cytokine performed using multiple Wilcoxon Rank Sum Tests, with Benjamini-Hochberg multiple test correction.
3.2.4 Untargeted Metabolomics

To assess the impact of MMF-only and MMF + Vancomycin on the murine gut metabolome, we used UHPLC-MS to identify and compare fecal pellet metabolites of Control vs. MMF-only and MMF-only vs MV treatment groups. In the Control vs. MMF-only trial, 106 metabolites were successfully annotated from our library of 412 validated compounds. Of these, 37 appeared to be differentially affected in the MMF-only group as compared to Controls by visual inspection. In the MMF-only vs. MV trial, 73 metabolites were successfully annotated. Of these, 23 appeared to be differentially affected in the MV group as compared to the MMF-only group by visual inspection. Of these differentially affected metabolites, guanosine, inosine, and stachyose were selected for further analysis. The ion intensity peak area (a proxy for ion concentration) of both guanosine and inosine decreased significantly following the addition of vancomycin ($p < 0.05$ and $p < 0.005$, respectively) while stachyose significantly increased ($p < 0.0005$). (Figure 3.8)
Figure 3.8. Selected fecal pellet metabolites differentially affected in mice treated with MMF-containing chow and vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 4) compared to mice treated with MMF-containing chow only (MMF-only, n = 4) from an untargeted metabolomics screen. Metabolites identified and measured using UHPLC-MS and an in-house library of 412 standard compounds. Intergroup comparisons were performed using a two-way ANOVA followed by Tukey’s Honest Significance Differences post hoc test with p < 0.05 denoted by ‘*’, p < 0.005 denoted by ‘**’ and p < 0.0005 denoted by ‘***’.
3.2.5 16S rRNA Sequencing

To determine which bacteria were affected by vancomycin in the GI tract of MMF-treated mice and how the bacterial landscape as a whole was affected, we employed 16S rRNA amplicon sequencing.

Vancomycin was found to have broad sweeping effects on the abundance and composition of the gut microbiota. Alpha-diversity (within sample diversity) was significantly decreased after the addition of vancomycin in the MV group compared to MMF-only as measured by Observed species (277.0 vs. 548.8, p = 2.8x10⁻⁴), Shannon (1.424 vs. 3.828, p = <1.0x10⁻⁷), and Simpson (0.5923 vs. 0.9191, p = 8.0x10⁻⁴) diversity indices on day 10, only 2 days after starting vancomycin administration. The Observed species index continued to be significantly reduced on Day 12, but not on Day 14 or 16. However, the Shannon and Simpson measures of alpha-diversity were both significantly lower in the MV group on all days after initiating vancomycin administration. There were no significant differences between either group on Days 0 or 8, the time-period where both groups consumed only MMF chow (Figure 3.9).
Figure 3.9. Results for Observed species, Shannon diversity and Simpson’s diversity indices measuring alpha-diversity of fecal microbiota in mice treated with either MMF (MMF, n = 2-4) or MMF and vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 4). Bacterial community composition was determined using 16S rRNA amplicon sequencing of bacterial genomic DNA extracted from mouse fecal pellets. Significant differences in diversity between groups was determined using a two-way ANOVA and Tukey’s Honest Significant Difference post hoc test with p < 0.1 denoted by ‘*’, p < 0.01 denoted by ‘**’, p < 0.001 denoted by ‘***’ for Observed diversity and p < 0.05 denoted by ‘*’, p < 0.005 denoted by ‘**’, p < 0.0005 denoted by ‘***’ for the Shannon and Simpson’s indices.
Vancomycin caused the MV bacterial community to be significantly distinct from the MMF-only bacterial community, as observed using a non-metric multidimensional scaling (NMDS) plot of Bray-Curtis dissimilarity, a measure of beta-diversity (between sample diversity). This effect was consistent across biological replicates as all MV samples clustered closely together, distinct from MMF-only samples, as early as day 10, and continued to become more different (p ≤ 0.1) despite no significant differences between the MMF-only and MV groups on Days 0 or 8 (start and end of MMF-only phase) (Figure 3.10).
Figure 3.10. Non-metric multidimensional scaling (NMDS) plots of Bray-Curtis dissimilarity of the fecal microbiota in mice treated with either MMF (MMF-only, n = 2-4) or MMF and vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 4). Bacterial community composition was determined using 16S rRNA amplicon sequencing of bacterial genomic DNA extracted from fecal pellets. Each data point represents an individual sample. Missing data points are due to removal of samples prior to analysis. Intergroup comparisons were performed for each time point using Adonis in R, a type of permutational multivariate analysis of variance, with Benjamini-Hochberg multiple test correction. p < 0.1 denoted by ‘*’. 
At the phyla level, we found both groups to be dominated by Firmicutes and Bacteroidetes, accompanied by a substantial abundance of Proteobacteria, after the initial 8 days of MMF treatment. However, after only 2 days of vancomycin exposure, a near complete depletion of Bacteroidetes, accompanied by an expansion in Firmicutes and Proteobacteria was observed in the MV animals. Following continued treatment, the gut of the MV mice became completely dominated by Firmicutes, with a small abundance of Proteobacteria remaining. The gut bacterial composition of the MMF-only group was relatively stable over the treatment period, but an increase in Proteobacteria accompanied by a decrease in Firmicutes was observed over time. (Figure 3.11)
Figure 3.11. Stacked bar plots showing mean relative abundance of microbial phyla in fecal pellets collected from mice treated with either MMF (MMF-only, n = 2-4) or MMF and vancomycin (introduced after 8 days of MMF-only treatment, MMF + Vancomycin, n = 4). Bacteria were identified using 16S rRNA amplicon sequencing of bacterial genomic DNA extracted from fecal pellets.
At the class level, we see that MMF (Days 0 – 8 in both groups) led to an expansion in Gammaproteobacteria, leading to a composition composed predominately of Bacilli, Bacteroidia, Clostridia, Erysipelotrichia, and Gammaproteobacteria. After the addition of vancomycin, the Bacteroidia, Clostridia, and Erysipelotrichia taxa were severely diminished, while Bacilli and Gammaproteobacteria flourished. By Day 16 the MV bacterial community was composed almost entirely of Bacilli, with a small portion of Gammaproteobacteria remaining, as compared to the MMF-only bacterial community which was composed primarily of Bacteroidia, Clostridia and Gammaproteobacteria taxa, with a small proportion of Bacilli (Figure 3.12).
Figure 3.12. Stacked bar plots showing mean relative abundance of microbial classes in fecal pellets collected from mice treated with either MMF (MMF-only, n = 2-4) or MMF and vancomycin (introduced after 8 days of MMF-only treatment, MMF + Vancomycin, n = 4). Bacteria were identified using 16S rRNA amplicon sequencing of bacterial genomic DNA extracted from fecal pellets.
Using the R package DESeq2 to statistically compare relative taxa abundance at the class level we found the taxa Bacteroidia, Clostridia, Erysipelotrichia, Verrucomicrobiae, and Mollicutes to be significantly depleted after the addition of vancomycin. Of these, only Bacteroidia, Clostridia, Erysipelotrichia were in relatively high abundance in the MMF-only animals compared to MV animals. The classes Bacilli and Betaproteobacteria were significantly increased after the addition of vancomycin. Gammabacteria increased initially (Day 10) but then returned to pre-vancomycin abundance by Day 16 (Figure 3.13).
Figure 3.13. Data from Figure 3.12 showing relative abundance of microbial classes found to be significantly differentially affected due to vancomycin treatment as compared to MMF-only treated animals. Bacteria were identified using 16S rRNA amplicon sequencing of bacterial genomic DNA extracted from murine fecal pellets. Differential abundance was determined using the R packages microbiomeSeq and DESeq2 by computing log2 fold change of relative abundance and using a cutoff value of $p < 0.05$. 
Considering the differentially depleted classes (Bacteroidia, Clostridia, and Erysipelotrichia), the genera that were both significantly differentially affected by vancomycin, and in relatively high abundance in MMF-only animals after 8 days of MMF treatment were *Bacteroides, Lachnospiraceae NK4A136 group, Roseburia*, and *Turicibacter*. Only successfully annotated genera (ASVs that were mapped to the SILVA database at the genus level) were included in calculating differential abundance (Figure 3.14). Of these genera, only *Bacteroides*, and *Lachnospiraceae NK4A136 group* had ASVs resolved to the species level. The most abundant of these bacteria were *Lachnospiraceae NK4A136 group bacterium, Bacteroides vulgatus, Bacteroides fragilis, Bacteroides cacae, Bacteroides uniformis, Bacteroides ovatus, Bacteroides nordii*, all of which underwent temporal variation in MMF-only animals, and were completely eliminated in MV animals, post-vancomycin (Figure 3.15).
Figure 3.14. Relative abundance of microbial genera from the bacterial classes Bacteroidia, Clostridia, and Erysipelotrichia which were found in relatively high abundance in MMF-treated mice and differentially affected by vancomycin treatment. Bacteria were identified using 16S rRNA amplicon sequencing of bacterial genomic DNA extracted from fecal pellets of mice treated with MMF (MMF-only, n = 2-4) or MMF and vancomycin (introduced after 8 days of MMF-only treatment, MMF + Vancomycin, n = 4). Differential abundance was determined using the R packages microbiomeSeq and DESeq2 by computing log2 fold change of relative abundance and using a cutoff value of p < 0.05.
Figure 3.15. Relative abundance of bacterial species from the genera *Bacteroides* and *Lachnospiraceae_NK4A136_group* found previously to be significantly reduced in mice treated with MMF and vancomycin (introduced after 8 days of MMF-only treatment, MMF + Vancomycin, n = 4) as compared to mice treated only with MMF (MMF-only, n = 2-4). Bacteria were identified using 16S rRNA amplicon sequencing of bacterial genomic DNA extracted from fecal pellets.
3.3 *In Silico* Predictions of GUS Genetic Content

We examined the proportional abundance of the bacterial GUS gene in Control, MMF-only, and MV treatment groups using the open source software PICRUSt. We found the predicted proportional abundance of GUS enzyme orthologs (KEGG Orthology: K01195, KEGG Enzyme: EC 3.2.1.31), to be comparable between groups before commencing MMF treatment. After 4 days of MMF treatment, the MMF-only group had an increase in the predicted proportional abundance of GUS enzyme orthologs (Day 0 - 4, p = 0.0057). From Days 4 to 8, the proportional abundance in the MMF-only group dropped slightly, but on Day 8 was not statistically different from Day 4 (p = 0.52), though it was also not statistically different from Day 8 Controls (p = 0.74). After removal of MMF, the predicted proportional abundance of GUS enzyme orthologs initially increased, but then returned to control levels by Day 16 (p = 0.48) (Figure 3.16).
**Figure 3.16.** PICRUSt-predicted proportional abundances of fecal GUS (KEGG Orthology: K01195; KEGG Enzyme: EC 3.2.1.31) in mice receiving control chow (Control, n = 4-6) or MMF (MMF-only, n = 4-6). MMF was removed on Day 8 to assess recovery from MMF-induced GI toxicity until Day 16 (Recovery, n = 4-6). Bars represent mean ± SEM and dots are individually recorded measurements. Intergroup comparisons over each time point were performed using a two-way ANOVA (P < 0.0005 = ‘***’).
Vancomycin reduced the predicted proportional abundance of GUS enzyme orthologs after only 2 days (Day 10). By Day 16 (8 days of vancomycin), this was reduced to nearly undetectable levels, despite continuation of the MMF treatment ($p = 1.2 \times 10^{-7}$). During the initial MMF-only treatment phase (Days 0 – 8), there were no differences between the MMF-only and the MV groups ($p = 0.33$) (Figure 3.17).
Figure 3.17. PICRUSt-predicted proportional abundances of fecal GUS (KEGG Orthology: K01195; KEGG Enzyme: EC 3.2.1.31) in mice treated with MMF (MMF-only, n = 2-4) or MMF and vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 4). Bars represent mean ± SEM and dots are individually recorded measurements. Intergroup comparisons over each time point were performed using a two-way ANOVA (P < 0.0005 = ‘***’).
3.4 In Fino Measurement of GUS Activity

We used a functional assay to measure GUS enzyme activity from fecal pellets and validate our in silico predictions. As predicted, MMF induced an increase in GUS activity over the first 4 days of treatment (MMF-only, Days 0 – 4, p = 0.027), followed by a slight decrease in activity from Days 4 – 8. Activity on Day 8 was not statistically different than on Day 4 (p = 0.93). Similar to the PICRUSSt prediction, activity was not statistically different between groups on Day 8 (p = 0.32) although there was a great amount of biological variation within the MMF-only group (Figure 3.18).
Figure 3.18. *In fimo* bacterial GUS activity in C57BL/6 mice receiving either control chow (Control, \( n = 6 \)) or MMF-containing chow (MMF-only, \( n = 5 \)). For extraction, fecal pellets were subjected to mechanical lysis and centrifugation. Bacterial GUS activity was measured by the rate of hydrolysis of phenolphthalein \( \beta \)-D-glucuronide. Intergroup comparisons for each time point were performed using a two-way ANOVA with \( p < 0.05 \) denoted by `*'.
3.5 *In Vivo* Fluorescence Imaging of GUS Activity

To localize GUS activity within the GI tract, we leveraged an *in vivo/ex vivo* fluorescence imaging technique developed by Chen et al.\textsuperscript{92} Imaging of *ex vivo* thoracic and abdominal organs showed that GUS activity took place solely in the GI tract and was localized almost exclusively to the cecum and colon (Figure 3.19). There were no significant differences in the levels of GUS activity in the ceca of the two groups, however, the MMF-only group showed a significantly higher level of activity in the proximal colon (Figure 3.20).
Figure 3.19. *Ex vivo* fluorescence imaging of the GI tract of control mice (A) and mice receiving MMF-containing chow (B). Intensity of fluorescence denotes the luminal concentration of fluorescein liberated from FDGlcU, an indicator of GUS activity. No differences were observed in the cecal activity (green ROI) between the two groups, however, activity was significantly increased in the proximal colon (red ROI) of the MMF-treated group. p < 0.05.
**Figure 3.20.** Fluorescence intensity of liberated fluorescein from FDGlcU indicating GUS activity in the cecum and proximal colon regions of interest of mice receiving either control chow (Control, n = 5) or MMF-containing chow (MMF, n = 5) from Figure 3.18. Data represent mean ± SEM. No differences were observed in the cecal activity between the two groups, however, activity was significantly increased in the proximal colon of the MMF-only group. p < 0.05 denoted by ‘*’.
3.6 GUS Activity Screen Against MPAG

An in vitro screening assay to assess the ability of different structural isoforms of bacterial GUS enzymes to metabolize MPAG to MPA and GlcA. Of the 23 isoforms tested, 16, each from a different species of bacteria, were able to catabolize MPAG, with varying rates of enzymatic activity. These 16 GUS isoforms fell into the structural categories of no-loop, loop 1, loop 2, and mini-loop 1. The Bacteroides genera appeared to be the most effective with GUS enzymes from 5 different species (B. uniformis, B. ovatus, B. nordii, B. fragilis, and 1 unannotated) displaying activity against MPAG (Figure 3.21).
Figure 3.21. Results from a preliminary screen showing the activity of different GUS isoforms against MPAG. Each isoform is represented by its bacterial producer and its structural classification (L1 = Loop 1; L2 = Loop 2; mL2 = mini-loop 2; NL = No Loop). Height of bar denotes the rate of apparent catalytic turnover ($k_{\text{cat}}^{\text{app}} (s^{-1})$) of MPAG to MPA. Data reproduced with permission from the laboratory of Dr. Matthew Redinbo (unpublished).
3.7 Targeted Metabolomic Quantification of Fecal and Serum MMF Metabolites

We assessed concentrations of the MMF metabolites MPAG, AcMPAG and GlcA in fecal pellets and serum of MMF-only and MV mice using UHPLC-MS. It is important to note, in this experiment, that the UHPLC technique used for fecal metabolite separation (HILIC-method) did not separate MPAG and AcMPAG. This metabolite is reported as MPAG, but is likely composed of both MPAG and AcMPAG. In serum however, the C18-method for UHPLC was used, and successfully separated MPAG and AcMPAG.

During the MMF-only phase in both groups (Days 0 – 8), we observed a relatively high average concentration of MPA in fimo (MMF-only; 35.2 µM ± 4.13 µM, MV; 33.73 µM ± 4.50 µM) along with a relatively low average concentration of MPAG (MMF-only; 1.85 µM ± 0.476 µM, MV; 1.53 µM ± 0.467 µM). After the addition of vancomycin, there was a significant increase in the average amount of MPAG (16.01 µM ± 0.923 µM, p = 0.013), with a significant decrease in average MPA (4.27 µM ± 0.725 µM, p < 1x10^{-7}) (Figure 3.22). There was not a significant difference in GlcA concentration between either group over the treatment period, although the concentration of GlcA on Day 4 in the MV group was transiently increased.

In serum, there were no significant differences between treatment groups for any metabolite on day 16. AcMPAG was not detected in the serum with this assay. (Figure 3.23).
Figure 3.22. Fecal pellet concentrations of the MMF metabolites MPAG/AcMPAG (MPAG), MPA, and glucuronic acid (GlcA) of mice treated with MMF-containing chow only (MMF-only, n = 4) or MMF-containing chow and vancomycin (introduced after 8 days of MMF only treatment, MMF + Vancomycin, n = 4) as measured using UHPLC-MS. Concentrations were determined from ion intensity peak areas using standard curves for each individual metabolite. Data represent mean ± SEM. The mean concentration of each metabolite before (Days 0 – 8) and after (Days 10 – 16) the introduction of vancomycin was compared independently in each treatment group using a two-way ANOVA with P < 0.0005 denoted by ‘***’.
Figure 3.23. Serum concentration of the MMF metabolites AcMPAG, GlcA, MPA and MPAG from mice treated with MMF-containing chow only (MMF-only, n = 4) or MMF-containing chow and vancomycin (introduced after 8 days of MMF-only treatment, MV, n = 4) as measured using UHPLC-MS. Concentrations were determined from ion intensity peak areas using a standard curve for each individual metabolite. Data represent mean ± SEM. There were no significant differences between treatment groups for any metabolite as determined using a Wilcoxon Rank Sum Test with a Benjamini-Hochberg multiple test correction. AcMPAG was not detected using this assay.
3.8 Direct Induction of GI Inflammation

MPA was administered directly into the colonic lumen of untreated mice via intrarectal injection. We observed a statistically significant loss in body weight in the 10% MPA group with respect to the vehicle control group (p = 0.0024). As well, although not statistically significant, there appears to be a trend towards a dose-dependent response as the 1% MPA group lost weight compared to the Vehicle Control group (105.6% +/- 2.8% vs. 109.2% +/- 0.40%), but did not lose as much weight as the 10% MPA group (101.9% +/- 0.47%) (Figure 3.24).

There were no significant differences found in tissue weight or colon length in the MPA or GlcA treated animals (Figure 3.25).

To assess inflammation, we also measured MPO activity in the colonic tissue of each treatment group. Although there were no significant differences between any of the three treatment groups (10% MPA, 1% MPA, GlcA) and the vehicle control group, there appeared to be a positive trend towards increased MPO activity in mice treated with MPA, but not GlcA (Figure 3.26).
Figure 3.24. Mean body weights of mice administered 1 % MPA (1.75 mM), 10 % MPA (17.5 mM), 10 % GlcA (17.5 mM) or 50 % DMSO (Vehicle) daily for 8 consecutive days. Percentage of administered MPA or GlcA is based upon the estimated concentration of MMF consumed daily in MMF treated animals. Bars represent mean ± SEM. Each treatment group was compared against the vehicle control using a Welch Two Sample t-test followed by the Benjamini-Hochberg procedure to correct for multiple comparisons and with P < 0.05 denoted by ‘*’.
**Figure 3.25.** Mean tissue weights from mice administered a daily dose of 1 % MPA (1.75 mM), 10 % MPA (17.5 mM), 10 % GlcA (17.5 mM), or 50 % DMSO (Vehicle) for 8 consecutive days. Percentage of administered MPA or GlcA is based upon the estimated concentration of MMF consumed daily in MMF treated animals. Bars represent mean ± SEM. Each treatment group was compared against the vehicle control using a Wilcoxon Rank Sum Test followed by the Benjamini-Hochberg procedure to correct for multiple comparisons. No significant differences were found between the groups.
Figure 3.26. Myeloperoxidase (MPO) activity of whole colonic tissue homogenates from mice administered a daily dose of 1 % MPA (1.75 mM), 10 % MPA (17.5 mM), 10 % GlcA (17.5 mM), or 50 % DMSO (Vehicle) for 8 consecutive days. Percentage of administered MPA or GlcA is based upon the estimated concentration of MMF consumed daily in MMF treated animals. Bars represent mean ± SEM. Each treatment group was compared against the vehicle control using a Wilcoxon Rank Sum Test followed by the Benjamini-Hochberg procedure to correct for multiple testing. No significant differences between the groups were found.
CHAPTER FOUR: DISCUSSION

MMF is frequently utilized for immune suppression post-transplantation, however, patients commonly experience dose limiting side effects including diarrhea, weight loss, abdominal pain, colitis and, rarely, an inflammatory bowel disease-like phenotype. The etiology of MMF-induced GI toxicity is not well understood, however, recent work has shown the microbiome is a required component for its development. This thesis developed a hypothesis that could explain MMF-induced GI toxicity and examined the ability of a single antibiotic, vancomycin, to both prevent and reverse MMF-induced GI toxicity in an established murine model. The effect of vancomycin on the intestinal microbiome in MMF-treated mice, and the relationship between these microbes and MMF toxicity was explored. In addition, we explored bacterial GUS catabolism of the MMF derivative MPAG producing increased concentrations of MPA in the colon as a mechanism for MMF-induced GI toxicity. We found that vancomycin eliminated many GUS-producing bacteria, reduced GUS activity and lowered the concentration of free MPA in the colonic lumen of MMF-treated mice, which coincided with reduced toxicity. These data suggest that the bacterial metabolism of the MMF metabolite MPAG, and most likely AcMPAG, to free MPA in the colonic lumen is responsible for the GI toxicity associated with MMF use.

4.1 Vancomycin Prevents and Reverses MMF-Induced GI Toxicity in the Mouse

Previous work by the Greenway and Hirota laboratories demonstrated that eradication of the gut microbiome in a murine model of MMF immunosuppression using a cocktail of broad-spectrum antibiotics was able to prevent and reverse MMF-induced GI toxicity. This approach
however, neither identified which bacterial taxa were responsible nor establish a microbial or molecular mechanism for this observation. The present study assessed the ability of each individual antibiotic (ampicillin, neomycin, metronidazole, and vancomycin) to prevent toxicity using the same murine model. We found that vancomycin was most effective in both preventing MMF-induced weight loss, during concurrent administration with MMF, as well as ameliorating incurred toxicity (manifested as significant weight loss and colonic inflammation) after 8 days of MMF. Re-establishment of control level body weight, cecum weight, liver weight, and spleen weight demonstrated that systemic toxicity was relieved by vancomycin treatment. Additionally, vancomycin was able to restore colon length, a non-specific marker of GI inflammation in models of colitis.100,101

To better understand how vancomycin reversed GI inflammation, we assessed its effect on the colonic tissue cytokine/chemokine profile of MMF-treated mice. Previously, Flannigan et al. demonstrated that MMF induced a local innate immune response based on upregulation of TNFα, IL-6, IL-1, KC (CXCL1), MIP-2α (CXCL2), MIP-1β, and MCP-1 (CCL2).26 Vancomycin appears to reverse this response as it significantly reduced the concentration of TNFα, IL-6, LIF, KC (CXCL1), MIP-2α (CXCL2), G-CSF, and M-CSF. These inflammatory mediators play a role in the stimulation of acute phase proteins (specifically, TNFα, IL-6, LIF),102,103 granulocyte and macrophage production (G-CSF, M-CSF),104 and the promotion of immune cell migration to the site of inflammation in response to bacterial antigens such as lipopolysaccharide (LPS) (KC, MIP-2α).105 Reduced expression of these inflammatory mediators suggests vancomycin is removing an inflammatory stimulus from the colonic lumen. This inflammatory stimulus was postulated to be either a pathogenic bacterium that expanded
due to MMF-induced dysregulation of the microbiome, such as pathogenic *Escherachia/Shigella* or a toxic molecule produced by the bacterial metabolism of MMF derivatives. Evaluating how the composition and function of the gut microbial community was altered by vancomycin provided insight into its mechanism of action. There was also an observed increase in four cytokines in the MV mice (IL-2, IP-10, MIG, and RANTES), which are involved in triggering an adaptive immune response. This may have been an off-target effect of vancomycin-induced upregulation of Toll-like receptors (TLR1 and TLR2) which bind pathogen-associated molecular patterns (PAMPs) from Gram-positive bacteria, such as Bacilli, a taxa that expanded greatly also due to vancomycin in MV treated mice, triggering an adaptive immune response. To understand the molecular basis for vancomycin-reduction in toxicity, we investigated its effects on the MMF-treated microbiome.

### 4.2 Vancomycin Eliminates GUS-Producing Bacteria in the GI Tract

Using 16S rRNA amplicon sequencing, we determined which bacteria thrived during MMF treatment (presumably due to their consumption of GlcA from the catabolism of MPAG as a food source) and were subsequently eliminated by the addition of vancomycin. Two treatment groups (MMF-only and MV) were included, allowing for inter-, intra-group and temporal comparisons. After 8 days of MMF-only treatment, there was a consistently high abundance of the bacterial classes Betaproteobacteria, Bacteroidia, Bacilli, Gammaproteobacteria, Erysipelotrichia, and Alphaproteobacteria. After initiating vancomycin treatment, the classes Bacteroidia, Clostridia, and Erysipelotrichia, were severely depleted, allowing for expansion of the classes Gammaproteobacteria and Bacilli, a response that is also observed in humans. The potential pathogenic bacteria *Escherichia/Shigella* (which cannot be distinguished based on 16S
rRNA sequencing) of class Gammaproteobacteria was previously thought to be a primary cause of inflammation following MMF-induce dysbiosis of the microbiota, however its expansion during vancomycin treatment, when inflammation and toxicity improved, suggests it may not the primary cause. Within the three depleted classes we identified the genera Bacteroides, Lachnospiraceae группы NK4A136, Roseburia, and Turicibacter as being severely reduced by vancomycin while still existing in relatively high abundance in the MMF-only treated animals. Both Bacteroides spp. and Roseburia spp. encode for GUS enzymes and have demonstrated GUS activity in vitro.

As previously discussed, bacterial GUSs are known to metabolize MMF-derived metabolites in the GI tract, producing active MPA and GlcA. The Bacteroides species B. fragilis, B. uniformis, B. ovatus, and B. nordii were all identified to be eliminated by vancomycin in the GI tract of MMF treated mice and possess GUSs that hydrolyze MPAG, as observed in our in vitro GUS activity screen (Figure 3.21). It is expected that MPAG would apply a positive selective pressure to bacteria encoding MPAG-compatible GUS, since the resultant GlcA can be used as a carbon source during anaerobic metabolism. Indeed, this was observed with B. fragilis, B. cacae, and B. vulgatus, all of which increased in abundance during MMF-only treatment (Figure 3.15). It is important to note that only a small percentage of amplicons were annotated at the genus or species level and only a small number of GUS enzymes have been assessed for activity against MPAG thus far. Therefore, it is highly likely that additional bacterial species and genera are involved with MPAG metabolism in our murine model that we were unable to identify based on the methodology that we used.

Vancomycin elimination of GUS-producing bacteria was inferred to reduce GUS activity in our MMF model. We demonstrated the validity of this inference in silico using PICRUSt to
predict the abundance of GUS gene content in the MMF-only and MV metagenomes, as well as an enzymatic functional assay to assess GUS activity in fimo.

4.3 GI GUS Activity is Modulated by MMF and Inhibited by Vancomycin

PICRUSt is a computational tool that predicts the functional content of a sample metagenome using 16S rRNA amplicon sequencing data. The software output consists of functional orthologs annotated in the Kyoto Encyclopedia of Genes and Genomes Orthology database (KEGG Orthology or KO) with a corresponding abundance for each individual KO. These KOs can also be collapsed into pathways, if desired, for higher level analysis. While PICRUSt metagenomic functional predictions are reported to be highly accurate, they are based on a number of assumptions and metagenomic content does not always reflect enzymatic activity due to variability in transcriptional and translational regulation. Therefore, PICRUSt data was verified using an in fimo enzymatic assay.

PICRUSt predicted an increase in the abundance of GUS orthologs in the GI tract of mice in response to MMF treatment (after 4 days) but, interestingly, gene content was reduced by Day 8. This activity pattern was supported by the in fimo assay where enzymatic activity initially increased after 4 days of MMF exposure, followed by a slight decrease after 8 days. Incidentally, we observed the effect of this activity pattern in our targeted metabolomics assay where the in fimo concentration of MPA, the product of GUS activity in MMF-treated animals, followed the same temporal variation with a transient increase on Day 4 of exposure that had returned to baseline by Day 8. Enzyme activity is linked, at least partially, to bacterial species abundance, as demonstrated by the parallel results of our in silico and in fimo assays for GUS activity. The pattern of temporal increases in enzyme activity, as opposed to a constant increase, may occur
due to the tendency of a stable ecological community to resist the development of states of low-diversity (disproportionate growth of a single taxa) through negative feedback loops. The genera *Bacteroides* appeared to be most highly involved in MPAG metabolism. This taxon may be growing disproportionately initially, influenced by the new source of carbon (GlcA), but is then impeded by a microbial negative feedback loop in the gut. The phenomenon of *Bacteroides* spp. ‘leaking’ the breakdown products of polysaccharide utilization (i.e. GlcA from GUS metabolism) to neighboring bacteria, supporting diverse growth within the gut community, may be fueling this negative feedback loop.

Using *ex vivo* fluorescence imaging, in conjunction with a fluorescently-active GUS substrate, we determined GUS activity to be localized to the cecum and colon. Importantly, MMF did not modulate GUS activity in the cecum, but had a substantial impact in the proximal colon. These data indicate sampling of luminal contents from the proximal colon, as opposed to fecal pellets, may lead to a better understanding of how MMF is impacting the gut bacterial community composition and function, and possibly reduce biological variation between replicates, as region-specific differences in community and function may be present. However, access to cecal or proximal colon contents would require sacrifice of the animal.

Despite the biological and temporal variation in GUS activity, it was shown convincingly *in silico* that vancomycin effectively eliminates the predicted abundance of GUS enzyme in MMF-treated mice, supporting our prediction from the 16S rRNA compositional analysis that vancomycin was eliminating GUS-producing bacteria. Additionally, the PICRUSt-predicted elimination of GUS activity by vancomycin was validated by the observed increase in fecal pellet concentration of the compound stachyose, a tetrasaccharide containing a β-D glycosidic bond (the same glycosidic bond conjugating GlcA to MPA in MPAG), found in our laboratory
mouse chow. Low concentrations of stachyose are found in the fecal pellets from MMF-only treated animals, likely due to its metabolism by GUS-expressing bacteria. The increase in stachyose concentration with exposure to vancomycin suggests that its metabolism by GUS is no longer occurring due to antibiotic-related elimination of GI GUS-expressers. In addition to the decrease in stachyose metabolism, abolishing GUS activity in the GI lumen was predicted to limit the hydrolysis of MPAG, resulting in its accumulation. This was tested by measuring the concentration of the metabolites MPAG, MPA and GlcA in the GI tract of these animals.

4.4 Reduced GUS Activity Reduces Hydrolysis of MPAG to MPA

Although the normal rate of MPAG deconjugation by bacterial GUS in the GI tract is not known, it is expected that a substantial portion of MPAG is metabolized to MPA and GlcA based on high levels of MPA enterohepatic recirculation. Therefore, the ratio of MPA:MPAG in the expelled fecal pellets of MMF-treated mice would likely be high. Subsequently, a reduction in GUS activity caused by vancomycin treatment would disrupt this interaction, inverting the MPA:MPAG ratio. This prediction was confirmed using UHPLC-MS, which showed relatively high levels of MPA and relatively low levels of MPAG in vivo during MMF-only treatment. Vancomycin induced a sharp increase in MPAG concentration, in tandem with a drastic reduction in the concentration of MPA, supporting our prediction that vancomycin reduces bacterial metabolism of MPAG in the colonic lumen (Figure 3.22). Limitation of the release of MPA from MPAG by vancomycin in our model coincided with reduced GI and systemic toxicity, suggesting that MPA itself may be the major contributor to MMF-induced GI inflammation and toxicity. Additionally, the serum concentration of MPA was not significantly
reduced in the vancomycin treated animals, suggesting MMF toxicity is occurring primarily in the GI tract, and systemic toxicity is not a result of toxic doses of MPA in the blood.

4.5 MPA Alone May Induce GI Inflammation

Recent *in vitro* work by Heischmann et al. found that MPA depleted the intracellular guanosine pool in human intestinal LS180 cells, despite high levels of guanosine supplementation, affecting a number of cellular processes dependent on nucleotides including protein, lipid and fatty acid metabolism, potentially leading to reduced membrane integrity. Given that intestinal epithelial cells salvage guanosine from the GI lumen, their data suggest that MPA inhibits this process. Our observation that guanosine concentration in fecal pellets of MMF treated animals was greatly reduced by vancomycin suggests that reducing the interaction between MPA and colonic epithelial cells allows for increased uptake of luminal guanosine. Similarly, the decrease in luminal inosine suggests an increase in its uptake by epithelial cells, potentially for use in *de novo* purine synthesis since the inhibition of IMPDH by MPA is no longer occurring. Qasim et al. demonstrated that MPA directly modulated intestinal epithelial tight junctions in Caco-2 cell monolayers by upregulating myosin light chain kinase (MLCK) leading to increased phosphorylation of myosin II regulatory light-chain and increased tight junction permeability. This may be occurring via MPA activation of the midkine-dependent PI3K pathway. Our murine model supports the hypothesis of MMF-induced epithelial barrier dysfunction as Flannigan et al. found MMF exposure to lead to an increase in serum LPS concentration, an indirect measure of intestinal epithelial barrier function, suggesting that MMF caused impaired barrier function. Together, these observations suggest that MPA
may trigger local GI and systemic toxicity by disrupting GI epithelial barrier function, potentially allowing toxic bacterial metabolites to cross into the tissue and circulation.

We attempted to directly assess the effect of MPA on the colon using IR administration of MPA into the colonic lumen. While we did not observe inflammation and toxicity comparable to our in vitro studies, there did appear to be inducible weight loss in a dose-dependent manner, and a trend towards increased granulocyte infiltration (based on increased MPO activity), suggesting that MPA may be capable of directly inducing GI toxicity and supporting our previous observations. It is possible that the experimental concentration of MPA used was sub-optimal and that greater systemic inflammation may occur when a higher dose is used. As well, IR injections are only capable of administering MPA into the distal colon, as opposed to the cecum or proximal colon where our ex vivo imaging experiments demonstrated the majority of GUS activity is localized. Opting for MPA instillation directly to the cecum and/or proximal colon via percutaneous cannulation would potentially greatly improve this experimental trial and more accurately reflect the in vivo release of MPA into the colonic lumen, potentially demonstrating the adverse effect of MPA on enterocytes in vivo.
CHAPTER FIVE: CONCLUSION AND FUTURE DIRECTIONS

5.1 Conclusion

The present study expanded on previous work that demonstrated the requirement for an intact microbiome for the development of MMF-induced GI toxicity in the mouse. We established that, of the four individual antibiotics tested, vancomycin was able to both prevent and reverse MMF toxicity, as demonstrated by increased body weight, tissue weight, colon length, and a reduction in innate cytokines and chemokines in vancomycin-treated animals. Amplicon sequencing revealed that vancomycin treatment reduced the proportional abundance of many GUS-producing bacteria, including those capable of metabolizing MPAG \textit{in vitro}, such as \textit{Bacteroides} spp., that flourished with MMF exposure. This translated to a reduction in GUS activity as shown by inferred \textit{in silico} and functional \textit{in fimo} analyses of fecal metabolite concentrations. Reduced GUS activity resulted in decreased hydrolytic conversion of MPAG to MPA in the cecum and proximal colon as demonstrated directly using targeted metabolomics and this diminished the opportunity for negative interactions between MPA and GI epithelial cells. Reduced epithelial exposure to MPA by vancomycin treatment was associated with the amelioration of MMF-induced GI toxicity, suggesting that MPA may be the molecule responsible for GI irritation, damage, and loss of barrier function. MPA-induced local inflammation may contribute to systemic inflammation by impairing GI epithelial barrier function, resulting in the passage of bacterial toxins into the tissue and systemic circulation. The work presented in this thesis supports the hypothesis that the bacterial metabolism of colonic MPAG is responsible for the GI toxicity that is seen clinically with MMF exposure.

A recent animal study demonstrated that rats with reduced biliary excretion of MPAG due to either genetic or xenobiotic (i.e. cyclosporine A) impairment of the organic anion
transporter MRP2, leading to reduced GI excretion of MPAG and thus epithelial exposure to MPA, experienced less GI toxicity. As well, in humans a genotype variant in the ABCC2 gene (coding for MRP2) was correlated with MMF-induced GI intolerance. Both studies suggest the increased transport of MPAG to the GI tract via the biliary tract is associated with adverse GI effects of MMF, further supporting our hypothesis. The combination of genetic predisposition in the host with increased activity of the MRP2 transporter leading to increased supply of MPAG into the GI tract with a high abundance of GUS-expressing bacteria may explain the GI toxicity of MMF in human patients and could also explain the toxicity seen with other commonly prescribed drugs that undergo hepatic glucuronidation as part of their metabolism.

5.2 Future Directions

While the present study demonstrated that the removal of bacteria capable of metabolizing MPAG results in reduced toxicity, it will be important to show, in order to demonstrate causality, that the introduction of GUS-producing bacteria results in GI toxicity. Creating a gnotobiotic mouse model using bacteria capable of in vivo MPAG metabolism, such as B. fragilis, B. uniformis, B. ovatus, and/or B. nordii, will be an important experiment in revealing the components required for MMF-induced GI toxicity. Furthermore, it is unclear if the GI and systemic toxicity (i.e. weight-loss) is the result of MPA alone, or if MPA-mediated injury of the GI epithelium simply permits the induction of inflammation via a secondary toxic metabolite, pathogen, or pathway. In vitro modeling of MPA toxicity, perhaps with human intestinal organoids, might help to address this mechanistic question.

Extending our observations in the mouse to humans will be an important step in translating our work. Recent in silico observations by Lee et al. suggest that MMF-associated
diarrhea is associated with an upregulation of GUS-expressing bacteria in humans\textsuperscript{130} but this remains to be experimentally validated. Assessing the GUS activity of human patients receiving MMF for immunosuppression using our \textit{in fimo} GUS activity assay, as well as the creation of human microbiota-associated mouse models\textsuperscript{129} will help to validate our hypothesis in humans, allowing us to work towards a treatment of MMF-induced GI toxicity suitable for clinical use.

Recovery of the gut microbiome after vancomycin exposure but in the continued presence of MMF may also provide insight into the potential of vancomycin as a therapy, if the non-GUS active microbiome is able to persist. While, vancomycin is exceedingly effective in the mouse, it is likely not a routinely viable option in human patients, due its widespread devastation of gut microbiota (Figure 3.12), and the subsequent potential short- and long-term risks of opportunistic infection (e.g. \textit{Clostridium difficile} colitis) and impaired microbiome function.\textsuperscript{109} Therefore, work towards a preventative therapy for MMF toxicity in humans would likely require a narrow-spectrum therapy, such as a specific GUS enzyme inhibitor. The use of a targeted GUS inhibitor would potentially allow for high specificity of inhibition, with low-risk of off-target effects. However, development of such an inhibitor may be difficult due to the broad range of GUS structural isoforms, cellular localization, and activity against MPAG, impacting the efficiency of MPAG metabolism \textit{in vivo}.\textsuperscript{93} A more targeted study, uncovering which bacterial species and strains, as well as which GUS isoforms, are most active against MPAG in both the mouse and humans will contribute to the development of an effective and targeted inhibitor.

As with all microbiome research conducted with animal models, the translation to human subjects is in question, due to compositional and functional differences between the murine and human microbiomes. Substantiating the conclusions in the present study in human subjects will
be incredibly important and necessary to develop an effective therapeutic strategy to reduce MMF-induced GI toxicity and improve overall patient health outcomes.
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**Supplementary Material**

**Table S1.1.** Barcoded forward and reverse primers used for the PCR amplification of the 16S rRNA gene in bacterial genomic DNA extracted from fecal pellets of MMF-only and MMF + Vancomycin treated mice. Primers were adapted from the Surette Laboratory,\(^6^9,^7^0\) originally modified from Bartram et al.\(^7^1\) All forward primers used a P7 Illumina adaptor. The reverse primer used a P5 Illumina adaptor.

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