

Mesalamine and Immunosuppressant Treatment in Crohn's Disease

By Kevin W. Sun¹

¹College of Biological Sciences, University of Minnesota – Twin Cities

Abstract: The effects of medicines are not always well understood, and some may worsen the diseases that they are intended to treat. The purpose of this study was to determine if the use of mesalamine (MM) and/or immunosuppressives (IS) causes an amplification or mitigation of the dysbiosis that the gut microbiome experiences during Crohn's Disease (CD). CD is an inflammatory bowel disease that results in chronic inflammation of the gastrointestinal tract. It was hypothesized that MM and IS mitigate the dysbiosis caused by CD individually, as well as when used together. The research subset bacterial DNA samples from the Gevers et al. study into five distinct groups based on CD and medication status (i.e. presence/absence of MM and/or IS) (Gevers et al. 2014). These were analyzed to determine if there were statistically significant differences of the alpha, beta, and taxonomic diversities between CD patients taking different medications. The study concluded that MM is likely able to mitigate CD dysbiosis and be kept as a treatment, while IS likely do not mitigate dysbiosis and may even amplify it. These findings are significant for medical practitioners to consider as a factor for what treatments should be used against CD.

1. Introduction

Crohn's Disease (CD) is a disorder that affects 1.4 million Americans (Bandzar et al., 2013). With no known cure for CD, most research has focused on understanding how it affects the body and improving treatments for the disease (Bandzar et al., 2013). Researchers have found that CD is linked with changes in levels of certain bacterial species in the gut (Gevers et al., 2014). After analyzing gastrointestinal bacteria samples from children with CD, literature suggests

that having CD is strongly correlated with dysbiosis in the gut microbiome, where dysbiosis is defined as a microbiome imbalance that sees increased levels of Enterobacteriaceae, Pasteurellaceae, Veillonellaceae, and Fusobacteriaceae (EPVF bacteria), and decreased presence of Erysipelotrichaceae, Bacteroidales, and Clostridialesare (EBC bacteria) (Gevers et al., 2014). This is a serious issue because it can lead to adverse health conditions, such as

chronic fatigue, digestive problems, and acid reflux (Gevers et al., 2014).

This study analyzed two drugs that are highly relevant in treating CD: mesalamine (MM) and immunosuppressants (IS). Mesalamine is an aminosalicylate drug (Morgan et al., 2012). It is known to be an antioxidant and can decrease inflammation in the intestines, mainly by inhibiting the NFκB protein complex, as well as inhibiting the production of eicosanoid, a compound that increases inflammation (Morgan et al., 2012). Thus, it is a widely used medicine for mitigating CD. Understanding how it affects the progression of dysbiosis would be highly valuable. If it increases dysbiosis, providers should consider switching to alternative medicines. Notably, researchers have found that mesalamine can lead to large reductions in *Escherichia* and *Shigella*, which are both types of *Enterobacteriaceae* that are increased in CD dysbiosis (Morgan et al., 2012).

The other compounds that were analyzed in this study were immunosuppressants. Immunosuppressants are often used in CD treatment because CD causes chronic inflammation in the intestines, which is a condition that immunosuppressants fight (Cosnes et al., 2005). They have become very common in CD treatment; in a group of CD patients from 1978–82, none used immunosuppressants, while each patient in a group from 1998–2002 had a 56% chance of using immunosuppressants, and this number continues to grow (Cosnes et al., 2005). Additionally, scientists have invested large amounts of research into producing new

immunosuppressants, which is time-consuming (Cosnes et al., 2005). Therefore, it is crucial to acquire a better understanding of how immunosuppressants can affect the dysbiosis resulting from CD and if they should continue to be used in CD treatment.

While previous research has shown that MM and IS are effective at reducing inflammation, whether these drugs mitigate dysbiosis has not been extensively studied. Thus, the aim of this study was to determine if the use of mesalamine and/or immunosuppressives cause an amplification or mitigation of the dysbiosis that the gut microbiome experiences during Crohn's Disease. It was predicted that mesalamine and immunosuppressives mitigate the dysbiosis caused by CD individually as well as when they are used together, as supported by literature that has found dysbiosis decreases when intestinal inflammation decreases, and both compounds have been found to decrease inflammation (Lewis et al., 2015).

2. Methods

2.1 Initial Data Processing

For this study, all data was taken from the Gevers et al. paper (Gevers et al., 2014). The researchers used Illumina sequencing on the 16S gene to identify bacteria found in the ileum, rectum, and fecal samples of healthy and CD patients (Gevers et al., 2014). They then sorted this data into OTUs (AKA

Group	Description	Sample Size Before Rarefaction	Sample Size After Rarefaction
Healthy	Healthy non-CD patients (not using MM or IS)	424	335
CD	CD, No MM and No IS	601	532
IS	CD, No MM and Yes IS	27	23
MM	CD, Yes MM and No IS	96	44
MM+IS	CD, Yes MM and Yes IS	18	13

Table 1: Description of the five treatment groups and their sample sizes before and after rarefaction. This table contains a description for what each treatment group is and what the sample size for that group was before and after rarefaction. Rarefaction greatly decreased the sample size of IS. The final sample sizes of IS, MM, and MM+IS were all fairly low. Rarefaction was completed by loading the Gevers OTU Table and Metadata into RStudio, then removing all data samples that did not have mesalamine MM or IS data, and then separating the remaining data into the five groups listed here.

Operational taxonomic unit, which are categories of similar sequence variants of the 16S rDNA marker gene sequence) using closed-reference OTU picking (Gevers et al., 2014). To get the dataset ready to be analyzed for the research study, the data was filtered, sorted, and rarefied. This was completed by loading the Gevers OTU Table and Metadata into RStudio, then removing all data samples that did not have mesalamine (MM) or immunosuppressants (IS) data, and then separating the remaining data into the five groups detailed in Table 1.

The data was then transferred to QIIME 2, which was used to produce a rarefaction curve from the data. Rarefaction is a technique that adjusts for differences in sample size across different datasets. It was determined that the rarefaction level that would best balance species richness with

sample size would be a depth of 1000 sequences. Table 1 displays what samples were before rarefaction, and what they were after rarefaction (Table 1).

2.2 Diversity Tables

Using QIIME 2, alpha and beta diversity tables were produced from the data with the previously determined rarefaction depth of 1000 sequences. The Shannon Index was used for the alpha diversity (AD) metric and Bray-Curtis was used for the beta diversity (BD) metric. This produced all the data analysis needed to create alpha diversity boxplots as well as a Principal Coordinates Analysis (PCoA) plot.

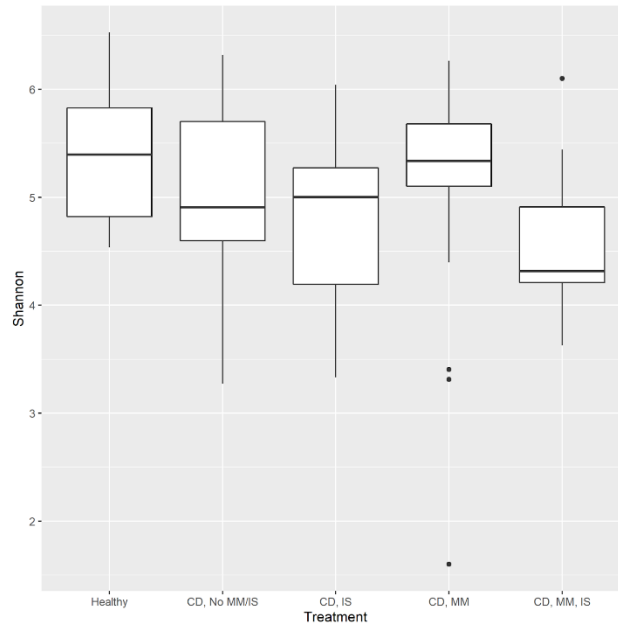


Figure 1. Mesalamine was highly effective at mitigating the decrease in alpha diversity associated with CD. Alpha diversity of microbial communities by the five treatment groups using Shannon metric comparisons (Healthy : n = 335, CD: n = 532, IS: n = 23, MM: n = 44, MM+IS: n = 13). The five box plots represent the five treatment groups outlined in the methods section. The data was then analyzed using a Kruskal-Wallis rank sum test (p-value = 0.161).

2.3 Statistical Analysis

A figure containing a box plot of alpha diversity for each of the five groups listed in the “Initial Data Processing” section was produced using the alpha diversity table from QIIME 2 in RStudio. A Kruskal Wallis test was run on the alpha diversity data to determine its statistical significance since MM was found to not be normally distributed by a Shapiro-Wilk Normality Test.

A PCoA plot figure was produced in RStudio using the Beta Diversity table. An Adonis test was run to determine if the differences in beta diversities were significantly different because the data was nonparametric and there were more than two groups to analyze. An Adonis test only gives one p-value and R² for the data as a whole, so the project cannot comment on

whether individual groups are significantly different from each other.

The Gevers et al. paper identified several key bacterial taxa that play a key role in CD dysbiosis (Gevers et al., 2014). To analyze these taxa, RStudio was used to calculate how the different compounds (MM and IS) caused changes in the abundance of these taxa during dysbiosis. These taxa were: Enterobacteriaceae, Pasteurellaceae, Erysipelotrichales, and Clostridiales (Gevers et al., 2014). These taxa were chosen because they have been identified as taxa that undergo major shifts during dysbiosis (Gevers et al., 2014). All of these taxa have been found to undergo significant shifts during dysbiosis and having abnormal levels of each is indicative of abnormal gut function; other taxa affected by dysbiosis were not analyzed due to time constraints (Gevers et

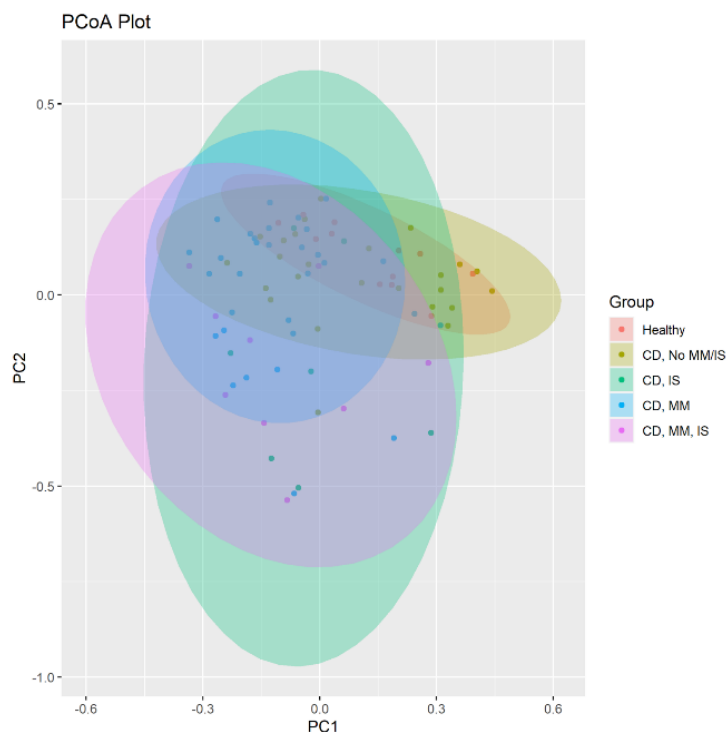


Figure 2: Mesalamine and Immunosuppressants explain ~10% of variability in samples and MM gut microbiomes resemble Healthy microbiomes the closest. The gut microbiomes of the five groups were compared on a PCoA using a Bray-Curtis index (Adonis test: $p = 0.001$, $R^2 = 0.097$). The MM group is clustered around the Healthy group indicating low beta diversity. Results are significantly different, as shown by the p-value. However, the R^2 values indicate that these groups have low explanatory power for the variability found in the data.

al., 2014). A table was generated that displayed each group's relative abundance of each of the taxa as a fold change from the Healthy Group's relative abundance, and a Wilcox test was used to test for significance between the mean relative abundances of each taxa between different treatment groups because the data was nonparametric.

3. Results

3.1 Highly Significant Microbial Changes are Not Present between Treatment Groups.

First, using the calculated Shannon diversity metrics, the alpha diversity of the samples was graphed on a box plot for each of the five groups. A Shapiro-Wilk test demonstrated that the data was not normalized ($p = 8.151e-06$), therefore statistical testing of the differences in mean alpha diversity was done using the Kruskal-Wallis rank sum test ($\chi^2 = 6.5613$, $df = 4$, $p = 0.161$). This analysis of the alpha diversity box plot figure revealed that there were no statistically significant differences in alpha diversity between treatment groups (Fig. 1). The Kruskal Wallis statistical test compared alpha diversity levels among all five of the groups to determine if MM and/or IS affected the alpha diversity of patients, since CD dysbiosis is typically characterized by a decrease in alpha diversity. This is because dysbiosis usually involves a few bacterial taxa greatly increasing in population while other taxa decrease in population, which lowers alpha diversity (Gevers et al., 2014). The effects of MM and IS on alpha diversity are one way to see if they mitigate, amplify, or do not affect CD dysbiosis.

A PCoA plot was then graphed and calculated with a Bray-Curtis distance matrix. Examination of the principal coordinates analysis plot (PCoA) revealed a mostly overlapping cluster formation of the five subgroups (Fig. 2). An Adonis statistical test

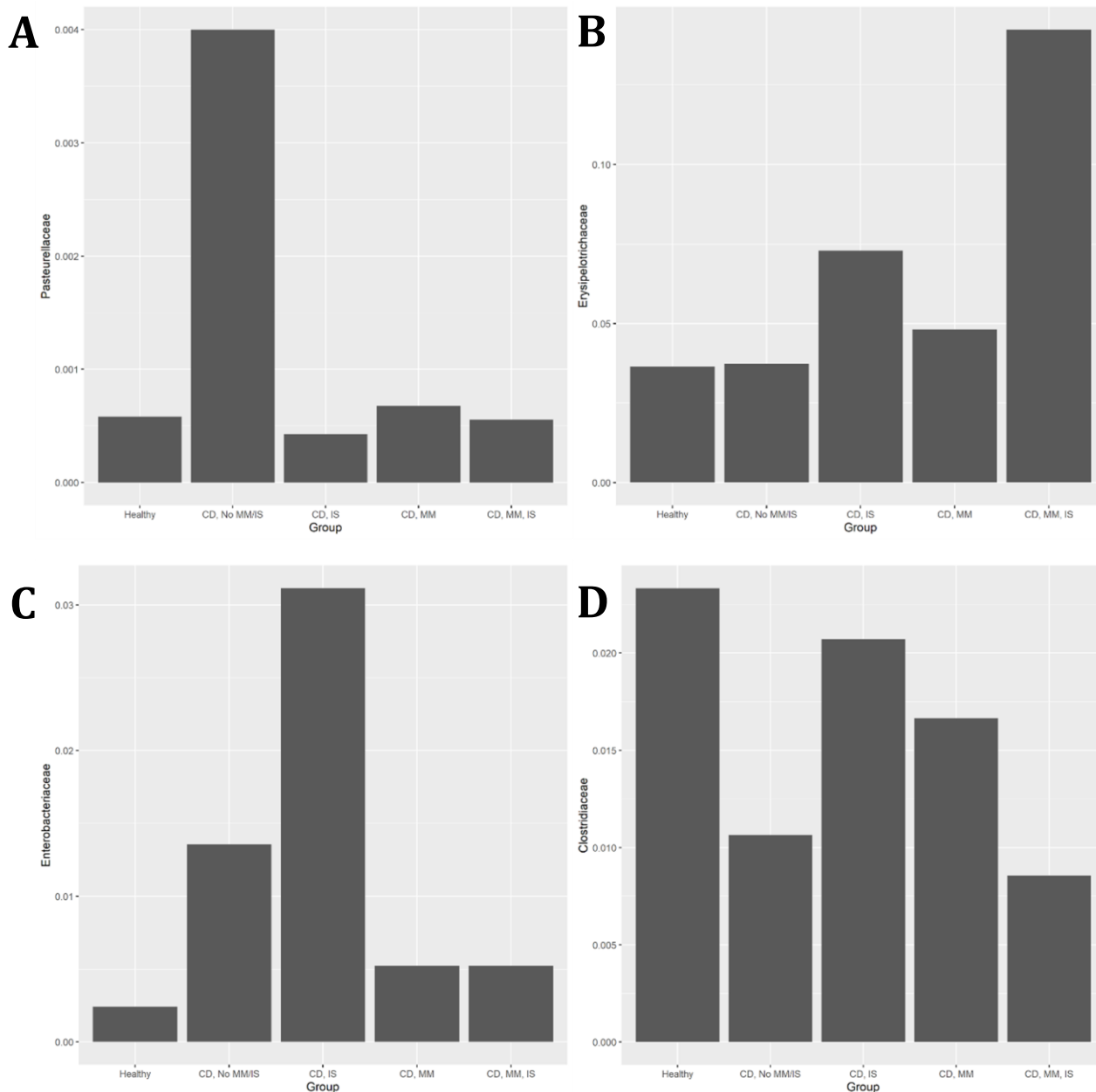


Figure 3. Mesalamine is generally effective at mitigating dysbiosis of specific taxa. On average, MM was shown to have relative abundance levels closest to Healthy. MM's relative abundance was (A) 1.16 times that of Healthy group's for Pasteurellaceae, (B) 1.32 times that of Healthy's for Erysipelotrichaceae, (C) 2.17 times that of Healthy group's for Enterobacteriaceae, and (D) 0.71 times that of Healthy group's for Clostridiales. IS and MM+IS had a greater difference from Healthy in at least one of these taxa.

demonstrated that the microbial compositions of the subgroups were statistically different from one another, however, the groupings that were chosen do not explain most of the variability in the data ($p = 0.001$, $R^2 = 0.097$). From the PCoA, MM

appears to be clustered around Healthy, while the other three groups are very widely dispersed around the plot.

Plots that displayed the relative abundance of the Pasteurellaceae (abbreviated as 'P'; Fig. 3A),

Group	Percent of Healthy Group's Alpha Diversity
Healthy	100%
CD	90.85%
IS	92.59%
MM	99.13%
MM+IS	79.96%

Table 2: Comparison of mean alpha diversity of each group to Healthy Group's alpha diversity The table expresses each group's alpha diversity as a percent of the Healthy Group's alpha diversity. Alpha Diversity was calculated automatically by QIIME 2. Notably, MM's mean alpha diversity is very close to Healthy's (99.13%). IS's alpha diversity is fairly close to Healthy's as well (92.59%).

Erysipelotrichaceae (abbreviated as 'Er'; Fig. 3B), Enterobacteriaceae (abbreviated as 'En'; Fig. 3C), and Clostridiaceae families (abbreviated as 'C'; Fig. 3D) in the microbiomes of each group were then created. Because this data is non-parametric, a Wilcox test was used to test for significance

between the mean relative abundances of each taxa between different treatment groups. Most of these data points were found to not be statistically significant (using $p < 0.05$ as the standard).

3.2 Mesalamine Tends to be More Effective at Mitigating Dysbiosis than Immunosuppressants.

Results from Figure 1 demonstrate that mesalamine was highly effective at mitigating the decrease in alpha diversity associated with CD, with the MM group's mean alpha diversity being 99.13% of the Healthy group's mean alpha diversity (Fig. 1). Table 2 compares the mean alpha diversity of each group to the Healthy group (Table 2).

IS also prevented mean alpha diversity from falling below that of CD patients who received no treatment, but was

Group	Fold change from Healthy Group's Relative Abundance (P)	Fold change from Healthy Group's Relative Abundance (Er)	Fold change from Healthy Group's Relative Abundance (En)	Fold change from Healthy Group's Relative Abundance (C)
Healthy	1.00	1.00	1.00	1.00
CD	6.82	1.02	5.70	0.46
IS	0.72	2.00	13.12	0.89
MM	1.16	1.32	2.17	0.71
MM+IS	0.94	3.91	2.19	0.37

Table 3: Fold change from Healthy Group's relative abundance

This table expresses each group's relative abundance of each of the taxa as a fold change from the Healthy Group's relative abundance. Fold change was calculated automatically by QIIME 2. Notably, MM group's relative abundance was always only around 0.71 to 2.17 times that of the Healthy group's, and MM+IS was around 0.37 to 3.91 times that of Healthy's, while all other groups differed from Healthy by significantly more in at least one taxa.

not able to preserve alpha diversity as well as MM. Another notable result is that MM+IS's mean alpha diversity is only 79.96% of the Healthy group's, which is very low compared to all other groups.

The finding that MM is effective at mitigating dysbiosis is corroborated by results from Figure 2, which demonstrate that MM remains closely clustered around Healthy, while IS and MM+IS are extremely spread out around the PCoA (Fig. 2). This indicates low beta diversity between Healthy and MM, meaning the microbial compositions of the two groups are similar.

The finding that MM is effective at mitigating dysbiosis is also supported by Figure 3, which indicates that the MM group's relative abundance was around 0.71 to 2.17 times that of the Healthy group's, and MM+IS was around 0.37 to 3.91 times that of Healthy's, while all other groups differed from Healthy by significantly more in at least one taxa (Fig. 3 & Table 3).

4. Discussion

4.1 Direction of Modulation

The aim of this study was to determine if the use of mesalamine and/or immunosuppressives causes an amplification or mitigation of the dysbiosis that the gut microbiome experiences during Crohn's Disease. It was hypothesized that the alpha diversity of medicated patients (MM, IS, and MM+IS) would be higher than those of non-medicated patients (CD) but lower than healthy people (Healthy) as past studies have found that dysbiosis decreases when intestinal inflammation decreases, and both compounds have been found to decrease

inflammation (Lewis et al., 2015; Morgan et al., 2012; Cosnes et al., 2005).

This part of the hypothesis, however, was inconclusive. Alpha diversity between the groups did not significantly vary, (p -value > 0.05 , Fig. 1). This lack of statistically significant differences (at a 5% significance level, which is the standard used throughout this paper) between the groups may have been due to small sample sizes since the dataset did not contain many patients who used MM or IS. Subsetting and rarefaction caused the sample size of the smallest group (MM+IS) to only be 13 samples. Larger sample sizes would give smaller p -values and thus are more likely to yield adequate sample size for conducting statistical analysis. Current results indicate that MM and IS do not significantly affect alpha diversity in CD patients, however, further studies with larger sample sizes are necessary to test this conclusion.

However, mesalamine was highly effective at mitigating the decrease in alpha diversity associated with CD, with the MM group's mean alpha diversity being 99.13% of the Healthy group's mean alpha diversity (Table 2). IS demonstrated effectiveness as well, with its mean alpha diversity at 92.59% of the Healthy condition. MM+IS was extremely ineffective, with its mean at 79.76% of Healthy's, indicating that these drugs may not be effective when used together. It is also possible that patients who use both drugs have a more severe CD, which induces their physicians to prescribe them both drugs, thus causing their alpha diversity to be lower on average.

4.2 Magnitude of Modulation

The results indicated that these drugs may not significantly modulate the effects of dysbiosis in CD patients. While the beta diversities between the different groups in the study were statistically significant (p -value < 0.05 , Fig. 2), i.e., there were significant differences in the microbiome compositions of the different groups, the R-squared value indicated that a significant amount of variation in distances is explained by the grouping used by the study ($R^2 = 0.097$, Fig. 2). 9.7% of the variation is explained by these groupings, which is reasonable given the complexity of the microbiome, although there could also be factors behind the variations in distances. These factors could be other drugs that the patients were taking that this study did not take into account, such as steroids, which were also reported in the Gevers et al. dataset (Gevers et al., 2014). Therefore, the hypothesis correctly identifies that MM and IS play some role in modulating dysbiosis in CD patients, but this effect is relatively weak.

From the data, MM remains closely clustered around Healthy, while IS and MM+IS are extremely spread out around the PCoA (Fig. 2). This means that there is low beta diversity between Healthy and MM, meaning that the microbial compositions of the two groups are similar. This indicates that mesalamine is able to preserve many of the microbial populations from being affected by dysbiosis because types of bacterial taxa and their relative abundances are likely fairly similar between Healthy and MM patients. This is consistent with past studies, since mesalamine has been effective for treating

CD (Lewis et al., 2015; Morgan et al., 2012). Past studies have recommended mesalamine as a treatment for CD patients who are allergic to a similar drug, sulfasalazine, and this study would support this recommendation (Bandzar et al., 2013; Tremaine et al., 1994). The results also indicated that immunosuppressants cannot preserve microbial populations from being affected by dysbiosis, which contradicts another study that immunosuppressants are effective for treating CD (Cosnes et al., 2005; Mao et al., 2017). This study suggested that immunosuppressants without anti-TNF therapy (a method of reducing chronic inflammation) are ineffective for treating CD, but using them together is effective, reducing CD hospitalizations by 50% and surgery by 33–77% (Mao et al., 2017). In the dataset, no anti-TNF therapy was used, so the results are consistent with the finding that immunosuppressants without anti-TNF are not effective for CD treatment (Gevers et al., 2014; Mao et al., 2017).

4.3 Specific Taxa

The results also demonstrated that Pasteurellaceae, Erysipelotrichaceae, Enterobacteriaceae, and Clostridiales undergo significant changes in population during CD dysbiosis. All of these taxa have been found to undergo significant shifts during dysbiosis and having abnormal levels of each is indicative of abnormal gut function; other taxa affected by dysbiosis were not analyzed due to time constraints (Gevers et al., 2014). MM is best able to prevent these shifts since its relative abundance was generally around only 0.71 to 2.17 times that

of the Healthy group's (Fig. 3 & Table 3). MM+IS was also fairly effective, as its relative abundance was around 0.37 to 3.91 times that of Healthy's for each taxa, while all other groups differed from Healthy by significantly more in at least one taxa (Fig. 3 & Table 3). This indicates that MM, and MM+IS to a lesser extent, are able to mitigate dysbiosis since they maintain bacterial levels similar to those of healthy patients. This is consistent with past findings that these taxa are good predictors of whether a drug mitigates or amplifies dysbiosis (Morgan et al., 2012; Gevers et al., 2014). These studies found that antibiotics amplified dysbiosis by, for example, causing about 0.01 times change in relative abundance of Erysipelotrichaceae from that of Healthy and a more than 10 times change of Enterobacteriaceae from that of Healthy, as well as larger changes from Healthy in the other two taxa (Morgan et al., 2012; Gevers et al., 2014).

4.4 Conclusions and Future Research

In the end, the research demonstrates that MM and IS may have small modulating effects on dysbiosis in CD patients, but the exact scale and direction of these modulations are unclear and require further exploration. This study seems to indicate that MM and IS only explain a part of any modulations that occur. This may explain why some of the results were found not to be statistically significant. Alternatively, MM and IS may simply have little to no effect on dysbiosis. This is significant because it could mean that MM and IS are not effective for treating dysbiosis and should be replaced with other drugs. Future studies should

explore other compounds used to treat CD, such as sulfasalazine, another aminosalicylate which has been shown to have higher efficacy than mesalamine at treating CD, although the reasons for this are not well understood (Lim et al., 2016). Using IS with anti-TNF therapy (a method of reducing chronic inflammation) to treat CD should also be studied because two studies, one of which utilized a network meta-analysis, found that this combination was more effective than IS alone, possibly because the combination was able to avoid triggering adverse immune responses (Hazlewood et al., 2015; Mao et al., 2017). These studies are necessary because they would explore other methods for treating CD that are based on existing treatments but have the potential to be more effective (Lim et al., 2016). Targeted studies of these medicines would help the medical community review the treatments for CD. Not many studies specifically look at how effective certain medicines are for treating CD, especially emerging treatments (Lim et al., 2016; Hazlewood et al., 2015). Studying these treatments on a wider scale is necessary to test their effectiveness, especially for mitigating dysbiosis. Reducing dysbiosis is an important goal because it serves as the main driver of inflammation in CD, one of the disease's main symptoms (Gevers et al., 2014). Effective treatment against CD inflammation would greatly improve the lives of CD patients since inflammation causes chronic pain and discomfort in CD patients (Lim et al., 2016; Hazlewood et al., 2015). Understanding how medicines modulate dysbiosis in CD patients is key to ensuring that these medications do not worsen the conditions of the patients, as

well as discovering that certain medicines are highly effective at combating dysbiosis.

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