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Andrew Pahnke March 23rd, 2022

The effect of Farnesoid X Receptor agonism on the gut microbiome and mortality during cholestatic liver disease

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Chronic liver diseases such as cholestatic liver diseases and non-alcoholic fatty liver disease are prevalent issues in today's world. The last decade has witnessed an explosion of insights into the role of gut microbiota in cholestatic liver disease; however, much remains unknown. The liver begins production of bile acids which break down fats in the diet. Cholestatic liver diseases tremendously alter the bile profile of the patient, but little is known about how this would affect the gut microbiota. Using a mouse model of cholestatic liver disease, multidrug resistance gene 2 knockout (*Mdr2*$^{-/-}$) mice, we studied how a high-fat diet (HFD) alters gut microbiota and disease outcomes during cholestatic liver disease progression. *Mdr2*$^{-/-}$ mice were used because cholestatic liver disease is known to disrupt bile synthesis and transport in this mouse model. Bile acid synthesis can be activated by the Farnesoid X receptor (FXR) signaling pathway in mice. GW4064 is an agonist of the FXR pathway which influences the production of bile acids in mice. Our aim is to examine how the administration of GW4064 to *Mdr2*$^{-/-}$ mice will influence their bile profile and their gut microbiota. Gut commensal bacteria *Lactobacillus* colonization of the intestine indicates a healthy microbiome. Our data suggest that the addition of the GW4064 agonist had an adverse effect on mouse mortality and *Lactobacillus* colonization levels within the gut. This could be due to a negative impact of the FXR agonist on commensal bacteria counts which allowed for pathogenic proliferation; however, I am unable to make that claim as there were no visible changes in mRNA expression due to the GW4064 agonist.

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1. Introduction & Aims

1. Primary Sclerosing Cholangitis

Chronic liver diseases have become a prominent issue in the United States and western world in synchrony with the obesity crisis[1]. Cholestatic liver diseases and non-alcoholic fatty liver disease (NAFLD) are examples of chronic liver disease that have been shown to affect the gut microbiome and vice versa[2]. Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease that does not have a known cure or origin. PSC is characterized by the scarring of the biliary duct in the liver which prevents the flow of bile acids into the intestinal tract. Sclerosing cholangitis can be caused by many conditions including autoimmunity, heritable disorders, and various infections that cause bile duct blockage[3]. Cholestasis is the condition of toxic bile acid buildup within the liver as a result of the inability to secrete bile acids[4].

The *ABCB4* gene locus encodes for the canalicular phospholipid transporter known as multidrug resistant gene 2 (MDR2) in mice or multidrug resistant gene 3 (MDR3) in humans[5]. When defective, this gene causes a liver disease progression leading to cholestatic liver disease[2]. The MDR2/3 transporter encodes for a biliary transporter that moves phospholipids from the liver into duodenum. When MDR2 is knocked out in mice, the bile acid buildup causes toxicity and liver damage resembling primary sclerosing cholangitis[4] (PSC).

In clinical patients, PSC manifests with symptoms of jaundice, pruritus, and gastrointestinal discomfort in roughly 60% of cases[6]; however, these symptoms can vary drastically from patient to patient. A measurement of liver health can be done with serum alkaline phosphatase analysis along with bilirubin analysis[6].

2. Gut Dysbiosis

PSC can have many downstream effects as a result of the decreased bile flow. One of these is disruption of the gut microbiome[2]. The gut microbiome is heavily influenced by genetics as well as environmental factors such as diet[7].

In particular, gut dysbiosis is a shift in the proportion of bacterial families within the gastrointestinal tract. Dysbiosis can lead to inflammation and bloating within the gut which are markers of IBD. In non-alcoholic fatty liver diseases, the ratio of *Bacteroides: Firmicutes* is increased leading to more intestinal inflammation[7].

Dysbiosis can cause major health issues leading to death within mouse models[2]. A common measurement of gut health is the abundance of the *Lactobacillus* bacterial family[8]. *L. reuteri* and *L. gasseri* are both commensal and bile resistant gut bacteria[9, 10]. *Lactobacillus spp.* are known to prevent potentially inflammatory bacteria such as *E. coli* or *Klepsiella spp.* from proliferating in the gut environment[8].

In recent years, gut dysbiosis has been linked with deterioration of the gut barrier causing different forms of leaky gut[11]. This in turn can lead to bacterial translocation causing inflammation and other complications throughout the body[11]. The gut-liver portal vein allows for a high risk of bacterial liver translocation and/or bacterial metabolites from the gut causing a highly mounted immune response[11]. This link between the gut and liver has a feedback mechanism involving bile[11].

Primary sclerosing cholangitis in particular has low diversity seen the fecal microbiome of patients[10]. There have been previous studies showing the translocation of *Lactobacillus gasseri*

bacteria into the liver of *MDR2*$^{-/-}$ mice which provides more evidence of a unique environment in this model of PSC[2]. There have also been reports of increased *Proteobacteria* colonies such as *E. coli* which are associated with gut inflammation and IBD[10]. Not many studies have looked into the relationship between gut dysbiosis and PSC-model bile acid signaling regulation.

3. Bile Acid Synthesis

The liver produces bile acids by processing serum cholesterol into cholic acid (CA) and chenodeoxycholic acid (CDCA)[6]. This is done through the use of enzyme cholesterol 7 alpha-hydroxylase (CYP7A1) which is the rate limiting step in primary bile acid production. CA and CDCA are the primary bile acids found in mammals, whereas CA and muricholic acid (MCA) are found in rodents. While producing bile acids, the liver can store conjugated bile acids in the gallbladder for later secretion. Either taurine or glycine is conjugated to the bile acid for secretion into the bile duct. Bile acids are capable of hormone function as well by activating receptors such as the Farnesoid X Receptor[12]. Currently, the FXR receptor is a promising pathway to ameliorate different cholestatic liver diseases[12-16].

As opposed to the primary bile acid pathway, there is also the alternative bile acid pathway which is characterized by the use of enzyme CYP27A1[17]. This pathway has shown to produce almost entirely CDCA in humans and MCA in mice[17]. It has also been shown that in fibrotic and cirrhotic livers that the alternative pathway is favored[17].

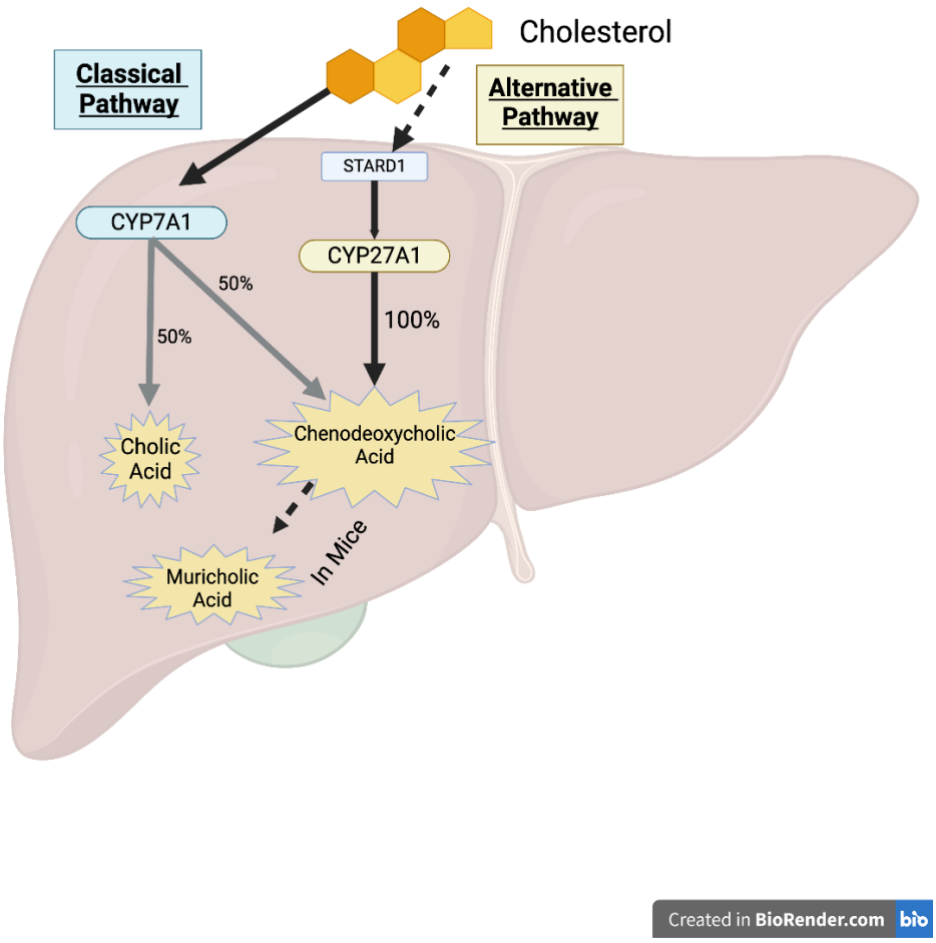

Figure 1: Bile Acid Synthesis Pathways

The classical pathway (left side) is characterized by rate-limiting enzyme CYP7A1 which catalyzes the 7alpha-hydroxylation reaction of cholesterol. The product 7alphaHC can then be turned into cholic acid or chenodeoxycholic acid in a 50:50 ratio. The alternative pathway (right side) starts with the entrance of cholesterol into the mitochondria of a hepatocyte by STARD1. Once inside the mitochondria, CYP27A1 catalyzes the 27alpha-hydroxylation reaction of the cholesterol putting it on a path only leading to chenodeoxycholic acid. In mice, further processing in the liver converts CDCA into MCA.

4. Farnesoid X Receptor Regulation

Treatment of PSC has been long sought after through employing the use of the Farnesoid X Receptor (FXR); however, the gut microbiome in this context has not been explored. The FXR pathway is a negative feedback mechanism that begins with the Farnesoid X Receptor in the lower ileum. FXR is expressed throughout the entire small intestine, but the highest

concentrations of expression are seen in the liver and ileum[14]. Here is where roughly 95% of bile acids are already broken down and reabsorbed into circulation back towards the liver while the remainder is excreted in feces[18].

The FXR is stimulated by bile acids causing the release of fibroblast growth factor 19 (FGF19) in humans and FGF15 in mice. FGF 19 travels to the liver where it is able to activate fibroblast growth factor receptor 4 (FGFR4). FGFR4 inhibits CYP7A1 which prevents the initial step in cholesterol metabolism.

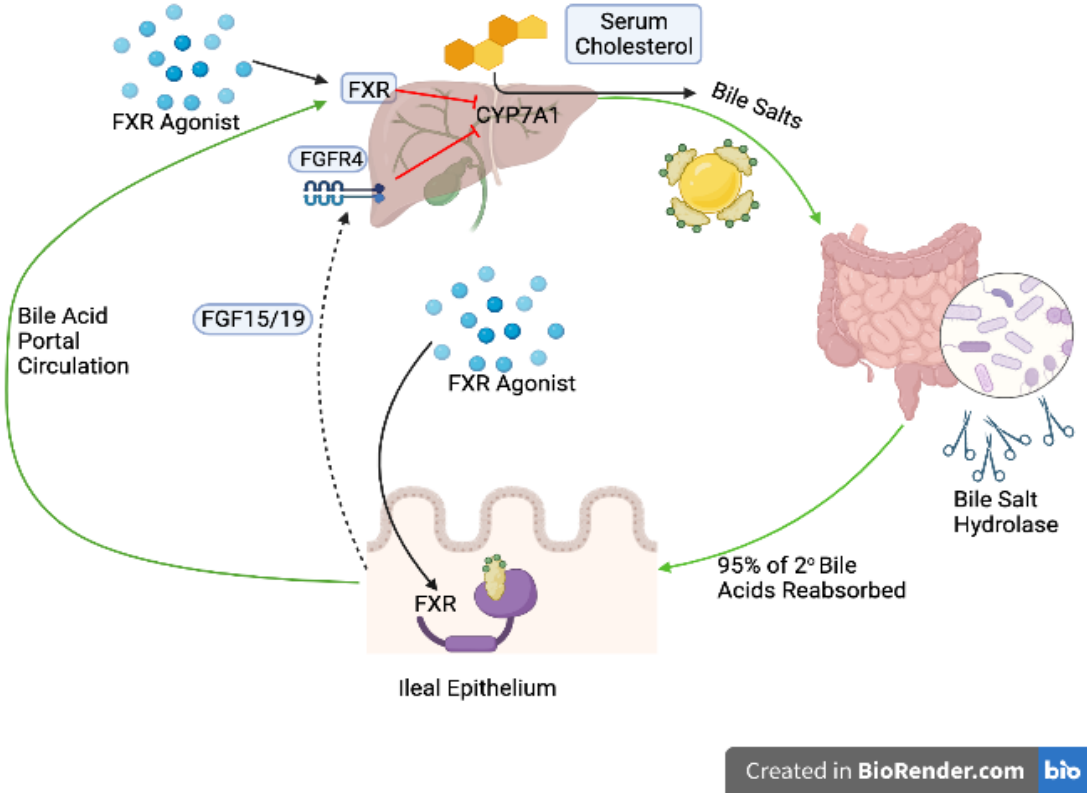

Figure 2: Farnesoid X Receptor Signaling

The FXR pathway begins with bile salts being secreted from the liver after synthesis from cholesterol. In the gut, various bacteria species produce bile salt hydrolases which breakdown the primary bile acids into secondary bile acids. These bile acids can stimulate the Farnesoid X Receptor causing fibroblast growth factor 15 in mice and 19 in humans to be secreted into portal circulation. Most of the bile acids get reabsorbed whereas roughly 5% get excreted in the feces. FGF15/19 activate FGFR4 in the liver which in turn inhibits the CYP7A1 enzyme. This enzyme is further inhibited by the stimulation of liver FXR through bile acid circulation back to the liver.

In the mouse model of *Mdr2*$^{-/-}$, we hypothesize that the lack of bile acid secretion due to the absence of bile phospholipid transporters would lower expression of FXR. This in turn would prevent FGF15/19 secretion therefore allowing CYP7A1 expression to be unregulated.

5. Our Aims and Objectives

In this experiment, I hypothesize that the FXR agonist GW4064 will be able to ameliorate liver toxicity by reducing bile acid production through inhibition of the primary bile acid synthesis pathway. If so, less liver damage could possibly help the gut microbiome environment.

The FXR receptor is a promising target because the gut microbes will deal with even less bile secretion than before. This will help determine if the FXR pathway is a viable target for individuals with cholestatic liver disease and have possible symptoms of gut dysbiosis. Previous studies have shown that FXR agonism can benefit cholestatic liver diseases[15], but it has never been done with synthetic agonist GW4064.

Our main method of observation will be utilizing bacterial colony counts on sheep's blood agar dishes. This observation has been shown to give reliable information on the status of the gut microbiome within mouse model. The most common sample to culture is stool which provides a wide glimpse at the GI tract health.

This mouse model of *Mdr2*$^{-/-}$ has been shown to exhibit 2 separate diseases. While it is commonly used to study primary sclerosing cholangitis, it also exhibits symptoms of gut dysbiosis as a result of the liver disease. The addition of this FXR agonist is intended to benefit both diseases present within this mouse model.

We hope to see if the gut-liver axis is strongly affected by the addition of a synthetic FXR agonist. This will be characterized by a stable population of *Lactobacillus spp.* bacteria and higher expression of FGF15 in the ileum.

1. Methods

12-week-old Male Mdr2$^{-/-}$ Mice	**Diet**	**Intraperitoneal Injection**	**Injection Frequency**	**Predictions**	**Sampling Methods**
Control N=3	Regular Chow	None	None	Normal Phenotype	Liver, ileum, and feces samples were taken post-mortem and homogenized in 5uL of phosphate buffered saline solution. The samples were all cultured on blood-agar at 10^{-3}, 10^{-4}, 10^{-5} concentrations. Liver and ileum samples were stored in Trizol solution for mRNA extraction and qPCR.
HFD Control N=5	High Fat Diet	Vehicle (10%DMSO, 90% Corn Oil)	2 Times a week	Gut dysbiosis and liver damage caused by HFD introduction	
Experimental Group N=5	High Fat Diet	50mg/kg GW4064 (10%DMSO, 90% Corn Oil)	2 Times a week	Less gut dysbiosis and liver damage as a result of the GW4064 FXR stimulation.	

Table 1: Experiment Layout

1. Animal Experiments

Mdr2$^{-/-}$ (friend virus B NIH [FVB].129P2-Abcb4^{tm1Bor}/J) double knockout mice were obtained from Jackson Laboratory and established true breeding lines. We fed the mice a standard chow (control; Labdiet 5001) or High-fat diet (HFD), matched to Paigen's Atherogenic

Rodent diet without sodium cholate (Research Diets 99020201) to 12-weeks-old *Mdr2*$^{-/-}$ male mice for 8–12 weeks ad libitum. In one HFD treatment group, FXR agonist GW4064 (GW, 20mg/kg, Sigma Aldrich) was administered mixed with 90% corn oil and 10% DMSO vehicle preparation intraperitoneally once a week. Control HFD group was injected with vehicle preparation only with no GW4064.

2. Standard Bacteriological Culture of Liver, Ileum, and Feces

Stool, liver, and ileum samples were weighed and homogenized within 5uL of sterile phosphate buffer solution after mouse sacrifice. Stool and ileum samples were plated at 10^{-3}, 10^{-4}, and 10^{-5} dilutions on sheep's blood agar plates. Liver samples were plated at 150uL and 350uL on sheep's blood agar for presence of translocated bacterial colonies.

3. Quantitative Polymerase Chain Reaction

Liver and ileum tissues were homogenized in 1mL of Trizol reagent (Zymo Research, Irvine, CA). The samples were then processed into an isolated sample of mRNA which was then reverse transcribed into complementary cDNA. This was done using a complementary cDNA isolation kit (Applied Biosystems, Carlsbad, CA). The PCR reactions were completed with the following thermocycling format. Starting 95°C for 10 minutes and followed by 40 cycles by 95°C for 15s, 60°C for 60s, and 72°C for 30s. A 7900HT Fast Real-Time PCR system (Applied Biosystems) was used for all reactions. Depending on tissue integrity, some samples were left out of analysis while additional samples have been added to control groups from simultaneous experiments. The list of primers can be found in the **Supplementary Table.**

4. Statistical Analysis

In this study, data were analyzed using Mann Whitney U test, student t test or one-way analysis of variance (ANOVA) using Graph Pad Prism 9 software.

2. Results

1. Mouse Mortality

In this study, 12-weeks-old *Mdr2*$^{-/-}$ mice were fed either regular chow (control, n=3) or HFD (n=5). In one HFD group (n=5), GW4064 was administered to determine how FXR agonist will influence the disease outcomes in *Mdr2*$^{-/-}$ mice. Interestingly, following 5-9 weeks of HFD treatment, *Mdr2*$^{-/-}$ mice with GW treatment (red) were found to have a higher mortality than HFD-fed (blue) or regular chow-fed (black) *Mdr2*$^{-/-}$ mice (Figure 3). The moribund mice showed

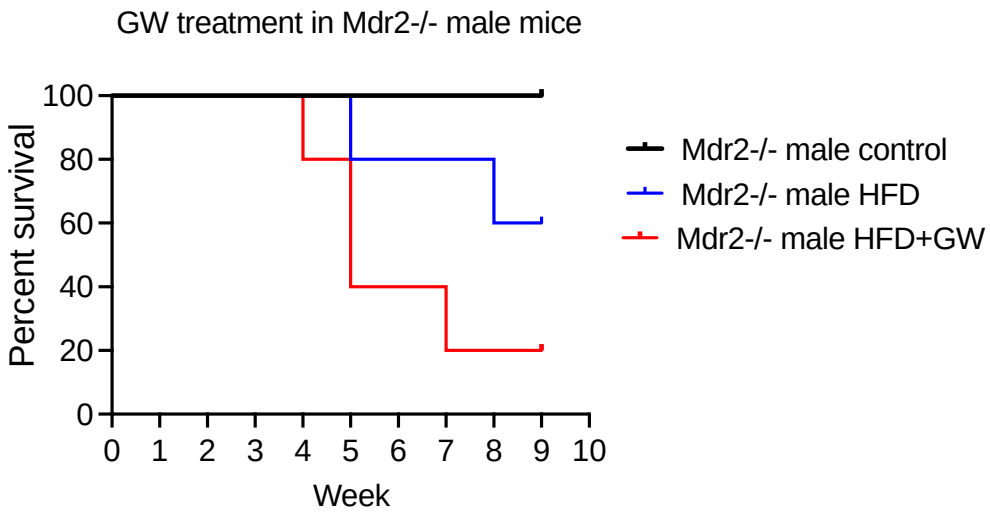

Figure 3: Mortality of *MDR2*$^{-/-}$ *Mice*

GW4064 treatment increased mortality of HFD-fed *Mdr2*$^{-/-}$ mice compared to control and HFD control mice. The graph is representative of 2 independent experiments (n=3-5 mice/group). The statistical analysis was performed by using ANOVA.

ruffled hair, slow movement, heavy breathing with an internal bleeding within the chest cavity (data not shown). Moreover, 80% mortality (4/5) was observed in GW treated mice compared

to 40% (2/5) in HFD only group and no mortality in control diet group by 9th week of treatment. In addition, HFD only group showed delayed mortality compared to GW treatment group. Taken together, GW treatment increased the mortality rate in HFD-fed *Mdr2*$^{-/-}$ mice.

2. *Bacterial Colony Counts*

Following animal harvest, we collected fecal specimens and ileum tissues from mice and processed for the standard bacteriological culture to examine the gut microbiota profile. Interestingly, we found that gut commensal bacteria *Lactobacillus* was significantly decreased in both ileum tissue and fecal samples in HFD-fed and HFD+GW treated groups (Figure 4). Since mortality rate was higher in GW treated group, the loss of *Lactobacillus sp.* was earlier in those mice. Within feces (B), there were some outliers in the GW+HFD group with higher amounts of *Lactobacillus*. Interestingly enough, the mouse that survived GW treatment, still have a significant amount of *Lactobacillus* within feces and ileum.

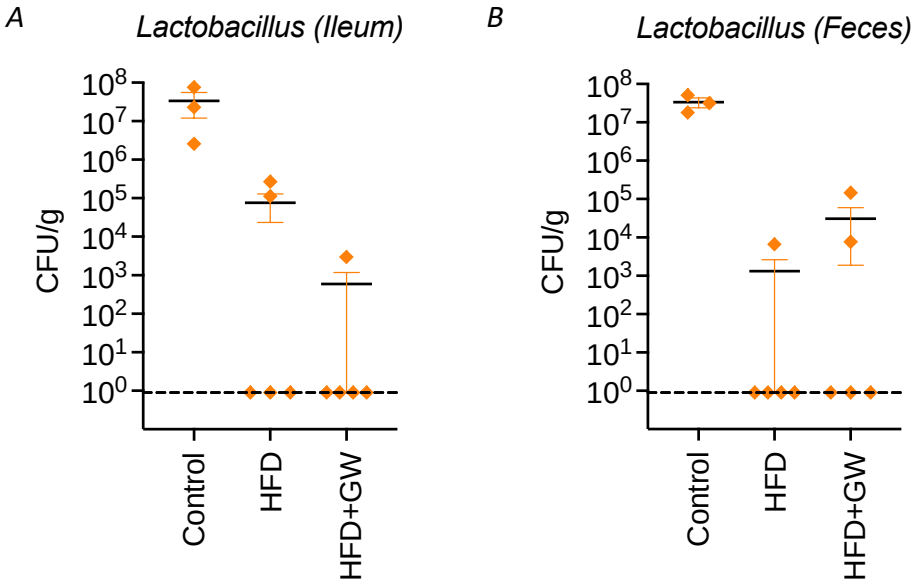

Figure 4: *Lactobacillus*

Colony forming unit (CFU) of *Lactobacillus* per gram of ileum (A) and feces (B) after HFD and HFD+GW4064 treatments. Statistical analysis was done by using student t test. No significant differences were seen between control and HFD+GW in either ileum (p=0.08) or feces (p=0.14). Error bars represent the standard error of the mean.

Seen below, the loss of *Lactobacillus* in ileum and feces was found to be correlated with enrichment of a gut pathobiont *Enterococcus faecalis* (*E. faecalis* or *Enterococcus*) in both HFD and HFD+GW treated groups (Figure 5).

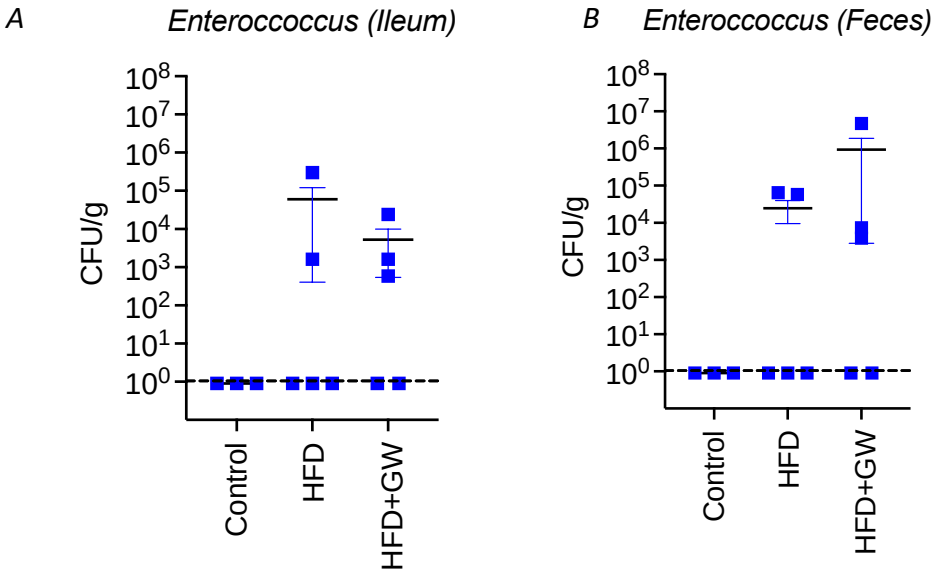

Figure 5: *Enteroccoccus*

Colony forming unit (CFU) of *Enterococcus* per gram of ileum (A) and feces (B) after HFD and HFD+GW4064 treatments. Statistical analysis was done by using student t test. No significant differences were seen between control and HFD+GW in either ileum (p=0.43) or feces (p=0.48). Error bars represent the standard error of the mean.

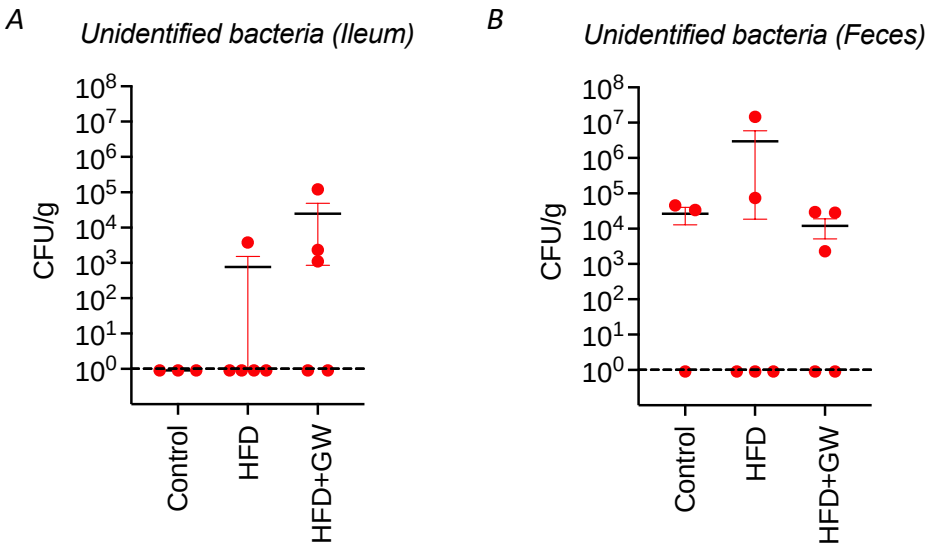

Figure 6: Unidentified Bacteria

Colony forming unit (CFU) of unidentified bacteria per gram of ileum (A) and feces (B) after HFD and HFD+GW4064 treatments. Statistical analysis was done by using student t test. No significant differences were seen between control and HFD+GW in either ileum (p=0.46) or feces (p=0.23). Error bars represent the standard error of the mean.

Moreover, we detected one unidentified bacteria colony enriched in both ileum tissue and feces of HFD and HFD+GW treated groups (Figure 6). Our future aim is to determine whether these unidentified bacteria play a critical role in disease pathogenesis during cholestatic liver disease progression.

3. mRNA Expression

Within our mouse groups, FGF15 appeared to be expressed the most in control mice followed by HFD then GW4064+HFD (Figure 7). This trend is surprising due to the expected FXR stimulation as a result of the GW4064 agonist injections. It could be due to the mobilized FGF15 into liver portal circulation targeting liver FGFR4 receptors.

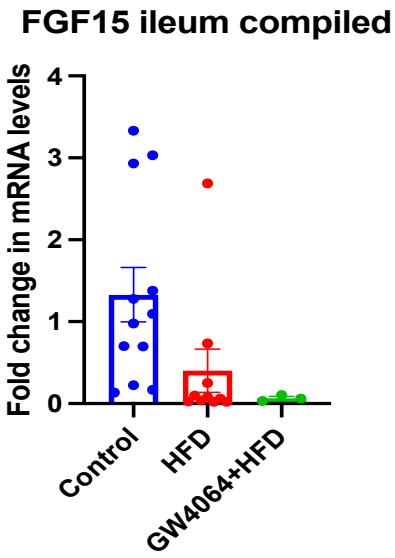

Figure 7: FGF15 mRNA Expression

GW4064 treatment mice have a lower trend of mRNA expression of FGF15 in the ileum as compared to HFD and control. Statistical analysis was done by using one way ANOVA analysis. Error bars represent the standard error of the mean.

Seen below in figure 8, FXR expression in the ileum has remained static. However, it seems that CYP7A1 expression is highest within the HFD mouse group with a lower trend in GW4064+HFD group and a trending higher expression level than the control group. CYP7A1 expression was expected to be lowest in the GW4064+HFD group as a result of FXR stimulation.

FXR expression was expected to be higher in the experimental agonist group as well but marginally so because receptor expression is difficult to change from exogenous supplements.

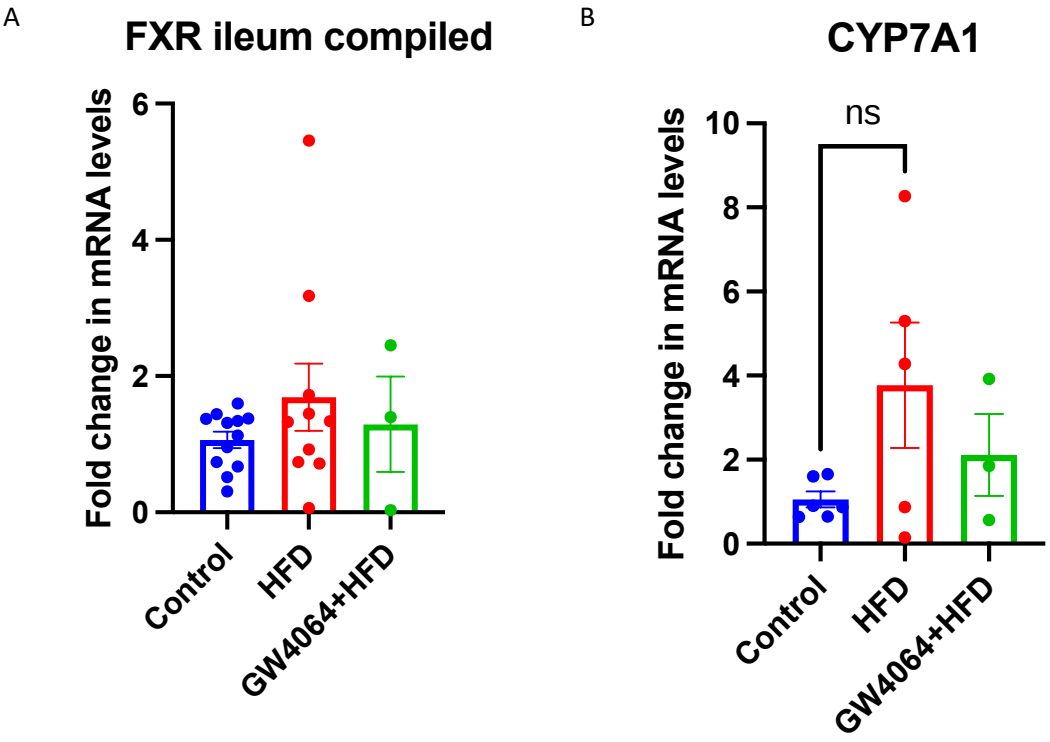

Figure 8: FXR and CYP7A1 mRNA Expression

No significant differences in mRNA expression for FXR (A) or CYP7A1 (B) between the treatment groups. Statistical analysis was done by using one way ANOVA analysis. Error bars represent the standard error of the mean.

3. Discussion & Future Directions

Based on the above data, Cholestatic liver disease and the gut microbiome seem to be correlated to bile acid signaling levels. While expression levels remained insignificant between the mouse experimental groups, there was a trend of higher CYP7A1 expression for both the GW4064+HFD, and the HFD group. This is contrary to our hypothesis of CYP7A1 inhibition due to FXR pathway activation.

This FXR experiment has shown that male mice given a HFD and the GW4064 agonist have higher mortality rates than just HFD mice. We saw extremely low *Lactobacillus spp.* counts in this experimental group of mice, so we suspect that a dysbiosis event caused this potentially pathogenic gut environment.

A possible mechanism to this quick mortality could have been the lack of any high fat diet metabolism within these mice due to a smaller bile acid pool. However, due to the lack of difference in mRNA expression between these groups of mice, we cannot conclude that the GW4064 agonist had only on-target effects.

This study was limited by the extreme nature in which the male HFD+GW4064 mice had died which could have added confounding variables to the gut microbe populations. Due to this unexpected mortality, the n-value for the HFD+GW4064 was low which inhibited our statistical analysis. In addition, during the second trial of the experiment, HFD group mice became sick earlier than usual and had low mRNA expression counts especially in FGF15 expression.

Previously, there have been experiments showing bacterial translocation to the liver from the gut.[2, 11]. However, we did not observe any bacteria present in this study (data not shown). Gut leakiness is a contributing factor in this phenomenon, and we were unable to include that variable in this study.

In future experiments, we plan on analyzing the bile acid profile within the stool and serum of all mouse groups. This information would help determine if there are differences in the hydrophobic/hydrophilic nature of the bile acids within the various mouse groups. If the GW4064 agonist caused inhibition of the primary bile acid synthesis pathway in this cholestatic liver, we would expect to see higher levels of MCA due to reliance on the alternative bile acid synthesis pathway.

In addition, this experiment would benefit from the insertion of a positive control group. This could be done by implementing bile acids CDCA or MCA which are known FXR agonists into

the diet of the positive control mice. This would give us a reference for FXR stimulation provided by the GW4064 agonist. We would expect to see similar expression levels in the FXR associated mRNA between the positive control group and the experimental group.

An FXR antagonist such as ursodeoxycholic acid could be tested as well to examine the relationship between bile-acid composition and the gut microbiome environment in this model of PSC within mice[12]. In an antagonistic model, we would expect to see less FGF15 expression leading to increased bile acid synthesis. It is unclear in PSC whether a larger bile acid pool or smaller bile acid pool is more beneficial for the patient.

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5. Supplemental Table

1	FXR mouse	Forward	5'-TCCGGACATTCAACCATCAC-3'
		Reverse	5'-TCACTGCACATCCCAGATCTC-3'
2	CYP7A1	Forward	5'AGCAACTAAACAACCTGCCAGTACTA3'
		Reverse	5'-GTCCGGATATTCAAGGATGCA-3'
3	FGF15	Forward	5’-ACGGGCTGATTCGCTACTC-3’
		Reverse	5’-TGTAGCCTAAACAGTCCATTTCCT-3’