THE ANTI-INFLAMMATORY EFFECTS OF LUMINAL LACTOBACILLUS RHAMNOSUS GG PERFUSION VIA REGULATION OF JNK RELATED PATHWAY

by

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ABSTRACT OF THE THESIS

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Master Thesis Director:

Ronaldo P. Ferraris, Ph.D

Lactobacillus rhamnosus GG (LGG) is a well studied and commercially available probiotic, known to be able to alleviate intestinal disorders. From previous research in Dr. Ferraris's lab, we discovered that by direct perfusion of active LGG to the jejunum of the small intestine, we were able to promote the production of anti-inflammatory cytokines IFN-γ, IL10, IL2, IL4, IL5, and IL6 ($P<0.05$) in four hours. To further study the properties of the LGG perfusate, we conducted a feeding study, where we gavage fed *Mus Musculus* (30 mice, 22.3±2 g Body Weight) with either Saline or LGG (6.6×10^8 CFU/mL in Saline) two times a day for days, then perfused with bacterial LPS or Poly I:C to the small intestine to determine whether prior consumption of LGG prevented bacterial or viral induced inflammation (n=5).

Unfortunately, the results generated were statistically insignificant, suggesting that gavage feeding LGG may have failed to stimulate an intestinal reaction, probably because of stress and dilution with endogenous bacteria.

We performed another perfusion experiment followed by transcriptional (RNAseq) analysis of the small intestinal mucosa of LGG- and Saline-perfused mice. Results of the RNAseq were analyzed by IPA (Ingenuity Pathway Analysis), a software that is able to predict the activities of molecular pathways affected by LGG. Among pathways predicted by IPA to be affected by LGG perfusion, “LPS/ IL-1 Mediated Inhibition of RXR (Retinoid X receptor) Function” has the most significant P value.

The “LPS/IL-1 Mediated Inhibition of RXR Function” pathway is a mitogen-activated protein kinase (MAPK) pathway, with JNK as the MAPK. Bacterial LPS would activate JNK by phosphorylation. The pJNK would in turn inhibit RXR activation, thus inhibiting the production of its downstream associated enzymes and transporters. According to our RNAseq data, IPA predicted this pathway inhibited with LGG perfusion. In contrast to LPS, LGG would decrease pJNK, resulting in an increase in downstream transcription.

There were a total of 25 effectors (enzymes and transporters) associated with xenobiotic metabolism downstream of RXRα. Among the effectors, the mRNA levels of 9 genes (CYP2A6, CYP2C8, CYP2C9, CYP3A5, CYP3A7, ALDH, PAPSS2, SULT, GST) were computed based on data from RNAseq while those of 12 genes (MGMT, SOD3, FMO2, UGT, MRP2, MRP3, MRP4, OATP2, CES, MAOA, CAT,MDR1) were measured by qPCR. Two receptors within the RXR complex (CAR,PXR), a known downstream substrate of JNK (c-JUN), and an upstream receptor of the pathway were also measured by qPCR. The phosphorylation of JNK and EGFR were analyzed with western blotting.

From RNAseq, the mRNA expression of 9 effectors (CYP2A6, CYP2C8, CYP2C9, CYP3A5, CYP3A7, ALDH, PAPSS2, SULT, GST) showed an increase of >1.5 fold with LGG perfusion compared to Saline Perfusion ($P<0.05$). From our qPCR results, downstream of RXR, when compared with saline perfused samples, the mRNA expression of 10 effectors (SOD3, FMO2, UGT, MRP2, MRP3, MRP4, OATP2, CES, MAOA, MDR1) increased more than 1.2 fold with LGG perfusion ($P<0.05$). The phosphorylation of JNK decreased in LGG perfused samples when compared with that of control.

In conclusion, we have discovered that, in contrast to the known effects of LPS, LGG has the ability to increase xenobiotic metabolism in the small intestine through the inhibition of the “LPS/IL-1 Mediated Inhibition of RXR Function” pathway. More experiments are needed to furtherevaluate the effect of LGG on RXR-mediated pathways.

Acknowledgements

I would like to gratefully acknowledge the help, support, guidance, and supervision of my dear mentor Dr. Ronaldo Ferraris. I have worked in Dr. Ferraris's lab for a year, and am very thankful for everything that I have learned here. I am very grateful to lab member Sarah Pearce and John Veltri for technical assistance and consistent moral support.

I would also like to thank Dr. Gao and Dr. Bonder for their helpful advise and encouragement.

Finally, I would like to dedicate this to my parents. Thank you mom and dad for always being there for me and helping me to find my way in life!

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List of Abbreviation

LGG : Lactobacillus rhamnosus GG

IFN-γ : Interferon γ

IL22/6/12β/15/10 : Interleukin 22/6/12β/15/10

TLR2/3/4/9 : Toll Like Receptor 2/3/4/9

TGF-β : Transforming Growth Factor Beta

LPS : Lipopolysaccharides

Poly I:C : Polyinosinic:polycytidylic Acid

IPA : Ingenuity Pathway Analysis

RNAseq : RNA sequencing

MAPK : Mitogen-Activated Protein Kinase

JNK : c-Jun N-terminal kinase

RXRα : Retinoid X receptor alpha

CYP : Cytochrome P450

ALDH: Aldehyde Dehydrogenase

PAPSS2 : 3'-Phosphoadenosine 5'-Phosphosulfate Synthase 2

SULT : Sulfotransferase

GSTM : Glutathione S-Transferase Mu

MGMT : O-6-Methylguanine-DNA Methyltransferase

SOD3 : Superoxide Dismutase 3

FMO2 : Dimethylaniline monooxygenase [N-oxide-forming] 2

UGT : Uridine 5’-Diphospho-Glucuronosyl Transferase

MRP2/34 : Multidrug Resistance-associated Protein 2/3/4

OATP2 : Organic Anion-Transporting Polypeptide

CES : Carboxyl Esterase

MAOA : Monoamine Oxidase A

CAT : Catalase

MDR1 : Multi Drug Resistance Protein 1

CAR : Constitutive Androstane Receptor

PXR : Pregnane X Receptor

EGFR : Epidermal Growth Factor Receptor

NOS : Nitric Oxide Synthase

Akt : Protein kinase B

MKK3/4 : Mitogen-Activated Protein Kinase Kinase 3/4

ATF2 : Activating Transcription Factor 2

ERK : Extracellular Signal–Regulated Kinases

Chapter 1

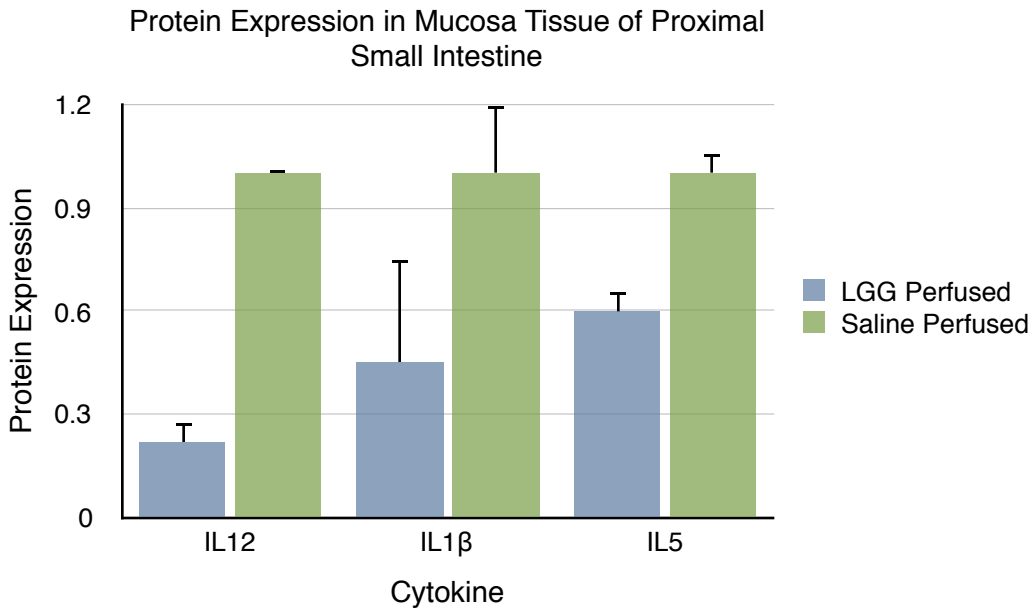
Introduction

Lactobacillus rhamnosus GG (LGG) is currently the most well studied strain of probiotic, and due to its strong resistance to bile and stomach acid, it is also the most commercially available strain of probiotic.

LGG promotes the production of immunoglobulins (Gao et al,.2015), affects the phosphorylation of numerous tyrosine kinases (Yan, F. 2011), and regulates the production of various cytokines (Wang et al,.2016). However, these studies have mostly been based on cell culture models. To study the direct and specific interaction between LGG and the mucosal layer of the small intestines, our lab perfused wild type mice with either LGG or saline. When LGG was introduced to the small intestine, the production of pro-inflammatory cytokine proteins IL-12, IL-1β, and IL-5 decreased significantly (n=6; Figure I.1). Moreover, mRNA levels of anti-inflammatory cytokine IL-22, iNOS, IL-10, TGF-β increased significantly (n=4, Figure I.2).

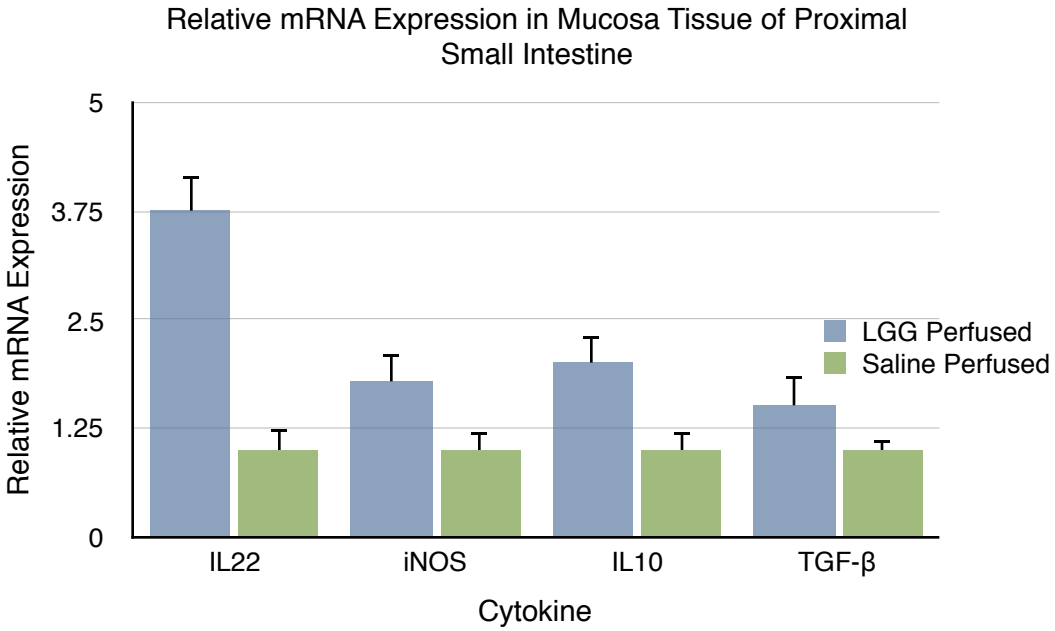
Since direct introduction of LGG to the small intestine had significant response, we decided to move forward to a feeding study, to evaluate the effects of orally administered LGG in the presence of LPS and Poly:IC induced inflammation within the small intestine,

We hypothesized that LGG, when introduced to the small intestine through oral intake prior to LPS and Poly:IC challenge, is able to quench induced



Multiplex ELISA analysis of protein supernatant of mucosal scrapings either perfused with saline solution or LGG for 4 hours. P<0.05. n=4 animals/treatments

Figure 1.1



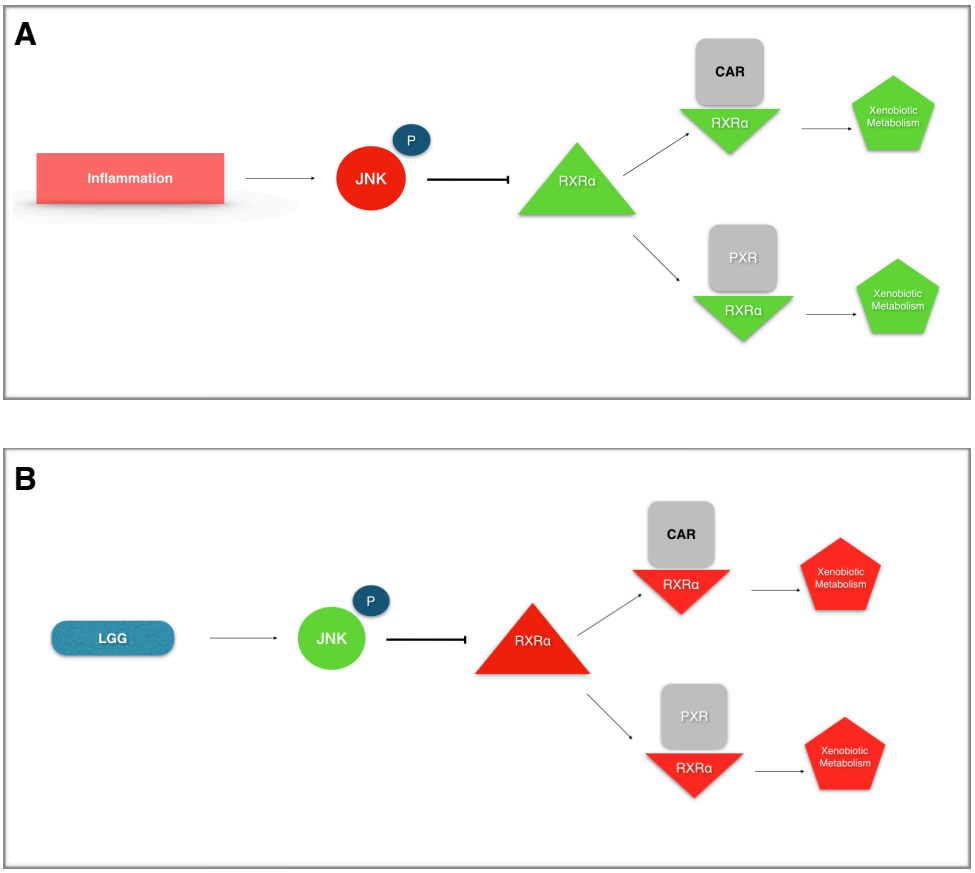
mRNA analysis of protein supernatant of mucosal scrapings either perfused with saline solution or LGG or unperfused for 4 hours. P<0.05. n=4 animals/treatments

Figure 1.2

inflammation by reducing pro-inflammatory and increasing anti-inflammatory cytokine production. However, after several month of feeding and sample analysis, there were no LGG effects on mRNA levels of all cytokines or Toll like receptors (TLRs).

After the insignificance of the feed study, we decided to conduct another perfusion experiment. Samples of mucosal scrapings from LGG- and saline-perfused mouse intestines generated from this experiment were submitted for RNAseq or whole transcriptome shotgun sequencing which determines levels of transcripts. Ingenuity Pathway Analysis (IPA) was then used to analyze the data that identified transcripts of genes that were significantly upregulated or downregulated with LGG perfusion. With algorithmic analysis, IPA was able to determine that direct LGG perfusion highly significantly changed the mRNA production of 441 genes (fold change>1.5*, P*<0.01) in the small intestine. LPS/ IL-1 Mediated Inhibition of RXR Function, Glutathione-mediated Detoxification, and Aryl-Hydrocarbon Receptor Signaling (*P*<0.01) were, most significant affected molecular pathways most significantly affected by LGG perfusion.

According to IPA's analysis, by LGG luminal perfusion, the inhibition of the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway has the most significant P value (P=2.54E-6). Thus, I hypothesize that perfusion of the probiotic LGG through the lumen of the small intestine, will inhibit the pJNK-mediated inhibition of this pathway of this pathway (Figure I.3). My specific aim is to analyze the effect of LGG perfusion on mRNA levels of RXR-regulated genes downstream of pJNK.



RXRα resides in the nucleus. In order for nuclear transcription factors PXR and CAR to initiate transcription, they would need to dimerize with RXRα.

A. *Under bacterial inflammation, the pathway of "LPS/IL-1 Mediated Inhibition of RXR Function" will be activated by LPS, the phosphorylation of its' MAPK, JNK will increase. As activated JNK marks RXRα for degradation and nuclear export, there will be less RXRα in the nucleus. Hence the activities of the PXR RXRα and the CAR RXRα complex will decrease, and the function of Xenobiotic metabolism will be inhibited.*
B. *We hypothesize, with LGG perfusion, the pathway of "LPS/IL-1 Mediated Inhibition of RXR Function" will be inhibited, and the phosphorylation of its' MAPK JNK will decrease. With less pJNK, there will be more RXRα in the nucleus. Hence the activities of the PXR RXRα and the CAR RXRα complex will increase, and the function of Xenobiotic metabolism will be activated.*

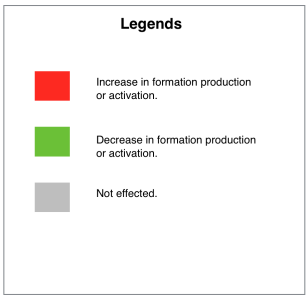


Figure 1.3

Chapter 2
Literature Review

2.1 *Lactobacillus Rhamnosus GG*

The human body contains a vast amount of micro-organisms, around 10^{13} to 10^{14}. A large percentage of these organisms are bacteria that colonize in the human gut, and are referred to as the intestinal microbiota or the intestinal micro biome (Staffas et al,.2017). Probiotics, which include lactic acid bacteria, are now mostly recognized by the general public, and are live microorganisms that have great health benefits to the host body when introduced in adequate amounts.

Probiotics have been used for the prevention and treatment of a large number of intestinal disorders, such as diarrhea, irritable bowel syndrome, ulcerative colitis, gluten intolerance (Gamallat et al,.2016), colon cancer, acute gastroenteritis, antibiotic-associated diarrhea (AAD) and necrotizing enterocolitis (NEC) (Guarino et al,.2015).

Probiotic bacteria are proposed to benefit the human intestine by three mechanisms. First, some probiotics can inhibit or exclude pathogens by direct action or through influence of the commensal microbiota. Second, probiotics can enhance epithelia barrier functions by activating or inhibiting certain signaling pathways, such as the MAPK, NF-kB, Akt dependent pathways, which could led to the increase of tight junction functions and the increase of cellular activities, such as cytokine production and cell cycle regulation. Third, most probiotics are able to modulate host immune responses (Segers et al,.2014).

In general, the beneficial effects of probiotics come from their ability to compete with other organisms for binding sites in the intestine. This allows them to participate in the up regulation of anti-inflammatory cytokines and the down regulation of pro-inflammatory cytokines, the increase in nutrient uptake, and the decrease in intestinal permeability (Gao et al,.2015).

Lactobacillus rhamnosus GG (LGG), is the most characterized naturally occurring probiotic. It is a gram negative bacteria, and has been used in clinical trials and as food supplements for more than fifty years. It was first isolated from the fecal samples of two adults, Sherwood Gorbach and Barry Goldwin, thus explaining the GG in the name of the probiotic strain. LGG is widely used in the production of nutrition supplements. Commercialized products include Culturelle, LGG Chr Hensen, and Phillip's Probiotics. LGG has shown to reduce apoptosis of intestinal cells, and significantly reduce inflammation in the intestines (Yoda et al,.2014).

Most probiotics strains are very vulnerable to gastric acids and bile salts (Li et al,.2012). However, in the presence of 19.4mM glucose, LGG survived after 90min of simulated gastric acid and bile exposure. And the percentage of survival increased with the concentration of glucose (Wang et al,.2016). This makes LGG less likely to be destroyed in the stomach when applied orally, and is able to be successfully delivered to the small and large intestine.

One of LGG's key features is its strong adhesive ability to the mucus of the small and large intestine. When administered orally, LGG can be recovered from the feces for up to one week after administration in adults (Segers et al,.

2014). Colonic biopsies even showed that LGG's colonization in the colon persists longer than the results indicated by fecal recovery. The excellent adhesive capacity of LGG to the gut mucus is due to its pili's structure (Figure L. 1). This 1 μm long and 5 nm thin structure is coded by the SpaCBA gene. It functions as nanosprings withdrawing forces and act as a mechanical zipper, thus promoting close interactions with host cells, such as the intestinal epithelial cell (Segers et al,.2014).

Figure 2.1

More and more studies demonstrated LGG's ability to alleviate inflammation by modulating cytokines and affecting signaling pathways in vitro. It is believed that the pili plays a vital part in the immunomodulatory role of this probiotic. But more studies are needed to fully understand the intestinal beneficial mechanisms of LGG (Gao et al,.2015).

2.2 Choosing Ingenuity Pathway Analysis

The Ingenuity Pathway Analysis (IPA), is a tool that involves the application of massive gene expression cause-effect relationship networks discovered in previous literature to analyze the gene expression data. The IPA

utilizes multiple important statistical algorithms, such as the Upstream Regulator Analysis (URA), which determines the likelihood of upstream regulators that are connected to the analyzed genes; the Causal Network Analysis (CNA), which generalize URA outcomes and link them to pathways; the Downstream Effects Analysis (DEA), which connect the analyzed genomic data to their downstream altered biological functions and diseases (Koch et al,.2011). In conclusion, IPA measures gene expression variation, and predicts cellular functions in an activity based manner, in that the prediction of the activity of a pathway regulator is via the measurement of genes that is known to be differentially expressed in a specific direction (Koch et al,.2011). This gives the program significant advantage when compared with the other analytical tools that only look into statistical enrichment in overlap to the analyzed genes.

2.3 MAPK pathways

The effects of LGG on the intestines, is mediated through the MAPK pathways (Hommes et al,.2003). Interests in the information railroads from the cell surface membrane to the cell nucleus has been expanding over the past few years. Knowledge of these information pathways has increased dramatically. Mitogen activated protein (MAP) kinase signal transduction pathway is among one of the best currently studied signaling pathway. MAP kinases are evolutionarily conserved enzymes, and is associated with many important regulators, such as the P53 protein. The pathway play a variety of crucial roles, such as cell migration, cellular differentiation, apoptosis, and many aspects of the

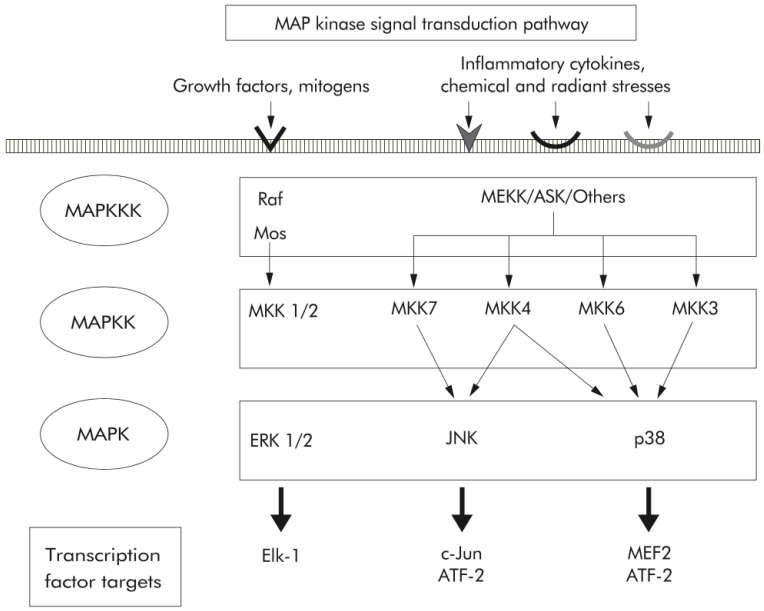


Figure 2.2

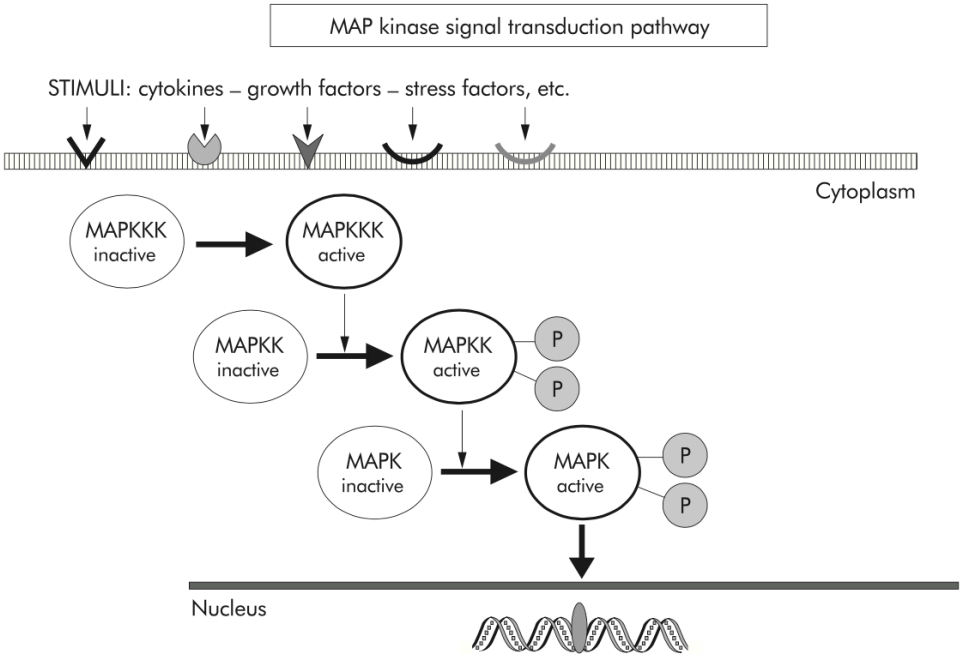


Figure 2.3

Table 1 Mitogen activated protein (MAP) kinases

MAP kinase Isoform	Alternative name	Substrate	Phenotypes of MAP kinase knockout mice.
ERK 1	p44 MAPK	MAPKAP-K1, MNKs, MSKs, Elk1	Defective T cell development
ERK 2	p42 MAPK	MAPKAP-K1, MNKs, MSKs, Elk1	
ERK 3α	p63 MAPK	MNKs, MSKs	
ERK 3β	human ERK3	MNKs, MSKs	
ERK 4	ERK1b	MNKs, MSKs	
ERK 5		MNKs, MSKs	
ERK 7		MNKs, MSKs	
JNK 1	SAPKγ	c-Jun, JunD, ATF-2, Elk1	Defective T cell differentiation to Th2 cells
JNK 2	SAPKα	c-Jun, JunD, ATF-2, Elk1	Defective T cell differentiation to Th1 cells
JNK 3	SAPKβ	c-Jun, JunD, ATF-2, Elk1	Resistance to excitotoxic neuronal cell death
p38α	CSBP, SAPK2 MPK2, RK, Mxi2	MAPKAP-K2/3, MSKs, ATF-2, Elk1, MEF2c	Placental defect, insufficient production of erythropoietin
p38β	p38–2, p38β_2	MAPKAP-K2/3, MSKs, ATF-2	
p38γ	ERK6, SAPK3	ATF2	
p38δ	SAPK4	ATF2	

ATF-2, activating transcription factor 2; ERK, extracellular signal regulated kinase; JNK, c-Jun NH_2 terminal kinase; MAP, mitogen activated protein; MAPK, MAP kinase; MAPKAP, MAP kinase activated protein kinase; MEF, myocyte enhance factor; SAPK, stress activated protein kinase; MNKs, MARK activating protein kinases; MSKs, mitogen and stress activated protein kinases; CSBP, cytokine suppressive anti-inflammatory binding protein; RK, reactivating kinase.

Table 2.1

immune mediated inflammatory responses (Gamallat et al,.2016). Thus, many drugs have been developed with MAPK pathway down stream regulators as their targets. Such drugs are used in the treatment for cancer as well as other diseases.

The MAPK pathways can be stimulated by cytokines, growth factors, and stress by phosphorylation (Figure L.2, Figure L.3). This stimulation then activates MAPKKK, which then activates MAPKK, then activates MAPK, which then effects cellular transcription and regulate cellular behavior (Gamallat et al,.2016). By far, we have found five distinct groups of MAP kinases in mammalian cells: Extracellular signal regulated kinases (ERKs) 1 and 2; c-Jun amino-terminal kinases 1 and 2 and 3(JNK1/2/3); p38 isoforms α/β/γ/σ; ERKs 3 and 4; and ERK5. But the most extensively studied vertebrate MAPKs are the ERK1/2, JNKs, and P38 (Krämer et al,.2014), which are shown in Figure L.2. The common substrates of the MAPKs are shown in Table L.1.

One of the first characterized MAP kinase cascade would be the ERK signaling module. In this specific MAPK pathway, the Raf protein function as upstream MAPKKK, and the MEK1/MEK2 as MAPKK. After being activated by phosphorylation, ERK1/2 not only target transcription factors, but also cytoplasmic proteins and membrane proteins. Although the precise mechanism of ERK1/2's effect on cellular physiology is still not fully known, knocking out ERK1/2 in mice, or its upstream effectors MEK and Raf, usually resulted in death (Gamallat et al,.2016).

Cellular stress is able to stimulate the activation of the JNK pathway, which then regulates cell apoptosis and proliferation. Similar to the ERK1/2 pathway, MKK7 and MKK4 act as MAPKK upstream proteins. However, the MAPKKK activators of JNK compose of a large group of more than 12 intercellular proteins. Mice with the JNK genes disrupted, experienced severe immune diseases. The JNK signaling pathway is believed to be involved in a large number of pathological conductions, such as inflammation diseases, cancer, heart diseases, and stroke (Gamallat et al,.2016).

The P38 signaling cascade share many similarities with the other MAPK pathways. MKK3, MKK4 and MKK6 function as the pathway's MAPKKs, and a large group of proteins acting as the MAPKKKs. The P38 MAPK plays a vital role in a wide range of immune response regulations, and is mostly activated by pathogens and inflammation associated cytokines.

2.4 MAPKs

ERK, P38, JNK, located throughout the human body, once activated, is able to phosphorylate various substrate proteins including transcription factors such as Elk-1, c-Jun, ATF2, and p53. In this study, we focused our attention on JNK.

2.4.1 ERK

ERK1/2 are distributed through out the whole cell. But once activated, the majority of activated ERK1/2 get transferred and accumulate in the nucleus. ERK is know to promote cellular proliferation and growth. It is able promote intestinal tumorigenesis by stabilizing the oncogene c-Myc (Zou et al,.2017).

2.4.2 P38

P38, connected with other pathways, governs cell survival and death in response to inflammation and stress. When activated, this MAPK is responsible for the phosphorylation of more than 100 proteins. Amon them are transcription factors, cell cycle and apoptosis regulators, growth factor receptors. Such as p53, ATF, cyclin D1, CDK inhibitors, Bcl-2, FGFR (Korhonen, Timo 2006).

2.4.3 JNK

The MAP kinase JNK consists of ten isoforms derived from three genes, JNK1 (four isoforms), JNK2 (four isoforms), and JNK3 (two isoforms) (Bruzzese

et al,.2004). Each gene is expressed as 46 KDa or 55 KDa protein depending on the variance of the gene. And there have been no functional difference between the 46 KDa protein and 55 KDa isoform protein kinases. JNK1 and JNK2 are expressed universally, while JNK3 is predominantly expressed in the brain (Bruzzese et al,.2004). The MAP kinase is activated through the dual phosphorylation threonine (Thr) and tyrosine (Tyr) residues within a Thr-Pro-Tyr motif. And this phosphorylation is stimulated by cellular stress, including but not limited to, growth factors, radiation, UV, inflammatory cytokines, oxidative stress, and G coupled receptors.

JNK is believed to be involved in the regulation of inflammation, apoptosis, and proliferation. JNK disrupted mice has defects in immune responses and cellular apoptosis. Two JNK activators, MKK4 and MKK7, have been identified. Whereas MKK7 is a specific activator of JNK, MKK4 can also phosphorylate p38 MAPK. Whereas MKK7 is a specific activator of JNK, MKK4 can also phosphorylate p38 MAPK. As with the other MAPKs, the membrane proximal kinase is a MAPKKK, typically MEKK1/4, or a member of the mixed lineage kinases (MLK) that phosphorylates and activates MKK4/7. Loss of MKK4 or MKK7 reduced JNK activation in response to UV and anisomycin. However, MKK7 is essential for activating JNK in cells incubated with pro-inflammatory cytokines (Bruzzese et al,.2004).

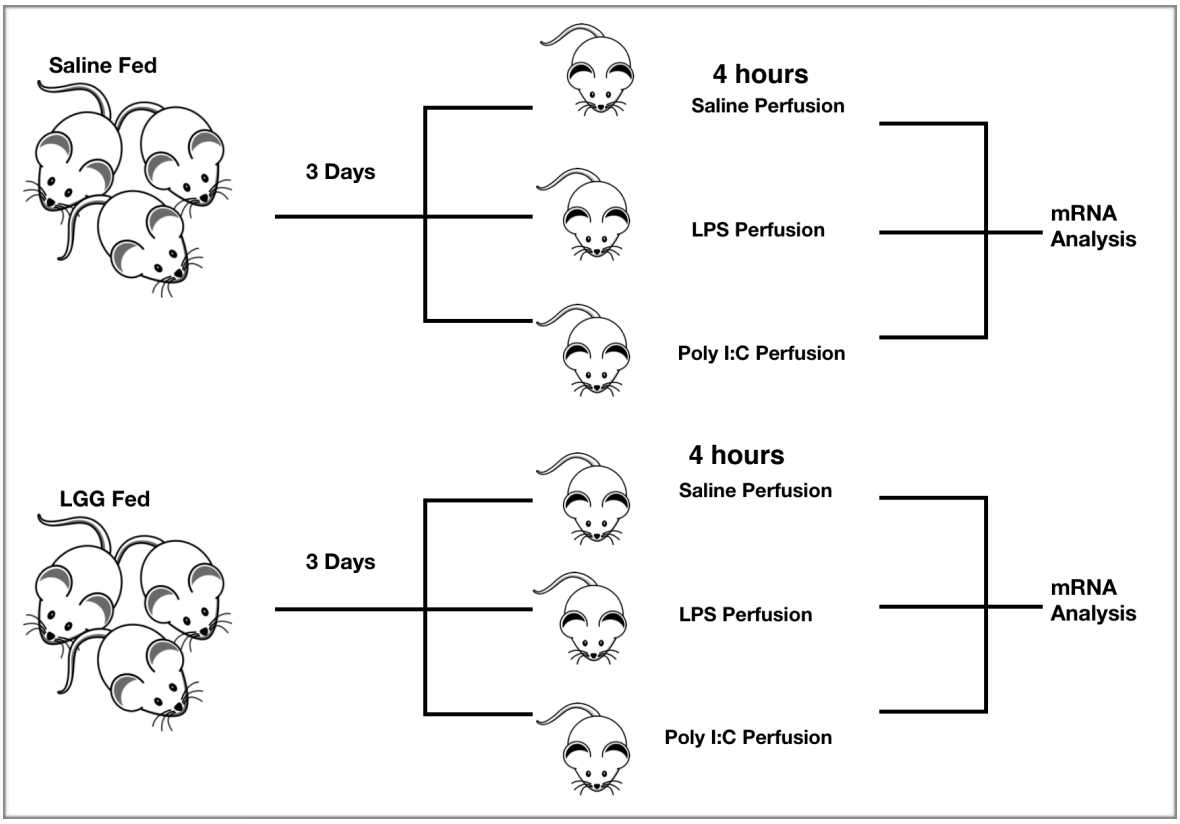
Currently, we did not discover any MKs that are exclusively activated by JNKs, but transcription factors such as c-Jun, STAT-3, ATF-2 are its' well known substrates.

Chapter 3

Material and Methods

Experimental Design

Design for the feeding experiment are shown in the figure below (Figure M.1).



Feed Study Experiment Design

Use 6 mice per experiment. Gavage feed mice for a continuous three days. On the fourth day, surgically perfuse the mice for 4 hours with either Saline, LPS, or Poly I:C solution. Their samples are then used for mRNA analysis.

Figure 3.1

Animals

Rutgers University Institutional Animal Care and Use Committee approved all procedures involving animals. Wild type Mus Musculus were used for the study. The animals were raised at germ free conditions with autoclaved food and water, and at thermal-neutral condition. A maximum of 4 mice were allowed to be housed in a single cage. A total number of 35 mice were used for the perfusion experiments. Animal's body weight range from 19.9g to 27.9g with an average of 23.62g.

To maintain a constant colony, breading cages were made. One male and two females of different parents were assigned to each breading cage. Newborn mice of one week or older were separated from the parents and assigned new cages. The newborn were at least two weeks of age before they were used for experiments. When the colony grew out of capacity, excessive mice where given food and water, and euthanized by professional animal care-takers.

Treatments

Mice were assigned to the following feeding and surgical treatments:

1. Gavage fed with LGG solution, two times a day, for three days. And the ileum of the mice was surgically perfused for 4 hours with Saline, or LPS, or Poly.IC perfusate on the fourth day.
2. Gavage fed with control solution (KRBeg buffer), two times a day, for three days. And the ileum of the mice was surgically perfused for 4 hours with Saline, or LPS, or Poly:IC perfusate on the fourth day.

Feeding

The mice were gavage fed with either 13.3mg/mL Inulin solution made with KRBreg and Inulin, or LGG solution with a bacteria concentration of 6.6 x 10^8 CFU/mL made with a commercially available LGG pill and KRBeg buffer.

The animals were fed two times a day, 200μL of feeding solution per fed, once at 10am and once at 5pm, for three days.

Mice Saline, LPS, Poly:IC Perfusion

65 ml of KRBeg buffer was used as the control perfusate. 65ml LPS Perfusate was made with LPS KRBeg buffer to a concentration of 5μg/mL. And the Poly I:C perfusate was made by adding Poly I:C KRBeg buffer to a concentration of 100μg/mL. All three perfusates were then translocated to 60ml syringes. Both syringes were installed to the Syringe Pump, the push speed was set to 15ml/hour, and surgical IV tubes were installed to the syringes. A heating pad and a rectal thermometer were connected to the Thermo-Regulator with the system's temperature set to 36°C. Thus if the mice's body temperature drops below 36°C, the Thermo-Regulation System will heat the heating pad until the animal's body temperature reaches 36°C.

For each set of experiments, 2 to 4 mice were used, and all animals were weighed and anesthetized prior to surgery. An initial does of 0.1 mL (0.1 mL/25g of body weight) Ketamine (20mg/mL) were applied to the mice intraperitoneally with 1mL syringe and 25G needle. The state of mice were tested by touching whiskers or pinching the tail, if the mouse reacts to these stimuli 90 seconds

later, an additional 0.02mL to 0.04mL of Ketamine (20mg/mL) were given. Once the mice were fully anesthetized, their limbs and tail were taped to the heating pad, and the rectal thermometers were inserted into mouse to measure body temperature.

The abdomen of the animal was disinfected with ethanol prior to incision. An initial transverse 0.8cm incision approximately 1cm inferior to the rib cage was made to the skin and peritoneum, exposing the small intestine. 37°C KRBeg buffer soaked cotton tipped applicators were used to tease out the entire small intestine. Two small incisions were made to the small intestine. The first incision was made at the distal end of the duodenum (the pancreas was used as a reference point), and the second incision was made at the distal end of the ileum (the colon was used as a reference point). The two incisions were approximately 8-10cm apart. Large blood vessels were avoided when making the incisions to prevent blood loss. Immediately after incisions, a large gavage needle was inserted from the first incision into the small intestine (only the tip of the gavage needles were inserted into the small intestine). A syringe containing 36°C KRBeg buffer was attached to the gavage needle, and was used to flush out the fecal matters from the intestine. After the flushing, a second large gavage needle was inserted to the second incision in the same manner. (according to their corresponding incision, the needles will be referred as: first gavage needle and second gavage needle). With both of the gavage needles inserted, the IV tube was attached to the first gavage needle. The process was repeated for each mice, and the perfusion pump was started after all animals were ready.

Periodically, 2-4 drops of Xylazine (2.5mg/mL) were applied to the exposed small intestine to keep the animal in an unconscious state throughout the experiment. At the end of the 4 hour perfusion, the perfused small intestine and approximately 5cm of the un-perfused small intestine were separated from the animal, the tissue were immediately flushed with RNAlater and put on ice, and the animal was sacrificed. Either whole tissue or the mucosa (collected by gently scraping the lumen of the intestine with glass slides) was collected from the separated small intestine, and snap frozen. 2ml of blood was collected from the heart of the carcass and snap frozen. All samples were labeled accordingly, and stored in -80°C.

RNA Extraction

RNeasy Micro Kit from Qiagen was used to extract RNA from all tissue samples. The RNA concentration were then measured on NanoDrop. RNA samples were labeled accordingly and stored at -80°C.

cDNA Making

iScript™ cDNA Synthesis Kit were used to make cDNA from RNA samples. The cDNA samples were labeled accordingly and stored at -4°C.

qPCR

SuperMix were made with Maxima SYBR green qPCR Master Mix kit. Specific primers of interests were designed and ordered through Integrated DNA

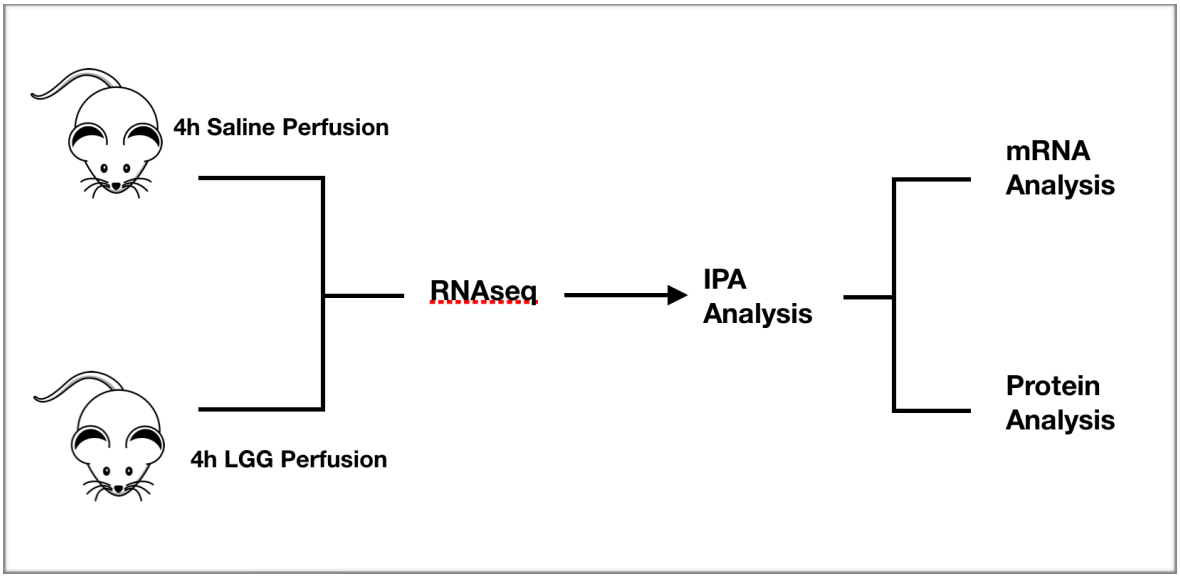
Technologies (IDT) (**Primer sequence listed in Appendixes**). Made cDNA was mixed with SuperMix and used for RT-qPCR with Mx3000P. Mx300P generated data was analyzed with Microsoft excel and StatsView. All samples were standardized to β-actin mRNA expression.

Statistic

All data were statistically analyzed using StatsView version 1.4. Data was analyzed using a two way anova model.

Experiment Design

Use 2 mice for each experiment. Surgically perfuse each mouse with either LGG or Saline solution for 4 hours. Extract and RNA from collected



Perfusion Study experimental design

Figure 3.2

samples and perform RNAseq. Analyze the RNAseq data with IPA (Ingenuity Pathway Analysis). Perform protein and mRNA analysis based on IPA's results. (Figure M.2)

Treatments

Mice were assigned to the following surgical treatments:

1. The ileum of the mice was surgically perfused for 4 hours with LGG perfusate.
2. The ileum of the mice was surgically perfused for 4 hours with Saline perfusate.

Mice LGG Perfusion

65mL LGG Perfusate has a bacteria concentration of 3.3×10^8 CUF/mL. It is made with a commercially available LGG pill and KRBreg buffer. As each LGG capsule also contain 200mg of Inulin beside the bacteria, the control perfusate were made by dissolving 400mg of Inulin into 65 ml of KRBreg buffer. Both perfusates were then translocated to 60ml syringes. Both syringes were installed to the Syringe Pump, the push speed was set to 15ml per hour, and surgical IV tubes were installed to the syringes. The rest of the procedure are as described in the Mice Saline, LPS, Poly:IC Perfusion.

Protein Extraction

Lysis buffer were made with: T-PER + Protease inhibitor tablet + Phosphatase inhibitor tablet. Tissue samples were removed from -80°C storage, and added 2mL ice cold lysis buffer. The tissue sample was then homogenized for approximately 30 seconds or until the tissue was ground up. The homogenate was then placed on ice for 10 minutes, and centrifuged at 10,000 r.p.m for 15 minutes at 4°C. The supernatant was collected after the centrifuge, they were according labeled and stored at -80°C.

BCA

Pierce BCA Protein Assay Kit from ThermoScientific and a spectrophotometer was used to analyze extracted protein concentrations.

Western Blot

Protein samples were all standardized to the same concentration with lysis buffer. 4 x Laemmli Sample Buffer and BME(β-mercaptoethanol) were mixed 9 : 1, and used as loading buffer. Standardized samples were then mixed with the loading buffer and left at room temperature for 2 hours for denaturation. Western blot running buffer was made with 10% 10 x Tris/Glycine/SDS Buffer and 90% milli-q water. Western blot transfer buffer was made with 10% 10 x Tris/Glycine/ SDS Buffer, 20% methanol, and 70% milli-q water. Loading buffer and protein samples were mixed1 : 3, and left at room temperature for 1-2 hours for denaturation. Denatured proteins samples were loaded to Mini-Protean TGX

Gels in running buffer, and run under 89V for 2-3 hours. Proteins on the gel were then transferred under 100V for 2 hours in the cold room (to avoid overheating) to a Nitrocellulose Membrane. The membrane was then blocked with 5% BSA solution in TBST for a hour (made with 10% 10 x TBS, 0.1% Tween and 89.9% milli-q water). The blocked membrane was then incubated with primary EGFR antibodies at 4°C overnight. The membrane was then washed with TBST for three times, around 10 minutes each for each wash, and incubated in secondary anti-rabbit HRP conjugated secondary antibody at room temperature for about a hour. Then the membrane was washed again for three times with TBST , around 10 minutes each wash, and excited with Chemiluminescence and imaged with LAS 4000.

RNAseq

8 of our samples were chosen according to there RNA quality for RNAseq. The experiment was performed by the Graduate School of Biomedical science.

IPA

Ingenuity Pathway Analysis was provided by the Graduate School of Biomedical science. Data acquired from RNAseq were imported into IPA and analyzed. According to IPA's statistical algorithms, a list of predicted to be effected pathways, as well as their relative p values were provided.

Chapter 4

Results

4.1. LGG Feed Study

Mice (n=15) experienced Saline Gavage Feeding showed an average decrease of 1.67% in body weight. Mice (n=15) experienced LGG Gavage Feeding showed an average decrease of 6.69% in body weight. (Table 4.1.1) (P=0.14).

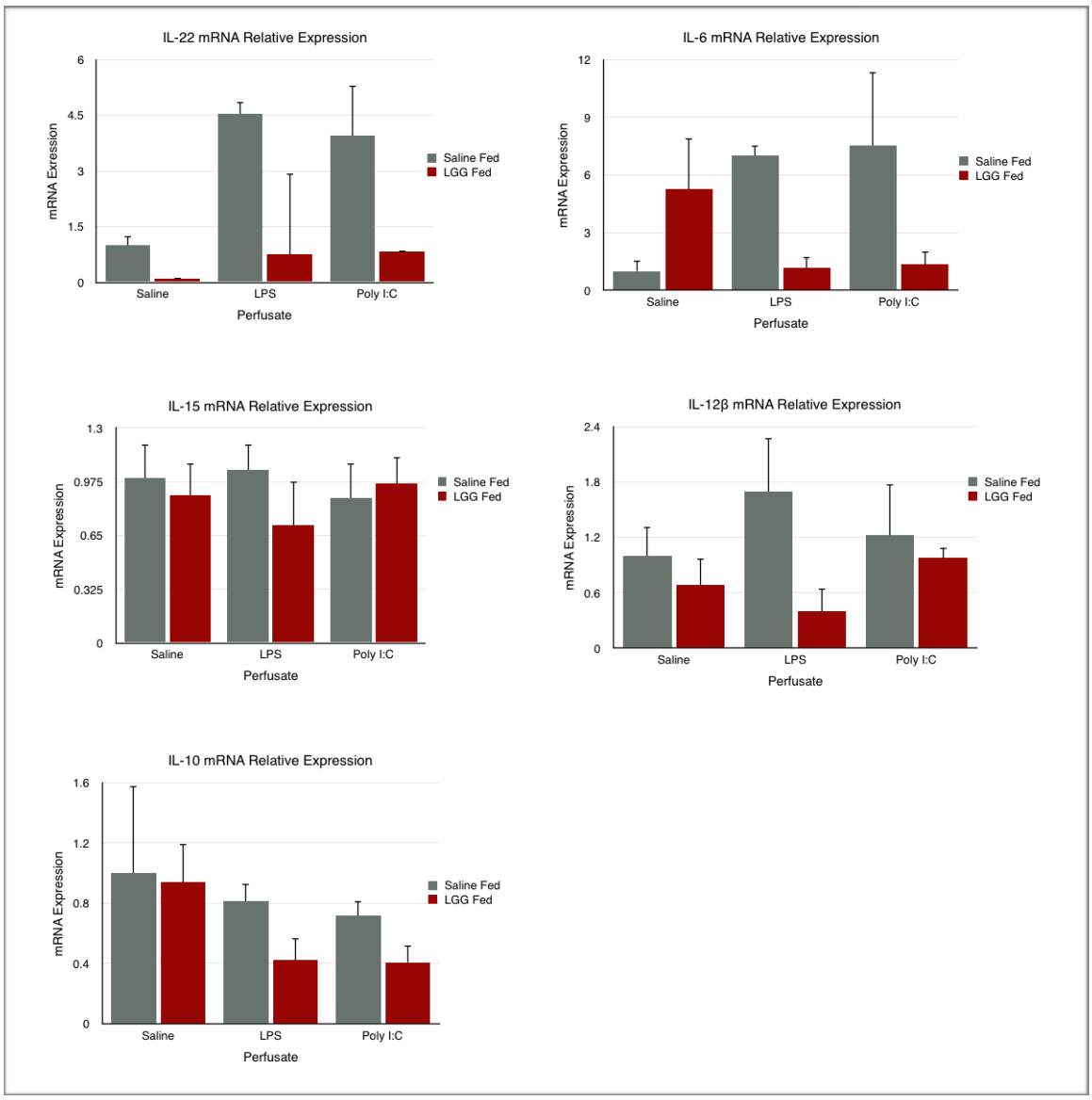
Average Food Intake & Weight Change

	Average Food Consumed per cage (3 mice) over 3 days (g)	Average Weight Change per mice over 3 days (g)	Average Percentage Change in Food per cage (3 mice) over 3 days	Average Percentage Change in Weight per mice
Saline Fed	28.76	-0.39	-13.6%	-1.67%
LGG Fed	26.08	-1.95	-8.2%	-8.60%

Table 4.1.1

4.1.1 Interleukin Analysis

The mRNA expression anti-inflammatory interleukins IL-22, IL12β, IL-10; as well as pro-inflammatory interleukins IL-6 and IL-15 were detected with qPCR and analyzed the StatView (Figure 4.1.1). Due to high P values of the data, the results are considered statistically insignificant (Table 4.1.2)



Anti-inflammatory IL-22, IL12β, IL-10; as well as pro-inflammatory IL-6 and IL-15 mRNA analysis of mucosal scraping samples either fed with Saline (perfused with Saline, LPS, Poly I:C), or fed with LGG (perfused with Saline, LPS, Poly I:C). n=6, P>0.05

Figure 4.1.1

Results of Interleukin Analysis

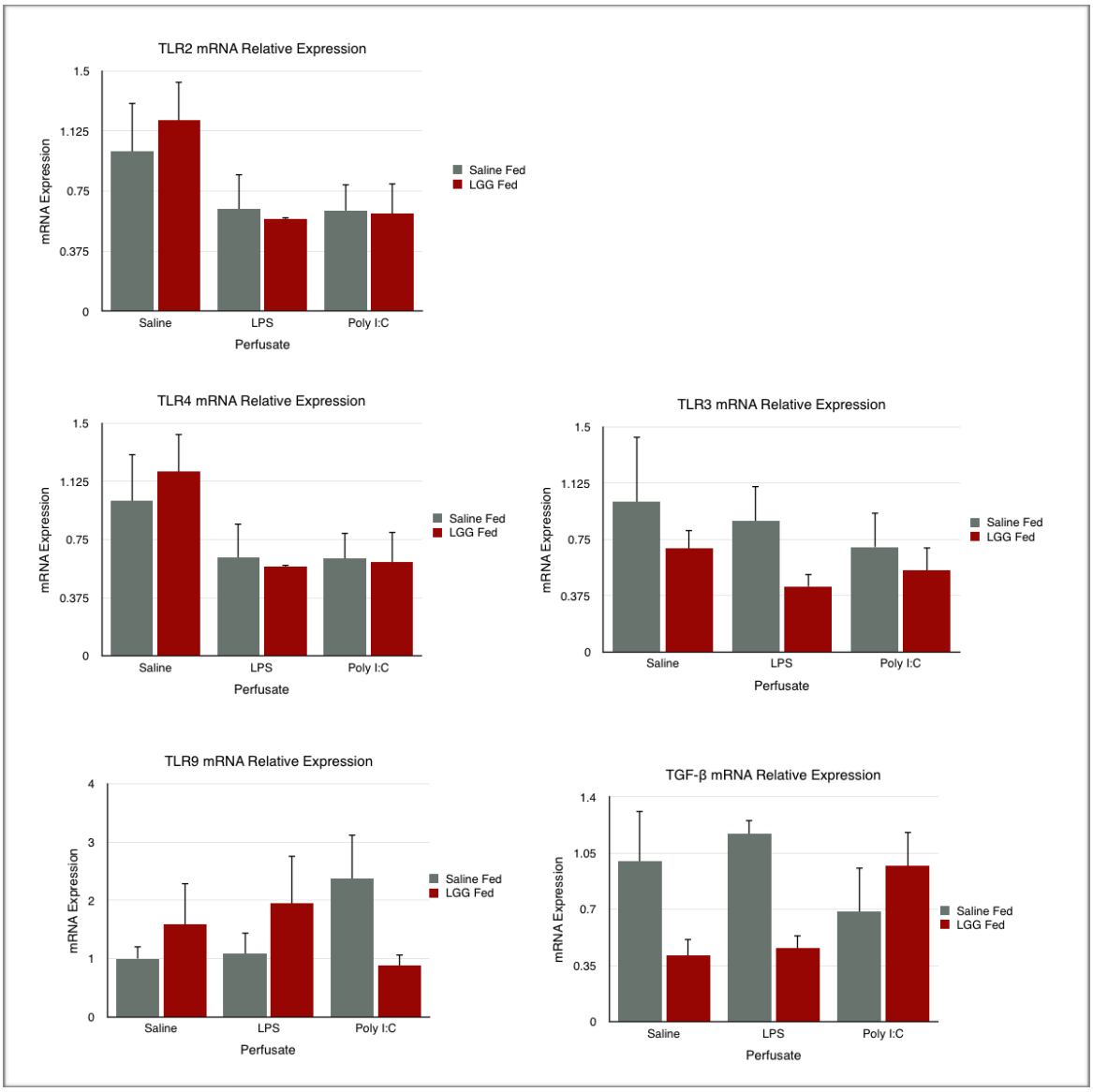
Interleukin	P Value of Feeding as the Effect	P Value of Perfusate as the Effect	P Value of Feeding and Perfusate
IL-22	0.2151	0.087	0.609
IL-6	0.5024	0.728	0.148
IL-12β	0.1304	0.731	0.059
IL-15	0.3319	0.571	0.227
IL-10	0.2710	0.222	0.796

P value of Feeding as the effect, P value of Perfusate as the effect, and P value of Feeding and Perfusate interaction were calculated by StatView. The values are all greater than 0.05, hence the results are considered insignificant. n=6

Table 4.1.2

4.1.2 Toll-Like Receptor (TLR) and Transforming Growth Factor (TGF) Analysis

TLR2, TLR3, TLR4, TLR9, and TGF-β were analyzed for mRNA expression. TLR2 recognizes gram positive bacteria, such as LGG. TLR3 recognizes Poly:IC, of which mimics virus RNA. TLR4 recognizes LPS secreted by gram negative bacteria. And TLR9 reacts to both bacterial and viral stimulations. TGF-β is able to prevent the activation of T cells and stimulate the apoptosis of B cells. However, the results all have a large P value, thus can be considered statistically insignificant (Table 4.1.3).



Toll Like Receptor and Transforming Growth Factor mRNA analysis of mucosal scraping samples either fed with Saline (perfused with Saline, LPS, Poly I:C), or fed with LGG (perfused with Saline, LPS, Poly I:C). n=6, P>0.05

Figure 4.1.2

Results of Toll Like receptors and Transforming Growth Factor Analysis

Toll Like Receptors and Transforming Growth Factor	P Value of Feeding as the Effect	P Value of Perfusate as the Effect	P Value of Feeding and Perfusate Interaction
TLR2	0.366	0.129	0.978
TLR3	0.116	0.535	0.785
TLR4	0.336	0.443	0.595
TLR9	0.905	0.860	0.118
TGFβ	0.271	0.983	0.0522

P value of Feeding as the effect, P value of Perfusate as the effect, and P value of Feeding and Perfusate interaction were calculated by StatView. The values are all greater than 0.05, hence the results are considered insignificant. n=6

Table 4.1.3

4.2 “LPS/IL-1 Mediated Inhibition of RXR Function” Pathway Analysis

According to our previous work and Ingenuity Pathway Analysis (IPA), the canonical pathway of “LPS/IL-1 Mediated Inhibition of RXR Function” is predicted to be inhibited with luminal *Lactobacillus rhamnosus GG* (LGG) perfusion. With a P value of 2.54E-6, the pathway is considered the most significant among all other predicted to be LGG affected pathways. With our interest of the pathway focused on the two nodes of Xenobiotic Metabolism and IPA’s results, the activities of downstream effectors show to be inhibited in the presence of inflammation (Figure 4.2.1), and show to be activated with the introduction of LGG perfusion (Figure 4.2.2).

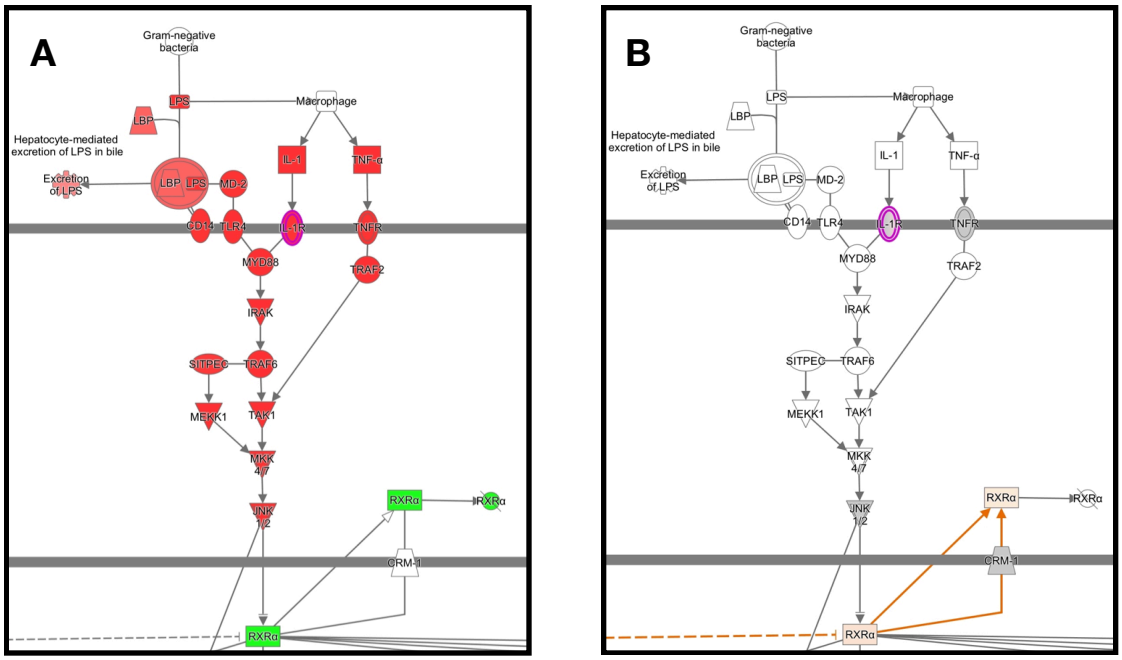
4.2.1 mRNA Analysis

mRNA expression of downstream regulators from the two nodes of Xenobiotic Metabolism as well as certain up stream effectors of the pathway were analyzed by qPCR or by previous RNAseq, and the results analyzed by StatsView.

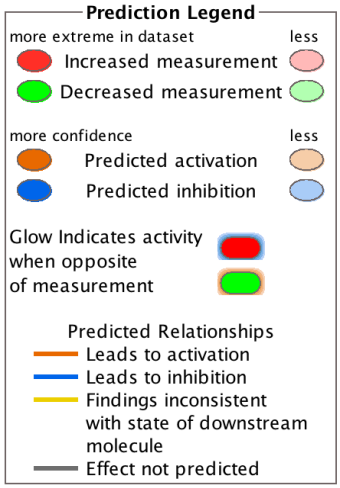
4.2.1.1 Up Stream Receptor and Kinase mRNA qPCR Analysis

IL1RL1 (Interleukin 1 Receptor Like 1), a receptor for Interleukin 1; c-JUN, a downstream regulator activated by phosphorylated JNK; CAR (Constitutive Androstane Receptor), a nuclear receptor and endobiotic as well as xenobiotic sensor; and PXR (Pregnane X Receptor), a nuclear receptor that senses the

presence of foreign toxic substances and up regulate down stream proteins in response. All of these receptors and kinases were analyzed for mRNA expression. Due to the large P values of the results, they are considered

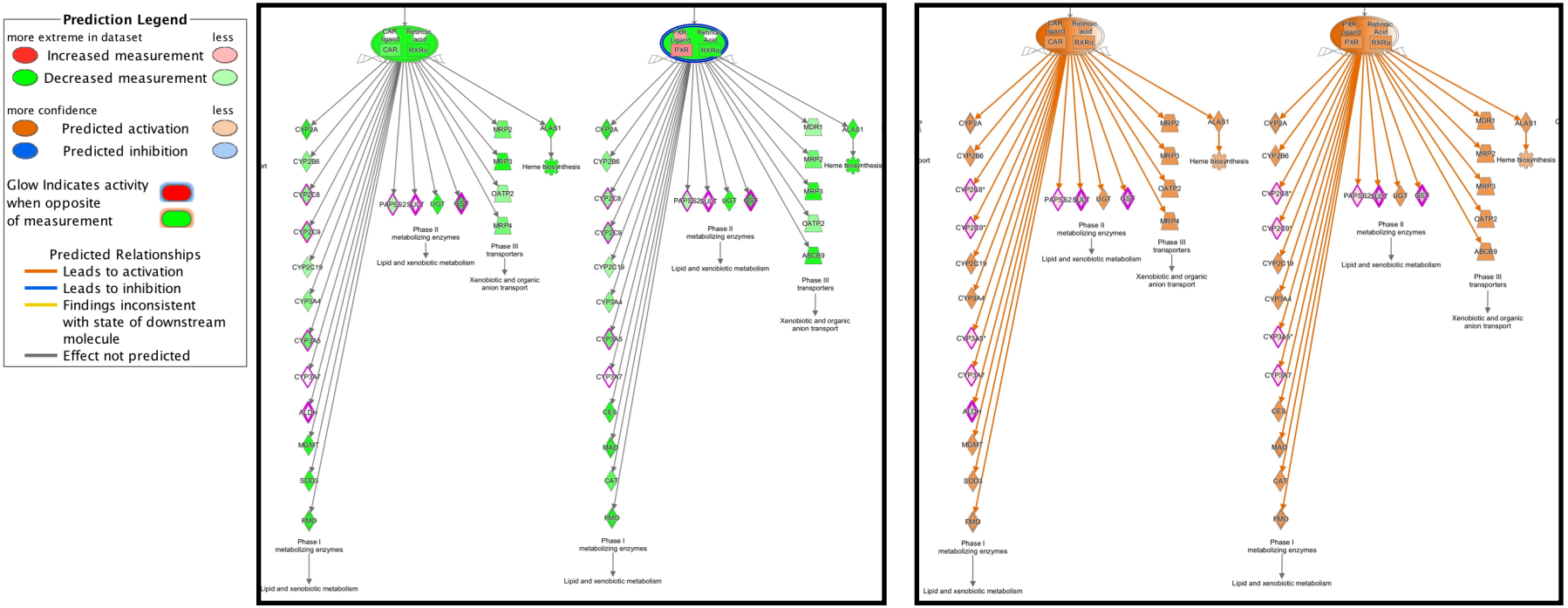


A. In the presence of inflammation, IPA shows that the the upstream (upstream of RXRα) potation of the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway is activated. And that RXRα is inhibited by this activation.
B. From the RNAseq data, IPA shows that with LGG perfusion, the upstream (upstream of RXRα) potation of "LPS/IL-1 Mediated Inhibition of RXR Function" is no longer activated in the presence of inflammation. And that RXRα is activated.



insignificant.

Figure 4.2.1



A. In the presence of inflammation, IPA shows that the the Xenobiotic Metabolism and Transport (downstream of RXRα) potation of the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway is inhibited.
B. From the RNAseq data, IPA shows that with LGG perfusion, the Xenobiotic Metabolism and Transport (downstream of RXRα) potation of "LPS/IL-1 Mediated Inhibition of RXR Function" is activated in the presence of inflammation.

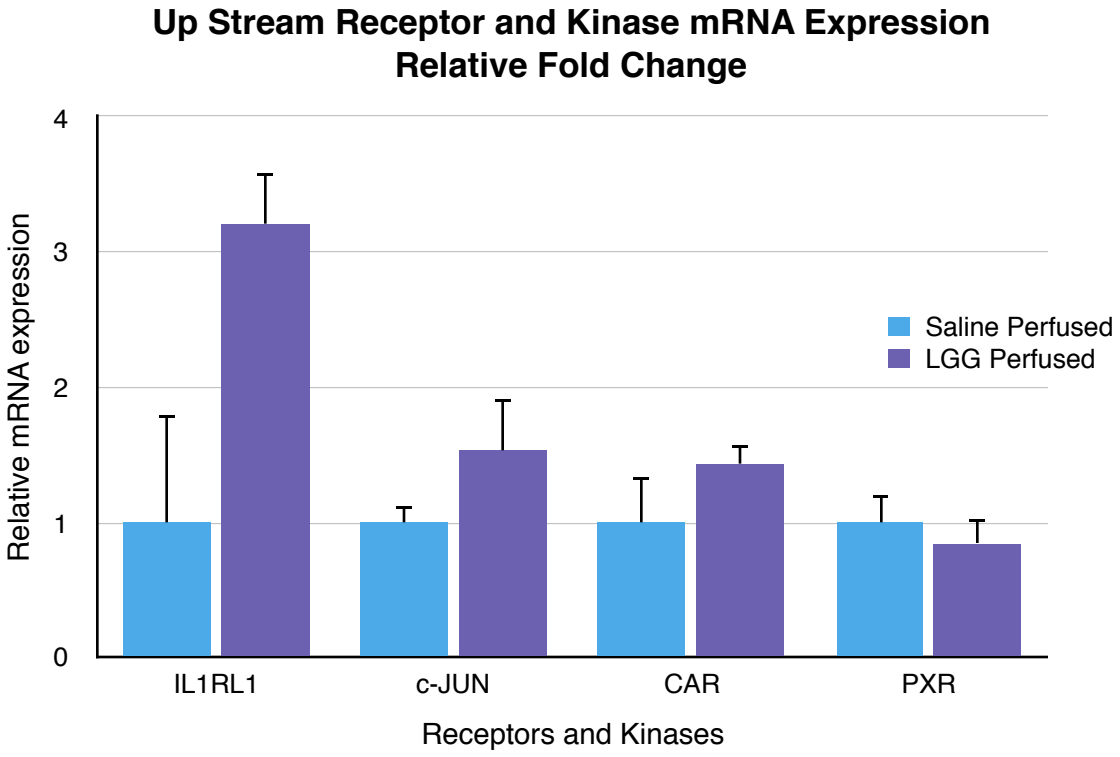
Figure 4.2.2

Up Stream Receptor and Kinase mRNA Expression Analysis

	P Value
IL1RL1	0.1790
c-JUN	0.2960
CAR	0.2610
PXR	0.5840

Kinase and receptor analysis result are not significant due to high P value. n=4

Table 4.2.1



mRNA expression levels of LGG perfused samples are standardized to its corresponding control. According to our hypothesis, the activity of upstream regulators from the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway should decrease with LGG perfusion.

Figure 4.2.3

4.2.1.2 Down Stream Enzyme and Transporter mRNA qPCR Analysis

The mRNA level of 11 downstream effectors were analyzed (Table 4.2.2) :

FMO2 (Dimethylaniline monooxygenase [N-oxide-forming] 2), a family member of the flavin-containing monooxygenases, is a catalyst for the NASPH-dependent oxygenation of various xenobiotics.

SOD3 (superoxide dismutase 3), an antioxidant enzyme that protect the tissue from oxidative stress; MGMT (O-6-Methylguanine-DNA Methyltransferase), a DNA repair protein that is involved in cellular defense from toxicity.

UGT (Uridine 5'-diphospho-glucuronosyltransferase), is a cytosolic glycosyltransferase that catalyze the addition of glucuronic acid moiety to xenobiotics, an important process for further xenobiotic metabolism.

OATP2 (Organic Anion-Transporting Polypeptide), an organic anion transporter that is responsible for transporting anions and foreign compounds located in the basolateral membrane.

MRP2 (Multidrug Resistance-associated Protein 2), is a member of the superfamily of ATP-binding cassette (ABC) transporters that is responsible for the transport various molecules across extra- and intra-cellular membranes. The protein is also involved in multi-drug resistance.

MRP3 (Multidrug Resistance-associated Protein 3), this anion transporter share similar functions with MRP2, as they belong to the same family.

MRP4 (Multidrug Resistance-associated Protein 4), the anion transporter belongs to the ABC transporter family.

CES (Carboxyl Esterase), is an enzyme that is responsible for the hydrolysis or transesterification of various xenobiotics.

MAOA (Monoamine Oxidase A), belongs to the family of MAO, is an enzyme that catalyze the oxidative deamination of amines.

CAT (Catalase), is an antioxidant enzyme that is important in the defense against oxidative stress.

MDR1 (Multi Drug Resistance Protein 1), is also an anion transporter that belongs to the ABC transporter family and share similar functions to MRP2/3/4.

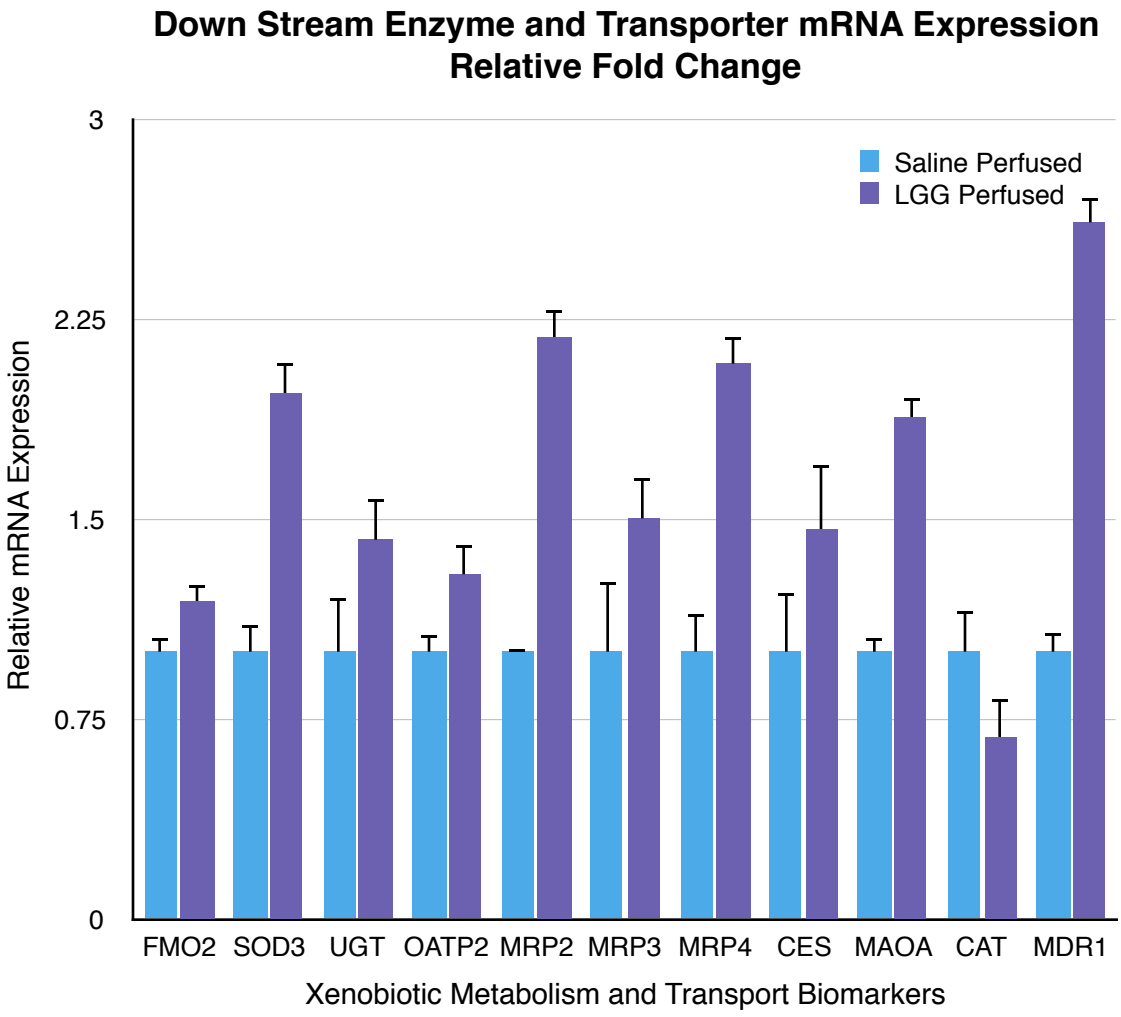
Down Stream Enzyme and Transporter mRNA qPCR Analysis

	Saline Perfused	LGG Perfused	P Value
FMO2	1 ± 0.054	1.196 ± 0.062	0.0500
SOD3	1 ± 0.104	1.976 ± 0.104	0.0006
UGT	1 ± 0.069	1.423 ± 0.113	0.0187
OATP2	1 ± 0.011	1.293 ± 0.098	0.0410
MRP2	1 ± =0.168	2.184 ± 0.158	0.0040
MRP3	1 ± 0.147	1.501 ± 0.107	0.0020
MRP4	1 ± 0.221	2.081 ± 0.239	0.0290
CES	1 ± 0.053	1.463 ± 0.065	0.0030
MAOA	1 ± 0.150	1.887 ± 0.146	0.0010
CAT	1 ± 0.150	0.681 ± 0.146	0.0390
MDR1	1 ± 0.070	2.611 ± 0.032	0.0001

mRNA expression levels of LGG perfused samples are standardized to its corresponding control (Saline Perfused). P Values are calculated with StatView.

Table 4.2.2

A total of 11 downstream enzyme and transporters were analyzed. 10 showed an increase from 20% to 160%, and 1 show a decrease with LGG perfusion. With perfusate as the effect, the set of data has a P value less than 0.05, which was considered significant (Table 4.2.2, Figure 4.2.4).



mRNA expression levels of LGG perfused samples are standardized to its corresponding control. The activity of Xenobiotic metabolism and transport biomarker from the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway increased with LGG perfusion. n=4. P<0.05

Figure 4.2.4

4.2.1.3 Down Stream Enzyme and Transporter mRNA RNAseq Analysis

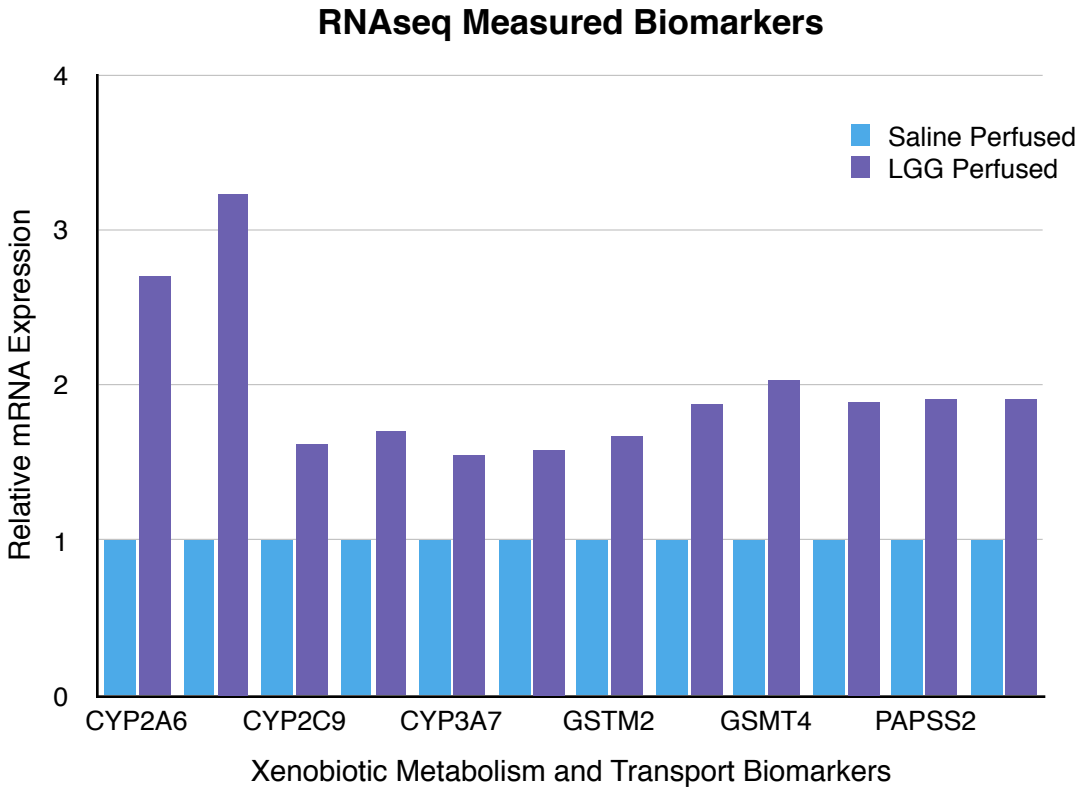
According to the RNAseq experiment conducted by the lab. Four members of the oxidase CYP (Cytochrome P450) family: CYP2C8, CYP2C9, CYP3A5, CYP3A7; five members of the GSTM (Glutathione S-Transferase Mu) family: GSTM1, GSTM2, GSTM3, GSTM4, GSTM5; one member of the SULT (Sulfotransferase) family: GAL3ST2 (Galactose-3-O-Sulfotransferase 2); and enzyme PAPSS2 (3'-phosphoadenosine 5'-phosphosulfate synthase 2) showed a mRNA expression fold change of >1.5 ($P<0.05$) in LGG perfused samples when compared to saline perfused control samples (Table 4.2.3, Figure 4.2.5).

Confirmed RNAseq Results

	Saline Perfused	LGG Perfused	P Value
CYP2A6	1	2.704	1.05*E-02
CYP2C8	1	3.232	3.58*E-04
CYP2C9	1	1.621	4.62*E-04
CYP3A5	1	1.709	1.91*E-04
CYP3A7	1	1.550	8.48*E-04
GSTM1	1	1.579	2.89*E-03
GSTM2	1	1.660	1.80*E-04
GSTM3	1	1.881	1.31*E-03
GSMT4	1	2.023	6.84*E-04
GSTM5	1	1.886	1.79*E-04
PAPSS2	1	1.912	4.85*E-04
SCARB1	1	1.903	4.45*E-03

mRNA expression levels of LGG perfused samples measured by RNAseq are standardized to its corresponding control (Saline Perfused). P Values are calculated with StatView. Data confirmed with qPCR.

Table 4.2.3



mRNA expression levels of LGG perfused samples are standardized to its corresponding control. The activity of Xenobiotic metabolism and transport biomarker from the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway increased with LGG perfusion. n=4. P<0.05

Figure 4.2.5

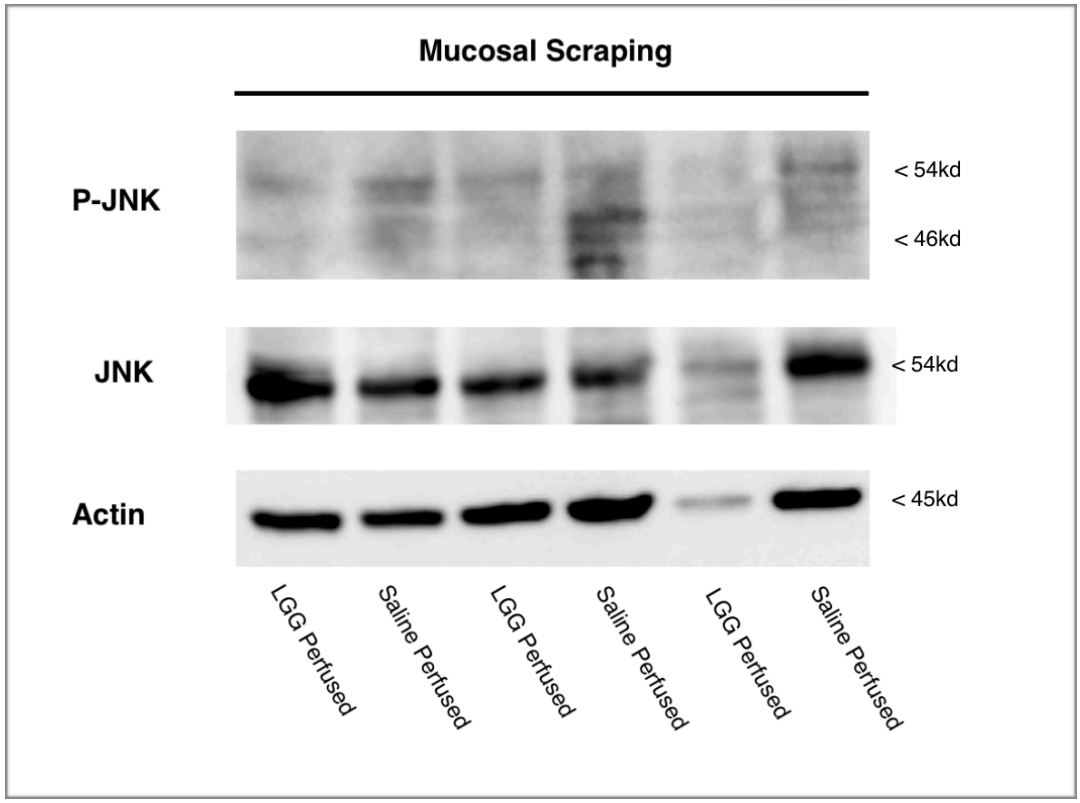
4.2.2 Protein Analysis

c-Jun N-terminal kinase (JNK) is the MAP Kinase regulating the “LPS/IL-1 Mediated Inhibition of RXR Function” pathway. Epidermal Growth Factor Receptor (EGFR), a receptor known to be associated with the amelioration of inflammation in the presence of two *Lactobacillus rhamnosus GG* (LGG) derived protein: p40, p75 (Yan, F. 2011).

4.2.2.1 JNK Analysis

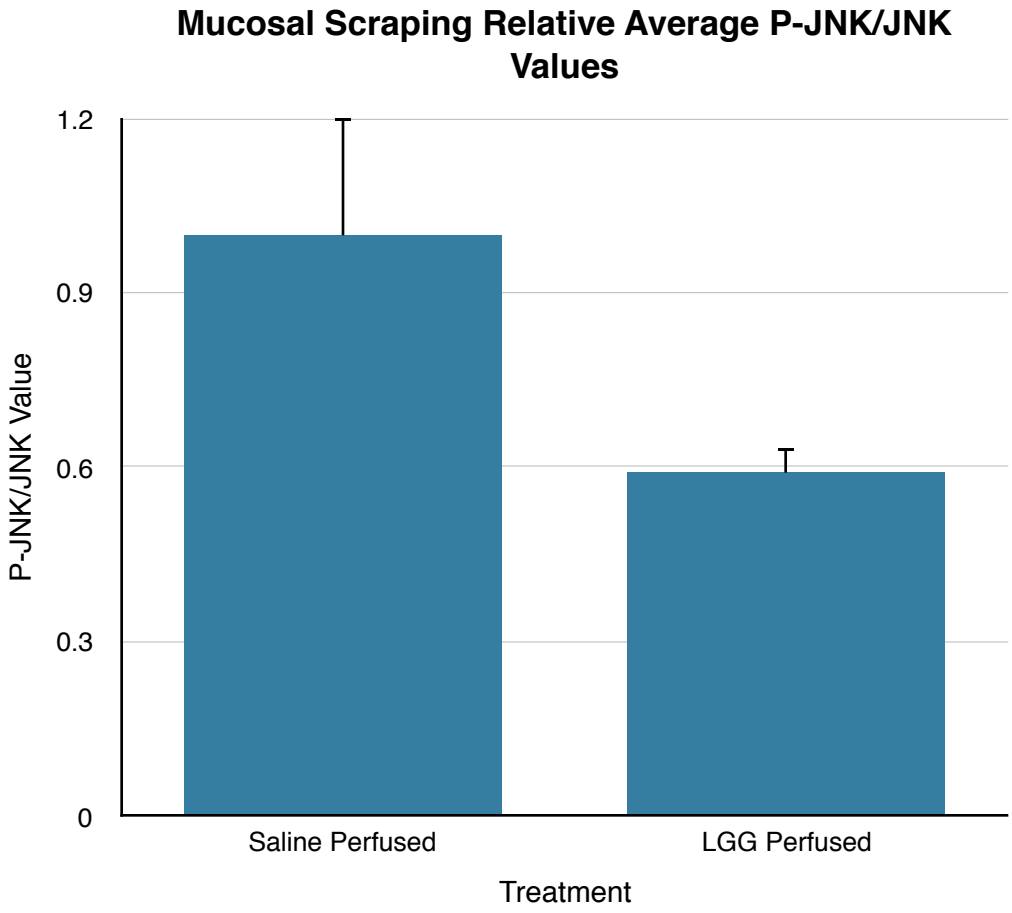
Total protein expression and protein phosphorylation of JNK were detected using JNK and p-JNK antibodies (Cell Signaling Technology, MA) with western blotting.

In mucosal scraping (MS) samples, there were less phosphorylated JNK protein in LGG perfused samples than saline perfused samples, not noticeable difference in the amount of total JNK protein or actin except for one LGG perfused sample, which had a less loaded amount of protein (Figure 4.2.6). Band intensities on the western blot membranes were quantified by ImageJ. LGG perfusion treated samples had smaller p-JNK/JNK value than saline perfused samples (Figure 4.2.7). Indicating that phosphorylation of JNK decreased with the contact of live LGG in the small intestinal mucosal.



Protein phosphorylation and total protein expression of JNK was analyzed with Western Blotting. β-Actin was used as house keeping protein. LGG perfusion decreased the production of P-JNK. n=3.

Figure 4.2.6



Blot was quantified with ImageJ. The value of P-JNK/JNK were used to compare protein phosphorylation. To account for total JNK's effect on p-JNK, the value of the band intensity of p-JNK divided by JNK (p-JNK/JNK) was used to compare protein phosphorylation between different treatments

The average P-JNK/JNK value of all Saline perfused and LGG perfused samples were calculated. The average P-JNK/JNK value for LGG perfused samples is lower than that of the Saline perfused samples. n=3.

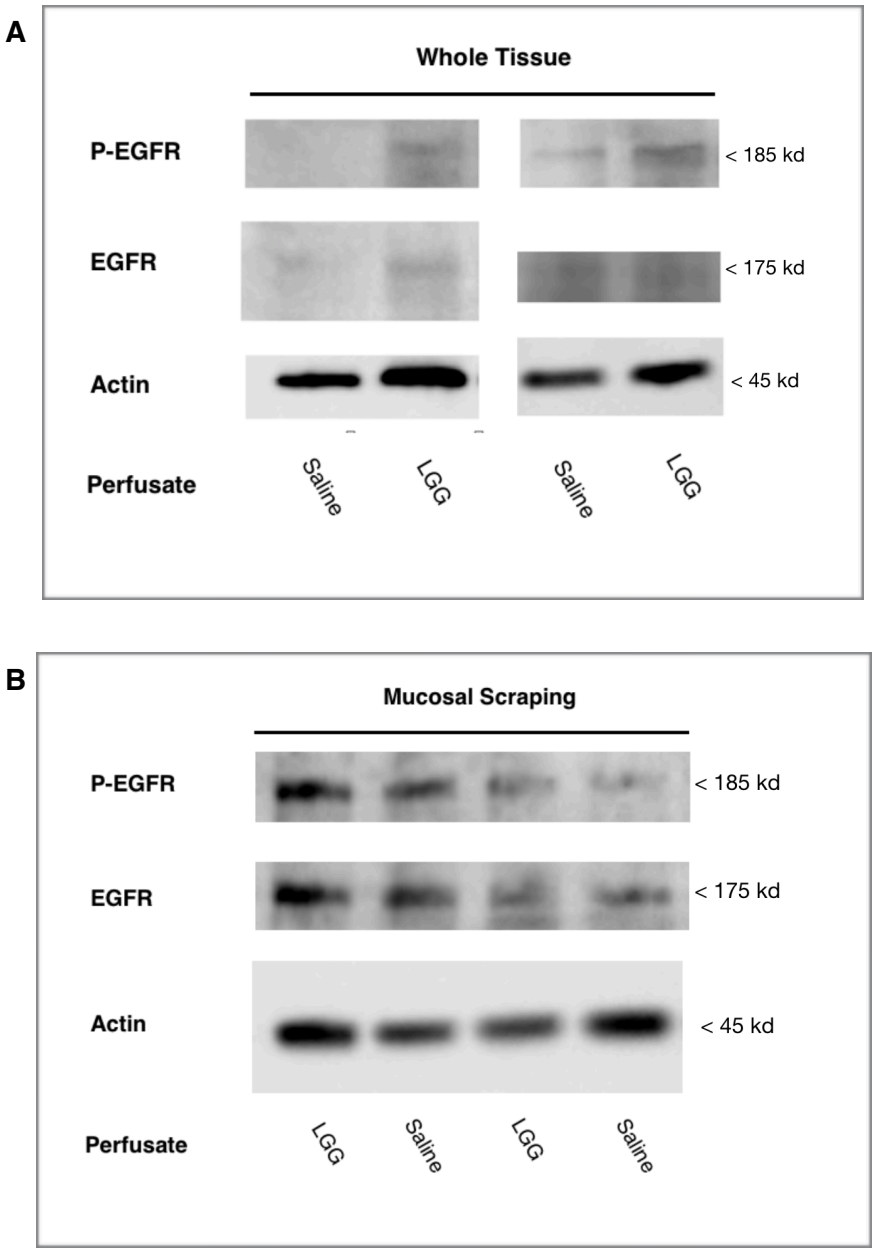
Figure 4.2.7

4.2.2.2 EGFR Analysis

Both whole small intestinal tissue samples and small intestinal mucosal scrapings collected from surgical perfused mice were analyzed by western blotting.

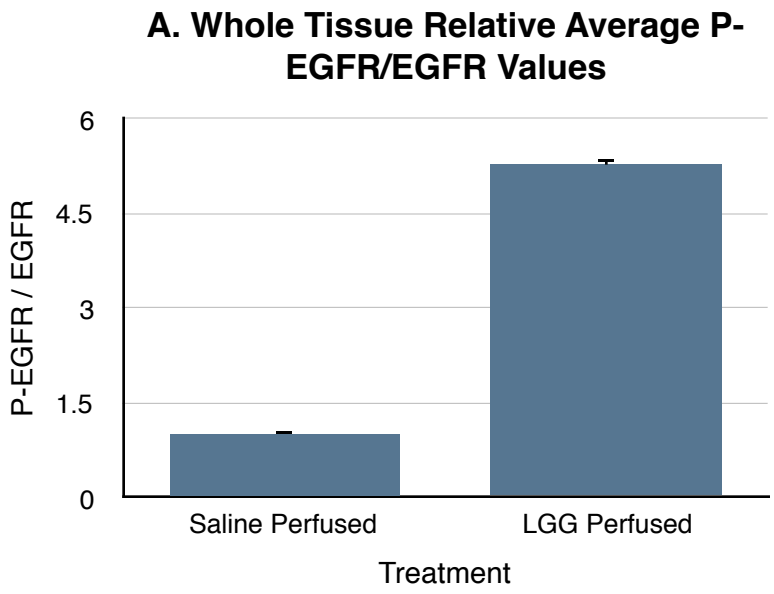
In whole tissue (WT) samples, there were more phosphorylated EGFR protein in LGG perfused samples than saline perfused samples, a slight noticeable difference in the amount of total EGFR protein, no change in actin expression (Figure 4.2.8). Band intensities on the western blot membranes were quantified by ImageJ. LGG perfusion treated samples had greater p-EGFR/ EGFR value than saline perfused samples (Figure 4.2.9). Indicating that phosphorylation of EGFR increased with the contact of live LGG in whole tissues.

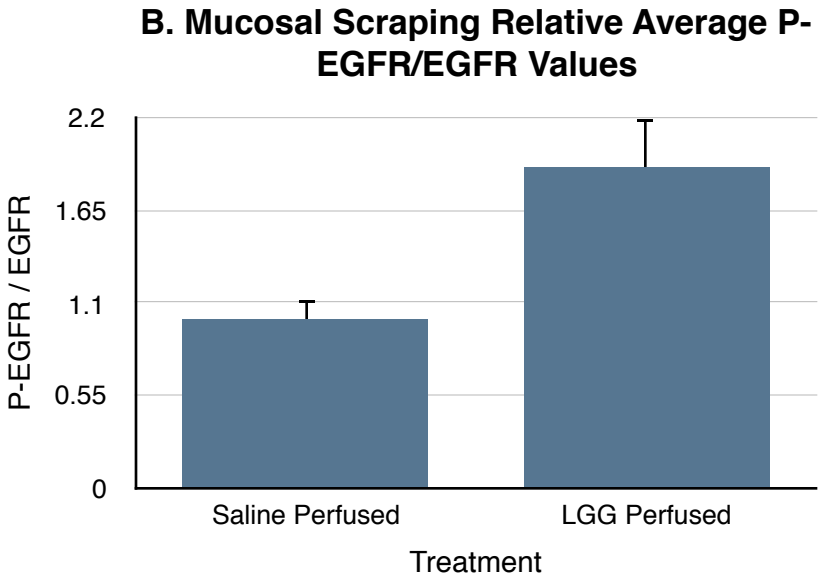
Mucosal scraping (MS) samples had a similar result. There was a noticeable increase in phosphorylated EGFR in LGG perfused samples than saline perfused samples, but not total EGFR or actin (Figure 4.2.8). Indicating that phosphorylation of EGFR increased with the contact of live LGG in the mucosal.



Protein phosphorylation and total protein expression of EGFR in whole tissue samples (A) as well as mucosal scraping samples (B) were analyzed with Western Blotting. β-Actin was used as house keeping protein. LGG perfusion increased the production of P-EGFR in both whole tissue and mucosal scraping samples. n=2.

Figure 4.2.8





Western Blots were quantified with ImageJ. The value of P-EGFR/EGFR were used to compare protein phosphorylation. The images were then quantified with ImageJ. To account for total EGFR's effect on p-EGFR, the value of the band intensity of p-EGFR divided by EGFR (p-EGFR/EGFR) was used to compare protein phosphorylation between different treatments.

The average P-EGFR/EGFR value of all Saline perfused and LGG perfused whole tissue (A) and mucosal scraping samples (B) were calculated. In both A and B, the average P-EGFR/EGFR value for LGG perfused samples is higher than that of the Saline perfused samples. n=2.

Figure 4.2.9

Chapter 5

Discussion

5.1 LGG Feed Study

Lactobacillus Rhamnosus GG (LGG) has shown to ameliorate inflammation when surgically perfused through the small intestine, in that it can up regulate the production of anti-inflammatory cytokines and down regulate the production of pro-inflammatory cytokines (Davies et al,.2012). The purpose of this study is to see whether LGG can yield similar anti-inflammatory results when administered through the digestive tract. After the administration of either LGG or saline, we introduced inflammation to the small intestine by the perfusion of Lipopolysaccharides (LPS) or Polyinosinic:polycytidylic acid (Poly I:C).

When feed is taken as the only effect, and in the presence of inflammation initiated by surgical perfusion, all 10 cytokines analyzed (both pro-inflammatory and anti-inflammatory) showed a P value greater than 0.05, thus results are statistically insignificant. Mice that were saline fed suffered an average weight loss of 1.67%, and LGG fed suffered an average weight loss of 6.69% ($P>0.05$). All weight change data also have a relatively high P value(>0.05), and were not able to generate a clear or significant trend. In conclusion, the results generated are considered to be statistically negative data.

By gavage feeding the subjects two times a day, 200μL per feed of LGG (concentration= 6.6×10^8 CFUs/mL, or Saline), we introduced 2.64×10^8 CFUs to the digestive tract of the subjects per day for 3 days. This amount of bacteria may not be enough to elicit a significant response in the intestines, as the intestines

are populated with billions of other microorganisms (Brown et al,.2000). Previous studies conducted feeding studies with *Lactobacillus* that generated significant data, fed the subjects (pigs or mice) an average of 10^8 to10^9 CUFs per day (Su et al,.2017, Wang et al,.2017). The probiotics in these studies were also introduced with food rather than gavage feeding (Su et al,.2017).

However, the mice intestines are colonized by endogenous bacteria, it may take longer than 3 days for the fed LGG to cause a significant reaction in the small intestine. As we have not conducted an experiment indicating that our method of three day gavage feeding is enough for the LGG to populate the small intestine, we can not determine that any reaction witnessed was caused by the fed LGG.

Gavage feeding is a viable method of agent delivery to the digestion tract, and has been used clinically and in research for many years. In our experiment, we gavage fed the mice two times a day for a consecutive of three days. This high frequent garage feeding may introduce a lot of stress to the animals (Arantes-Rodrigues et al,.2012). Since every animal react to foreign agents and stress differently, this may explain the high standard errors in both saline and LGG fed data, resulting in the insignificant P values.

From various past academic breakthroughs, we have learned that statistically insignificant responses or discoveries may actually be biologically significant. Since different animals may react to stress and LGG differently, and to determine whether the gavage feeding response is biologically significant, a couple of additional steps could be taken into future studies:

1.To decrease stress given to the animals, we may have to increase the amount of LGG concentration in each fed, and feed once a day for 6 to 10 days before performing perfusion and analyzing samples.

2. Evaluate LGG's population in the small intestine with qPCR as well as other methods, to determine whether the reaction witnessed is actually induced by the presence of LGG.

3. Collect and analyze multiple segments of perfused and unperfused tissue sample from the same mice in each treatment. By comparing perfused and unperfused samples from the same animal, we can study the small intestine's reaction to LGG when the endogenous bacteria are present and when flushed out by the perfusate. As each mice may react to treatments differently, more data from single animals will better give us an idea of whether the responses are biologically significant.

5.2 Pathway Analysis Study

With focus on the LGG luminal perfusion's effect on the xenobiotic metabolism nodes of the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway, mRNA and protein analysis were performed. 23 out of 27 effectors and receptors with functions related to xenobiotic metabolism (downstream of the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway) were analyzed by qPCR. JNK and EGFR phosphorylation as well as total protein were analyzed with western blots.

5.2.1 IPA's prediction of the LPS/IL-1 Mediated Inhibition of RXR Function pathway

Under inflammation, LPS or pro-inflammatory cytokines produced either by gram negative bacterial or by activated immune cells will activate LPS and cytokine receptors on the apical cellular membrane. They activate downstream signaling cascades, in the case of our pathway of interest (LPS/IL-1 Mediated Inhibition of RXR Function), the JNK signing cascade. The phosphorylated MAP kinase JNK, inhibits the activation of retinoid X receptor alpha (RXRα) and its' downstream effectors associated with xenobiotic metabolism (Trinder et al,. 2015). In other words, inflammation activates the pathway, and decreases xenobiotic metabolism.

Our RNAseq data and IPA analysis indicated that LGG perfusion inhibited the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway in the small intestine ($P<0.05$). This suggests that LGG perfusion ameliorated the decrease in xenobiotic metabolism caused by inflammation in the small intestine. Since pro-inflammatory cytokines are some of the main activators of the pathway, analyzed pathway's inhibition in the presence of LGG corresponds with our previous findings, that LGG decreases the production of pro-inflammatory cytokines in the small intestine (Figure I.1). This discovery proved that LGG ameliorates inflammation.

5.2.1.2 mRNA regulation by LGG perfusion

With the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway inhibited by LGG perfusion, we hypothesized a decrease in the mRNA production of its'

receptors. PXR and CAR forms heterodimerization complexes with RXR. The mRNA expression data of PXR ,CAR, and IL1RL1 (a upstream cytokine receptor for the “LPS/IL-1 Mediated Inhibition of RXR Function” pathway) are insignificant, as the P values for these data were greater than 0.05. Under normal circumstances, the activities of receptors are reflected through phosphorylation and not mRNA expression, thus the insignificance of these data had no negative effects on our other results.

As mentioned, a total of 25 effectors (enzyme and transporters) were involved in xenobiotic metabolism within the “LPS/IL-1 Mediated Inhibition of RXR Function” pathway, downstream of the RXRα receptor complexes. There are 20 effectors downstream of the CAR/RXRα complex, 21 effectors downstream of PXR/RXRα complex, and the two nodes share 16 effectors (Figure 4.2.2). Among the 25 effectors, 21 were analyzed either by qPCR or RNAseq. The 4 effectors that were not analyzed (CYP2B6, CYP2C19, CYP3A4, ABCB9) belonged to the subfamilies of the effectors that were analyzed, and share similar functions. Among the 21 downstream effectors that were analyzed, the mRNA expression of 19 enzymes and transporters increased with LGG perfusion compared to saline perfusion ($P<0.05$). Our result corresponds with IPA’s analysis, and clearly indicates that the xenobiotic metabolism function in the “LPS/IL-1 Mediated Inhibition of RXR Function” pathway, was indeed activated with LGG perfusion. This mechanism may explain other previous research findings. For example, *Lactobacillus rhamnosus GR-1* can modulate host xenobiotic metabolism, as children and pregnant women that were over-exposed

to pesticides reduced the bioaccumulation of mercury and arsenic when administered the probiotic. (Yoda et al,.2014)

5.2.2 Protein phosphorylation regulation by LGG perfusion

5.2.2.1 JNK Analysis

JNK is the MAPK in the “LPS/IL-1 Mediated Inhibition of RXR Function” pathway. When the pathway is activated during inflammation caused by gram-negative bacteria, JNK will be activated. As mentioned in the introduction, (Figure I.3) phosphorylated JNK, will normally inhibit downstream RXRα activity by increasing its degradation and nuclear export (Wang et al,.2017), thus decreasing the ability of the RXRα CAR complex and RXRα PXR complex to initiate nuclear transcription, decreasing enzymes and transporters in xenobiotic metabolism.

LGG perfusion decreased the phosphorylation of JNK in the mucosal of the small intestine (n=3) when compared to the saline perfused samples as control (Figure 4.2.6). With the phosphorylation of JNK reduced, downstream RXRα activity will increase, due to less RXRα degradation and nuclear export (Wang et al,.2015), thus increasing the ability of the RXRα CAR complex and RXRα PXR complex to initiate nuclear transcription, and increasing xenobiotic metabolism.

This result is strong evidence showing that the “LPS/IL-1 Mediated Inhibition of RXR Function” pathway is affected by LGG perfusion, increasing downstream xenobiotic metabolic functions. As another group has shown that the

probiotic *Lactobacillus* can decrease JNK phosphorylation under inflammation (Yu et al,.2015, Peng et al,.2017), these results corresponds with our qPCR and IPA analysis as well as the previous work of others.

5.2.2.2 EGFR Analysis

Previous research on LGG and its anti-inflammatory effects showed that, either LGG or LGG derived proteins may regulate intestinal homeostasis and prevent inflammation through the activation of the tyrosine receptor EGFR (Yan, F. 2011, Wang et al,.2017). Since our perfusion model is a novel method of introducing probiotic to the system (Davies et al,.2012), with previous studies as background, we wanted to observe EGFR's regulation in our experiments.

The phosphorylation of EGFR increased with LGG perfusion in both whole tissue samples (n=2), and mucosal scraping samples (n=2) when compared with the control (Figure 4.2.9). This result correspond with existing literature (Yan et al,.2014), and further proves that our method of perfusion is valid in initiating a reaction between the probiotic and the small intestinal tissue.

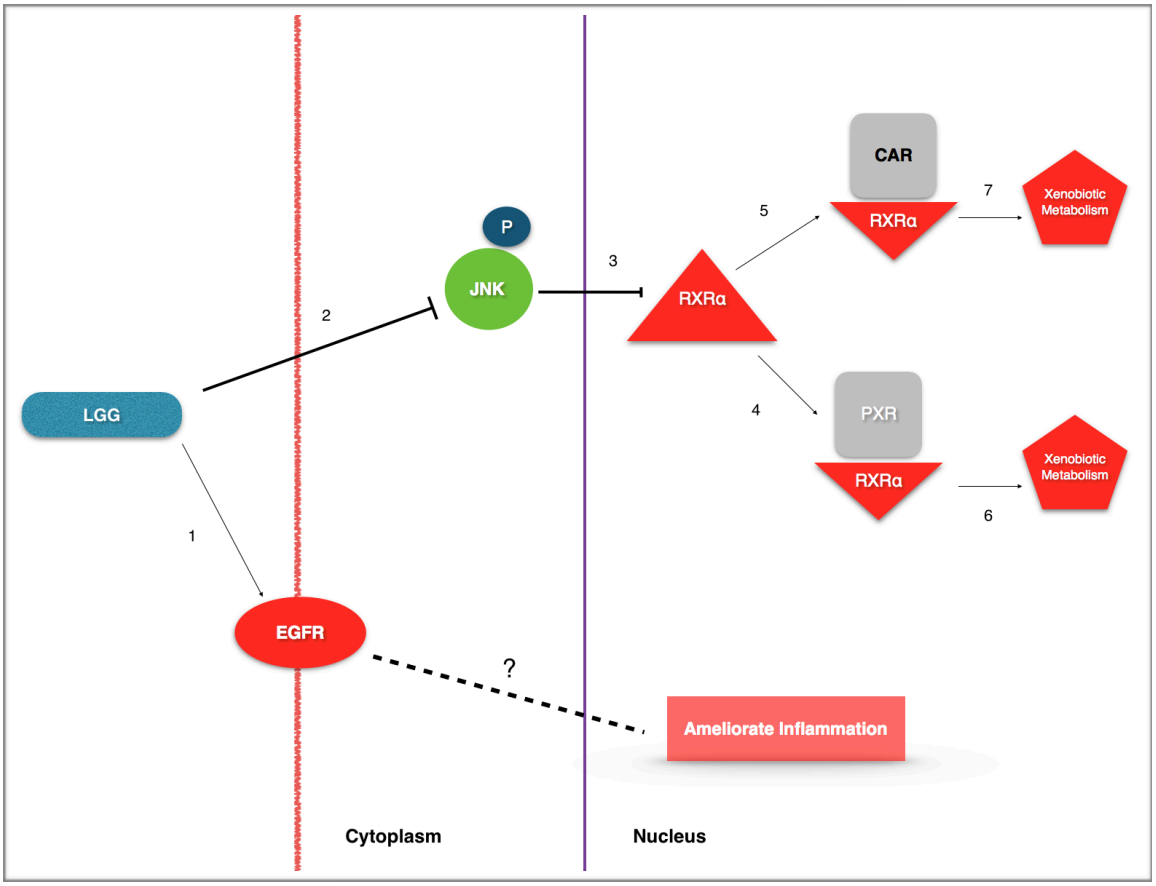
The anti-inflammatory effects caused by LGG or LGG derived proteins in the intestines have also shown to be EGFR dependent (Li et al,.2002), and that the activation of EGFR promotes IgA production. There may be a connection between the activation of EGFR and the mRNA expression regulations in the pro and anti-inflammatory cytokines observed in our prior studies. However, more experiments are needed to prove this claim, which could be one of the new directions of this project.

Chapter 6

Conclusion

When perfused through the lumen of the small intestine, *Lactobacillus rhamnosus GG* (LGG) or its derived proteins can up-regulate the mRNA expression of xenobiotic metabolism associated enzymes and transporters. And this up-regulation may be the result of the inhibition of the “LPS/IL-1 Mediated Inhibition of RXR Function” pathway, as the phosphorylation of the MAPK of the pathway, JNK, is inhibited by LGG perfusion (Figure 6.1). The phosphorylation of EGFR was increased with LGG perfusion, which may be associated with LGG’s amelioration of inflammation observed in our current and prior experiments (up-regulation of anti-inflammatory cytokine mRNA and down-regulation of pro-inflammatory cytokine mRNA).

To further provide proof to the thesis, we can introduce a JNK inhibitor (Anisomycin) and a JNK activator (SP600125) (Kojima et al,.2013) to our current perfusion model. Our theory will be correct if with JNK inhibited, LGG perfusion can no longer activate the “LPS/IL-1 Mediated Inhibition of RXR Function” pathway.



With LGG perfusion:

1. *Activation of EGFR on the apical membrane increased. This may be responsible for the amelioration of inflammation witnessed in our previous experiments.*
2. *Phosphorylation of JNK decreased.*
3. *With JNK's activation decreased, the degradation and nuclear exportation of RXRα decreased.*
4. *With the amount of RXRα increased in the nucleus, the formation of the RXRα PXR nuclear transcription complex increased.*
5. *With the amount of RXRα increased in the nucleus, the formation of the RXRα CAR nuclear transcription complex increased.*
6. *The transcription of the enzymes and transporters responsible for xenobiotic metabolism, that are downstream of the RXRα PXR nuclear transcription complex increased.*
7. *The transcription of the enzymes and transporters responsible for xenobiotic metabolism, that are downstream of the RXRα CAR nuclear transcription complex increased.*

Figure 6.1

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Appendixes

RNA Extraction Protocol (RNeasy Micro Kit, Qiagen)

1. Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 5 mg.
2. Disrupt the tissue and homogenize the lysate in Buffer RLT with theTissueRuptor, operate the TissueRuptor at full speed until the lysate is homogeneous.
3. Centrifuge the lysate for 3 min at full speed. Carefully transfer the supernatant to a new microcentrifuge tube (not supplied) by pipetting. Use only this supernatant (lysate) in subsequent steps.
4. Add 1 volume (usually 350 µl) of 70% ethanol to the lysate, and mix well by pipetting. Do not centrifuge. Proceed immediately to next step.
5. Transfer the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 10,000 rpm, and discard the flow-through.
6. Add 350 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow-through.
7. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube.
8. Add the DNase I incubation mix (80 µl) directly to the RNeasy MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.

9. Add 350 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow-through and collection tube.
10. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at10,000 rpm to wash the spin column membrane. Discard the flow-through.
11. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at 10,000 rpm to wash the spin column membrane. Discard the flow-through and collection tube.
12. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
13. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

cDNA Making Protocol (cDNA Synthesis Kit, BioRad)

Components (Per Tube)

5x iScript Reaction Mix	4µl
iScript Reverse Transcriptase	1µl
Nuclease-free water	Variable
RNA template (100 fg–1 µg total RNA)*	Variable
Total volume	20µl

Transfer Tubes to Thermal cycler and start reaction.

Reaction

Priming	5 min at 25°C
Reverse transcription	20 min at 46°C
RT inactivation	1 min at 95°C
Optional step	Hold at 4°C

PCR Protocol

Components (Per Tube)

Syber Green	10µl
Primer	1µl
Rox	0.04µl
RNAase free water	7µl
cDNA	2µl
Total	**20µl**

Transfer tubes from ice to thermal cycler and start reaction.

Reaction

Segment 1	95°C	10 min
Segment 2 (40 cycles)	95°C	15 s
	60°C	1 min
Total Time		1h,31min,31s

BCA Protocol (BSA Protein Assay Kit, Thermal)

1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)			
Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Table A.1

2. Use the following formula to determine the total volume of WR required: (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required.
3. Pipette 25µL of each standard or unknown sample replicate into a microplate well (working range = 20-2000µg/mL).
4. Add 200µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
5. Cover plate and incubate at 37°C for 30 minutes.
6. Cool plate to RT. Measure the absorbance at or near 562nm on a plate reader.
7. Subtract the average 562nm absorbance measurement of the Blank standard replicates from the 562nm measurements of all other individual standard and unknown sample replicates.

8. Prepare a standard curve by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Western Bolting

A. Solution Preparation

1. Standardize all protein samples to the same concentration with lysis buffer.
2. Make loading buffer with 4 x Laemmli Sample Buffer and BME(β-mercaptoethanol), 9 to 1.
3. Make Running Buffer with 10% 10 x Tris/Glycine/SDS Buffer and 90% milli-q water.
4. Make Transfer Buffer with 10% 10 x Tris/Glycine/SDS Buffer, 20% methanol, and 70% milli-q water.
5. Make Washing Buffer TBST with 10% 10 x TBS, 0.1% Tween and 89.9% milli-q water.
6. Make Blocking solution 5% BSA with 2.5 g Bovine Serum Albumin and 50ml TBST.

B. Sample Loading, Running, and Transfer

1. Denature samples by mixing Loading Buffer with protein samples 3 to 1, and leave at room temperature for 1-2 hours.
2. Put Mini-Protean TGX Gel in Running Buffer. Load 20μg of each sample wells of the gel.
3. Run gel under 89 volts for 2-3 hours.
4. After running, assemble gel with a Nitrocellulose Membrane and fix into western transfer kit with Transfer Buffer (assemble the complex so the protein can be transferred to the membrane). Transfer at 4 degrees, under 100V, for 1.5-2 hours.

C. Applying Antibodies and Imagining

1. After transfer, block the membrane in 5% BSA solution for 1 hour.
2. Incubate the blocked membrane in primary antibody, at 4 degrees, overnight.
3. Wash the membrane with TBST for 3 times, around 10 minutes each wash.
4. Incubate the membrane in secondary antibody, at room temperature, for 1 hour.
5. Wash the membrane again with TBST for 3 times, around 10minutes each wash.
6. Apply Chemiluminescence to membrane, let the membrane and the Chemiluminescence react in dark for 5 minutes, and image with LAS 4000.

Materials

Ketamine (20mg/mL)/Xylazine (2.5mg/mL)

Lactobacillus rhamnosus GG (Commercially available, 10 billion live bacteria per capsule)

1mL syringe (Becton & Dickinson, Franklin Lakes , NJ)

25G needle (Becton & Dickinson, Franklin Lakes , NJ)

Large Gavage Needles (Becton & Dickinson, Franklin Lakes , NJ)

Thermo-Regulation System (Harvard Apparatus, Holliston, MA)

Syringe Pump (Harvard Apparatus, Holliston, MA)

Water Circulator

Krebs-Ringer Buffe (KRBreg); Composition: 78 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2·5H2O, 1.2 mM MgSO4, 19 mM NaHCO3, 2.2 mM KH2PO4 (pH 7.4) (300 mOsm)]

Krebs-Ringer Buffer in DEPC H_2O (DEPC-KRBreg)

RNAlater

60mL Syringes (Becton & Dickinson, Franklin Lakes , NJ)

30mL Syringes (Becton & Dickinson, Franklin Lakes , NJ)

5mL Syringe (Becton & Dickinson, Franklin Lakes , NJ)

T-PER Tissue Protein Extraction Reagent (ThermoScientific, Waltham, MA)

Protease inhibitor tablet, Sigma Aldrich cOmplete protease inhibitor cocktail EASYpack (Thermalfisher Scientific, Waltham, MA)

Phosphatase inhibitor tablets, Sigma Aldrich PhosSTOP phosphatase inhibitor cocktail EASYpack (Thermalfisher Scientific, Waltham, MA)

Pierce BCA Protein Assay Kit (ThermoScientific, Waltham, MA)

iScript™ cDNA Synthesis Kit (BIO-RAD, Berkeley, California)

Mx3000P (Stratagene, La Jolla, CA)

Maxima SYBR green qPCR Master Mix, ROX solution provided (Thermofisher Scientific, Grand Island, NY)

4 x Laemmli Sample Buffer (BIO-RAD, Berkeley, California)

10 x Tris/Glycine/SDS Buffer (BIO-RAD, Berkeley, California)

Mini-Protean TGX Gels 4-20% (BIO-RAD, Berkeley, California)

Nitrocellulose Membrane (BIO-RAD, Berkeley, California)

10 x TBS (BIO-RAD, Berkeley, California)

EGFR antibody, rabbit (Invitrogen/ThermoFisher Scientific, Waltham, MA)

Anti-rabbit secondary antibody (Invtrogen/ThermoFisher Scientific, Waltham, MA)

Beta-Actin antibody, conjugated with HRP (Invtrogen/ThermoFisher Scientific, Waltham, MA)

SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, MA)

StatView (SAS, Cary, NC)