Temporal Modulation of Gut Microbiome and Metabolome by Morphine

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DEDICATION

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ABSTRACT

Opioids such as morphine have many beneficial properties as analgesics, however, opioids may induce multiple adverse gastrointestinal symptoms. It has been recently demonstrated that morphine treatment results in significant disruption in gut barrier function leading to increased translocation of gut commensal bacteria. However, it is unclear how opioids modulate gut homeostasis.

A mouse model was used to investigate the effects of morphine treatment on gut microbiome and metabolome. When phylogenetic profiles of gut microbes were characterized, the results revealed a significant shift in the colonic microbiome following morphine treatment when compared to placebo. At the species level, *Enterococcus faecalis* was associated with morphine-modulated gut microbiome alteration. Morphine treatment also resulted in dramatic changes in the fecal metabolomic profile. Through LC-MS based metabolomics profiling analysis, fatty acids and bile acids metabolism and in particular, deoxycholic acid (DCA) and phosphatidylethanolamines (PEs) was identified to be greatly affected by morphine treatment, implicating that changes in the microbiome community has functional consequences. In a longitudinal study, naltrexone, an opioid receptor antagonist, reversed the effect of morphine on bile acid metabolism, indicating morphine induced changes are opioid receptor dependent. Cross-correlation between gut microbiome and metabolome indicated association between bacterial communities and functional metabolites. Furthermore, morphine induced dysbiosis disrupts morphine metabolism and its enterohepatic recirculation. This study shed light on the effects of morphine on the microbiome-metabolome-host axis, and its role in gut homeostasis.

In a mouse model of *Citrobacter rodentium* infection, morphine treatment resulted in 1) the promotion of *C. rodentium* systemic dissemination, 2) increase in virulence factors expression with *C. rodentium* colonization in intestinal contents, 3) altered gut microbiome, 4) damaged integrity of gut epithelial barrier function, 5) inhibition of *C. rodentium*-induced increase of goblet cells, and 6) dysregulated IL-17A immune response. This is the first study to demonstrate that morphine promotes pathogen dissemination in the context of intestinal *C. rodentium* infection, indicating morphine modulates virulence factor-mediated adhesion of pathogenic bacteria and induces disruption of mucosal host defense during *C. rodentium* intestinal infection in mice.

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BACKGROUND-LITURATURE REVIEW

1. GUT HOMEOSTSIS

A. Concepts of gut homeostasis and dysbiosis

a. Gut homeostasis and health

The gut is a complex and dynamic network where interaction between host and gut microbiota establishes a balanced, symbiotic, and mutual beneficial relationship(Kau et al. 2011). Gut homeostasis refers to the state of resilience and resistance to external and endogenous disturbance(Lozupone et al. 2012). Gut homeostasis is established and maintained by commensal microbiota, functional barrier and tolerant immune response(E. M. Brown, Sadarangani, and Finlay 2013). Gut microbiota is the sum of all microorganisms within gastrointestinal tract, including bacteria, bacteria, archaea, eukaryotes and viruses(Gordon 2012). It is estimated that almost 10^{13}-10^{14} bacteria inhabit the gastrointestinal tract, which is two orders of magnitude more than host cells(Relman 2012). Unborn fetus lives in a basically sterile environment. During birth and thereafter, infants are exposed to the external environment whereby the gut microbial community is initialized, established and develop gradually(Dominguez-Bello et al. 2010). The gut microbiota becomes stable and adult-like at approximately 3-5 years old(Rodrı et al. 2015). It has been demonstrated that microbiota play important roles in modulating host neural and immune development,

morphogenesis, as well as resistance to diseases(Sommer and Bäckhed 2013). The mechanisms by which the gut microbiota maintains a healthy state and how microbial dysbiosis increases susceptibility to diseases is largely unknown.

b. Microbial dysbiosis and diseases

Microbial dysbiosis refers to a change of the structural and/or functional configuration of gut microbiota, which causes disruption of gut homeostasis and is associated with a variety of diseases, such as obesity, diabetes, autoimmune, allergic, inflammatory and infectious diseases,(Gordon 2012; Sommer and Bäckhed 2013). Recent rapid progress in metagenomics provides powerful tools to determine perturbation of the human microbiome as a contributor to diseases(Gordon 2012). Changes in composition or density of the microbiota may leads to higher susceptibility to a variety of pathogens and abnormal mucosal immune responses(Wells et al. 2011; Stecher and Hardt 2008). For example, antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium difficile* infection(Theriot et al. 2014). Meanwhile, dysregulation of host immune defense can also change gut microbiota during host-microbe interactions(E. M. Brown, Sadarangani, and Finlay 2013). For instance, *Salmonella enterica* serotype *Typhimurium* (*S. typhimurium*) causes acute gut inflammation, which can shift the gut microbiota in favor of the pathogen growth(Stecher et al. 2007). *S. typhinurium* induced intestinal inflammation can promote the production of respiratory electron acceptor, tetrathionate, which provides a

growth advantage for *S. typhinurium*(Winter et al. 2010). Although it is still unclear about causal-consequence relationship, a variety of diseases is associated with a vicious circle of microbial dysbiosis, gut barrier dysfunction, and dysregulated immune response. HIV-1 infection induced alteration of intestinal mucosal microbiome is associated with mucosal and systemic immune activation and endotoxemia(Dillon et al. 2014). Dysregulated immune response in inflammatory bowel disease has been shown to be associated with dysfunction of the intestinal microbiome(Morgan et al. 2012). Mechanistic links between gut microbial community dynamics, microbial functions and metabolic health are basically unknown and attract attention from both clinical therapeutics and basic research(Ha, Lam, and Holmes 2014).

The association between the signature of microbial dysbiosis and diseases demonstrates potential diagnostic application in monitoring onset and progress of the disease. Intestinal *Prevotella copri* can play a potentially diagnostic role based on its strong correlation with enhanced susceptibility to arthritis in a gut microbiome study of new-onset untreated rheumatoid arthritis (NORA) patients(Scher et al. 2013). After characterization of gut microbiome in patients with liver cirrhosis with healthy human subjects, 15 biomarkers have been identified as signature of alterations of the human gut microbiome in liver cirrhosis(Qin et al. 2014). Biomarkers specific to liver cirrhosis as a powerful tool in differential diagnosis have been confirmed by a comparison

with those for type 2 diabetes and inflammatory bowel diseases(Qin et al. 2014).

An altered microbiome is not only a marker of disease, but also actively contribute to pathogenesis of disease(Chassaing et al. 2012). The causal relationship is established when healthy host display the disease phenotype after the microbiome transplantation from diseased donors and controls into healthy germ-free hosts(Turnbaugh et al. 2006; Faith et al. 2014). In order to determine direct causal relationship between microbiome and disease, the approach of microbiome transplantation has been conducted in studying multiple diseases, such as colitis, type I diabetes, metabolic diseases(Elinav et al. 2011; Koren et al. 2012; Wen et al. 2008; Garrett 2013).

Another important unanswered question is the relationship between the microbiome and therapeutic treatment. Probiotics and prebiotics have a long history as therapeutic tool in treating diarrhea, vaginal yeast infections, urinary tract infections, irritable bowel syndrome, and preventing or reducing the severity of colds and flu(Leyer et al. 2009; Vouloumanou et al. 2009; Borges, Silva, and Teixeira 2014; Ciorba 2012). Recently, fecal microbial transplantation has been conducted effectively in treating recurrent *Clostridium difficile* infection(Weingarden et al. 2014). The administration of prebiotics (oligofructose) to genetically obese mice can decrease fat to muscle mass ratio, improve glucose and lipid metabolism, reduce plasma LPS, improve gut barrier function and increase enteroendocrine L-cell number in obese mice(Everard et al. 2011). Administration of oligofructose can induce

change of gut microbiota and increase the abundance of *Akkermansia muciniphila* by 100 fold in obese mice(Everard et al. 2011). Further study from the same research group shows that the abundance of *A. muciniphila* decreased in obese and type 2 diabetic mice, while administration of *A. muciniphila* reduces body weight, improves metabolic disorders and counteracts mucosal barrier dysfunction in obese mice(Everard et al. 2013). Remedies for gut dysbiosis and restoration of healthy gut homeostasis have become important therapeutic strategies to cure diseases.

B. Functional entities that constitute gut homeostasis

a. Gut microbiota

i. Resilience and resistance to disturbance

Resilience of gut homeostasis reflects physiological stability, which is the ability to resist change in response to potentially perturbing forces(Levine and D'Antonio 1999). Taxonomic and functional diversity of gut microbiota are crucial in conferring resilience in gut homeostasis(Lozupone et al. 2012). Low microbial diversity correlates with obesity(Yatsunenko et al. 2012; Ley et al. 2006), Inflammatory Bowel Disease (IBD)(Ben P Willing et al. 2010), and recurrent *Clostridium difficile*-associated diarrhea (CDAD)(Chang et al. 2008). It is still unclear if there is direct cause-consequence relationship between microbial diversity and resilience.

Mirroring principles in ecology, Catherine A. Lozupone, et al, proposed competition and feedback loops as mechanisms for resilience

in gut microbiota(Lozupone et al. 2012). Competing with commensal bacteria for the same resources, such as nutrients, is associated with pathogen colonization and eradication. For example, *Citrobacter rodentium* exhibits optimal growth on monosaccharides. *Bacteroides thetaiotaomicron* directly competes with *C. rodentium* for monosaccharides and decreases load of *C. rodentium* when only simple sugar diet is available(Kamada et al. 2012). Thus, nutrient competition can regulate *C. rodentium* growth and resistance against external invasion in the gut. Positive and negative feedback loops can drive primary succession and confer resilience in gut homeostasis(Lozupone et al. 2012). For example, microbial perturbation can cause negative or positive feedback loop which is delicately manipulated by metabolic activities and host pathways. Pathogen infection induced diarrhea or constipation may be regulated by changes in microbial metabolites and their downstream host pathways to control gut retention time and mucin secretion. Changes of gut retention time can regulate pathogen colonization and eradication. Although gut homeostasis can resist perturbation to some extent, the unhealthy state of microbial community could be also very stable and persist for years or decades, which partially explains why some Irritable Bowel Syndrome (IBS) or Inflammatory Bowel Disease (IBD) cases are recurrent and hard to cure. The therapeutic strategy is

focused on increased resilience of healthy microbiota and decreased resilience of unhealthy microbiota.

ii. Gut-liver axis

Liver is the major organ that metabolizes microbial products and toxin derived from gut microbes. Nutrients and metabolites are transported into liver via two afferent pathways, the hepatic portal system and the lymphatic system(Björkholm et al. 2009). Liver is the processing center and regulates the homeostasis of cholesterol, bile acids, choline metabolites, vitamins, lipids, phenols and short chain fatty acids(Nicholson et al. 2012). The metabolites are then transported from the liver to the intestine via the efferent bile ducts to close the loop and define the enterohepatic circulation(Björkholm et al. 2009). Mammalian-microbial co-metabolisms takes place in the gut-liver axis. An example is bile acids, which are primarily derived from cholesterol catabolism and become conjugated bile acid in the liver(Nicholson et al. 2012). The primary bile acids are transported as bile into intestines, where microbial enzymes help primary bile acids transform into secondary bile acids through dehydroxylation, dehydrogenation, and deconjugation(Shapiro et al. 2014). Intestinal bacteria can transform about 5-10% bile acids through degradation. It is worthy to be noted that secretion and reabsorption of bile acids through enterohepatic cycle take place by about eight times a day. It has been demonstrated that germ-free mice lack secondary bile

acids(Narushima et al. 1999) implicating the role of gut bacteria for the conversion of primary to secondary bile acid.

The liver is the largest immune organ and plays an important role in immune response to gut derived nutrients and other signals. Alteration of gut microbiome and disruption of gut barrier function may promote translocation of microbes into the portal circulation and increase bacterial load in the liver(Szabo et al. 2010). Damage of liver function impairs clearance of microbes from blood resulting in activation of nonmucosal immune responses(Arvaniti et al. 2010; Szabo 2015). Fibrosis and cirrhosis can cause multiple symptoms, such as portal hypertension, spleen enlargement, intestinal damage, and abnormal vascular channels, and impair the portal circulation, leading to increased risks of systemic infections of gut-derived and blood-borne pathogens(Arvaniti et al. 2010; Szabo 2015).

iii. Gut-brain axis

Preclinical evidences suggested that gut-brain interactions are implicated in modulation of peripheral enteric and central nervous systems. Alteration of gut microbiome is associated with brain disorders, such as autism, Parkinson's disease, depression and impaired cognition(Mayer, Tillisch, and Gupta 2015). It is largely unknown how gut microbiota modulate nervous system. The bidirectional communication between gut and both central and peripheral nervous system involve microbial metabolites, immune

response, neurotransmitters, and the hypothalamic pituitary adrenal (HPA) axis(Carabotti et al. 2015). The HPA axis plays a crucial role in modulating emotion or stress through neuro-immuno-endocrine mediators. For example, environmental stressors or inflammation can activate this system and induce release of cortisol, a stress hormone that affects many organs, including the brain(Carabotti et al. 2015). Recent studies by Hsiao and colleagues uncover a role of gut microbiota in modulating serotonin (5-hydroxytryptamine, 5-HT) biosynthesis(Yano et al. 2015). It suggests gut microbiota can directly regulate fundamental neurotransmitter 5-HT-related biological functions. Opioids as analgesic drugs can change transmission and perception of pain. However, it can also cause gastrointestinal symptoms, such as constipation, nausea, vomiting, and bloating. The study on the gut-brain axis may help elucidate mechanisms by which opioids modulate gut homeostasis.

b. Metabolites

Microbiome patterns and metabolome profiles can't simply map or reflect each other. The metabolic consequences and potential, rather than microbial patterns, allow us to understand the functional microbiota(Ursell et al. 2014). Gut metabolites play a significant role in the crosstalk between gut microbes and host biological functions, such as the maturation of the host immune system(Nicholson et al. 2012) and protection against pathogens(Lawley and Walker 2013). Healthy microbiota produce the SCFAs

as carbon sources for the host, synthesize vitamins and essential amino acids, transform bile acids, produce neurotransmitters and modify xenobiotics(Heinken and Thiele 2015). Based on the importance of microbial metabolites and non-invasive characteristics, dietary intervention presents huge potential in therapeutic strategy.

i. Bile acids

Bile acids are typical metabolites that involve mammalian-bacterial co-metabolism. After primarily produced by liver from cholesterol, bile acids are secreted as bile into intestine and transformed into secondary bile acids by gut microbes through dehydroxylation, dehydrogenation, and deconjugation(Shapiro et al. 2014). Germ-free mice lack secondary bile acids and exhibit decreased bile acid diversity when compared with conventionally raised mice(Sayin et al. 2013). Bile acids play crucial roles in absorption of dietary fats and lipid-like vitamins, maintenance of intestinal barrier function, orchestration of endocrine signals, regulation of triglycerides, cholesterol, glucose and energy homeostasis(Brestoff and Artis 2013). Different bile acids can bind the G protein coupled receptor RGR5 and nuclear receptor farnesoid X receptor (FXR)(Pols et al. 2011; Vavassori et al. 2009). Activation of RGR5 and FXR can negatively regulate NFκB signaling pathway and lead to attenuated inflammation. Recent studies reveal that bile acids can mediate resistance to *C. difficile* infection and maintain healthy gut microbiota(Buffie et al.

2014). It has been demonstrated that administration of *Clostridium scindens*, a bile acid 7α-dehydroxylating intestinal bacterium, can enhance resistance to *C. difficile* infection, indicating secondary bile play an important role in protection against *C. difficile*. The intervention using secondary bile acids and/or related biosynthesis bacteria may contribute to therapeutic strategies.

ii. Short chain fatty acids

Short chain fatty acids (SCFAs) are bacterial fermentation products from plant-derived polysaccharide, an essential component of human diet. SCFAs serve as energy source and inhibit autophagy in gut epithelial enterocytes(Donohoe et al. 2011). SCFAs can directly modulate leukocytes, for example, enhance chemotaxis in neutrophils and increase cytolytic activity, inhibit histone deacetylases in macrophages and reduce proinflammatory cytokines production, modulate methylation of the conserved non-coding sequence 1 (CNS1) in the FoxP3 locus and leads to regulatory T cells (Tregs) proliferation (Shapiro et al. 2014; Furusawa et al. 2013; P. M. Smith et al. 2013). SCFAs propionate and butyrate can modulate brain function, mood, and behaviors via gut-brain neural circuits (Burokas et al. 2015; De Vadder et al. 2014).

iii. Long chain fatty acids

Long chain fatty acids (LCFAs) are derived from dietary oils. A variety of LCFAs exhibit association with health risks and benefits. The

number of double bonds in LCFAs can affect cellular membrane fluidity and stability. More double bonds in phospholipid fatty acids can lead to higher membrane fluidity. Gut microbiota can modulate LCFAs quantity and composition. For example, *Bifidobacterium breve* can help transform linoleic acids into conjugated linoleic acids (CLA), which can reduce hepatic triacylglycerol content in host metabolism and inhibit atherosclerosis(Toomey et al. 2006; Gudbrandsen et al. 2009). Omega-6 and omega-3 fatty acids are considered essential dietary fatty acids for mammals, because they are necessary for physiologic growth and function but animals are unable to synthesize them in adequate quantities. The long chain polyunsaturated fatty acids (PUFAs), including omega 3 fatty acids have been shown to decrease inflammatory response via NFκB inhibition. Compared to omega-3 PUFAs, omega-6 PUFAs increased susceptibility to *C. rodentium* infection in mice. However, combination of omega-3 and omega-6 PUFAs supplementary diet has been shown reduce *C. rodentium* induced inflammation and inhibit epithelial intestinal alkaline phosphatase-mediated detoxification of LPS, leading to increased mortality from *C. rodentium* infection(Ghosh et al. 2013).

iv. Polysaccharides

Polysaccharides are not only structural components of gut microbes, but function as mediators between host-microbial interactions. The capsular polysaccharide A (PSA) of the commensal *Bacteroides*

fragilis can modulate Th1/Th2 balance and direct lymphoid organogenesis. Intestinal dendritic cells can present PSA to activate CD4+ cells and induce appropriate cytokine production (Mazmanian et al. 2005; Mazmanian, Round, and Kasper 2008). PSA of *B. fragilis* can promote tolerant immune response via induction of FoxP3+ regulatory T cells and suppression of Th17 cell reaction in a TLR2-dependent manner(Round et al. 2011). PSA-deficient *B. fragilis* are unable to colonize gut mucosal surface in germ free mice due to robust Th17 immune response(Round et al. 2011). So, PSA plays an important role in modulating gut homeostasis and host-microbial symbiosis.

v. Vitamins

Vitamins are important nutrients that modulates multiple cellular and organ functions. It has been well demonstrated that vitamin biosynthesis and metabolism rely on commensal intestinal microbiota. Vitamin A and its metabolite retinoic acid (RA) are critical in maintenance of epithelial integrity and gut microbiota diversity(Rojanapo, Lamb, and Olson 1980; Cha et al. 2010). Retinoic acid can regulate immune homeostasis by inducing histone acetylation of FoxP3 promoter(Kang et al. 2007). Interestingly, RA is required in the promotion of CD4+ T cell effector responses to infection, mucosal vaccination, T cell polarization shift from Th1/Th17 to Th2 and B cell isotype switch to IgA and IgE (Tokuyama and Tokuyama 1996; Hall, Cannons, et al. 2011). Vitamin A deficiency can cause impaired

humoral and cellular immune response, increased susceptibility to infection, and mucosal damage(Hall, Grainger, et al. 2011). Therapeutic administration of vitamins serves as a good way in modulating gut homeostasis.

c. The gut epithelium

The gut has a huge surface area (400 sq m), which is necessary to absorb nutrients and transport water, and the gut epithelial barrier contains only a single layer of epithelium, which prevents against microbial invasion and promotes host-microbial interaction(Lozupone et al. 2012). The intestinal epithelium is composed of a variety of histological and functional entities, such as enterocytes, goblet cells, Paneth cells, microfold (M) cells and enterochromaffin (ECC) cells. Goblet cells continuously produce and secrete mucins which form a thick mucus layer. Paneth cells can sense microbiota via MyD88-dependent TLRs and release antimicrobials such as defensins and lectins(Vaishnava et al. 2008). M cells facilitate the transport of microbes and particles from gut lumen to immune cells in the underlying Peyer's patches, where antigens can be transported to antigen presenting cells, such as dendritic cells (DC). Unlike their neighboring cells, M cells are not covered by the thick mucus layer. This unique property allows them to facilitate ingestion of antigen from intestinal lumen. The epithelial tissue functions as a selectively permeable barrier by delicate regulation of the tight junctions, which join together intestinal epithelial cells (IECs). ECCs are a type of enteroendocrine and neuroendocrine cells that secrete various hormones, like

serotonin, in response to chemical, mechanical or pathological stimuli from the intestinal lumen. Additionally, DCs can project dendrites through the intestinal barrier and directly sample antigens in lumen. DCs can then deliver pathogen to local T cell areas, or mesenteric lymph nodes (MLN) through lymphatic circulation. Besides DCs, that constitutively traffic, the lamina propria beneath the epithelium also include: mucosal macrophages, T cells, and IgA-secreting B cells.

d. The immune system

Intestinal immune defense is designed to allow immune surveillance of pathogens, while it operates with minimal disruption to the absorptive function of the gut(Hooper, Littman, and Macpherson 2012). In normal hosts, commensal bacteria activate homeostatic response by host immune system that permit coexistence with potential toxic bacterial products through down-regulating bacterial receptors, inhibiting pathogenic innate and adaptive immune responses, inducing antimicrobial peptides, and promoting mucosal barrier repair(Sartor 2008). Gut microbiota exert profound effects on the maturation and function of the immune system, thus it influences immune-mediated diseases, such as inflammatory bowel disease (IBD)(Morgan et al. 2012). Although the casual role in multifactorial autoimmune diseases is still outstanding, host-microbial interaction is implicated in the development of autoimmune diseases, such as type 1 diabetes (T1D), rheumatoid arthritis (RA), multiple sclerosis (MS)(Ruff and Kriegel 2015). Understanding pathogenesis of immune-mediated disease might create potential cures.

Pattern recognition receptors mediated innate immune recognition of the microbiota promotes maturation of immune system and host-microbial symbiosis(Chu and Mazmanian 2013). Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are sensors of microbes(Wells et al. 2011; Artis 2008). Cytokines, chemokines, neurotransmitters, mammalian and bacterial metabolites are key molecular mediators in modulation of immune homeostasis(Marchiando, Graham, and Turner 2010; Artis 2008; de Jonge 2013). Toll-like receptor-4 (TLR4) signaling in the intestine is required for intestinal response to epithelial injury and inhibit bacterial translocation in DSS induced acute colitis in mice(Fukata et al. 2005). An increase in gram-negative bacterial translocation into mesenteric lymph nodes (MLN) was seen in TLR4-/- mice more frequently than wild-type littermates given DSS(Fukata et al. 2005). Innate immune response is necessary for induction of antibody response to trivalent inactivated influenza vaccine (TIV) in humans(Oh et al. 2014). TLR5 mediated sensing of the gut microbiota impacts the magnitude of antibody response to the TIV by promoting plasma cells differentiation and increasing antibody titers(Oh et al. 2014).

Humoral immunity also plays an important role in gut homeostasis. Secretory IgA from gut epithelial cells is concentrated in the outer layer of colonic mucus and interacts with gut microbes(Rogier et al. 2014). IgA-mediated targeting of bacteria helps transport of commensals to Peyer’s patch and promotes immune surveillance of dendritic cells(Rol et al. 2012).

Antinuclear antibodies are signature of generalized autoimmune disease and aberrant adaptive immune system can cause immune-mediated tissue damage(von Mühlen and Tan 1995; William et al. 2002). Van Praet, et al. demonstrated that neonatal colonization of gut microbiota is associated with generalized autoimmune disease in adult life(Van Praet et al. 2015). Non-specific innate immune and specific adaptive immune interactively respond to virulent bacteria in gut(Collins et al. 2014). Intestinal infection induced IgG can selectively target virulent factors and bind pathogenic bacteria in gut, leading to pathogen elimination and host survival (Kamada et al. 2015).

e. Host genetics

Host genetic variation can directly cause significant alteration in gut microbiome and host-microbial interaction(Spor, Koren, and Ley 2011; Leifer et al. 2014). By using the well-powered twins study, Goodrich JK, et al, compared monozygotic (MZ) than dizygotic (DZ) twin pairs, and found microbiome are more highly correlated within MZ than DZ twin pairs, indicating host genetics can influence gut microbiome (Goodrich et al. 2014). Particularly, they found some microbes are more influenced by host genetics and considered heritable microbes. Interestingly, one of the cultured strains, *Christensenella minuta*, is more abundant in lean twins than obese ones. *C. minuta* can shape phenotype by leading to thinner body weight after adding *C. minuta* in obese mouse. It is still unknown what variation of genetics is associated with changes of heritable microbes, such as *C. minuta*. It has been demonstrated that a single gene, MEFV, encoding pyrin, associates with

changes of gut microbiota and leads to familial Mediterranean fever (Khachatryan et al. 2008; Ting, Kastner, and Hoffman 2006).

C. Variable factors that affect gut homeostasis

a. Age

Infants are born from a nearly sterile environment and expose to microbes results in their establishment of gut microbiota thereafter. During the first three years of life, bacterial diversity increases along with age and gradually reach a stable adult-like profile(Yatsunenko et al. 2012). Gut microbiome become more stable throughout adulthood(Yatsunenko et al. 2012). Although aging as a multidimensional process is poorly defined, the physiological alterations along with aging process affect gut microbiome(Nicholson et al. 2012). Gut microbiome of infants exhibit high levels of *Clostridium leptum* and *Clostridium coccoides*, while *Escherichia coli* and *Bacteroidetes* were highly represented in elderly subjects(Mariat et al. 2009). The ratio of *Firmicules* to *Bacteroidetes* is 0.4, 10.0 and 0.6 in infants, adults and elderly individuals respectively(Mariat et al. 2009). Elderly individuals are accompanied with a broad range of medical challenges, such as increased risk of infectious diseases, cancers, cardiovascular disease, Parkinson's disease and Alzheimer's disease. Rapid progress has been ongoing in cellular and organism levels of physiological function and neuroimmune changes in terms of aging process(Lynch, Jeffery, and O'Toole 2015). Age as a variable factor is inevitable and deeply involved in the interaction between host-microbes.

b. Natural birth vs. Caesarean section

Infants born vaginally and born via Cesarean section exhibit differential initialization of gut microbiota(Dominguez-Bello et al. 2010). Naturally born infants acquire their mother's vaginal microbiota, while C-section babies establish microbial communities resembling skin microbiota. It has been demonstrated that C-section links to higher risk of childhood diseases, such as obesity, asthma, and other immune-mediated disorders(Leung et al. 2015; Mueller et al. 2014; Francis et al. 2014). Correlations have been noted in mice born by C-section are more anxious and exhibit symptoms of depression(Reardon 2014). A large cohort study of 11096 mother-infant pairs gives evidence that children born by C-section may have a higher risk of cognitive and motor development delay at age 9 months(Khalaf et al. 2015). Delivery mode affects microbiome colonization early in life and may cause lifelong changes in physiological development and mental health examples of these disease states.

c. Pregnancy status

It has been well known that pregnancy involves significant changes in immune and metabolic syndromes. These immune and metabolic changes are never alone. Pregnancy status can also mediate changes in microbial patterns and lead to differential functional consequence. In the Koren, et al. study of 91 pregnant women, gut microbiota exhibited differential profiles from first (T1) to third (T3) trimesters, with an increase of Proteobacteria and Actinobacteria with a decrease in richness(Koren et al. 2012). T3 microbiota can cause

greater inflammatory response when compared to T1(Koren et al. 2012). T3 microbiota can induce greater adiposity and insulin insensitivity in germ free mice, when compared with T1 microbiota(Koren et al. 2012).

d. Diet

The human gut microbiota act as processing center of nutrition and vitamins for host intestinal absorption(David et al. 2014; G. D. Wu et al. 2011). On one hand, gut microbiota are rapidly and reproducibly shaped by diet intake. On the other hand, gut microbiota exploit counteractive efforts on nutrition consuming and human health. High-fat diet can shape distinct signatures of host-microbial metabolome and gut microbiome in mice(Walker et al. 2014). Gut microbiota from twins discordant for obesity can modulate metabolism in mice and induce marked weight gain in obese microbiota recipient mice when compared to lean microbiota recipient(Ridaura et al. 2013). Malawian twin pairs discordant for kwashiorkor, a severe acute malnutrition induced by inadequate nutrition intake, exhibit distinct signatures in gut microbiome(M. I. Smith et al. 2013). The combination of Malawian diet and kwashiorkor microbiome can induce kwashiorkor-like weight loss in recipient gnotobiotic mice(M. I. Smith et al. 2013). Diet intervention has been shown to be an effective method in treating human diseases, such as obesity and kwashiorkor(Zhao 2013; Garrett 2013).

There are epidemiological evidence that supports insufficient exposure to dietary and microbial metabolites is associated with increased incidence of immune-mediated diseases such as allergies, asthma, and autoinflammatory

diseases in Western countries(Thorburn, Macia, and Mackay 2014; Lukens et al. 2014). Retinoic acid, a vitamin A metabolite, can shape early intestinal response and attenuate inflammation by modulating mucosal T helper cell response and promote IL-22 production by γδ T cells and innate lymphoid cells(Mielke et al. 2013). Dietary compounds derived from cruciferous vegetables such as broccoli can activate the aryl hydrocarbon receptor (AhR), which play an important role in intraepithelial lymphocytes (IELs) maintenance in the intestinal epithelium and promote normal intestinal immune function(Li et al. 2011). SCFAs, such as acetate, propionate, and butyrate, are derived from dietary fiber and play a crucial role in gut homeostasis and immune tolerance(Thorburn, Macia, and Mackay 2014). By crossing the placenta or delivery via breast milk, SCFAs can also influence gene expression and the development of the immune system of the developing fetus(Thorburn, Macia, and Mackay 2014).

e. Xenobiotics

Morphine metabolism presents a fashion of host-microbial interaction (Stain-Texier, Sandouk, and Scherrmann 1998). The morphine metabolic pathway is primarily focused on glucuronidation to morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G) in the liver(Pacifici et al. 1982). Though M3G exhibits no analgesic effect, M6G is more potent than morphine(Frances et al. 1992). M6G and M3G can be hydrolyzed by β-glucuronidase in both intestinal mucosal cells and gut bacteria, and subsequently reabsorbed as morphine(Koster, Frankhuijzen-Sierevogel, and

Noordhoek 1985; Hawksworth, Drasar, and Hill 1971; Walsh and Levine 1975). Anaerobes, such as bacteroides and bifidobacteria, are major sources of β-glucuronidase (Walsh and Levine 1975). Since the β-glucuronidase mediated hydrolysis is dependent on the bacterial composition of the gut, changes of gut microbiota may affect the rate and extent of the hydrolysis of morphine glucuronide. Diet has been shown to significantly influence the intestinal bacterial β-glucuronidase activity(Reddy, Weisburger, and Wynder 1974). The fecal microbial β-glucuronidase activity in human subjects consuming Western high meat diet is higher when compared to nonmeat diet(Reddy, Weisburger, and Wynder 1974). However, it remains unknown how alteration of gut microbiota can affect β-glucuronidase activity and subsequent hydrolysis of morphine glucuronide

Host-microbiome metabolic interaction affects human drug metabolism significantly(Clayton et al. 2009). Clayton, *et al*, observed that high urinary level of *p*-cresol sulfate produced by certain gut bacteria is correlated to low urinary ratio of sulfated to glucuronidated acetaminophen, a wide used analgesics(Clayton et al. 2009). Further study conducted by the same group demonstrated that the host-gut microbial metabolic interaction leads to modifications of major xenobiotic-metabolizing cytochrome enzymes and alteration of bile acid metabolites(Claus et al. 2011).

Intestinal microbiota not only directly participate in xenobiotic metabolism in gut, but regulate xenobiotic metabolism in the liver, which is the central organ for xenobiotic metabolism. Xenobiotic metabolism in the

liver is regulated by nuclear receptor (NR), including Constitutive Androstane Receptor (CAR) and the Pregnane X receptor (PXR)(Meyer 2007). Bjorkholm et al. demonstrate that microbiota can regulate liver gene expression and alter xenobiotic metabolism by inhibiting CAR and PXR function (Björkholm et al. 2009). Germ free mice exhibit higher efficient xenobiotic metabolism and undergo 35% shorter time of anesthesia when compared to conventional raised mice following pentobarbital treatment(Björkholm et al. 2009).

Innate immunity links to xenobiotic metabolism and is implicated with host detoxification. TLR2 signaling can regulate gene expression in xenobiotic metabolism, such as multidrug transporter ABCB1/multidrug resistance (MDR)1 p-glycoprotein (p-gp)(Frank et al. 2015). TLR2-mediated synthesis and activation of ABCB1/MDR1 p-gp in murine and human $CD11b^{+}$-myeloid cells functionally preserve drug efflux activity, leading to protection against chemotherapy-induced cytotoxicity(Frank et al. 2015). This finding suggests that targeting TLR2 is a novel therapeutic approach in modulating side effects of xenobiotics, such as chemotherapy-induced intestinal mucositis.

It has been demonstrated that the xenobiotic sensor, pregnane X receptor (PXR) and Toll-like Receptor 4 (TLR4) play crucial roles in modulating gastrointestinal barrier function and xenobiotic toxicity(Venkatesh et al. 2014). Commensal bacteria-derived metabolite, indole 3-propionic acid (IPA), can regulate gastrointestinal permeability and inflammation, and

protects against indomethacin-induced intestinal injury in a PXR and TLR4 dependent manner(Venkatesh et al. 2014).

Antibiotics usage in early life can cause profound perturbation to gut microbiome and lead to long-term dysbiosis, which links to higher risk of childhood disorders, such as childhood obesity, autism and type 1 diabetes (T1D)(Mueller et al. 2014; McDonald et al. 2015; Benjamin P Willing, Russell, and Finlay 2011). Alteration of the gut microbiome caused by antibiotics can lead to shifts of gut metabolome, which increased susceptibility to opportunistic infections, such as *Clostridium difficile*(Theriot et al. 2014; Börnigen et al. 2013). Long term studies in mice and pigs given sub-therapeutic dose of antibiotics found profound shifts in gut microbiome can cause increased abundance of SCFAs and higher host adiposity(Cho et al. 2012; H. B. Kim et al. 2012).

f. Infections

Commensal bacteria, such as *Enterococcus faecium* and *Enterococcus faecalis*, colonize the gastrointestinal tract and are basically non-pathogenic(Vouloumanou et al. 2009). However, Enterococci with tolerance to environmental stress and antibiotics can cause clinical infections in elderly and immunocompromised patients(Leanti La Rosa et al. 2013). There is no clear evidence has been found explains the ecological success of these infectious disease causing strains.

Pathogen bacteria colonization and invasion always accompany by collapse of host homeostasis by dysregulating immune response, disrupting

gut barrier function and disturbing commensal microbiome. *Citrobacter rodentium* is a natural mouse pathogen that models intestinal infection by *Escherichia coli* in humans and causes attaching and effacing (A/E) lesions and colonic hyperplasia(Puente et al. 2004). *C. rodentium* colonization depends on virulence factor-mediated adhesion and provoke inflammation, which further disrupts gut microbiota and promotes the outgrowth of pathogenic bacteria (Lupp et al. 2007). Dextran sodium sulfate (DSS) chemically induced intestinal inflammation causes microbiota changes that resembles enteropathogenic infection(Lupp et al. 2007). Regulation of the virulence factor-mediated colonization and commensal-driven pathogen eradication may be potential therapeutic strategies.

It is worthy to be noted that bacteria might not be the only organisms that can manipulate microbiota to cause diseases, viruses have been shown to promote virus replication and systemic pathogenesis by disrupting microbiota(Kuss et al. 2011). In an infectious murine model of early HIV infection, EcoHIV can cause bacterial translocation, suggesting intestinal microbial dysbiosis might contribute to early HIV pathogenesis in the gut(Sindberg et al. 2014). Clinical studies show that HIV-1 infection induces intestinal microbiome alteration, mucosal and systemic immune activation, microbial translocation and endotoxemia in human patients(Dillon et al. 2014). The extent of gut microbial dysbiosis correlates with kynurenine levels and IL-6 plasma concentration, two established markers of disease progression(Vujkovic-Cvijin et al. 2013).

2. OPIOIDS MODULATION OF GUT HOMEOSTASIS

A. Opioids and opioid receptors

a. Opioids

Opioids refer to a large group of compounds and chemicals that share the characteristics of opium(Ninković and Roy 2013). Synthetic opioids resemble morphine in pharmacological effects(Degenhardt et al. 2013). Opioids are powerful analgesics being widely prescribed clinically, and as such, their use has a great impact on a large percent of world population(Docherty, Jones, and Wallace 2011). However, their clinical use has been limited by several serious adverse effects such as addiction, immunosuppression, and adverse gastrointestinal (GI) symptoms(Juurlink and Dhalla 2012; Gomes et al. 2011; Docherty, Jones, and Wallace 2011; Hilburger et al. 1997). Opioids can induce other CNS effects that include sedation, euphoria, and dysphoria(Coetzee 2013). There are epidemiological evidences that support higher mortality among older adults with opioid use disorders(Larney et al. 2015). Mortality rates from dependent opioids users are approximately 15 times higher than that of age- and sex-matched control subjects(Degenhardt et al. 2011).

b. Opioid receptors

Opioid analgesics can exert their pharmacological effects through binding to specific cellular receptors (i.e., μ-, κ-, and δ- opioid receptors) and alter the transmission and perception of pain(Chan 2008). Most of the clinical effects of opioids are μ receptor dependent(Ninković and Roy

2013). The clinical effects of opioids vary between the μ opioid receptor agonists (e.g., morphine, hydromorphone), partial μ agonists (e.g., buprenorphine), and agonist-antagonists (e.g., butorphanol)(Epstein and Dabvp 2013). While primary pharmacological effects of opioids are dependent on the distribution of opioid receptors in central nerve system, opioid receptors are also expressed in the peripheral nervous system and the gastrointestinal tract (GI), such as in the myenteric plexus of the enteric nervous system (ENS)(Farzi et al. 2015). In addition, opioid receptors are also expressed by the cells of the immune system such as T cells, B cells and macrophages(Ninković and Roy 2013; Sabita Roy et al. 2011). Thus, activation of opioid receptors not only affects perception of pain, but induces a variety of side effects attributing to regulation of neuro and immune systems.

B. Opioids and diseases

a. Opioids induce sepsis

Morphine treatment has been shown to cause lethal gut-derived sepsis in mice(Babrowski et al. 2012; Hilburger et al. 1997). The sepsis caused by morphine treatment are implicated with disruption of gut barrier function, higher bacterial translocation, increased bacterial virulence expression, and dysregulated immune response (Banerjee et al. 2013). Chronic morphine administration can directly activate *Pseudomonas aeruginosa* virulence expression and lead to lethal gut-derived sepsis in mice(Babrowski et al. 2012). Banerjee, et al. have demonstrated that

chronic morphine treatment induced severe inflammation and exacerbation of septicemia is mediated through miR-146a regulation(Banerjee et al. 2013).

b. Opioids increase susceptibility of infectious diseases

Opioids induce immunosuppression and bowel dysfunction leading to increased susceptibility to bacterial and opportunistic infections(Mora et al. 2012; Ross et al. 2008; MacFarlane et al. 2000). For instance, morphine increases susceptibility to *Streptococcus pneumoniae* lung infection by impairing host innate immune response(J. Wang et al. 2005). There are clinical evidences that support opioid administration is associated with a greater risk of *Clostridium difficile* infection(Mora et al. 2012; D. L. Keller 2013). Opioids are also known to exacerbate viral pathogenesis. For example, morphine together with HIV-1 tat can regulate tight junction and modulate blood-brain barrier permeability, which is correlated with a greater risk of developing HIV dementia in opiate using HIV-1 patients (Mahajan et al. 2008). Neuropathogenesis of the simian immunodeficiency virus (SIV) infection in rhesus macaques can be exaggerated by morphine treatment(Bokhari et al. 2011). It has been demonstrated in a HIV-1 model of co-infection with pneumococcal pneumonia, morphine potentiates neuropathogenesis through modulation of toll-like receptors in microglial cells(Dutta et al. 2012). In order to better study effects of morphine on early HIV pathogensis in gut, Sindberg, et al, have established a mouse model of EcoHIV infection following opioids treatment(Sindberg et al.

2014). In this infectious murine model, EcoHIV infection with opioid treatment can induce bacterial translocation, intestinal damage and proinflammatory immune response, resembling effects of opioids on early HIV pathogenesis in the gut(Sindberg et al. 2014).

c. Opioids and wound healing

Opioids play an important role in relief of chronic pain and post-surgical pain(Horn et al. 2002). Medical prescription of opioids in terminal patients is implicated with a greater risks of decubitus ulcers and bacterial infections(Egydio et al. 2012). Martin, et al. found that chronic morphine treatment is implicated with delayed wound healing by inhibiting LPS-induced angiogenesis and immune cell recruitment to the wound site(J. L. Martin, Charboneau, et al. 2010; J. L. Martin, Koodie, et al. 2010). A well-powered clinical trial shows that naltrexone, an opioid antagonist, can promote mucosal healing in active Crohn's disease(J. P. Smith et al. 2011). Topical treatment of naltrexone can also improve wound healing in type 1 diabetes rats(Immonen et al. 2013). Opioids induced delayed wound healing may be implicated with the unrepairable state of intestinal barrier dysfunction following morphine treatment.

C. Opioids and gut homeostasis

a. Opioids modulate intestinal function

The primary functions of gastrointestinal tract includes digestion, absorption, secretion, motility, immune surveillance and tolerance(Leppert 2015). Opioid administration is associated with multiple gastrointestinal

syndromes, such as constipation, leaky intestinal barrier function, bloating, nausea and vomiting(Harari, Weisbrodt, and Moody 2006). Morphine are known to inhibit protective mucus and bicarbonate secretion from the intestinal epithelium and human bronchi(Rogers and Barnes 1989). Opioids treatment can attenuate intestinal motility by inhibiting coordinated myenteric activity, and cause delayed transit time and a greater risk for bacterial translocation(Balzan et al. 2007). Morphine induced prolongation of intestinal transit time increases the intraluminal bacterial count and augments bacterial translocation(Erbil et al. 1998). Morphine can attenuate epithelial immune function by decreasing cytokines secretion from gut epithelium in response to enteric infections of entero-adherent *Escherichia coli* O157:H7 and entero-invasive *Salmonella enterica serovar Typhimurium*(Brosnahan et al. 2014). There are clinical evidences that support that opioid administration leads to dysregulated immune response, increased intestinal barrier permeability, bacterial translocation, higher risk of enteric infection, and gut-derived sepsis(Mora et al. 2012; Babrowski et al. 2012).

b. Opioids impair gut epithelial integrity

Disruption of gut epithelial integrity has severe consequences including bacterial translocation from the gut leading to proinflammatory immune response(Schulzke et al. 2009). Well organized transmembrane and paracellular tight junction proteins in polarized intestinal epithelium facilitate their selective barrier function. Meng et al. demonstrated that morphine can disrupt intestinal barrier function and damage tight junction protein

organization via modulation of myosin light chain kinase (MLCK) in a TLR dependent manner(Meng et al. 2013). Naltrexone, an opioid antagonist, has been shown to have therapeutic effect on mucosal healing in active Crohn's disease, indicating opioid receptors are implicated with maintenance of gut epithelial integrity(J. P. Smith et al. 2011).

c. Opioids modulate immune systems

Studies on modulation of immune systems by chronic opioid use and abuse have been well documented ever since 1996 and subsequent reviews(Sabita Roy and Loh 1996; Sabita Roy et al. 2011; Ninković and Roy 2013; Hutchinson, Shavit, and Grace 2011). It is well established that opioid receptors are expressed on cells of immune system, such as B cells, T cells, and macrophages(Eisenstein 2011). Thus, opioids exert their pharmacological effects not only as analgesics but regulators of immune function. Roy, et al, demonstrated that morphine suppresses macrophage colony formation in bone marrow and modulates NFκB activation in macrophages(Sabita Roy et al. 1991; S Roy et al. 1998). Morphine produces immunosuppressive effects, attenuates T cell maturation, alters cytokine secretion, decreases production of protein mediators of energy metabolism, signaling, and cell structure maintenance in nonhuman primates(J. N. Brown et al. 2012). Morphine induced neuro-immune interaction causes direct intestinal functional consequences. Meng et al. demonstrated that morphine disrupts gut barrier function through TLR-dependent manner(Meng et al. 2013). Chronic morphine treatment inhibits innate immune response, decreases Th1 cytokine

production and T cell activation, shifts to Th2 differentiation, and reduces antibody production and MHCII expression, leading to a greater risk of opportunistic infection and impaired pathogen elimination(Sabita Roy et al. 2011).

d. Gap of knowledge: opioid modulation of gut microbiome and metabolome

It has been well demonstrated that morphine treatment results in significant disruption in gut barrier function, leading to increased translocation of gut commensal bacteria(Meng et al. 2013; Das et al. 2011). The interaction of gut microbiota with host immune system is shown to be required to maintain the homeostasis of the mucosal immunity and preserve the integrity of the gut epithelial barrier(Hooper, Littman, and Macpherson 2012). However, it is unclear how opioids modulate the gut microbiome and metabolome. Current rapid progress in understanding of host-microbial interaction has redefined pathogenesis. Effects of morphine treatment on gut homeostasis in the context of bacterial or viral infections remain unknown. The study of morphine modulation of gut microbiome and metabolome will shed light on the effects of morphine on gut homeostasis and its role in the infectious disease scenarios.

CHAPTER 2

OPIOID TREATMENT INDUCES DISTINCT MICROBIOME AND METABOLOMIC SIGNATURES

INTRODUCTION

Morphine is the gold standard for pain management. Opioids analgesics are frequently prescribed in the United States and worldwide(Docherty, Jones, and Wallace 2011). However, serious side effects such as addiction, immunosuppression and gastrointestinal (GI) symptoms limit their use(Juurlink and Dhalla 2012; Gomes et al. 2011; Docherty, Jones, and Wallace 2011; Hilburger et al. 1997). Alteration in gut microbiome has been shown to contribute to bowel dysfunction and gut barrier disruption(Buccigrossi, Nicastro, and Guarino 2013). It has been well studied that morphine can cause gastrointestinal symptoms such as constipation, nausea, vomiting, bloating, and gut barrier dysfunction(Leppert 2015). However, it is still unknown how opioids modulate gut flora and its homeostasis within the host. Approximately, 10^{13}-10^{14} bacterial communities inhabit human intestinal tract(Relman 2012). The symbiotic relationship between commensal gut microbiota and the host is to achieve a balanced, mutually beneficial state. (Hooper, Littman, and Macpherson 2012). Gut homeostasis is maintained by commensal bacteria, functional barrier, and tolerant immune system and conveys the state of resilience and resistance to external and endogenous factors. Disruption of gut homeostasis leading to microbial dysbiosis is associated with disease and is implicated in a variety of diseases, such as obesity, diabetes, autoimmune, allergic,

metabolic, inflammatory and infectious diseases (Gordon 2012; Sommer and Bäckhed 2013).

Recent rapid progress in metagenomics provides powerful tools to determine perturbation of the human microbiome as a contributor to diseases(Gordon 2012). Changes in composition or density of the microbiota is associated with higher susceptibility to a variety of pathogens and abnormal mucosal immune responses(Wells et al. 2011; Stecher and Hardt 2008). Taxonomic and functional diversity of gut microbiota are crucial in conferring resilience in gut homeostasis(Lozupone et al. 2012). Low microbial diversity correlates with obesity(Yatsunenko et al. 2012; Ley et al. 2006), Inflammatory Bowel Disease (IBD)(Ben P Willing et al. 2010), and recurrent *Clostridium difficile*-associated diarrhea (CDAD)(Chang et al. 2008). It is still unclear if there is direct cause-consequence relationship between microbial diversity and resilience. It has been shown in our laboratory and other research groups that morphine disrupts intestinal barrier function and induces bacterial translocation in mice. Use of opioids are associated with an increased risk of *C. difficile* infection(Mora et al. 2012). Morphine treatment has been shown to activate the virulent factors of *Pseudomona* and induce gut derived sepsis. We recently demonstrated that morphine inhibition of endotoxin tolerance leading to sustained sepsis is mediated through modulation of miR-146a(Banerjee et al. 2013). Opioid exacerbation of gram-positive sepsis is rescued by IL-17A neutralization(Meng et al. 2015). It is not yet clear whether morphine treatment perturbs gut microbial homeostasis and thus resulting in increased growth of gut pathogenic bacteria and microbial translocation.

There are evidences suggesting that metabolites in gut play a significant role in the crosstalk between gut microbes and host biological functions, such as the maturation of the host immune system(Nicholson et al. 2012) and protection against pathogens(Lawley and Walker 2013). Disruption in bile acids metabolism is associated with susceptibility to *C. difficile* infection(Buffie et al. 2014). Gastrointestinal barrier function can be regulated by intestinal symbiotic bacterial metabolites via the Xenobiotic Sensor PXR dependent TLR4 signaling(Venkatesh et al. 2014). Healthy microbiota produce the short chain fatty acids (SCFAs) as carbon sources for the host, synthesize vitamins and essential amino acids, transform bile acids, produce neurotransmitters and modify xenobiotics(Heinken and Thiele 2015). We have confirmed that morphine-induced intestinal barrier dysfunction contributes to bacterial translocation(Meng et al. 2013). However, it is still unknown how morphine treatment modulates composition and abundance of gut metabolites. Metabolomic analysis allow for the determination and identification of small molecular metabolites within the gut lumen thus profiling the functional status of the microbiome in (Marcobal et al. 2013). The current study therefore is focused on identification of distinctness in morphine-modulated gut microbiome and their functional consequence through metabolomics analysis.

Host-microbiome metabolic interaction affects xenobiotics metabolism significantly(Clayton et al. 2009). Clayton, *et al,* demonstrated that the host-gut microbial metabolic interaction leads to modifications of major xenobiotic-metabolizing cytochrome enzymes and alteration of bile acid metabolites(Clayton et al. 2009; Claus et al. 2011). The morphine metabolic pathway is primarily through glucuronidation biotransforming to morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G)

in the liver(Pacifici et al. 1982). Though M3G exhibits no analgesic effect, M6G is more potent than morphine(Frances et al. 1992). M6G and M3G can be hydrolyzed by β-glucuronidase in both intestinal mucosal cells and gut bacteria, and subsequently reabsorbed as morphine(Koster, Frankhuijzen-Sierevogel, and Noordhoek 1985; Hawksworth, Drasar, and Hill 1971; Walsh and Levine 1975). However, the role of the gut microiome in morphine metabolism and elimination is relatively unknown.

The aim of the present study is to reveal the distinctness of gut microbiome and metabolome modulated by morphine. We characterize morphine-induced alteration of gut microbiome and metabolome in mice. Our results show that, when compared to placebo, morphine treatment induces decrease in microbial community diversity, and leads to distinct clustering and profiling of gut microbiome and metabolome. We establish that expansion of *Enterococcus faecalis* is a distinct feature associated opioid-induced gut microbiome alteration, and alteration in deoxycholic acid (DCA) and phosphatidylethanolamines (PE) associated with opioid-induced metabolomic changes. We find cross-correlation association between intestinal bacterial communities and functional metabolites. Furthermore, we determine an increased M3G/MS ratio in gut suggesting alteration of M3G deconjugating microbes may influence opioid metabolism and elimination. Collectively, these results reveal opioids-induced distinct alteration of gut microbiome and metabolome, may contribute to opioids-induced pathogenesis and morphine pharmacokinetics.

MATERIALS AND METHODS

Experimental animals

Pathogen-free C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). All animals were housed in a specific-pathogen-free facility. All animal experiments were done in accordance with the Institutional Animal Care and Use Committee's guidelines at the University of Minnesota (Protocol No. 1203A11091). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Animal treatment

Mice received morphine and pellet implantation method as described(Bryant et al. 1988). Using this method, plasma levels of morphine are in the 0.6–2.0-microg/ml range (range seen in opioid abusers and patients on opioids for moderate to severe pain). Furthermore, this model is commonly used in the study of opiate dependence and addiction(Bryant et al. 1988). Briefly, placebo or 25 mg morphine or 30 mg naltrexone pellets (National Institutes of Health [NIH]/National Institute on Drug Abuse [NIDA], Bethesda, MD) were inserted in a small pocket created by a small skin incision on the animal's dorsal side; incisions were closed using surgical wound clips (Stoelting, 9 mm Stainless Steel, Wooddale, IL).

Fecal sample collection and DNA extraction

Stool samples were collected into 1.7ml RNase/DNase-free tubes (Catalog #: C-2170, Denville Scientific, Holliston, MA, USA) at time points accordingly. The fecal

samples were immediately frozen on dry ice and then stored at -80 °C; DNA extractions from fecal matters were carried out using the PowerSoil DNA isolation kit (Catalog #: 12888-100, MO BIO Laboratories, Carlsbad, CA, USA). All extracted DNA samples were stored at -80°C until amplification.

Quantitative real-time PCR amplification

Fecal DNA samples (25 ng) were used as template for PCR amplification of the V4 region of the 16S rRNA gene. Degenerate primer sets were designed with Illumina index sequences on the 5 end of the reverse primer, which were specific to each fecal DNA sample and allowed for multiplexed sequencing. Primers also contained Illumina PCR primer sequences (reverse primer) and Illumina TruSeq Universal Adapter sequences (forward primers) for library creation. Primer sequences (16S-specific portion in bold) are Meta_V4_515F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGCCAGCMGCCGCGGTAA**) and Meta_V4_806R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACHVGGGTWTCTAAT**). The indexing primers are as follows. This step adds both the index and the flow cell adapters. [i5] and [i7] refer to the index sequence codes used by Illumina. The p5 and p7 flow cell adapters are in bold. Forward indexing primer: **ATGATACGGCGACCACCGA**GATCTACAC[i5]TCGTCGGCAGCGTC; Reverse indexing primer: **CAAGCAGAAGACGGCATACGA**GAT[i7]GTCTCGTGGGCTCGG. PCR reactions are performed using KAPA HiFidelity Hot Start Polymerase. PCR 1 (using the

Meta_V4_515F/Meta_V4_806R primer pair): 95°C 5 minutes, 20 cycles (98°C 20 seconds, 55°C 15 seconds, 72°C 1 minute), then hold at 4 °C. After the first round of amplification, PCR 1 products are diluted 1:100 and 5 ul of 1:100. PCR 1 is used in the second PCR reaction. PCR 2 (using different combinations of forward and reverse indexing primers): 95°C 5 minutes, 10 cycles (98°C 20 seconds, 55°C 15 seconds, 72°C 1 minute), then hold at 4°C.

DNA sequencing

The genomic DNA sequencing were performed by using Illumina MiSeq at the University of Minnesota Genomic Center (UMGC). Pooled, size-selected samples were denatured with NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 15% PhiX, and heat denatured at 96C for 2 minutes immediately prior to loading. A MiSeq 600 cycle v3 kit was used to sequence the sample. Nextera adapter sequences for post-run trimming

Read 1:

CTGTCTCTTATACACATCTCCGAGCCCACGAGACNNNNNNNNATCTCGTATG
CCGTCTTCTGCTTG

Read 2:

CTGTCTCTTATACACATCTGACGCTGCCGACGANNNNNNNNGTGTAGATCTC
GGTGGTCGCCGTATCATT

Sequence processing and analysis

Microbial operational taxonomic units (OTUs) and their taxonomic assignments were obtained using default settings in QIIME version 1.8.0 by reference-mapping at

97% similarity against representative sequences of 97% OTU in Greengenes (release GG_13_8), following which chimeric sequences were removed from subsequent analyses. Sequences showing 97% or greater similarity were clustered into operational taxonomic units (OTUs) using the USEARCH method and representative sequences were assigned taxonomies using the RDP classifier. Principal coordinate analysis (PCoA) of weighted UniFrac phylogenetic distances between communities, and Phylogenetic Diversity estimates were performed using the scripts in QIIME 1.8.0. To perform bootstrap, jackknife, and rarefaction analyses, the OTU table was rarefied at the cutoff value of 31000 sequences per sample. Bacterial taxa were arcsine-square-root transformed to stabilize variance and reduce heteroscedasticity. Differences in mean proportions of taxa were analyzed using Microbiome-Wide Association Study (MWAS) packages in R 3.2.1, the results of which were corrected for multiple testing using the false discovery rates (FDR) adjustment. FDR values <0.05 were considered significant. (Hu Huang and Dan Knights. (2015). MWAS R package. URL: https://github.com/danknights/mwas).

Microbiome phenotype analysis

Microbiome based functional phenotype analysis were performed by using BugBase software package, which relies on other software, such as PICRUSt and QIIME. (Tonya Ward and Dan Knight. URL: https://github.com/danknights/bugbase)

LC-MS analysis of fecal extracts

The method for LC-MS analysis of fecal extracts was based on previous study with slightly modification (Weingarden et al. 2014). Fecal samples were suspended in 1 ml of 50% acetonitrile (wt/vol) and extracted by vortexing and sonication for 10 min. The suspension was extracted twice by collecting supernatant after centrifuge at at 18,000 × *g* for 10 min, and after passage of the supernatant through a 2-μm filter, the filtrate was transferred to a HPLC vial and subjected to LC-MS analysis. A 5 μl of aliquot prepared from fecal extract was injected into an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) and separated in a BEH C18 column (Waters). The mobile phase was used a gradient ranging from water to 95% aqueous ACN containing 0.1% formic acid over a 10 min run. LC eluant was introduced into a Xevo-G2-S quadrupole time-of-flight mass spectrometer (QTOFMS, Waters) for accurate mass measurement and ion counting in negative-mode. Capillary voltage and cone voltage for electrospray ionization was maintained at -3 kV and -35 V for negative-mode detection. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution with mass-to-charge ratio (*m/z*) of 50-1,000 and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M-H]^-$ = *m/z* 554.2615) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx^{TM} software (Waters) in centroided format. Additional structural information was obtained tandem MS (MSMS) fragmentation with collision energies ranging from 15 to 40 eV. The concentration of bile acids in fecal samples were determined by calculating the ratio between the peak area of individual bile acids and the peak area of internal

standard, and then fitting with a standard curve using QuanLynx^{TM} software (Waters). Morphine sulfate standard was purchased from National Institute of Drug Abuse (NIDA) and M-3-G standard was purchased from Sigma.

Cross-correlation analysis

Cross-correlation between gut microbiome and metabolome data were performed using Microbiome Package, by following instruction online. Cross-correlation between phylogenotypes of microbiome and metabolites were Spearsman correlation. Taxa relative value were transformed as the base-10 logarithm of a number before correlated with metabolomic relative abundance value and FDR adjusted p-value (q-value). (Leo Lahti and Jarkko Salojarvi. (2014). Microbiome R package. URL: http://microbiome.github.com)

Microbial Culture

Enterococcus faecalis strain (SL11, MMH594::pREG969*lux*P_{gelE}) used in this study was routinely grown at 37°C without shaking in M17 medium (Oxoid Ltd., United Kingdom) supplemented with 0.4% (vol/vol) glucose (GM17) and spectinomycin was added at 500 ug/ml (Leanti La Rosa et al. 2013).

Statistics

Microbiome data were FDR adjusted p-value (q-value). Q-value < 0.05 was considered statistically significant. P-value in UniFrac distance comparison is Bonferroni-corrected. The tests of significance were performed using a two-sided

Student's two-sample t-test. Alternative hypothesis: Group 1 mean != Group 2 mean. Experimental values for fecal metabolites are expressed as means ± SEM. Statistical analysis was performed using t-test or ANOVA followed by Tukey's honestly significant difference test on significant threshold at $\alpha = 0.05$. P <0.05 was considered statistically significant.

RESULTS

1. Morphine treatment induces distinct changes in gut microbiome

Morphine treatment has been shown in our laboratory to induce bacterial translocation on day 2 post treatment and cause bacterial dissemination both in a mouse model and in human patients(Meng et al. 2013; Hilburger et al. 1997; Babrowski et al. 2012). It has been shown that microbial dysbiosis contributes to bowel dysfunction and susceptibility to infectious diseases(Buccigrossi, Nicastro, and Guarino 2013; Pham and Lawley 2014). To determine the effect of morphine treatment on gut microbiome profile, we analyzed gut microbiome based on Illumina sequencing of intestinal microbial 16S-rRNA genes. Hierarchical clustering of the samples was performed through the unweighted pair group method with arithmetic mean (UPGMA) using the Bray-Curtis similarity. The cladogram and dendrogram of a hierarchical cluster analysis showed distinct clustering in the morphine treatment group when compared to placebo. Housing animal with same treatment group in separate boxes does not result in individual differences (Figure 2.1). To compare microbial patterns, a principal coordinate analysis (PCoA) was used. A scatter plot based on an unweighted UniFrac distance matrics obtained from the sequences at OTU level with 97% similarity showed a distinct clustering of the community composition between the morphine and placebo treated groups. By using unweighted UniFrac distance to evaluate beta diversity (that is diversity between groups, comparing microbial community based on compositional structures), we found that morphine treatment results in a significant shift in fecal microbiome at day 3 post treatment compared to placebo treatment (Figure 2.2).

2. Morphine treatment leads to temporal modulation of gut microbiome

Opioid-induced adverse effects such as constipation are observed in patients less than 24 hours following morphine administration (McNicol, Midbari, and Eisenberg 2013). At 24 hours post treatment, mice receiving morphine revealed bacterial translocation to mesenteric lymph node (MLN) and liver(Meng et al. 2013). To determine time dependent changes in microbial composition following morphine pellet implantation, fecal samples were collected from the same animal at time 0, 24, 48 and 72 hours following morphine treatment and analyzed using 16S rRNA genes sequencing. Control animals were implanted with a placebo pellet. To determine the role of the opioid receptor in morphine induced effects, morphine implanted animals were also implanted with a Naltrexone pellet. Our time course study revealed distinct modulation of gut microbiome by morphine. Principal coordinates analysis of fecal samples from day 0, day 1, day 2, and day 3 post treatments showed that the microbial profile at day 0 in all treatment groups were similar and there were no distinct clustering. However, as early as 24 hours the microbiome from the morphine treatment group clustered distinctly from all other groups (Figure 2.3 & Figure 2.4). Naltrexone, an opioid receptor antagonist, antagonized morphine's induced alteration of gut microbiome (Figure 2.5 & Figure 2.6). Interestingly, animals treated with naltrexone alone clustered distinctly from the placebo group at day 3 following implantation suggesting that endogenous opioid may set a basal tone on the host microbial profile.

3. Morphine treatment increases pathogenic function and decreases stress tolerance of gut microbiome

To further determine if morphine treatment results in functional consequence following gut microbiome alteration, predicted metagenomic functional analysis was performed using a BugBase software package which is based on the PICRUSt analysis and KEGG metabolic OTUs from the GreenGenes reference database. Our results show that morphine treatment resulted in a significant increase in pathogenic bacterial strains (Figure 2.7B). Furthermore, there is a decrease in strains associated with stress tolerance indicating decreased resilience against perturbation to homeostasis (Figure 2.7B). These results implicate that healthy commensal microbiota might be less likely to outcompete outgrowth of pathogenic bacteria and eliminate pathogens colonization in the gut following morphine treatment. Less stress tolerance of the gut microbiota community means that the homeostasis is more "fragile" or susceptible.

4. Time course of morphine-modulated gut microbiome at genus level

Next, we performed a time course study in the phylogenetic analysis to determine whether certain strains changed post morphine treatment contributed to increased pathogenic function and decreased stress tolerance of gut microbiome. The analysis of OTUs on genus level shows that relative abundance of pathogenic bacteria (genus level) increased significantly at day 3 post morphine treatment compared to placebo treatment (Figure 2.8). Increased representative pathogenic bacteria include *Flavobacterium*, *Enterococcus*, *Fusobacterium*, *Sutterella*, *Clostridium* (Figure 2.8, Table 2.1). To avoid false positive paradox, multiple hypothesis tests and "multiple testing correction" were performed by using false discovery rate (FDR), Q-value (Noble 2009). The "Q-value" is the FDR based measure of significance which applies to multiple hypothesis tests and can

be calculated simultaneously (Storey 2003). The q-value is defined to be the minimum FDR at which the test is called significant (Q-value < 0.05).

5. Identification of association between gut microbial dysbiosis and bacteria at species level

In order to confirm the pathogenic strains that constitutes distinctness of gut microbiome alteration, we detected expression profiling of selected species specific 16SrRNA gene in gut microbiota by using quantitative real-time PCR. The result shows that expansion of *Enterococcus faecalis* is associated with morphine induced alteration of gut microbiome (Figure 2.9A). *E. faecalis* 16S-rRNA gene amplification was 100 times greater in morphine modulated gut microbiome than placebo treated gut microbiome. The effect of morphine on *E. faecalis* expansion was observed as early as day 1in morphine treated animals and sustained in all days tested post treatment (Figure 2.9B). The effect of morphine on *E. faecalis* abundance in the gut microbiome was antagonized by naltrexone treatment. This results suggest that expansion of *E. faecalis* can potentially act as a biomarker of gut dysbiosis following morphine treatment (Figure 2.9C).

6. Morphine induces distinct gut metabolomic profile

Microbial dysbiosis leading to a disruption of host-microbes homeostasis is not only alteration of microbial composition, but more importantly disruption of functional configuration of the microbiota(Sommer and Bäckhed 2013). The intestinal microbiota metabolizes input substrates from host, including diet and xenobiotics, into metabolites that can affect the host(Ursell et al. 2014). Although gut microbiome may

vary in different individuals, a core functional metabolic interaction with gut microbiota is essential for the host and alteration of the core functional microbiome is associated with different physiological states(Turnbaugh et al. 2009). Thus, identification of gut metabolomic profile is fundamental to reveal the functional changes of gut microbiome. To explore changes in metabolomic profile induced by morphine treatment, a time course study was carried out as described earlier. Fecal samples were collected from the same animal following treatment at day 0, 24, 48 and 72 hours and analyzed together using LC-MS. Gut microbiome analysis were also performed on the same fecal samples. Scores scatter plot of the partial least square discriminant analysis (PLS-DA) model of fecal samples were performed from morphine or placebo treated samples (Figure 2.10A). The result shows that morphine treatment induced a distinct gut a metabolomic profile when compared to placebo treated group. To identify metabolites contributing to separation of fecal samples from mice following morphine or placebo treatments, we performed a loading plot of principal components analysis model in which each dot represents a single molecular metabolite (Figure 2.10B).

7. *Morphine changes gut metabolomic profile gradually and shifts metabolites differentially*

Metabolic profiles following morphine treatment was determined in a time course study. Our results show that morphine treatment resulted in a gradual and differential shift in metabolites in a time dependent manner (Figure 2.11). To identified shifts of gut metabolome following morphine treatment we performed scores scatter plot of the partial least square discriminant analysis (PLS-DA) model and characterized the

metabolomics profiling of fecal samples from wild type mice (C57B6/J) with morphine or placebo treatment at 0, 24, 48 and 72hours following morphine treatment (Figure 2.11A). Morphine treatment revealed distinct clustering in the metabolome profile when compared with placebo treatment at each time tested. In order to reveal the details of metabolomic changes due to morphine treatment, we identified their chemical identities that increased and decreased following morphine treatment at day 3 post morphine treatment (Figure 2.11B). The result shows that morphine changes gut metabolomic profile gradually and shifts metabolites differentially in fecal matters taken from mice at prior to and day 1, day 2, day 3 post morphine or placebo treatment. Bile acids decreased gradually, while phosphatidylethanolamines (PEs) and saturated fatty acids increased as a consequence of morphine treatment (Figure 2.11B).

8. Naltrexone antagonized morphine induced gut metabolomic shift and reversed effect of morphine on bile acid metabolism

We used an opioid receptor antagonist, naltrexone, to determine whether it can inhibit effect of morphine on gut metabolomics profile. The result shows that naltrexone can inhibit morphine induced gut metabolomics shift and reverse the effect of morphine on bile acid metabolism (Figure 2.12A). Particularly, we established that the abundance of secondary bile acid, deoxycholic acid significantly decreased following morphine treatment and morphine induced decrease was antagonized by naltrexone (Figure 2.12B). The abundance of phospholipid, PE, which is a major component of cell membrane, was increased by morphine treatment, indicating increased cell injury, and morphine induced increase was reversed by naltrexone treatment (Figure 2.12B). This result indicate that

deoxycholic acid (DCA) levels and phosphatidylethanolamines (PE) can be used as biomarkers to indicate morphine-modulation of gut metabolome.

9. Morphine induced dysbiosis disrupts morphine metabolism and its enterohepatic recirculation

Morphine is conjugated to morphine-3-glucuronide (M3G) and morphine-3-glucuronide (M6G) in liver and excreted to gut via biliary tract. M3G is totally inactive, whereas M6G appears to display stronger analgesic activity than morphine(Lötsch and Geisslinger 2001). Intestinal de-conjugating bacteria transform M3G and M6G to morphine, which is reabsorbed back to systemic circulation. The enterohepatic circulation plays an important role in morphine elimination, which is characterized by a prolonged terminal elimination phase(Ouellet and Pollack 1995). In mice and rats, M6G formation is insignificant, so morphine is recycled significantly via M3G. We found that the ratio of M3G/MS serum concentration increases between day 1 and day 6 post-treatment (Figure 2.13) and also increases in feces between day 1 and day 2 post morphine treatment, indicating decreased M3G deconjugation to morphine in the gut (Figure 2.14).

10. Cross-correlation of morphine-modulated gut microbiome and metabolome

In order to further clarify if morphine-modulated gut microbiome alteration is associated with gut metabolomic changes following morphine treatment, we performed cross-correlation between gut microbiome and metabolome (Figure 2.15). The cross-correlation shows that cholic acids, and Octadecanedioic acid are negatively associated with *Enterococcus* and *Erysipelotrichaceae* at the family level. While

phosphatidylethanolamines (PE) are negatively associated with bacteroidales (Order level), and positively associated with *Erysipelotrichaceae*. Although the cross-correlation analysis is only statistical evidence, however statistical inference can help reveal the inherent causal relationship between gut microbiome and metabolome.

DISCUSSION

In this longitudinal study, we show that morphine treatment, when compared to placebo, induces a significant shift in gut microbiome and increased potential pathogenic bacteria. Phylogenetic analysis revealed that morphine treatment results in decreased alpha diversity and distinct clustering in a beta diversity plot when compared to placebo. Previous studies in our laboratory and other research groups have demonstrated that morphine induced increase risk in virulent bacterial infection, bacterial translocation and lethal gut-derived sepsis(Babrowski et al. 2012; J. Wang et al. 2005; Degenhardt et al. 2011,Meng et al. 2015). Our current results indicate that the increased abundance of potential pathogenic bacteria following morphine treatment may account for morphine induced bacterial translocation and sepsis.

Richness of microbial diversity is an indicator of microbial homeostasis and less microbial diversity is associated with microbial dysbiosis(Bäckhed et al. 2012). Morphine treatment is associated with a significant decrease in gut microbial alpha diversity, indicating microbial dysbiosis and higher risk for dysbiosis (Figure 2.15).

In this study, we reveal that *E. faecalis* is associated with morphine-modulated gut microbiome alteration in a time-course study. This result is alignment with serotyped species in our previous study on morphine-induced bacterial translocation and confirmed a significant prevalence of *Enterococcus* in all mesenteric lymph nodes (mLN), spleen, and liver isolates from morphine-treated animals(Meng et al. 2015). *E. faecalis* is associated with ulcerative colitis in human and opioid-induced sepsis in mouse model(Fite et al. 2013; Meng et al. 2015).This indicates that *E. faecalis* may act as biomarker of morphine induced sepsis and systemic bacterial translocation. In order to

investigate the role *E. faecalis* play in gut microbiome, we infected mouse in context of morphine treatment. In this *E. faecalis* infection experiment, we found that *E. faecalis* can decrease alpha-diversity and shifts beta-diversity of gut microbiome (Figure 2.6).

This study revealed morphine changes gut metabolomic profile gradually and shifts metabolites differentially. The differential changes of gut metabolomics profile may reflect alteration of gut microbiome and therefore contribute to host response following morphine treatment. Cross-correlation between gut microbiome and metabolome indicate association between bacterial communities and functional metabolites (Figure 2.15). Cholic acid is negatively associated with *Enterococcus* and *Erysipelotrichaceae*, but positively associated with *Bacteroidales*. On the contrary, PEs and stearic acid are positively associated with *Enterococcus* and *Erysipelotrichaceae*, but negatively associated with *Bacteroidales*. Our previous study determined that morphine treatment leads to intestinal barrier dysfunction. It was well demonstrated that bacterial metabolites regulate GI barrier function via Xenobiotic Sensor PXR-dependent TLR4 signaling pathway, suggesting alteration of gut microbiome and metabolome may contribute to gut barrier dysfunction following morphine treatment. Furthermore, bile acids can mediate host resistance to *C. difficile* infection. Decrease of bile acid may be associated with morphine-induced increase of pathogenic bacteria in gut, such as *E. faecalis*. The gut microbiome of mice that switch to high fat diet are characterized by *Erysipelotrichaceae* increase, and are associated with higher risk of infectious disease and inflammation(Greiner and Bäckhed 2011; Honda and Littman 2012). Taken together, the present study has elucidated the mechanism under which how morphine treatment results in pathological effects.

It has been well demonstrated that gut metabolites play an important link between gut microbes and host biological functions. Morphine treatment results in dramatic changes in the fecal metabolome, alters fatty acid and bile acid metabolism, and increases PEs levels in gut. PEs are the main lipid components of the inner bacterial membrane. An increase of PEs level is indicative of significant cell injury. PEs are associated with bacterial stress responses(R. Keller et al. 2015). In this study, analysis using BugBase software package suggested morphine treatment results in a decrease in stress tolerance. The increase of PEs in gut is associated with decreased stress tolerance.

Morphine metabolism and elimination plays an important role in determining drug pharmacokinetics and assessing their efficacy and adverse effects in clinical terms. To investigate whether gut microbiome alteration impacts morphine metabolism is of essence in clinical terms. We found that the ratio of M3G/MS serum concentration increases between day 1 and day 6 post-treatment (Figure 2.13) and also increases in feces between day 1 and day 2 post morphine treatment, indicating decreased M3G deconjugation in gut(Figure 2.14). The major glucuronide deconjugating bacteria are the strict anaerobes, *bacteroides,* and *bifidobacteria*, which express the β-glucuronidase activity(Stain-Texier, Sandouk, and Scherrmann 1998). In present study, we show that morphine treatment results in a decrease in *Bacteroidales,* suggesting that decrease M3G-deconjugation is a consequence of a decrease in deconjugating bacteria (Table 2.1). Our cross-correlation analysis results revealed that these *Bacteroidales* are positively associated with cholic acid and octadecenoic acids, but negatively associated with PEs, glucosides, and stearic acid, which is consistent with what we observed in morphine-induced alteration of metabolomic profile (Figure 2.11 & Figure 2.13). The

phosphatidylethanolamine-binding protein (PEBP)/(alternatively named Raf-1 kinase inhibitor protein or RKIP) , initially found to bind PEs has been shown to be associated with morphine derivatives(Atmanene et al. 2009). PEBP acts as a molecular shield and prevents morphine-3-glucuronide from rapid clearance(Goumon et al. 2006). These results revealed that loss of deconjugating bacteria, decreased bile acids and increased PEs in gut modulate morphine metabolism and elimination, which consequently affects clinical use of opioids in pain management.

For the first time, we show emergence of distinctness in bacterial profile in the gut microbiome and metabolome following morphine treatment. The present study revealed that morphine-induced gut microbiome and metabolome shifts are inhibited by naltrexone, an opiate receptor antagonist, indicating morphine induced changes are opiate receptor dependent. Peripheral opiate receptor antagonists, such as naltrexone, can be exploited as potential therapeutic approach to inhibit or rescue morphine-modulation of gut microbiome and metabolome. We identified *E. faecalis* as being associated with intestinal microbiome in response to morphine treatment, indicating potential application in therapeutics and non-invasive diagnostics. In this study, we investigated various effects of the intestinal microbiota on the biotransformation of opioids and other small molecular metabolites. By understanding and altering the intestinal microbiota one may be able to detect and minimize the adverse effects of xenobiotics. Thus one can consider the intestinal microbiota as another drug target. We also identified DCA and PEs as molecular metabolites that represent morphine-induced alteration of gut metabolome, indicating potential target of therapeutic intervention. This study shed light on the mechanism that morphine modulates gut homeostasis provides novel approaches to avoid

morphine adverse side effects. Furthermore, this study may help improve medical intervention concerning consequences of drug use/abuse and will provide potential therapeutic and diagnostic strategies for opioids-modulated intestinal infections as well.

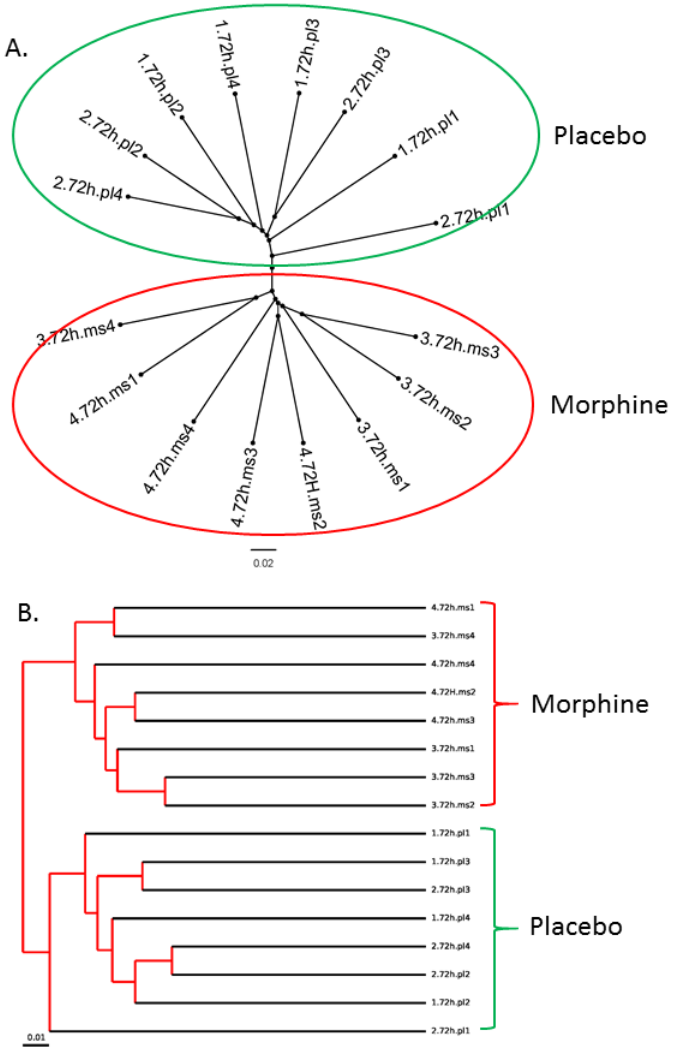


Figure 2.1 Morphine induced distinct clustering and distribution of gut microbiome. Housing animal in separate boxes does not result in individual differences. Fecal samples were collected from mice at day 3 post treatments. (A) The dendrogram of a hierarchical cluster analysis shows distinct clustering of morphine treated samples when compared to placebo. Hierarchical clustering of the samples with the unweighted pair group method with arithmetic mean (UPGMA) using the Bray-Curtis similarity additionally confirmed that samples from morphine treated mice clustered differently than those from controls. (B) The jackknifed tree with internal nodes shows the relationship among samples and reveals that samples from morphine treated mice clustering differently from that of placebo. The length of bar value explains how frequently a given internal node had the same set of descendant samples in the jackknifed UPGMA clusters as it does in the UPGMA cluster using the full data set. Annotation of sample names: For example, in the sample "1.72h.pl2", the number "1" prior to the first dot refers to animal box number; "72h" refers to 72 hours post treatment; "pl2" refers to the number 2 mouse in the box 1 that placebo treatment;

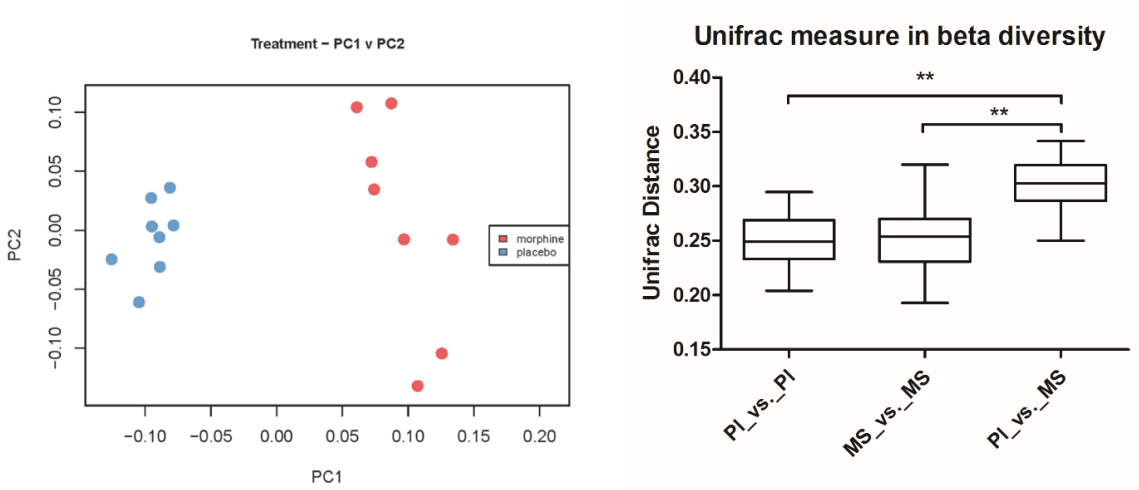


Figure 2.2 Beta diversity measures of gut microbiome following morphine or placebo treatment. Wild type mice were implanted with placebo, 25mg morphine pellets subcutaneously. Fecal matter were taken for analysis at day 3 post treatment. (A) Principal coordinates analysis (PCoA) of samples using the UniFrac metric at the OTU level. (B) UniFrac distance significant tests were performed using QIIME. The tests of significance were performed using a two-sided Student's two-sample t-test. *Parametric p-value (Bonferroni-corrected) <0.05, ** Parametric p-value (Bonferroni-corrected) <0.01.

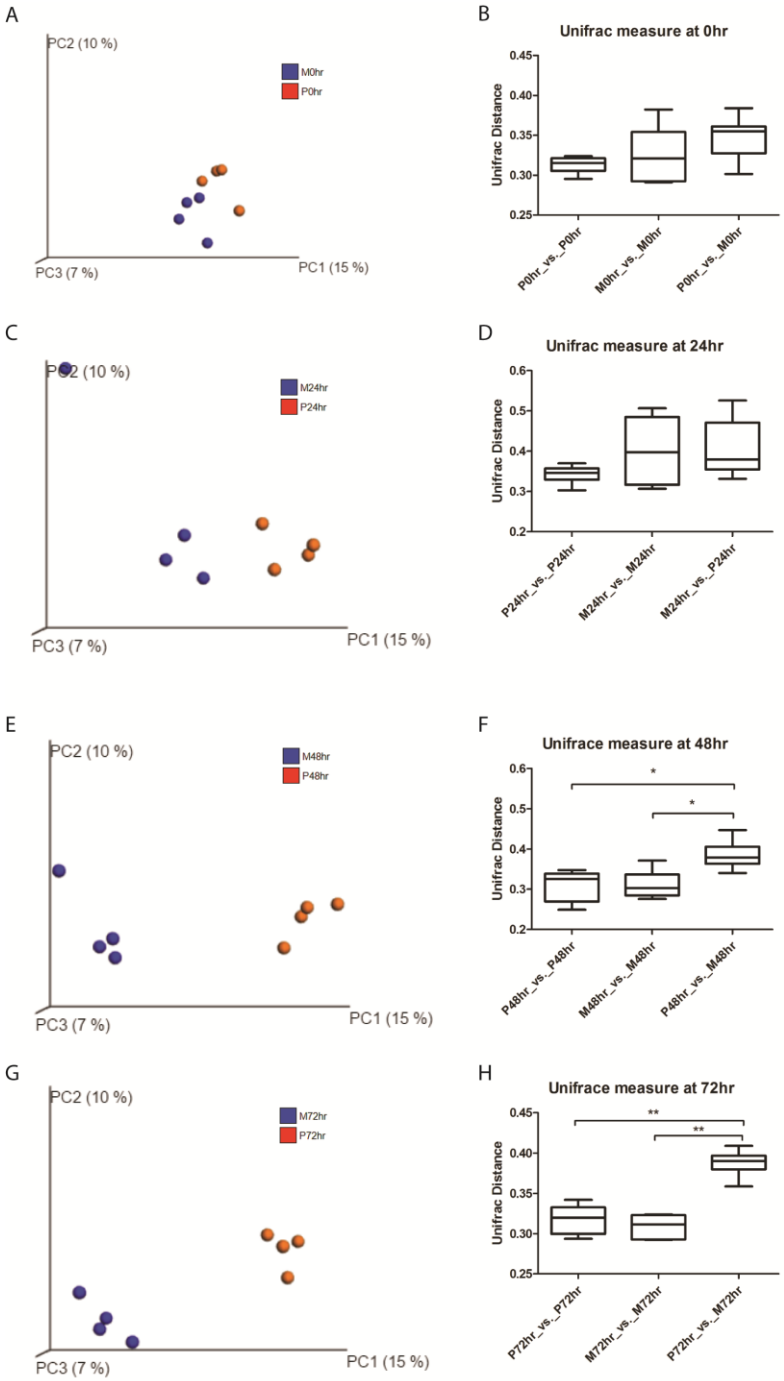


Figure 2.3 Beta diversity measures of gut microbiome following placebo or morphine treatment. Wild type mice were implanted with placebo, 25mg morphine pellets subcutaneously. Fecal matter were taken for analysis at pre-treatment, day 1, day 2, day 3 post treatment, respectively. Principal coordinates analysis of samples from pre-treatment (A), day 1 (C), day 2 (E) and day 3 (G) using the UniFrac metric at the OTU level. The tests of significance were performed using a two-sided Student's two-sample t-test. *Parametric p-value (Bonferroni-corrected) <0.05, ** Parametric p-value (Bonferroni-corrected) <0.01.

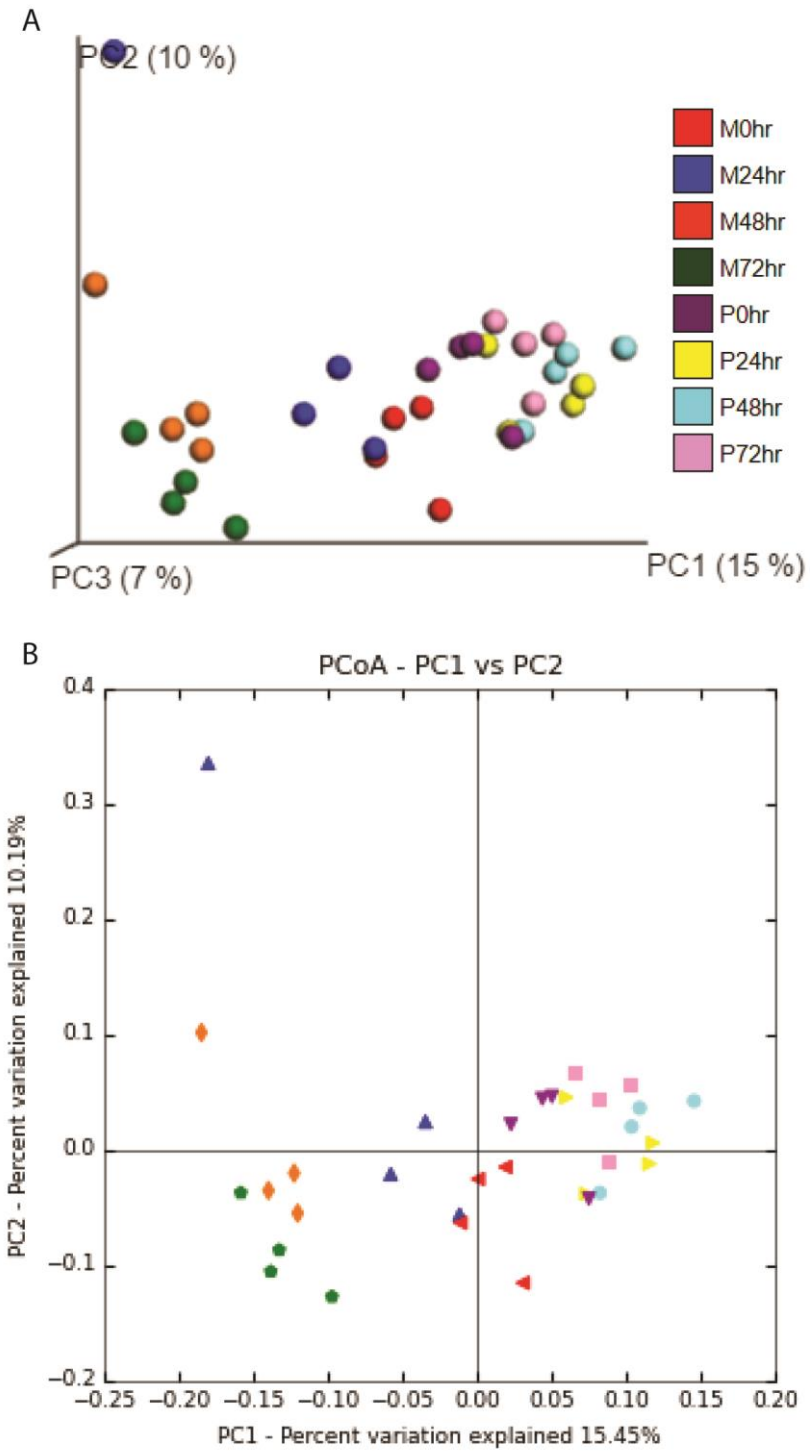


Figure 2.4 Beta diversity measures of gut microbiome following placebo or morphine treatment. Fecal samples from day 0, day 1, day 2 and day 3 post treatments collectively presented on the same PCoA plot in 3D (A) or 2D (B) visualization respectively.

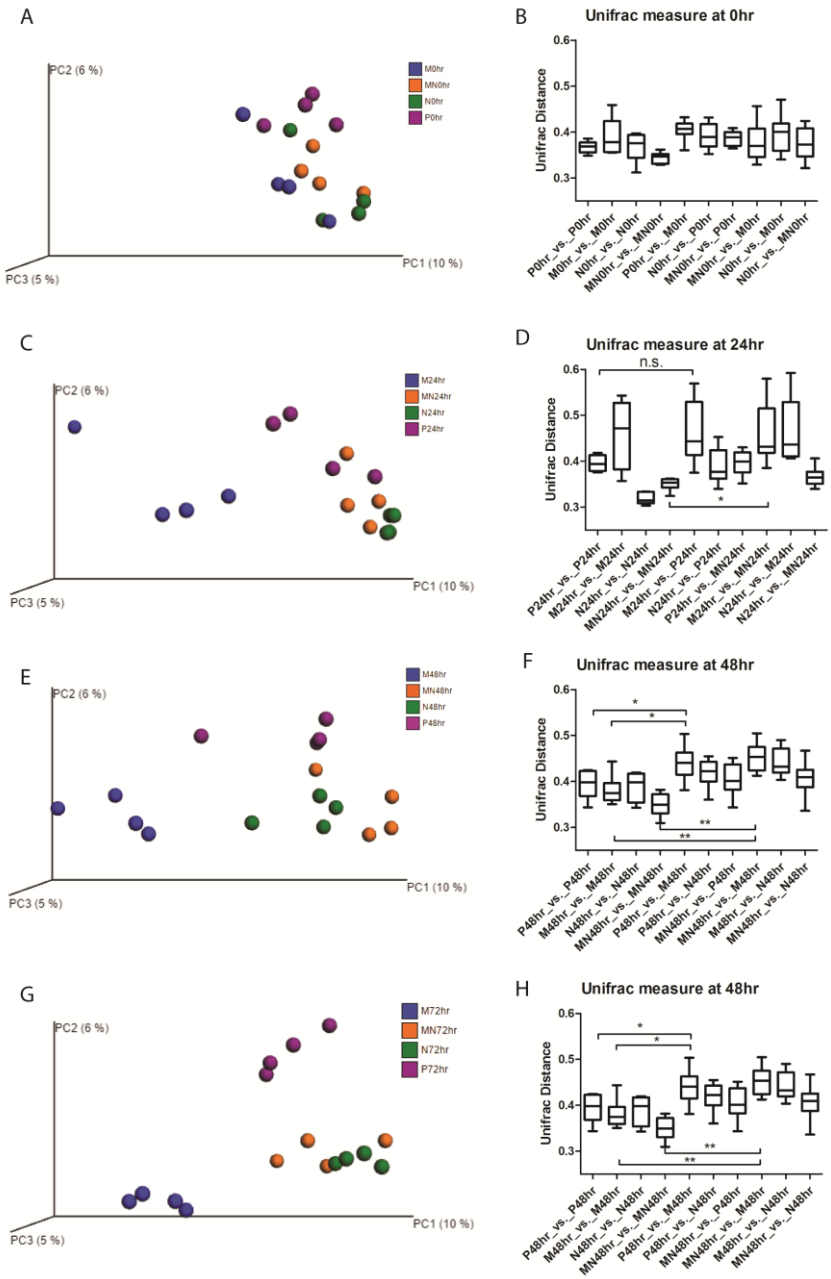


Figure 2.5 Beta diversity measures of gut microbiome following placebo, morphine, naltrexone, or morphine plus naltrexone treatment. Wild type mice were implanted with placebo, 25mg morphine, 30mg naltrexone, or morphine and naltrexone pellets subcutaneously. Fecal matter were taken for analysis at pre-treatment, day 1, day 2, day 3 post treatment, respectively. Principal coordinates analysis of samples from pre-treatment (A), day 1 (C), day 2 (E) and day 3 (G) using the UniFrac metric at the OTU level. The tests of significance were performed using a two-sided Student's two-sample t-test. *Parametric p-value (Bonferroni-corrected) <0.05, ** Parametric p-value (Bonferroni-corrected) <0.01.

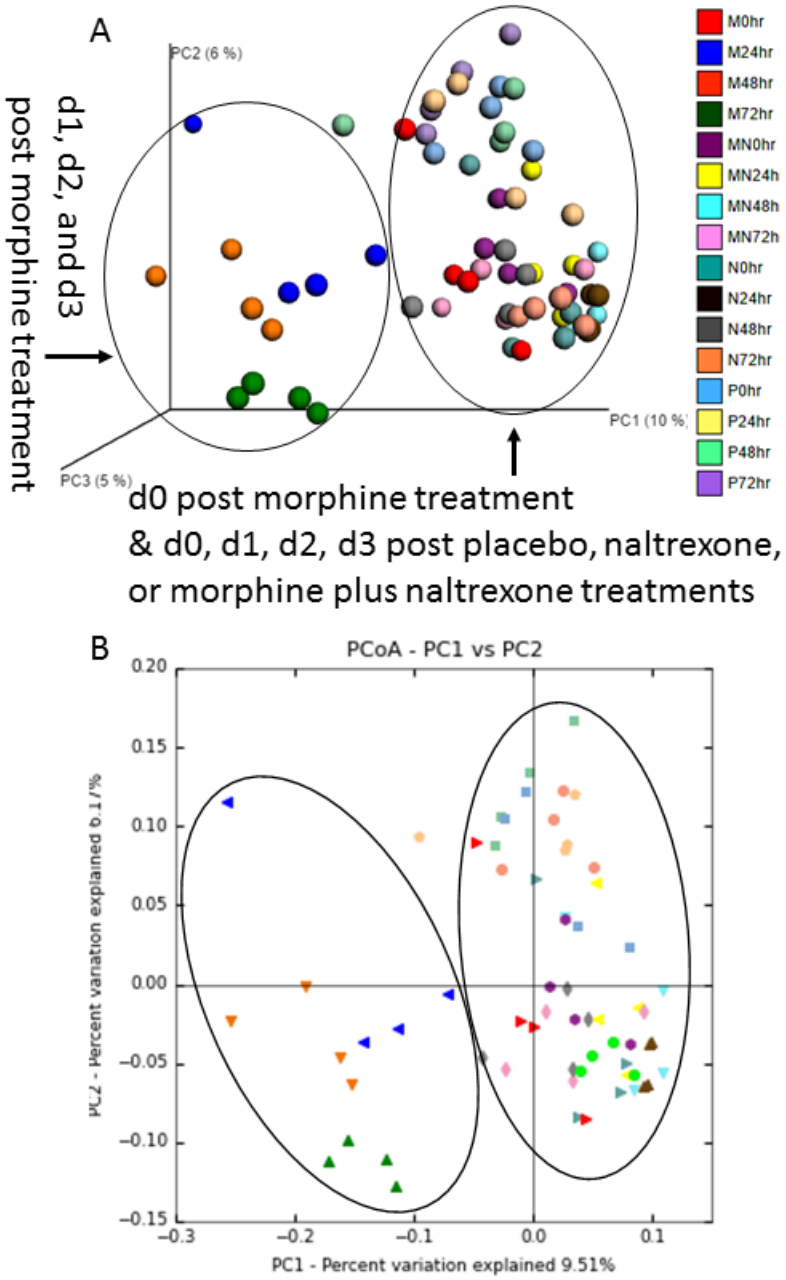


Figure 2.6 Beta diversity measures of gut microbiome following placebo, morphine, naltrexone, or morphine plus naltrexone treatment. Fecal samples from day 0, day 1, day 2 and day 3 post treatments collectively presented on the same PCoA plot in 3D (A) or 2D (B) visualization respectively.

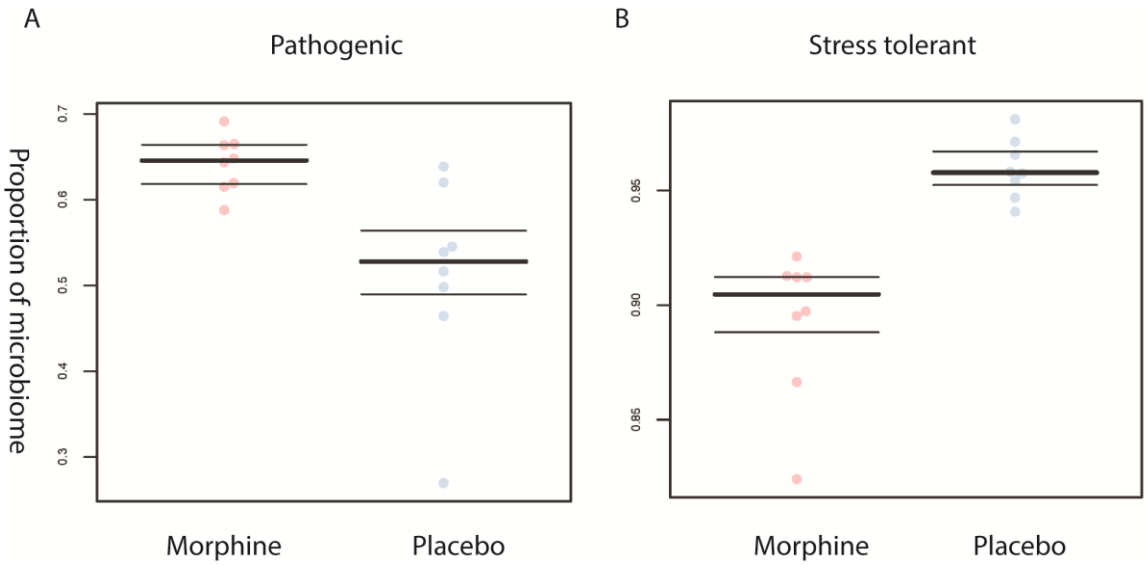


Figure 2.7 Microbiome based functional phenotype analysis. Morphine treatment results in a significant increase of pathogenic bacteria (A) and decrease of stress tolerance (B). Predicted metagenomic functional analysis were performed by using BugBase software package, which is based on the PICRUSt analysis and KEGG metabolic OTUs from the GreenGenes reference database. (A) FDR-corrected p-value is: 0.004662005; (B) FDR-corrected p-value is: 0.0001554002.

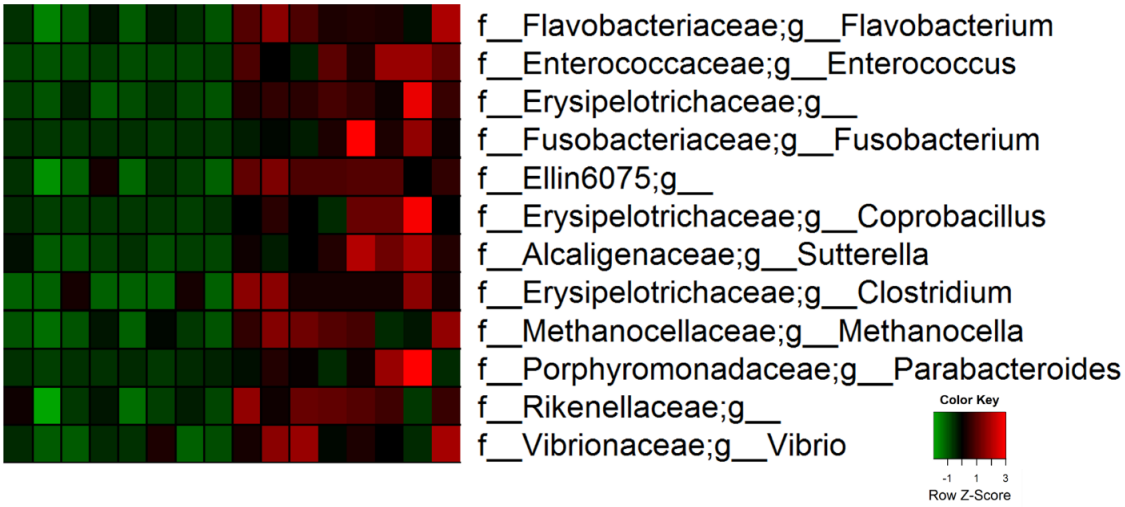


Figure 2.8 Morphine treatment results in a significant increase of pathogenic bacteria. Multiple hypothesis test with the given threshold (FDR=0.05) shows relative abundance of pathogenic bacteria (genus level) increase significantly at day 3 post-treatment with morphine treatment compared to placebo treatment. Increased (red color) representative pathogenic bacteria include *Flavobacterium, Enterococcus, Fusobacterium, Sutterella, Clostridium*.

Table 1.

Features	pvalue	qvalue	d3ms mean	d3pl mean
k_Bacteria;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium	0.000939	0.019635	0.084028	0.030900
k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Enterococcaceae;g_Enterococcus	0.000939	0.019635	0.008298	0.000883
k_Bacteria;p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_	0.000939	0.019635	0.007198	0.001056
k_Bacteria;p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_Fusobacteriaceae;g_Fusobacterium	0.000939	0.019635	0.038899	0.001976
k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Chromatiales;f_Ectothiorhodospiraceae;g_	0.001192	0.019635	0.000000	0.000061
k_Bacteria;p_Acidobacteria;c_[Chloracidobacteria];o_RB41;f_Ellin6075;g_	0.001315	0.019635	0.001117	0.000436
k_Bacteria;p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Coprobacillus	0.001359	0.019635	0.005758	0.000379
k_Bacteria;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_Sutterella	0.001359	0.019635	0.001871	0.000472
k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_;g_	0.001616	0.020691	0.000685	0.001560
k_Bacteria;p_WS3;c_PRR-12;o_Sediment-1;f_CV106;g_	0.001751	0.020691	0.000024	0.000391
k_Bacteria;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_;g_	0.001953	0.021154	0.000077	0.000012
k_Bacteria;p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Clostridium	0.002695	0.026951	0.000044	0.000008
k_Archaea;p_Euryarchaeota;c_Methanomicrobia;o_Methanocellales;f_Methanocellaceae;g_Methanocella	0.003163	0.029369	0.000569	0.000234
k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_Parabacteroides	0.003824	0.031493	0.001556	0.000250
k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_	0.003876	0.031493	0.058407	0.035380
k_Bacteria;p_Tenericutes;c_Mollicutes;o_Anaeroplasmatales;f_Anaeroplasmataceae;g_Anaeroplasma	0.006088	0.044013	0.000012	0.001774
k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae;g_Vibrio	0.006094	0.044013	0.000718	0.000262

Table 2.1 Taxonomic significance test in gut microbiome following morphine or placebo treatments. Taxa features were selected by their ranked individual hypothesis test p-values and false discovery rate, q-value. The statistical analysis were performed by using MWAS R Package v0.9.3.

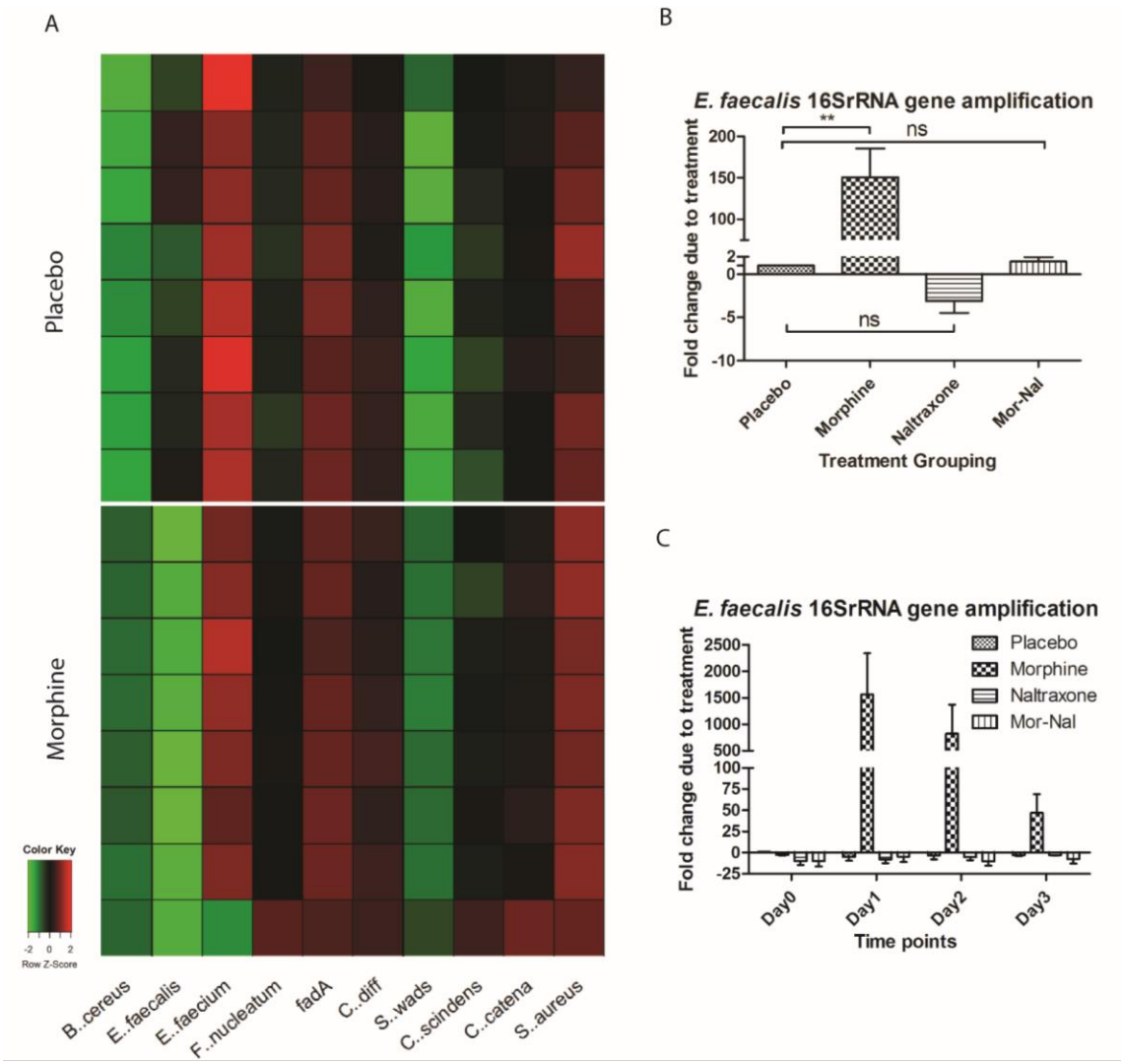


Figure 2.9 *Enterococcus faecalis* is biomarker of morphine induced alteration of gut microbiome. Real-time PCR expression profiling of species specific 16S-rRNA gene in gut microbiota. (A) The expression of species specific 16S-rRNA gene was profiled in stool samples from eight mice s.c. implanted with placebo (control) and eight with morphine (treatment) using a qRT-PCR assay. The heatmap was generated by the real-time PCR data presented as ΔCT (CT_species – CT_universal_16SrRNA). A greener color indicates higher level of amplification. (B) *E. Faecalis* 16S-rRNA gene amplification fold change due to treatments on day 3 post treatment. (C) *E. Faecalis* 16S rRNA genes amplification fold change due to treatments in a longitudinal study.

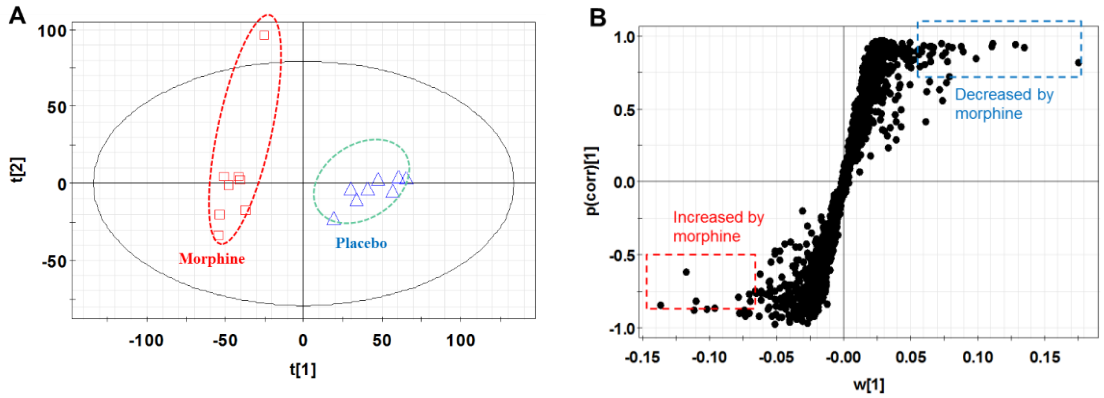


Figure 2.10 Metabolomic analysis of fecal matter and identification of metabolites changes. Mice were treated with 25mg morphine or placebo pellet subcutaneously. Fecal matter were taken for analysis at 3 days post treatment. (A) Scores scatter plot of the partial least square discriminant analysis (PLS-DA) model of fecal samples from wild type mice (C57B6/J) with morphine (□) or placebo (Δ) treatment. The t[1] and t[2] values represent scores of each sample in principal components 1 and 2, respectively. (B) Loadings plot of principal components analysis model. Metabolites contributing to separation of fecal samples from mice following morphine and placebo treatment were labeled, and their chemical identities are confirmed.

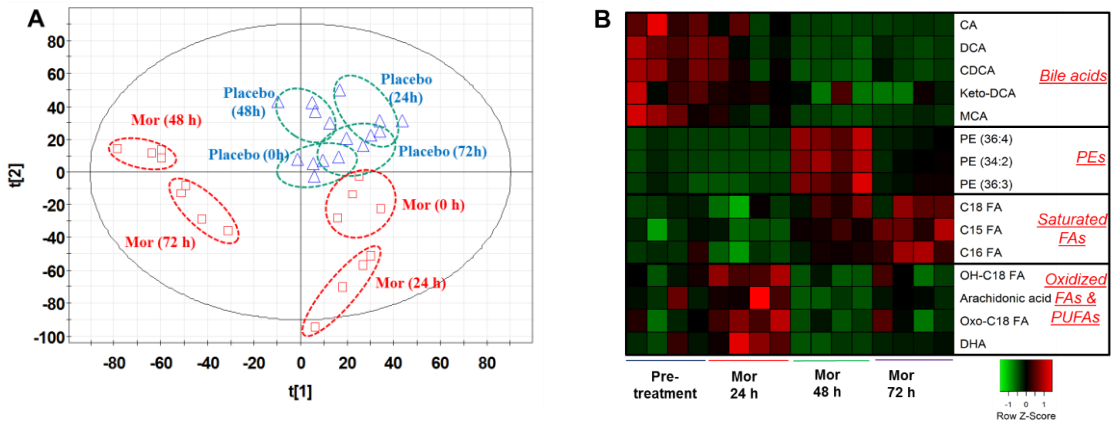


Figure 2.11 Metabolomic analysis of fecal matter and identification of significant shifts of gut metabolome following morphine treatment. In a longitudinal study, fecal matters were taken from mice at prior to and day 1, day 2, day 3 post morphine or placebo treatment. (A) Scores scatter plot of the partial least square discriminant analysis (PLS-DA) model of fecal samples from wild type mice (C57B6/J) with morphine (□) or placebo (Δ) treatment. The t[1] and t[2] values represent scores of each sample in principal components 1 and 2, respectively. (B) Heatmap plot of significant associations with morphine treatments and the loading of indicator metabolites in fecal matter from mice. All relative abundances are row z-score normalized for visualization.

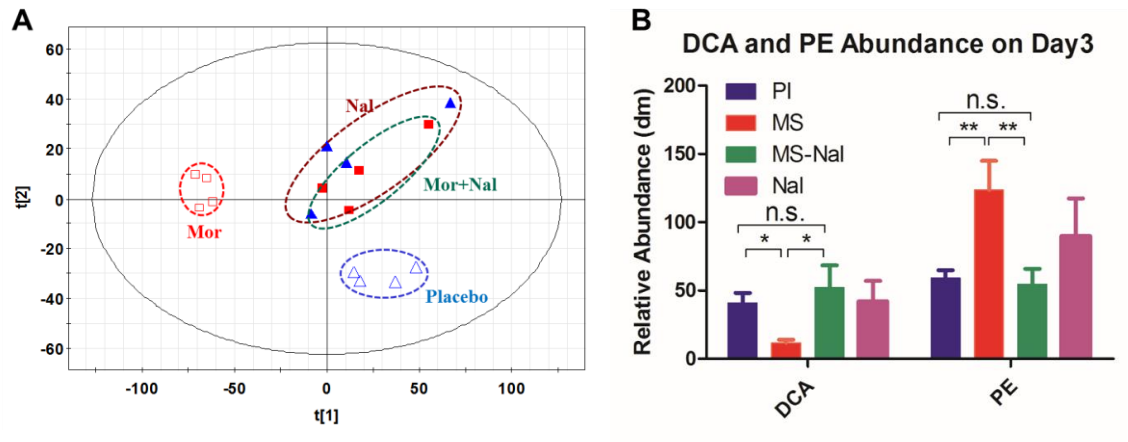


Figure 2.12 Metabolomic analysis of fecal samples and measurement of effects of naltrexone on morphine induced gut metabolomic shifts. Mice were treated with placebo, 25mg morphine, 30mg naltrexone, or morphine + naltrexone pellets subcutaneously. Fecal matter were taken for analysis at 3 days post treatment. (A) Scores scatter plot of the partial least square discriminant analysis (PLS-DA) model of fecal samples from wild type mice (C57B6/J) with morphine (□) or placebo (Δ) treatment. The t[1] and t[2] values represent scores of each sample in principal components 1 and 2, respectively. (B) Relative abundance analysis of metabolites reveals reversed naltrexone, an opioid receptor antagonist, reversed the effect of morphine on loading of deoxycholic acid (DCA), a secondary bile acid, and phosphatidylethanolamines (PEs), a class of phospholipids found in biological membranes.

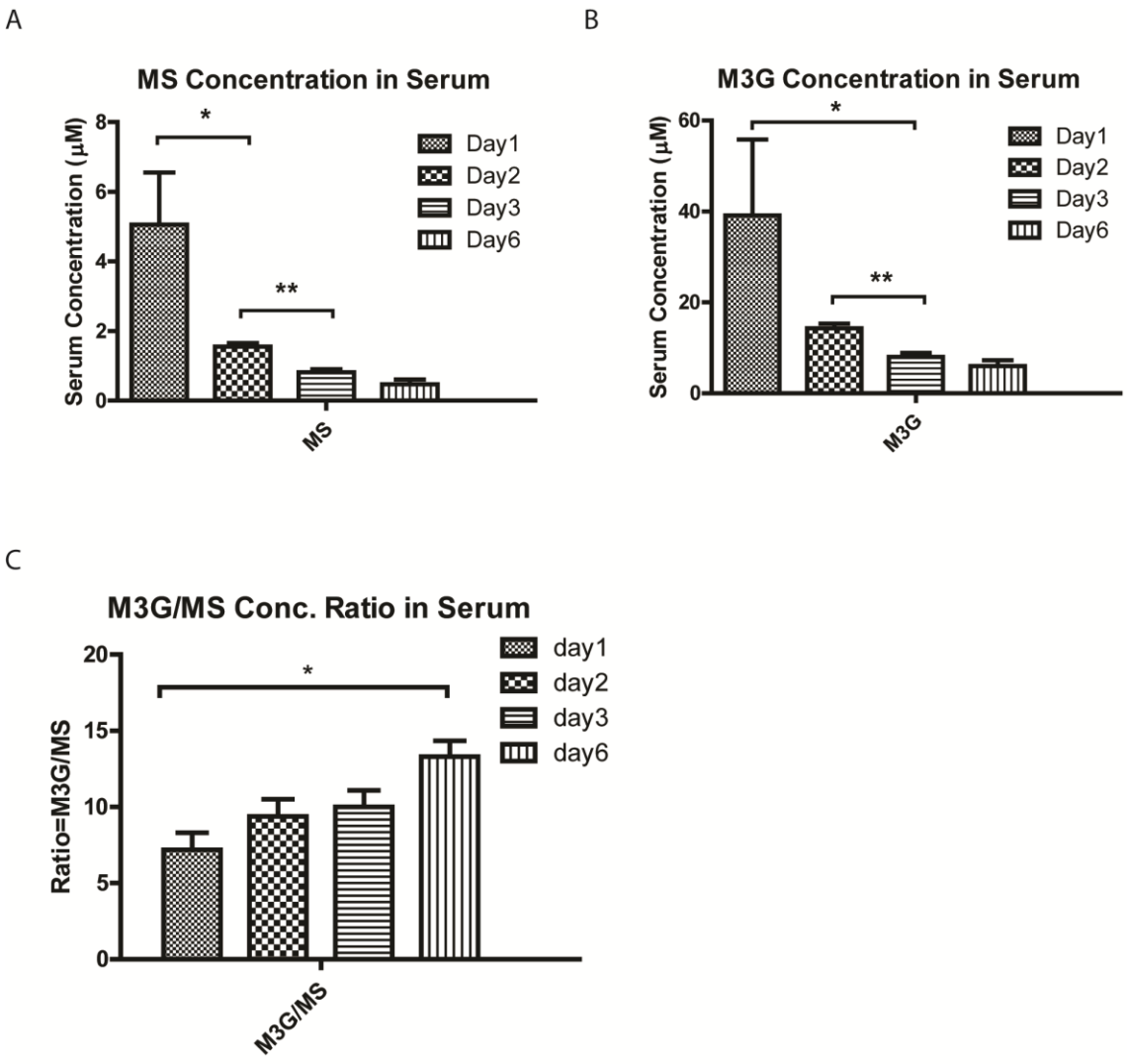


Figure 2.13 The ratio of M3G/MS serum concentration increases post morphine treatment. LC-MS analysis identified MS and M3G concentration in mouse serum. Statistical significance tests were performed using Student t test. $P<0.05$.

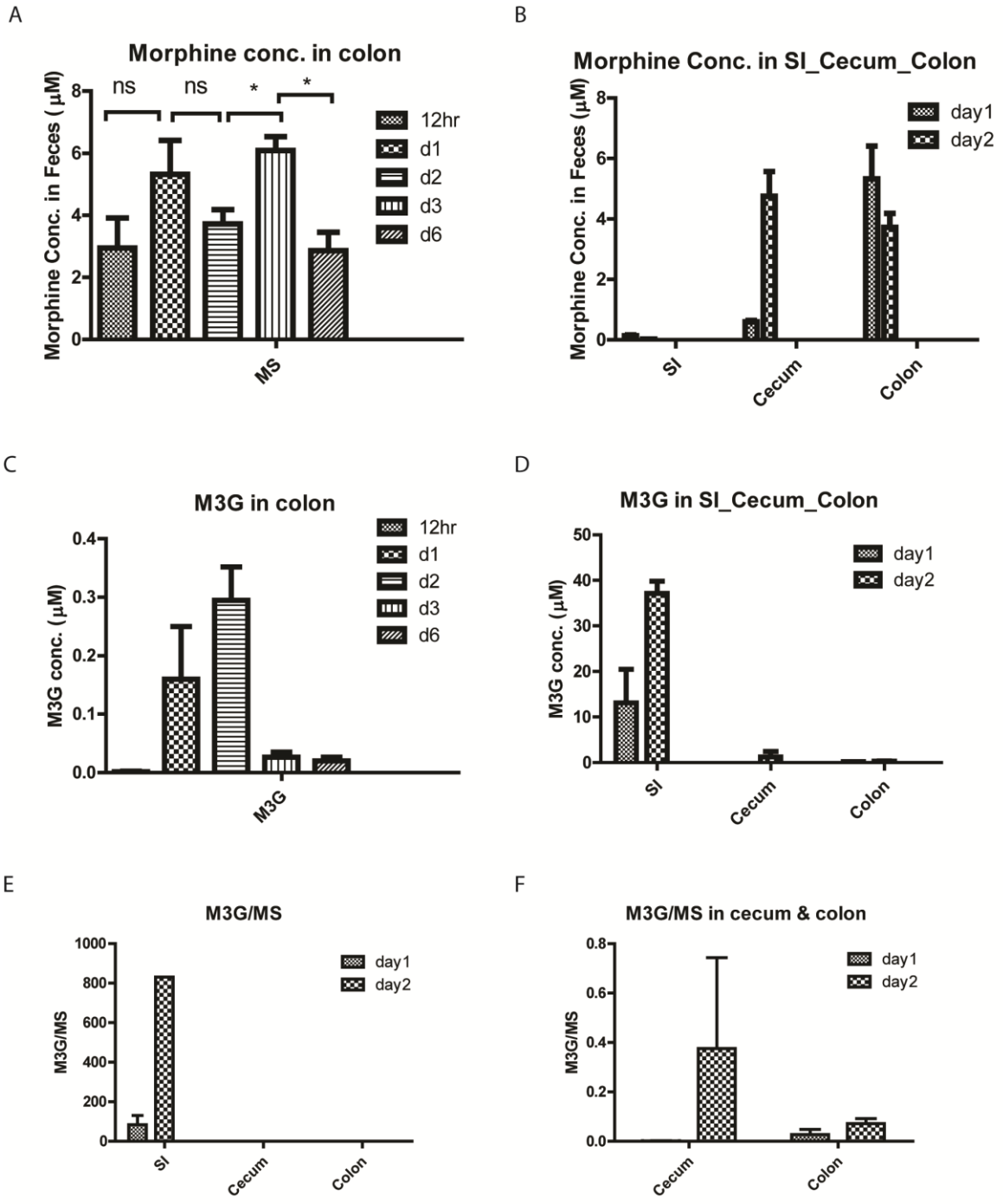


Figure 2.14 The ratio of M3G/MS fecal concentration increases post morphine treatment. LC-MS analysis identified MS and M3G concentration in mouse feces. Statistical significance tests were performed using Student t test. $P<0.05$.

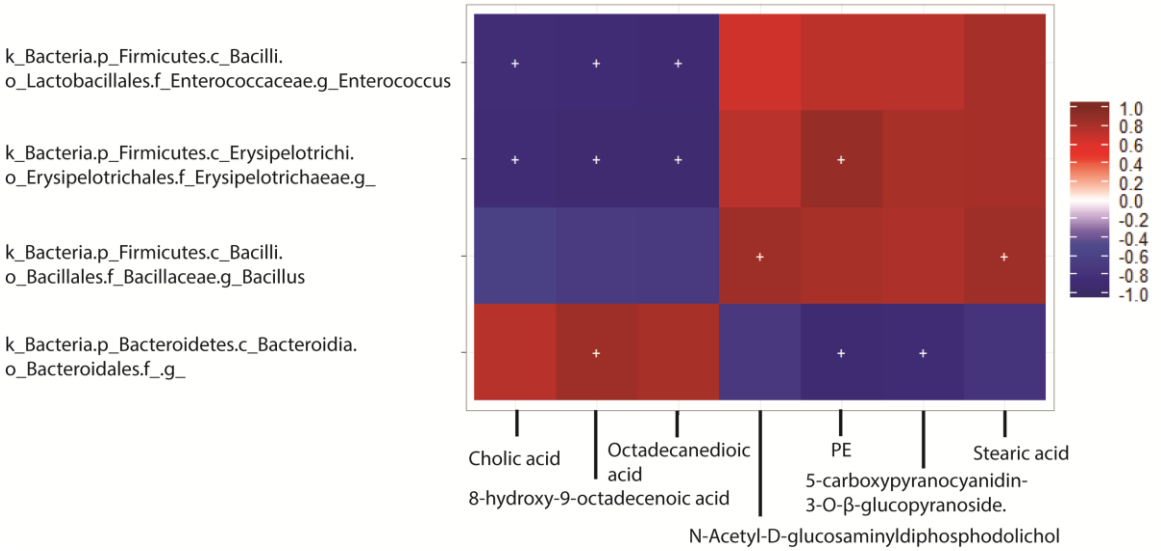


Figure 2.15 Cross-correlation analysis between gut microbiome and metabolome. Cross-correlation between phylogenotypes of microbiome and metabolites were Spearsman correlation. Taxa relative value were transformed as the base-10 logarithm of a number before correlated with metabolomic relative abundance value and FDR adjusted p-value (q-value). Significance was considered as q-value < 0.05.

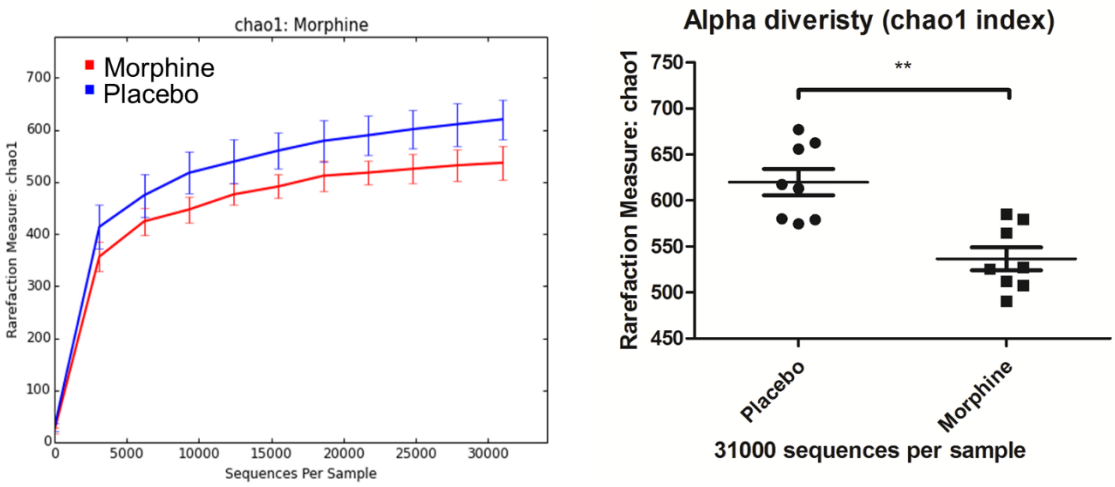


Figure 2.15 Alpha diversity in gut microbiome following morphine or placebo treatment. (A) Alpha diversity were assessed by using chao1 index. Morphine treatment (n=8) results in decreased alpha diversity compared to controls (n=8) measured by using chao1 index. The OTU table were rarefied at the cutoff value of 31000 sequences per sample. (B) t-test was conducted on chao1 index. ** indicates significantly different, *P* value=0.0030.

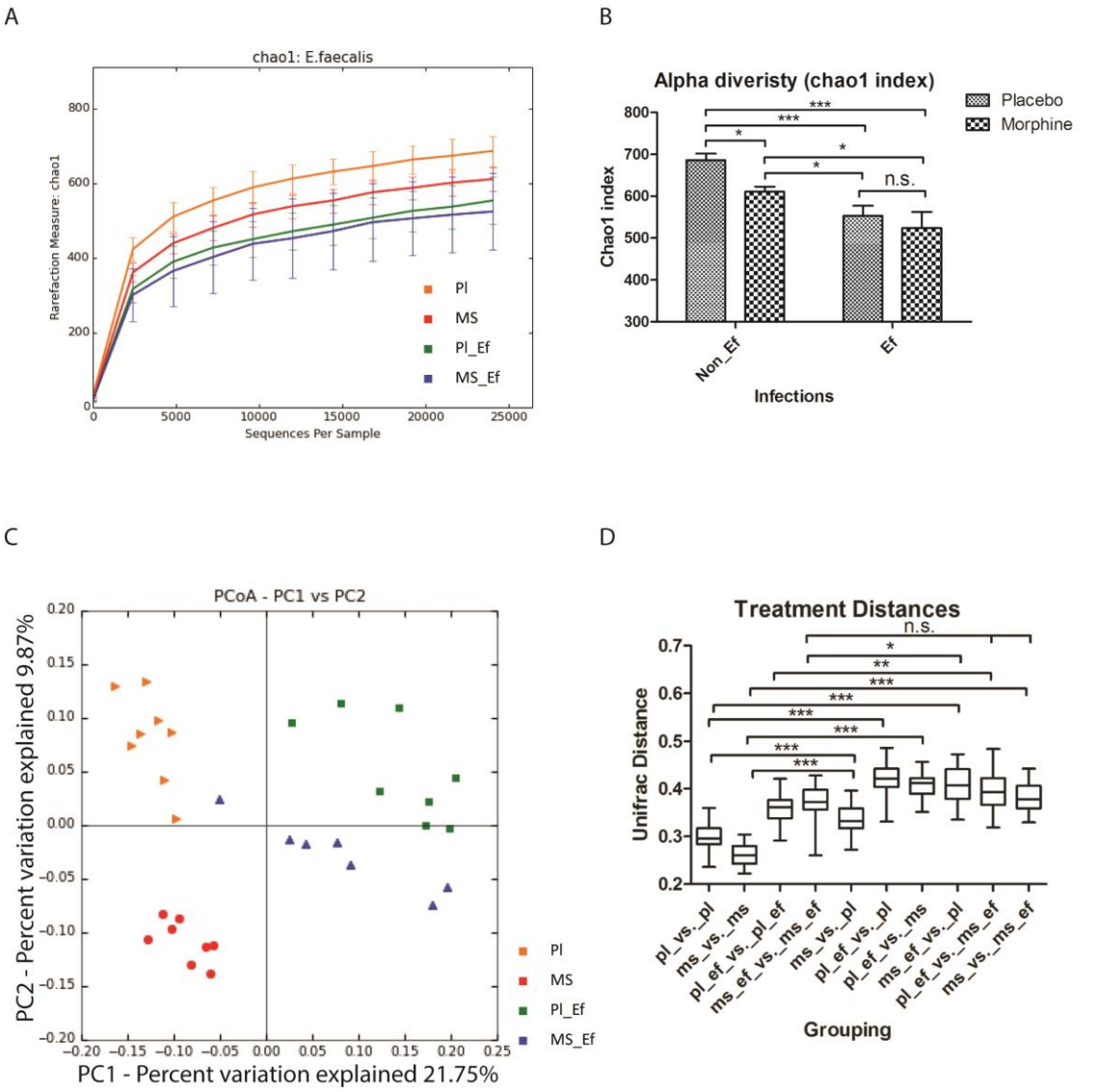


Figure 2.16 *E. faecalis* infection alters gut microbiome following morphine treatment. (A) Morphine decrease alpha diversity in context of *E. faecalis* infection. (B) Significant analysis of Alpha diversity (Chao1 index) by using two-way ANOVA. Interaction between morphine treatment and *E. faecalis* infection accounts for 1.62% of the total variance. The *P* value =0.3616. The interaction is considered not significant. Morphine treatment accounts for 8.44% of the total variance. The P value = 0.0434. The effect is considered significant. *E. faecalis* infection accounts for 37.09% of the total variance. The effect is considered extremely significant. (C) Morphine induced distinct gut microbiome clustering in context of E. faecalis infection. (D) Significant analysis showed measures of beta diversity using UniFrac distance metrics. *E. faecalis* infection dominated the alteration of gut microbiome and leads to a less different beta diversity clustering when compared to samples without *E. faecalis* infection.

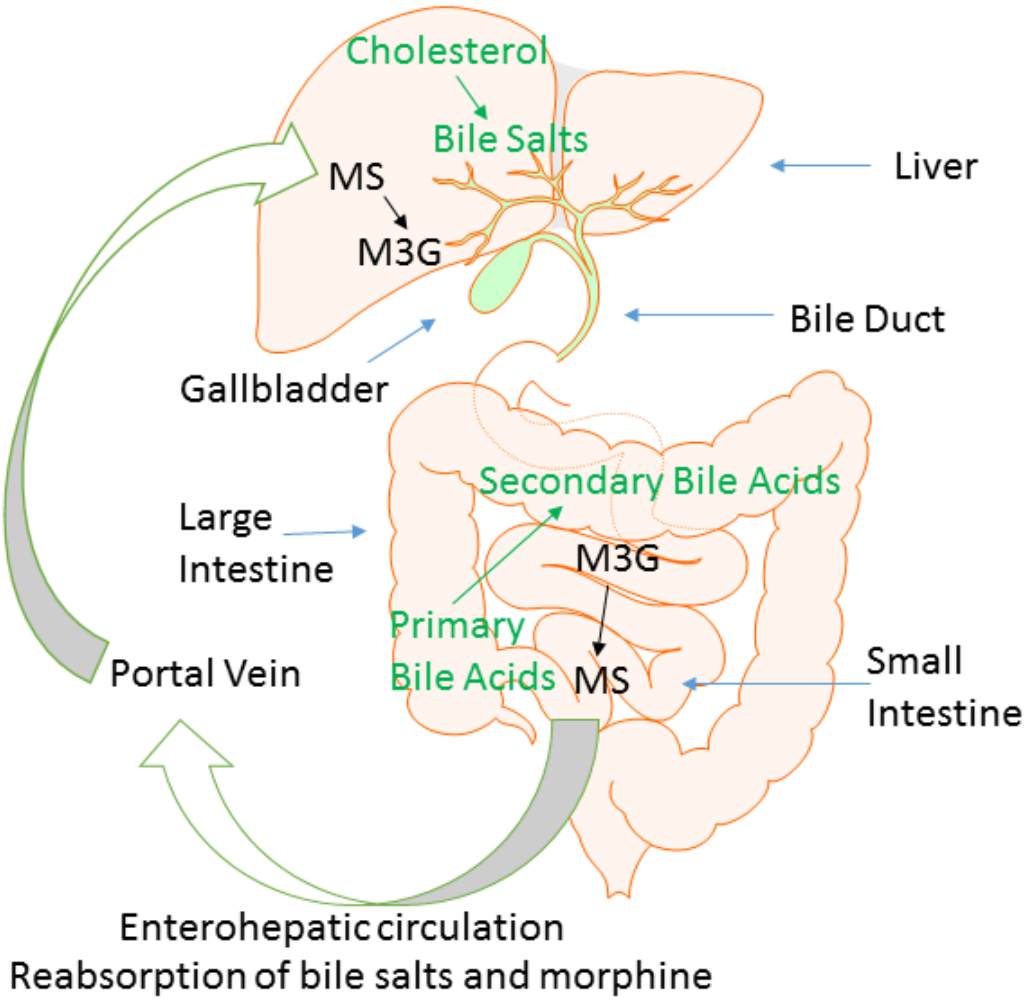


Figure 2.17 Model of metabolism and biotransformation of morphine and bile acids. In liver, cholesterol were transformed to primary bile acids, and morphine were conjugated to M3G. In gut, intestinal bacteria transform primary bile acids and M3G into secondary bile acids and morphine respectively. Bile acids and morphine are reabsorbed and recycled via enterohepatic circulation.

CHAPTER 3

MORPHINE TREATMENT POTENTIATES CITROBACTER RODENTIUM VIRULENCE, SYSTEMIC DISSEMINATION AND EXACERBATES GUT DYSBIOSIS

INTRODUCTION

Risk of lethal bacterial infection is a tremendous concern for hospitalized patients(Fagundes-Neto and de Andrade 1999; Larney et al. 2015). Opioids induce immunosuppression and bowel dysfunction leading to increased susceptibility to bacterial and opportunistic infections(Mora et al. 2012). Chronic morphine has also shown to lower host defense to enteric bacteria such as *Salmonella enterica* and *Pseudomonas aeruginosa*(Feng et al. 2006; Babrowski et al. 2012), induce spontaneous sepsis in mice(Hilburger et al. 1997), increase mortality following *Acinetobacter baumannii* infection or lipopolysaccharide (LPS) treatment in mice(Breslow et al. 2011).We have recently shown that morphine induced bacterial translocation in mice by compromising intestinal barrier function(Meng et al. 2013). The aim of the studies is to investigate if opioids use increase virulence and susceptibility to a common hospital acquired infection.

The interaction between gut microbiota and intestinal epithelial surface play important roles in preventing the outgrowth of pathogenic organisms and maintaining gastrointestinal homeostasis(Marchiando, Graham, and Turner 2010; Wells et al. 2011). The disruption of commensal gut microbiota contributes to pathogenic bacteria

colonization in gut(Blaschitz and Raffatellu 2010; Balzan et al. 2007). Other than composition of bacterial species in gut microbiota, bacterial virulence is pathophysiologically important in intestinal bacterial infection. In the early stage of intestinal infection, bacterial virulence genes are expressed and required for pathogen growth on the gut epithelium surface(Kamada, Chen, and Núñez 2012). Pathogen bacterial colonization and invasion are always accompanied by collapse of host homeostasis by dysregulating immune response, disrupting gut barrier function and disturbing commensal microbiome. Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) are important causes of diarrheal disease and are human specific(Bergstrom et al. 2010). *C. rodentium* is a natural mouse pathogen that models intestinal infection by enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) in humans and causes attaching and effacing (A/E) lesions and colonic hyperplasia in human and animal hosts leading to diarrhea (Luperchio et al. 2000). To facilitate colonization and invasion, *C. rodentium* delivers virulence factors, such as translocated intimin receptor (Tir) and virulence genes regulator, Ler protein. *C. rodentium* colonization depends on virulence factor-mediated adhesion and provoke inflammation, which further disrupts gut microbiota and promotes the outgrowth of pathogenic bacteria (Lupp et al. 2007). Regulation of the virulence factor-mediated colonization and commensal-driven pathogen eradication may be potential therapeutic strategies. However, it is unclear whether and how morphine modulates pathophysiologically important functional changes in bacterial virulence.

Here, by using a mouse-model of *C. rodentium* infection, we determined effects of morphine on gut homeostasis and host resistance against *C. rodentium* infection.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All animals were maintained in pathogen-free facilities and all procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. 8–10 week old animals were used for our studies. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Bacterial strains

C. rodentium strain (DBS 100) was obtained from American Type Culture Collection (ATCC; Manassas, VA). Nalidixic acid resistant *C. rodentium* was selectively screened out on LB agar plate with 20ug/ml Nalidixic acid. A spontaneous mutant of C. rodentium DBS100 resistant to nalidixic acid (20μg/ml) would be obtained by growing DBS100 at 37°C in LB medium supplemented with nalidixic acid at 5μg/ml for 7 h before spreading the liquid culture onto LB agar plates supplemented with nalidixic acid at 20μg/ml. The selective LB agar plates and broth would be made by adding nalidixic acid up to 20μg/ml in order to selectively culture *C. rodentium* in the future study (Gueguen and Cascales 2012). The nalidixic acid solution was made with Mili-Q water in fume hood and the stock solution was sterilized through a 0.22um filter using a 10-ml syringe with in the sterile hood. Kanamycin resistant *C. rodentium* was obtained from Dr. Bruce A. Vallance laboratory at University of British Columbia, Vancouver, Canada (Bhinder et al. 2013). Bioluminescent strains of *C. rodentium* were constructed by introducing plasmid pT7 carrying the entire lux operon from *Photorhabdus luminescens*.

For selective culture of *C. rodentium*, kanamycin (SKU 60615, Sigma) was added into LB broth or agar plates at the concentration of 30 ug/ml.

Animal treatment and bacterial oral infection

Mice received placebo or 25mg morphine pellet implantation as described(Bryant et al. 1988). Placebo or 25 mg morphine pellets (National Institutes of Health [NIH]/National Institute on Drug Abuse [NIDA], Bethesda, MD) were inserted in a small pocket created by a small skin incision on the animal's dorsal side; incisions were closed using surgical wound clips (Stoelting, 9 mm Stainless Steel, Wooddale, IL). Mice were subcutaneously treated with placebo or 25mg morphine pellet for 24hr prior to infection. Mice were infected with $1x10^9$ *C. rodentium* in 200ul medium via oral gavage(Bhinder et al. 2013). Mice were sacrificed for study at day 5 post infection with *C. rodentium*.

Colony forming Units

Bacterial culture on blood agar plates without antibiotics or LB agar plates with 20ug/ml Nalidixic acid or 30ug/ml Kanamycin over night at 37ºC.

Histology of animal tissues

Animal intestinal tissues were collected at appropriate time points, fixed in 10% buffered formalin and embedded in paraffin. Paraffin specimens were cut into 5µm sections and mounted on microslides. Hematoxylin and eosin (H&E) and Alcian blue staining were performed on paraffin embedded sections, and stained slides were reviewed using Leica DM5500B Microscope.

Fluorescence in situ hybridization (FISH).

Detection of commensal bacteria: Use Alexa-Fluor 488 (FITC)-labeled Eub338 (5'-GCT GCC TCC CGT AGG AGT-3'), which is a universal probe complementary to the 16s RNA of virtually all bacteria were performed to determine the infiltration of bacteria into the colonic epithelium. This allows direct visualization of bacteria within the colonic mucosa. Blue color indicates nucleus staining with DAPI; green color indicates bacteria staining with FITC.

Detection of *C. rodentium*: Paraffin-embedded sections were deparaffinized and rehydrated as described above. Sections were incubated overnight at 37°C in the dark with Texas red-conjugated EUB338 general bacterial probe (5'-GCT GCC TCC CGT AGG AGT-3') and an AlexaFluor 488 conjugated GAM42a probe (5'-GCC TTC CCA CAT CGT TT-3') that recognizes bacteria that belong to the γ-Proteobacter class diluted to a final concentration of 2.5 ng/μl each in hybridization solution (0.9 M NaCl, 0.1 M Tris pH 7.2, 30% Formamide, 0.1% SDS). Sections were then washed once in the dark with hybridization solution for 15 minutes with gentle shaking. This step was repeated once with wash buffer (0.9 M NaCL, 0.1 M TRIS pH 7.2), and sections were placed in dH2O, and then mounted using ProLong Gold Antifadereagent with DAPI (Molecular Probes) and imaged using Leica DM5500B Microscope.

mRNA expression primers

Ler F 5'-AAT ATA CCT GAT GGT GCT CTT G-3' ; R 5'-TTC TTC CAT TCA ATA ATG CTT CTT-3'; Tir F 5'-TAC ACA TTC GGT TAT TCA GCA G-3'; R 5'-GAC ATC CAA CCT TCA GCA TA-3'. rrsA (16SRNA); 5'-AGG CCT TCG GGT TGT

AAA GT-3’ and 5’-ATT CCG ATT AAC GCT TGC AC-3’. Quantitative real-time polymerase chain reaction (PCR) was performed on an Applied Biosystems 7500 Realtime PCR Detection system. All samples were run in triplicate, and relative mRNA expression levels were determined after normalizing all values to 16S rRNA.

Flow cytometric analysis

Cells from mesenteric lymph nodes (mLNs) were isolated and fixed for flow cytometry. CD4+, CD17A+ and CD17F+ were stained for analysis.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software Inc.). Differences between two groups were evaluated using Student’s t test (parametric) or Mann-Whitney U test (non-parametric). For the multiple comparisons, statistical analysis was performed using one-way ANOVA (parametric) or Kruscal-Wallis test (non-parametric), and Bonferroni test for parametric samples. Differences at $P<0.05$ were considered significant.

RESULTS

1. Morphine treatment increases C. rodentium systemic dissemination

High risk of bacterial infection in hospitalized patients is a significant concern especially in those patients that are on opioids for pain management. (Sabita Roy et al. 2011; Larney et al. 2015). To determine whether morphine treatment can increase dissemination of *C. rodentium* following infection, we infected mice with *C. rodentium* at day 1 post morphine treatment mimicking bacterial infections in opioids users. C57/BL6J wild type mice were implanted with 25mg morphine or placebo pellet subcutaneously. Mice were infected with 200ul (~10^9) *C. rodentium* through oral gavage right after morphine treatment. Mesenteric lymph nodes (MLNs), spleen and liver were collected and homogenized in 1 ml, 2 ml and 5 ml PBS respectively at day 5 post infection. 100ul suspensions and 100 ul collected blood were cultured on LB agar plates with 50ug/ml Nalidixic acid over night at 37ºC. The colony forming units (CFUs) were counted. At day 5 post *C. rodentium* infection, placebo-implanted mice showed very few colonies growing on the antibiotic selective LB agar plates, indicating no systemic dissemination. However, mice receiving morphine revealed an increased number of CFUs, indicating systemic dissemination of *C. rodentium* into mesenteric lymph nodes, spleen, liver and blood circulation (Figure 3.1).

2. Morphine treatment facilitates bacterial adherence

The first stage of intestinal bacterial infection requires bacterial adherence to the gut epithelial surface(Baumgart et al. 2007; H. M. Martin et al. 2004). To determine whether bacterial adherence to intestines were increased by morphine treatment, we

determined commensal bacterial adherence to intestines following morphine treatment. C57/BL6J wild type mice were implanted with 25mg morphine or placebo pellet subcutaneously. After 24 hours, duodenum, jejunum, ileum, and colon were collected and washed, then homogenized with 5ml PBS. 100 ul tissue suspensions were cultured on blood agar plates over night at 37^{o}C. Bacterial colonies were quantified and described as colony forming units (CFUs). Compared with placebo treatment, morphine increased commensal bacterial adherence to jejunum, ileum and colon, but not to duodenum (Figure 3.2A). To visualize the bacterial adherence to intestinal epithelium, we determined bacterial adherence through fluorescence *in situ* hybridization (FISH) on frozen tissue sections by using FITC-labeled universal probe EUB-388. Small intestine were excised and processed to 5um thickness cryostat sections for FISH staining. Blue color indicates nucleus staining with DAPI and green color indicates bacteria staining with FITC (Figure 3.2B&C). The FISH results revealed morphine treatment increased bacterial adherence to small intestine and disrupted histological structure of mucosal surface of small intestine.

Furthermore, to determine *C. rodentium* bacterial adherence to intestines, we conducted FISH on tissue sections and visualized the g-Proteobacteria class to which *C. rodentium* belongs by using GAM42a probe. The FISH results revealed morphine treatment increased *C. rodentium* adherence to small intestines and colon, when compared to placebo treatment (Figure 3.3).

3. Morphine treatment increases C. rodentium virulence

Pathogen colonization in the intestine has been shown to be controlled by bacterial virulence and through competition with gut commensal microbiota(Kamada et al. 2012; Kamada, Seo, et al. 2013). To investigate if morphine treatment increases *C. rodentium* virulence, we determined virulence factors, ler and tir, mRNA levels by quantitative polymerase chain reaction in fecal pellets of mice infected with *C. rodentium* at day 5 post infection. Expression was normalized to that of the 16S rRNA genes. Expression of virulence factors, Ler and Tir, were significantly increased in the morphine treatment group when compared to placebo. Same effects were seen in fecal samples of small intestines, cecum and colon (Figure 3.4).

4. Morphine increases C. rodentium load in fecal matter and shifts gut microbiome.

Expression of *ler* and *tir* is essential for pathogen colonization in the intestines of mice, we next asked the question whether morphine can increase *C. rodentium* growth in intestine contents and modulate gut microbiome in the context of *C. rodentium* infection. To determine the *C. rodentium* load, we collected fecal matters and homogenized in PBS at day 5 post infection. 100 ul suspensions were cultured on LB agar plates with kanamycin over night at 37 ^{o}C. Colony forming units (CFUs) were quantitated. Results revealed that morphine treatment increases *C. rodentium* growth in intestinal contents (Figure 3.5). Fecal bacterial DNA were isolated and purified for microbiome analysis. Alpha diversity analysis results revealed, when compared to placebo, C. rodentium infection decreases alpha diversity of the gut microbiome. However, morphine treatment did not further decrease alpha diversity of gut microbiome in the context of infection(Figure 3.6A). Beta diversity analysis results revealed morphine treatment shifts

gut microbiome in the context of *C. rodentium* infection, indicating that the relative bacterial abundance and composition were changed (Figure 3.6B).

5. *Morphine treatment disrupts integrity of epithelial barrier function in the context of C. rodentium infection*

The gut has a large surface area (400 sq m), which is necessary to absorb nutrients, and the gut epithelial barrier contains only a single layer of epithelium(Lozupone et al. 2012). Thus, intestinal immune defense is designed to allow immune surveillance of pathogens, while it operates with minimal disruption to the absorptive function of the gut(Hooper, Littman, and Macpherson 2012). To begin to understand early events that occur in morphine-treated mice that can precipitate increased *C. rodentium* infection, we evaluated the extent of inflammation and epithelial damage at day 5 post infection. Histologic evaluation of the extent of epithelial damage revealed that morphine treatment disrupts morphological structure of mucosal surface of intestines (Figure 3.7 & 3.8). Histological evaluation of the tight junction protein ZO-1 on sections of jelly-rolled small intestines and colons revealed morphine treatment decreased tight-junction organization between intestinal epithelial cells (Figure 3.9). However, *C. rodentium* infection alone does not result in histological damages in intestinal mucosal epithelial integrity and barrier function.

6. *Morphine treatment inhibits C. rodentium-induced increase of goblet cells.*

Goblet cells secrete mucus to protect the lining of the intestine. To determine whether morphine modulate expression of goblet cells in the mucosal lining of the small

intestine and colon, we evaluated goblet cells on intestinal sections by using Alcian staining on jelly-rolled intestinal sections. In each section, goblet cells were counted on 20 microvilli on epithelial surface of small and large intestines. Following Alcian blue staining, intensely blue material, interpreted as mucus, is evident within the goblet cells lining the intestinal epithelium. Faint blue material (mucus) is also present within the adjacent intestinal lumen. Alcian staining results revealed that morphine treatment can inhibit *C. rodentium* induced increase in the number of goblet cells in small intestines and large intestines, although morphine alone does not decrease the number of goblet cells when compared to placebo (Figure 3.10 & 3.11).

7. Morphine treatment disrupts C. rodentium induced IL17a immune response

At early phase of infection, *C. rodentium* induces an IL-17 dependent bacterial clearance in wild type mice (Geddes et al. 2011). To determine whether morphine treatment modulates *C. rodentium* induced IL17 immune response in mice, we determined the IL17 immune response by flow cytometry. Cells from mesenteric lymph nodes (mLNs) were isolated and fixed for flow cytometry. CD4+, CD17A+ and CD17F+ were stained for analysis. The flow cytometry results revealed that morphine treatment disrupts *C. rodentium* induced IL-17A immune response at day 5 post infection (Figure 3.12 A, C, D). Morphine treatment also decreased the percentage of CD4+ cells in mesenteric lymph nodes in the context of *C. rodentium* infection (Figure 3.12C). Neither morphine treatment nor *C. rodentium* infection regulated IL-17F immune response (Figure 3.12B).

DISCUSSION

Opioid users and abusers are at a higher risk for infectious diseases (Mora et al. 2012; Sabita Roy et al. 2011). Animals treated with morphine exhibit greater susceptibility to enteric infections with strains, such as *Salmonella enterica*, *Listeria monocytogenes*, *Vibrio cholerae*, and *Pseudomonas aeruginosa*(Asakura et al. 2006; Cray, Tokunaga, and Pierce 1983; Babrowski et al. 2012; MacFarlane et al. 2000). To date, opioid analgesics are the most commonly prescribed medications for pain management. As such, the correlation between opioids usage and increased susceptibility to pathogenic strains need to be well investigated. When orally inoculated into wild type mice, *C. rodentium* can't colonize in their hosts and cause pathology(Kamada et al. 2012). In wild type mice, commensal gut microbiota can outcompete invading *C. rodentium* and inhibit their colonization in host. Intact intestinal barrier function and host immune response to pathogens may also contribute to eradication of *C. rodentium* from wild type mice. In present study, for the first time, we demonstrate that morphine increases *C. rodentium* virulence and potentiates systemic dissemination in wild type mice.

In this study, we utilize a *C. rodentium*-mouse model to determine morphine treatment increases, Tir and Ler, two remarkable virulence factors of *C. rodentium*. While establishing colonization, *C. rodentium* delivers a variety of virulence factors, such as translocated intimin receptor (Tir), into epithelial cells by means of the type III secretion system in order to facilitate their adherence and invasion(Yi and Goldberg 2009). EspFU directly binds and activates N-WASP, leading to actin polymerization events. The expression of most virulence genes in *C. rodentium* is controlled by a regulator, Ler

protein, a member of the histone-like nucleoid structuring (H-NS) protein family(Puente et al. 2004). Thus, Ler is a virulence factor that regulates *C. rodentium* virulence. It has been demonstrated that morphine treatment can activate the virulent factors of *Pseudomonas* and induce gut derived sepsis(Babrowski et al. 2012). Increased bacterial virulence may promote *C. rodentium* colonization in gut and results in microbial dysbiosis. It has been shown in our study, morphine treatment increases *C. rodentium* growth in gut and changes composition of gut microbial communities. *C. rodentium* infection and morphine treatment result in distinct gut commensal microbiome. In return, microbial dysbiosis enhances bacterial infections(Pham and Lawley 2014).

The gut homeostasis is reached through normal microbes-host interactions. The disruption of barrier function and increased growth of pathogenic bacteria are significant disturbance to gut homeostasis. In normal hosts, commensal bacteria activate homeostatic response by host immune system that permit coexistence with potential toxic bacterial products through down-regulating bacterial receptors, inhibiting pathogenic innate and adaptive immune responses, inducing antimicrobial peptides, and promoting mucosal barrier repair(Sartor 2008). This study showed that morphine treatment increases bacterial attachment to intestines in wild type mice in comparison with placebo treatment, suggesting morphine can modulate the interaction of microbiota and intestinal surface. Our study shows that morphine treatment resulted in increased expression of the virulence factors, Ler and Tir. It has been shown morphine treatment increases infiltration/adherence of bacteria to the gut epithelium by using fluorescence *in situ* hybridization (FISH) on intestinal cryostat sections. However, *C. rodentium* infection alone is unable to damage tight junction protein ZO-1 and disrupt intestinal barrier

function. Morphine treatment disrupts epithelial barrier function and results in impaired morphologic mucosal structure in the context of *C. rodentium* infection. The histological evidence reveals disruption of intestinal barrier function by morphine treatment may contribute to systemic dissemination of *C. rodentium.*

Intestinal mucus is secreted by goblet cells in the epithelial lining of mucosal surface, and forms the first line of host defense against invading pathogens, prevent pathogen colonization and remove the adherent load from mucosal surface(Babrowski et al. 2012; Benjamin P Willing, Russell, and Finlay 2011). Commensal microbiota facilitates host barrier function through upregulation of the mucus layer(Kamada, Chen, et al. 2013). *C. rodentium* induces increased amount of goblet cells, however, morphine treatment decreases the amount of goblet cells in villi of mucosal epithelial surface in the context of *C. rodentium* infection, indicating mucus excreting goblet cells are attenuated. How morphine modulates goblet cells is still unclear. It has been shown an innate immune regulatory pathway, NLRP6, regulates goblet cell mucus secretion(Wlodarska et al. 2014). NLRP6 deficient mice are unable to clear *C. rodentium* from mucosal surface and exhibit goblet cell impairment. NLRP6 inflammasome plays an important role in maintaining gut commensal microbiota and regulates gut microbial ecology(Chen et al. 2011; Elinav et al. 2011). However, how morphine treatment modulates NLRP6 pathway remains unknown. It is worthy to be noted that future study should be done to elucidate whether morphine modulate goblet cell and gut microbiome through NLRP6 pathway.

Intestinal commensal microbiota and host immune system co-evolved for millions of years and developed the symbiotic relationship to achieve a balanced, mutually beneficial state(Chu and Mazmanian 2013). Innate barriers ensure a tolerant immune

response to the microbiota(Hooper, Littman, and Macpherson 2012). At early phase of infection (4-7 days post infection), *C. rodentium* induces an IL-17 response in the cecum and colon (Geddes et al. 2011). It was well demonstrated that robust IL-17A secretion is crucial for *C. rodentium* clearance in late stage of infection(Z. Wang et al. 2014; Geddes et al. 2011). Furthermore, it has become clear that rapid IL-17A-dependent innate immune response is necessary for maintaining gut homeostasis (Sonnenberg and Artis 2012). Morphine treated mice with *C. rodentium* infection exhibited low CD4+ T cell counts and decreased CD4+ T cell expression of the proinflammatory cytokine IL-17A, compared with infected placebo group. This study demonstrates that morphine treatment inhibits *C. rodentium*-induced IL-17A immune response, thus suppressing gut mucosal immune protection against invading *C. rodentium.*

It has been demonstrated that innate lymphoid cells (ILCs) are major IL-17/IL22 producing immune cells and play important roles in intestinal homeostasis(Rubino, Geddes, and Girardin 2012; Hepworth et al. 2013). However, it is unknown whether morphine modulate ILCs proliferation and recruitment during *C. rodentium* infections. Previous study in our laboratory shows that morphine disrupts positive signals interleukin-23 (IL-23)/IL-17-mediated pulmonary mucosal host defense against *Streptococcus pneumoniae* infection(Ma et al. 2010). It has been demonstrated that retinoic acid receptor related orphan receptor γτ positive (RORgt+) ILCs regulate intestinal homeostasis by integrating negative signals such as IL-25, which is independent of IL-23(Gladiator et al. 2013; Hepworth et al. 2013). However, it is unknown whether morphine modulates ILCs by regulating negative signal IL-25 expression and IL-25

responsive DC. Because of bidirectional relationship between gut microbiota and host immune system, it is difficult to determine the primary effects of morphine.

To summarize, we report that morphine treatment in a mouse model of *C. rodentium* infection results in 1) promotion of *C. rodentium* systemic dissemination, 2) increase in virulence factors expression and *C. rodentium* colonization in intestinal contents, 3) altered gut microbiome, 4) damaged integrity of gut epithelial barrier function, 5) goblet cells differentiation, 6) dysregulated IL17A immune response. This is the first study to demonstrate that morphine promotes pathogen dissemination in the context of *C. rodentium* intestinal infection. This study demonstrates and further validates and establish a positive correlation between that opioid drug use/abuse, increase risk of infections. These results indicate morphine modulates virulence factor-mediated adhesion of pathogenic bacteria and induces disruption of mucosal host defense during *C. rodentium* intestinal infection in mice, suggesting over-prescription of opioids may increase the risk in the emergence of pathogenic strains and should be used cautiously.

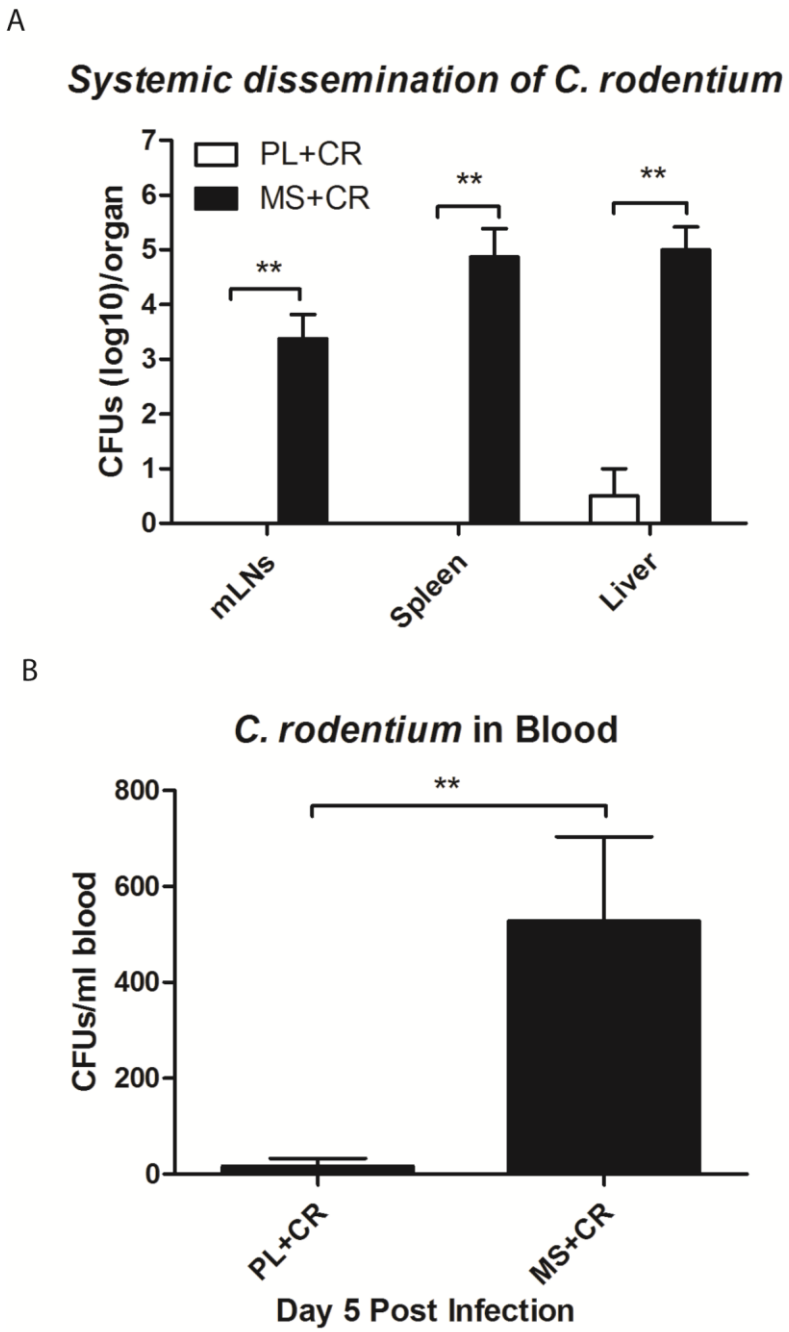
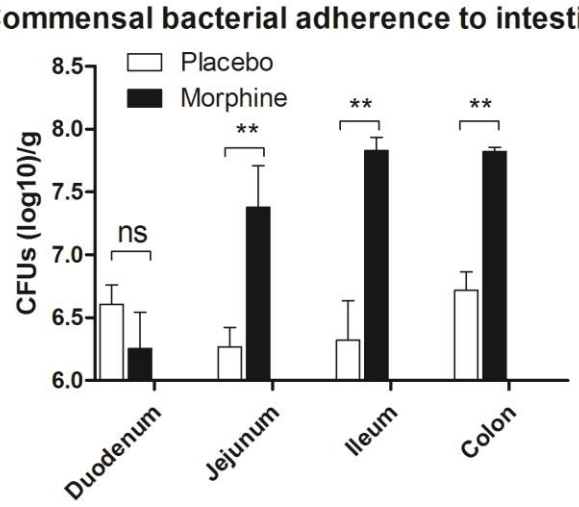


Figure 3.1 Morphine increases systemic dissemination of *C. rodentium*. Wild type mice were infected with Kanamycin resistant *C. rodentium* via oral gavage. Each mouse were infected with $1x10^9$ *C. rodentium* in 200ul. Bacterial load is determined by bacterial culture on LB agar plates with 30 ug/ml Kanamycin over night at 37°C. Bacterial colonies were quantified and described as colony forming units (CFU). N=5 and above for the quantitative data. ** indicates $p< 0.01$ in comparison with the matching treatments.

A



B C

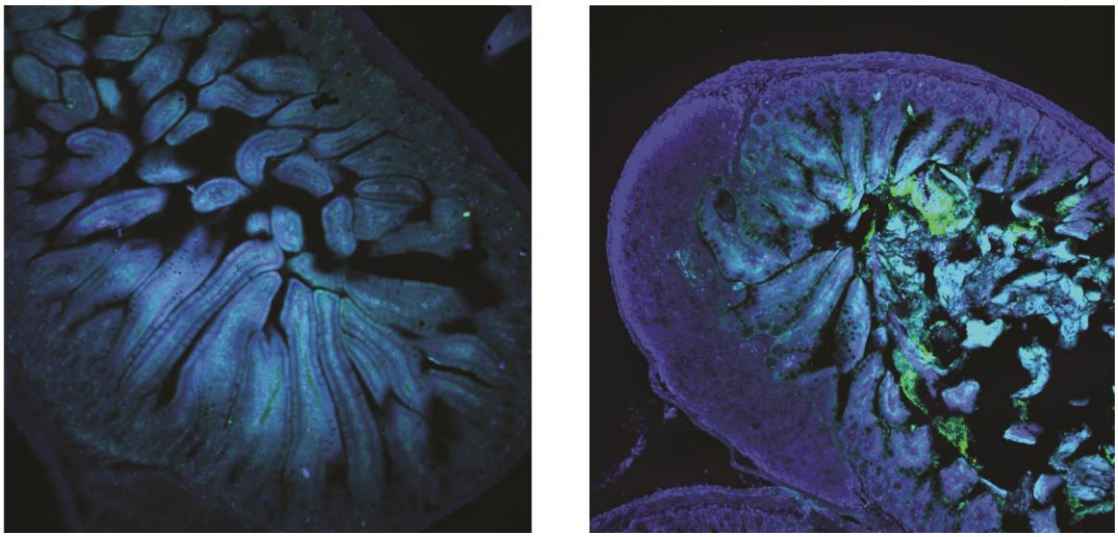


Figure 3.2 Morphine increases commensal bacterial adherence to intestines. (**A)** Bacterial adherence to intestines is determined by bacterial culture on blood agar plates over night at 37^{o}C. Bacterial colonies were quantified and described as colony forming units (CFU). N=5 and above for the quantitative data. ** indicates $p< 0.01$ in comparison with the matching treatments. (**B)** and **(C)** Fluorescence *in situ* hybridization (FISH) determines bacterial adherence to the intestinal epithelium using FITC-labeled universal probe EUB-388. Wild type mice (B6/129PF1) were subcutaneously treated with placebo (B) or 25mg morphine (C) pellets for 24hr. Small intestine were excised and processed to 5um thickness cryostat sections for FISH staining. Blue color indicates nucleus staining with DAPI; green color indicates bacteria staining with FITC. Representative photomicrographs of FISH stained slides at 10x.

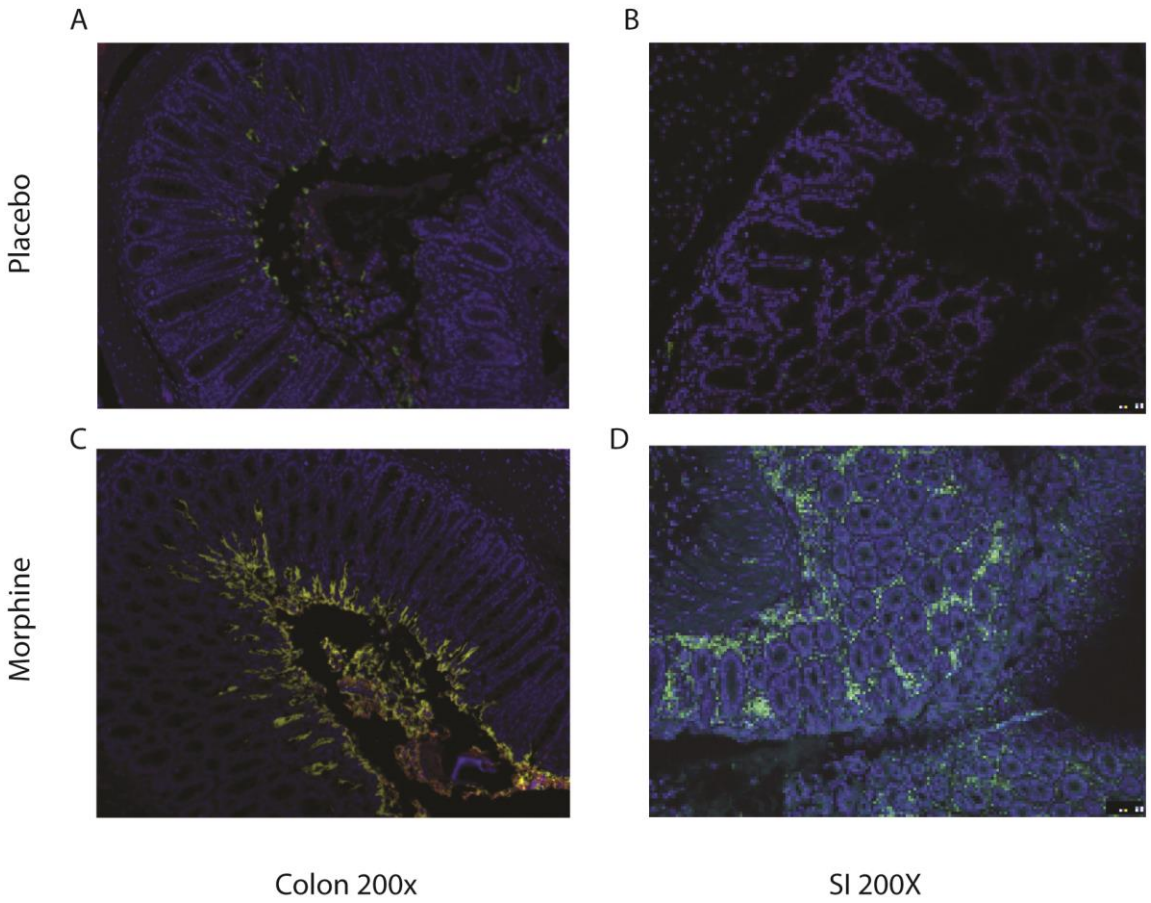


Figure 3.3 Morphine increases *C. rodentium* adherence to intestines**.** Visually blue color indicates nucleus staining with DAPI, red color is all true bacteria (EUB338 probe), and green color indicates bacteria staining with FITC, the *γ-Proteobacteria* class to which *C. rodentium* belongs (GAM42a probe), yellow color is merge of red and green.

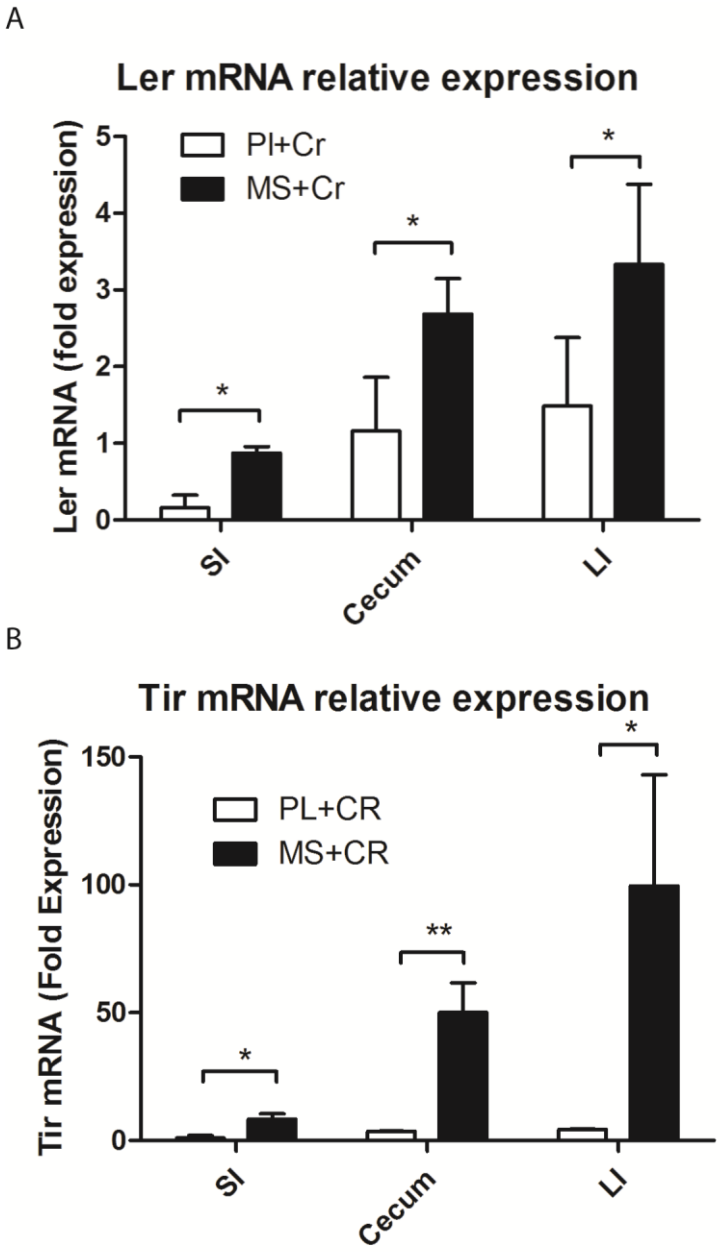


Figure 3.4 Morphine increases the expression of virulence factors of *C. rodentium*. **(A)** *ler* and **(B)** *tir* mRNA levels were determined by quantitative polymerase chain reaction in fecal pellets of mice infected with C. rodentium at day 5 post infection. Data represent mRNA expression relative to that of the 16S rRNA gene. Results are means ± SEM of individual mice (n=4). Results are representative of at least two experiments. **P*<0.05, ***P*<-.01.

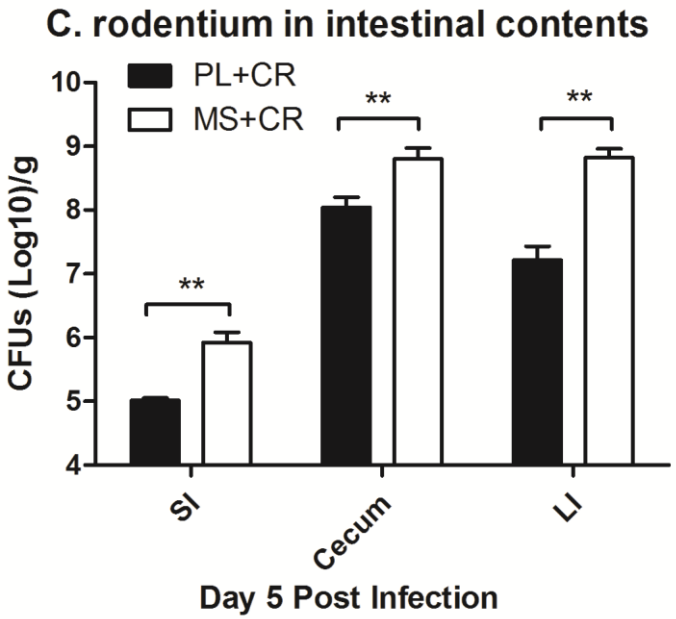


Figure 3.5 Morphine increases *C. rodentium* bacterial load in the intestinal contents at day 5 post infection. WT mice (n=7) were infected with 1x10^9 colony-forming units (CFUs) of C. rodentium, and pathogen load in feces were determined over the indicated time. Mice were subcutaneously treated with placebo or 25mg morphine pellet for 24hr prior to infection. Data points are means ± SEM. Results are representative of at least two independent experiments. **P<0.01, Student t test.

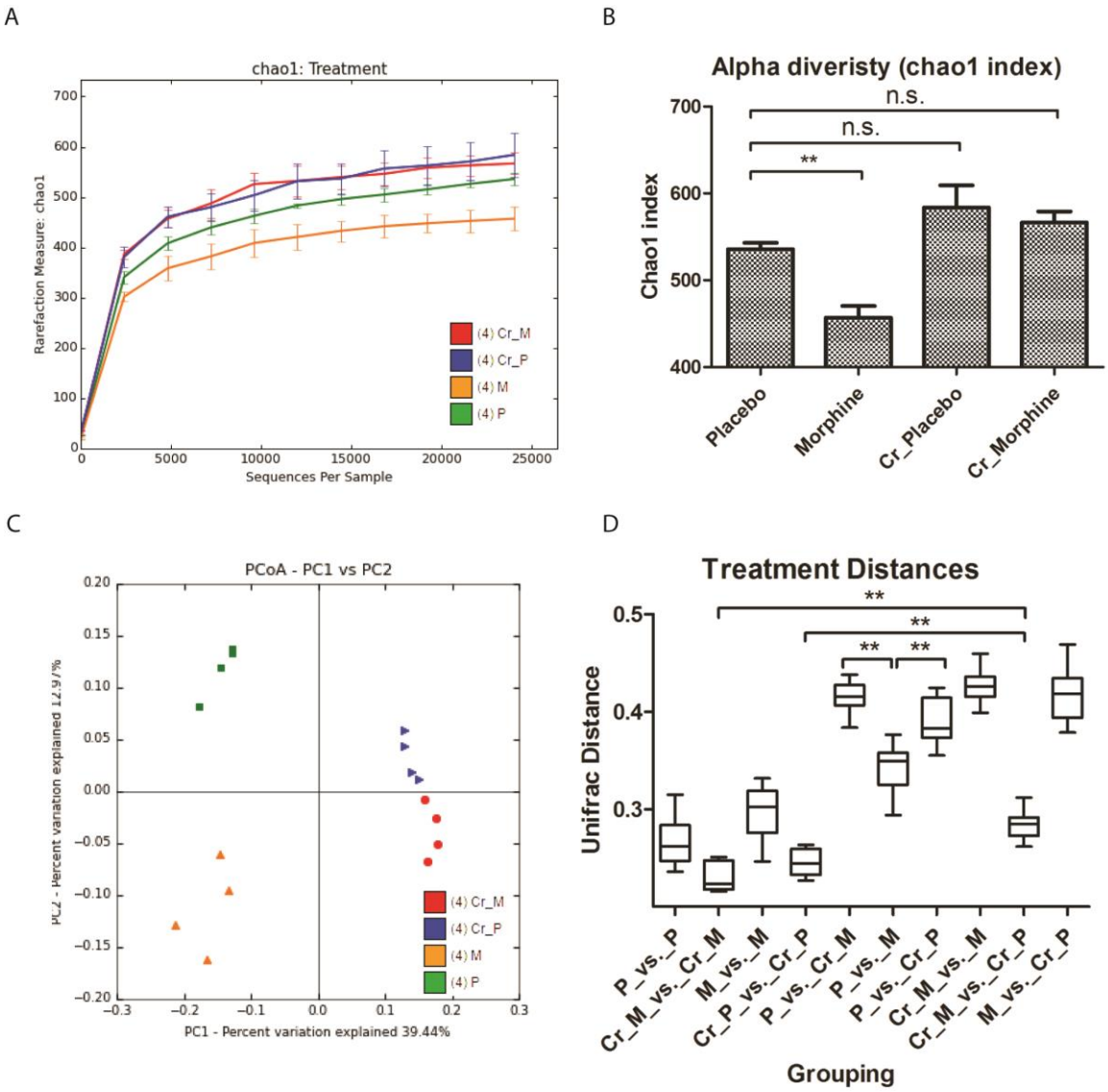


Figure 3.6 Morphine treatment alters gut microbiome in the context of *C. rodentium* infection. (A) Alpha diversity were assessed by using chao1 index. (B) t-test was conducted on chao1 index. (C) Principal coordinates analysis (PCoA) of samples using the UniFrac metric at the OTU level. (D) UniFrac distance significant tests were performed using QIIME. ** indicates significantly different, *P* value<0.01.

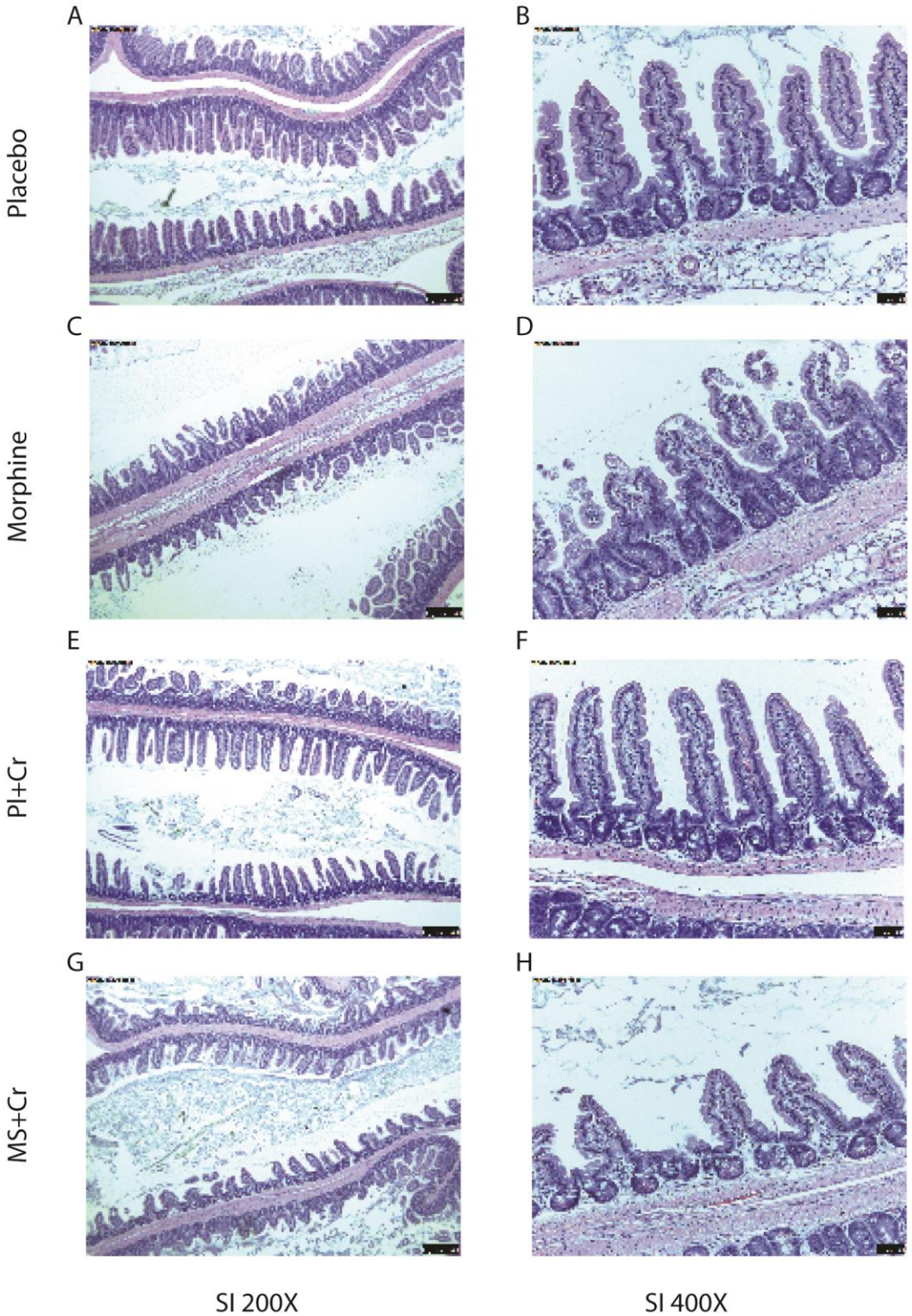


Figure 3.7 Morphine treatment disrupts morphological structure of mucosal surface of small intestine. H&E staining were performed on paraffin embedded sections of large intestines.

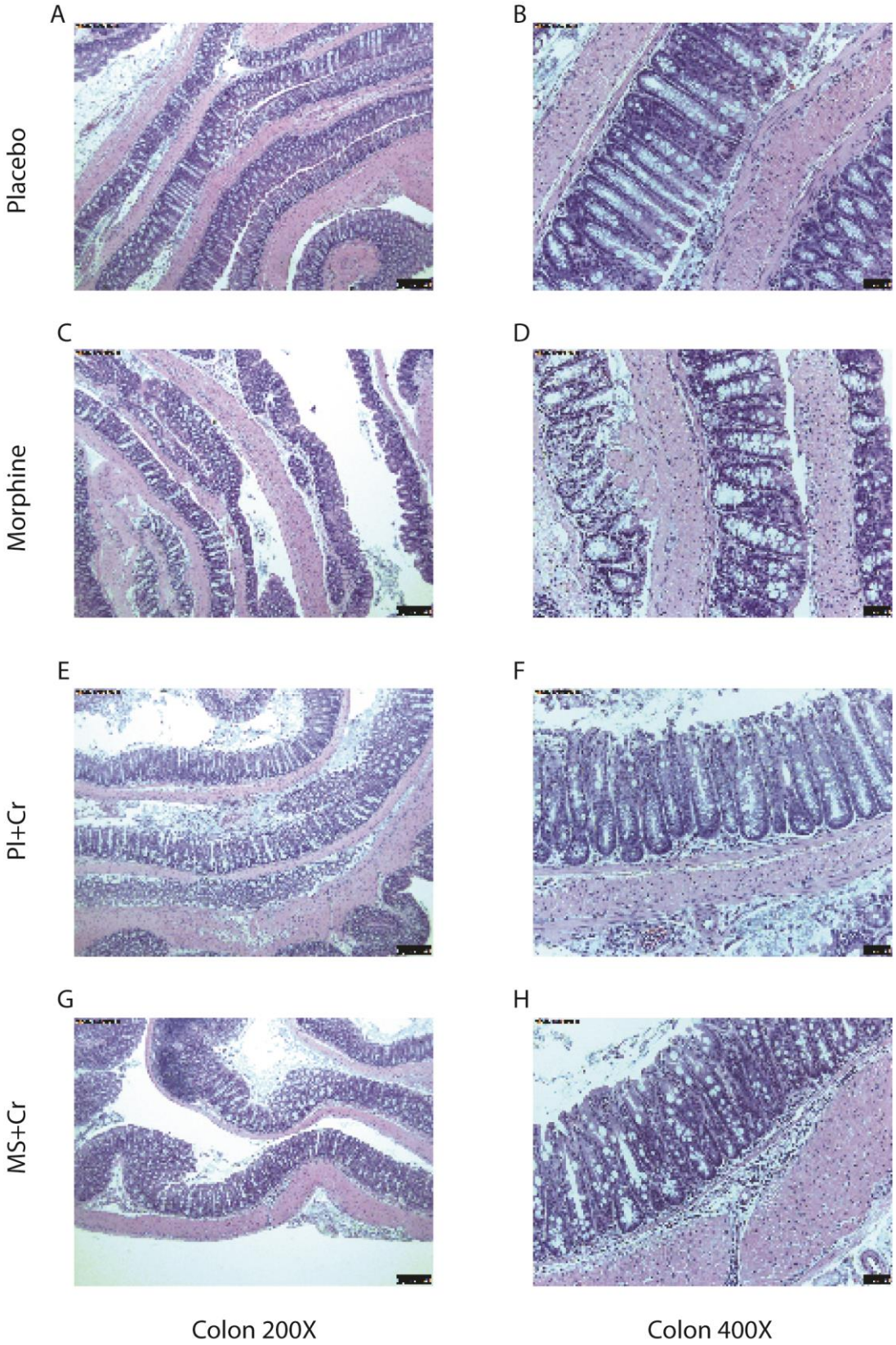


Figure 3.8 Morphine treatment disrupts morphological structure of mucosal surface of colon. H&E staining were performed on paraffin embedded sections of large intestines.

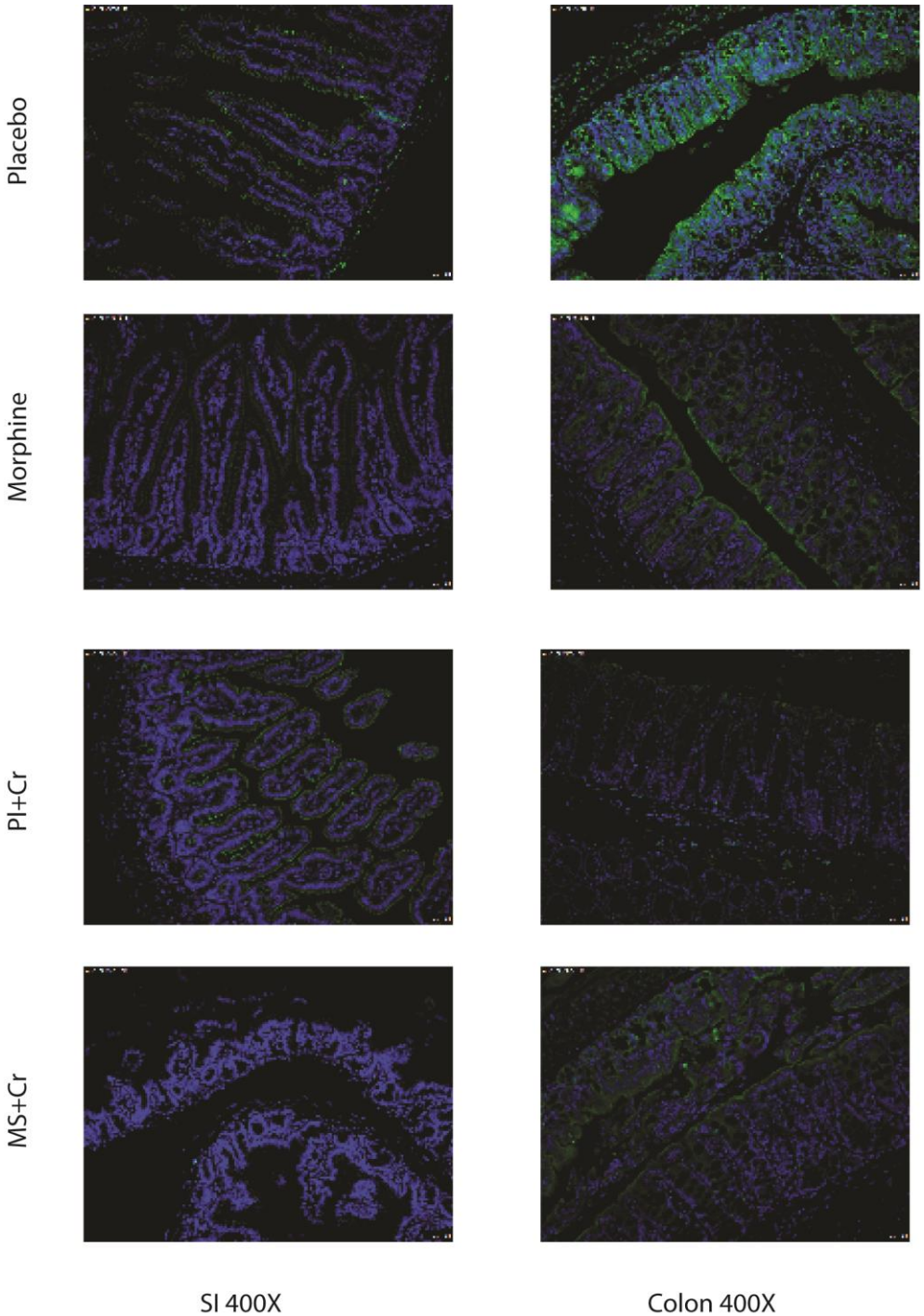


Figure 3.9 Morphine treatment disrupts ZO-1 tight junction organization in epithelium**.** Wild type mice were implanted with 25 mg morphine pellet subcutaneously and orally infected with *C. rodentium*. The small intestines and colons were excised and fixed. Images were analyzed by Leica fluorescent microscope.

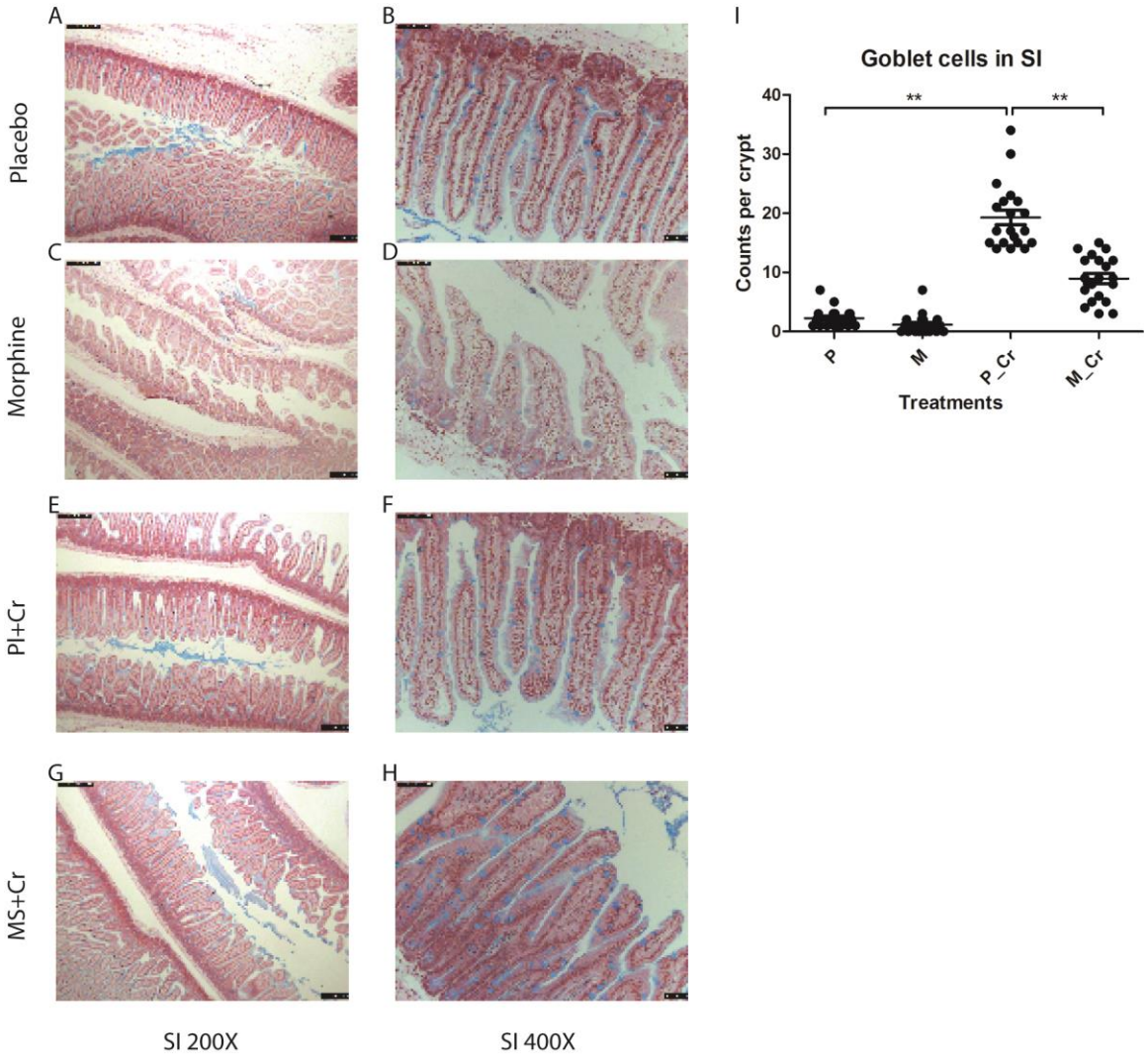


Figure 3.10 Morphine reduces *C. rodentium* infection induced proliferation of goblet cells in small intestine. Alcian blue staining of additional sections of the small intestines resulted in bright blue staining of the cytoplasm of the goblet cells lining the intestinal epithelium. Goblet cells were evaluated by using Alcian staining on jelly-rolled intestinal sections. In each section, goblet cells were counted on 20 microvilli on epithelial surface of large intestines. Significance test were performed using Student t test. *$P \leq 0.05$, **$P \leq 0.01$.

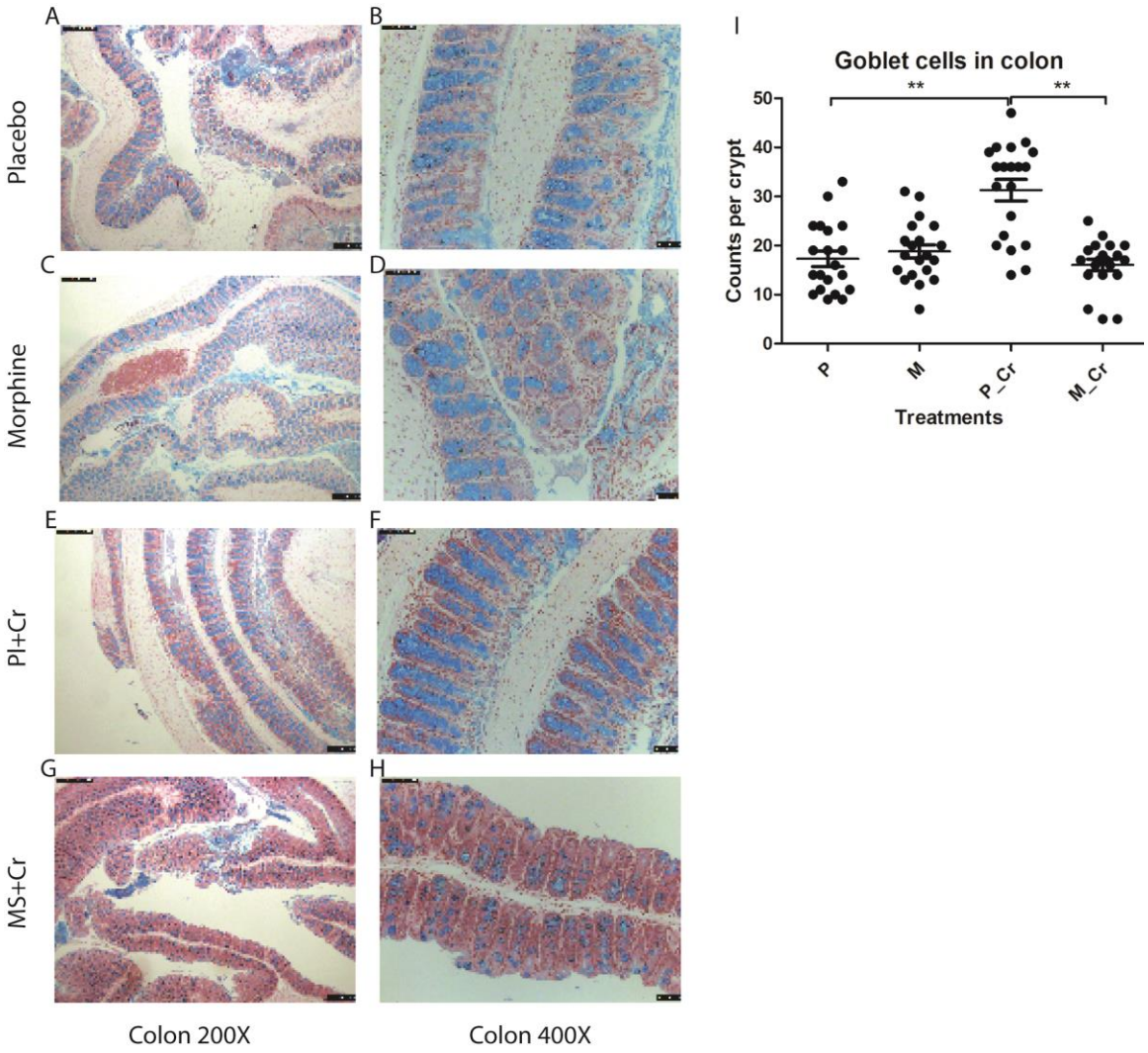


Figure 3.11 Morphine reduces *C. rodentium* infection induced proliferation of goblet cells in colon. Alcian blue staining of additional sections of the large intestines resulted in bright blue staining of the cytoplasm of the goblet cells lining the intestinal epithelium. Goblet cells were evaluated by using Alcian staining on jelly-rolled intestinal sections. In each section, goblet cells were counted on 20 microvilli on epithelial surface of large intestines. Significance test were performed using Student t test. *$P \leq 0.05$, **$P \leq 0.01$.

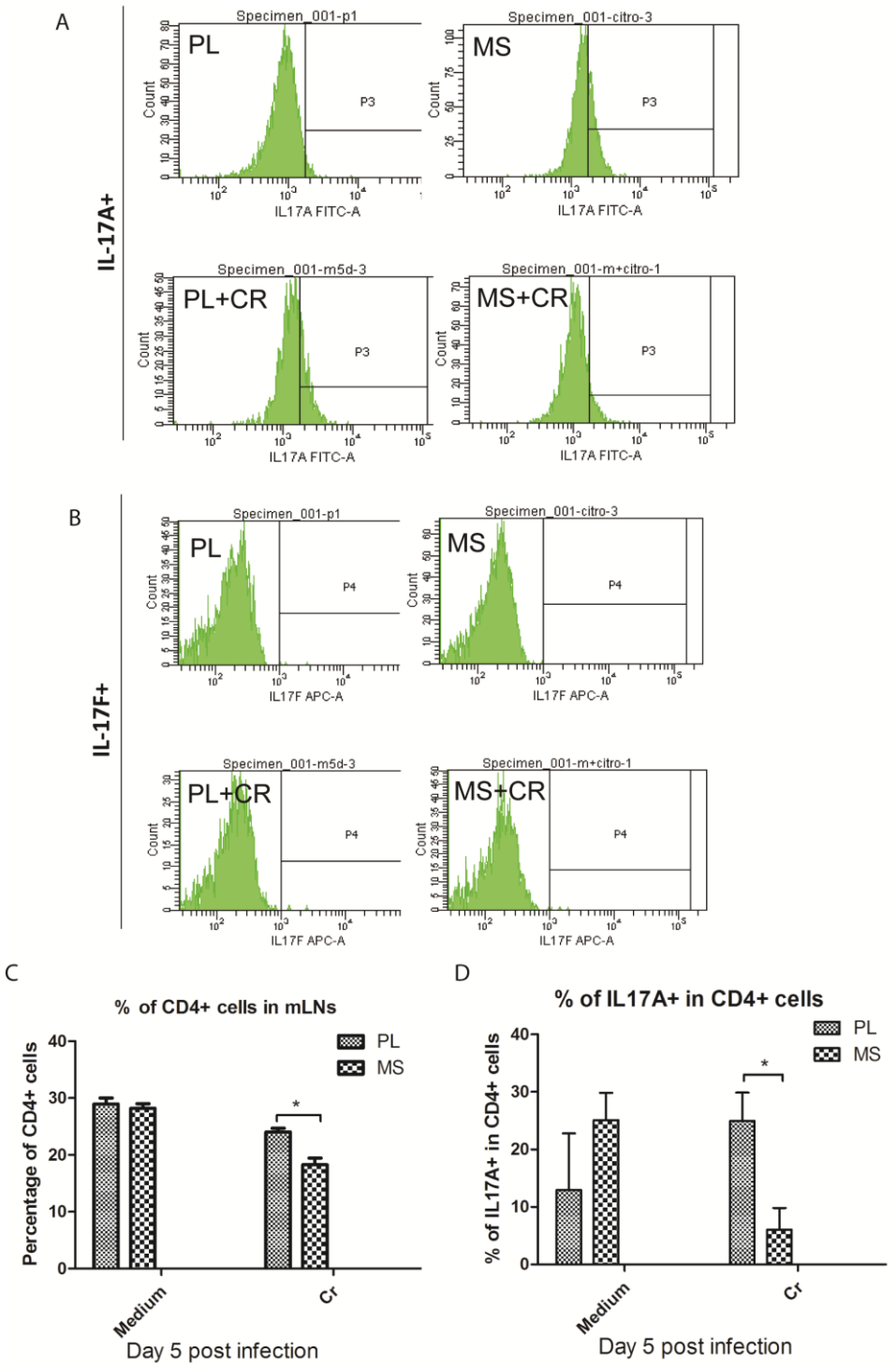


Figure 3.12 Morphine disrupts infection induced IL-17A immune response. Flow cytometry analysis of cells isolated from mesenteric lymphnodes (mLN). WT mice were subcutaneously treated with placebo or 25mg morphine pellet for 24hr prior to infection. Mice were sacrificed at day 5 post infection. (N=3 and above), $P<0.05$, student t test.

CONCLUSION

Opioids are frequently prescribed for pain management in the United States and worldwide(Docherty, Jones, and Wallace 2011). In the United States, approximately 100 million people suffer from chronic pain(Stayner and Copenhaver 2012), and 5% of the U.S. population use opioids drug prescription(Boudreau et al. 2009). However, serious side effects such as addiction, immunosuppression and gastrointestinal symptoms limit their use. These GI symptoms include constipation, bloating, nausea, vomiting. Previous studies in our laboratory and other research groups have demonstrated that morphine disrupts intestinal barrier function and induces bacterial translocation in mice(Meng et al. 2013). Use of opioids are associated with an increased risk of *C. difficile* infection in human patients(Mora et al. 2012). Morphine treatment can activate the virulent factors of *Pseudomona* and induce gut derived sepsis(Babrowski et al. 2012). Our laboratory's study has confirmed that morphine induced sepsis is mediated through modulation of miR-146a(Banerjee et al. 2013). Our study revealed that opioid exacerbation of gram-positive sepsis is rescued by IL-17A neutralization(Meng et al. 2015). It has been shown that the disruption of barrier function and increased growth of pathogenic bacteria are major factors that disrupts gut homeostasis(Marchiando, Graham, and Turner 2010; Estes et al. 2010; Sartor 2008; Kamada and Núñez 2013).

Gut homeostasis refers to a symbiotic relationship between the commensal microbiota and the host(Chassaing et al. 2012; Faust et al. 2012). It is maintained through interactions between gut microbiota and the host. An estimated 10^{14} bacteria colonize within the human intestinal tract(Nieuwdorp et al. 2014) which is 10 times greater than amount of human cells. There are more than 10 thousand unique species in gut

microbiota, and their genetic load are 150 times greater than human genome(Honda and Littman 2012). The total mass of human gut microbiota is about 2 to 6 pounds(Forsythe et al. 2010). Changes in the composition of the microbiota can contribute to diseases, such as obesity, diabetes, autoimmune diseases, inflammatory bowel disease (IBD), and infectious diseases(Zhao 2013; Wen et al. 2008; Severance, Yolken, and Eaton 2014; Bassett et al. 2015). However, very little is known how exogenous (diet, drugs, and infectious microorganism) and endogenous (the immune system, genetic background) factors modulate the formation and homeostasis of gut microbiota. Humans and animals are born into the world in a state free from microbial contamination, particularly within their gastrointestinal tract(van de Pavert et al. 2014). During birth and thereafter, they are exposed to microbes. Gut microbiota make important contributions to the health and well being of animals and humans, for example, nutrition metabolism, immune development, and CNS development(Ochoa-Repáraz and Kasper 2014; Cullender et al. 2013). Particularly, babies acquire microbes from their mothers. Infants born by C-section have a different microbiota composition as compared with vaginally delivered newborn infants(Dominguez-Bello et al. 2010). Vaginally delivered infants come in contact with the maternal vaginal and faecal microbiota which results in the gut colonization by microbes from mother's birth canal. In infants born by C-section, the establishment of gut microbiota is delayed. Breastfed infants exhibit significant differences in gut microbiota as compared with formula fed(Ding and Schloss 2014). Breast milk has been shown to be an excellent and continuous source of bacteria.

Gut microbiota play an important role in maintaining gut homeostasis. The symbiotic relationship between commensal microbiota and the host achieve a balanced,

mutually beneficial state. Alteration in gut microbiome and metabolome has been shown to contribute to dysregulated immune response, bowel dysfunction and gut barrier disruption(Vangay et al. 2015; Diehl et al. 2013). However, the effect of opioids on gut microbiota has not been investigated. In this study, we use a mouse model of morphine treatment. In the experiment, pellets were implanted subcutaneously to mice. Fecal samples were collected at day 0, day 1, day 2 and day 3 post treatment. Then, fecal DNA were extracted, 16s-rRNA genes were amplified by using V4 primers, then proceeded by Illumina MiSeq sequencing. The gut microbiome profile exhibits a distinct signature following morphine treatment when compared to placebo treatment.

Alpha diversity refers to diversity of species within a habitat unit(The Human Microbiome Project Consortium. 2012; Walter and Ley 2011). It answers questions: What is there? How much is there? Morphine treatment results in decreased alpha diversity when compared to controls. Richness of microbial diversity is an indicator of microbial homeostasis and less microbial diversity is associated with microbial dysbiosis (Antharam et al. 2013). Morphine treatment is associated with a significant decrease in gut microbial alpha diversity, indicating microbial dysbiosis and higher risk for dysbiosis and infectious diseases.

Beta diversity refers to diversity between habitat units(Schloss 2010). It answers question: How similar or different are samples? Each dot represents a sample, the position and distance between dots reflect similarity of microbiome profile. The closer clustering means higher similarity and less difference. Our study shows that morphine treatment results in a significant shift in fecal microbiome when compared to placebo treatment. A cladogram of phylogenetic tree shows distinct clustering by comparing

morphine treatment and placebo. Furthermore, a dendrogram of gut microbiome reveals that housing animals in separate boxes does not result in individual differences, when compared with effects of treatments.

Then, we use an opiate receptor antagonist, naltrexone, to determine if it can inhibit effect of morphine on gut microbiome. Our study shows that prior to treatment all fecal samples have similar gut microbiome. On day 1 post treatment, morphine treated groups were distinct from morphine, naltrexone, naltrexone plus morphine treatment. In this time course study, we found naltrexone antagonizes morphine-induced alteration of gut microbiome. Interestingly, animals treated with naltrexone alone clustered distinctly from the placebo group at day 3 post treatment suggesting that endogenous opioid may set a basal tone on the host microbial profile.

To further determine if morphine treatment results in functional consequence following gut microbiome alteration, predicted metagenomic functional analysis was performed using a BugBase software package. Our results show that morphine treatment resulted in a significant increase in pathogenic function of gut microbes. Furthermore, there is a decrease in stress tolerance of gut microbiome indicating decreased resilience against perturbation to gut homeostasis. These results implicate that morphine-treated gut microbiota might be less likely to outcompete outgrowth of pathogenic bacteria and eliminate pathogens colonization in the gut. Less stress tolerance of the gut microbiota community means that the homeostasis is more “fragile” or susceptible.

By using phylogenetic analysis, we found morphine treatment results in a significant increase of potential pathogenic bacteria. The analysis of OTUs on genus level shows that relative abundance of potential pathogenic bacteria (genus level) increased

significantly at day 3 post morphine treatment compared to placebo treatment. Multiple-test with the given threshold (FDR=0.05) shows relative abundance of pathogenic bacteria of genus level increase significantly at day 3 post-treatment with morphine treatment compared to placebo treatment. Increased representative potentially pathogenic bacteria include *Flavobacterium*, *Enterococcus*, *Fusobacterium*, *Sutterella*, *Clostridium*, etc.

In order to confirm the pathogenic strains that constitutes distinctness of gut microbiome alteration, we detected expression profiling of selected species specific 16SrRNA genes in gut microbiota by using quantitative real-time PCR. The result shows that expansion of *Enterocuccus faecalis* is associated with morphine induced alteration of gut microbiome. *E. faecalis* 16S-rRNA gene amplification was 100 times greater in morphine modulated gut microbiome than placebo treated gut microbiome. The effect of morphine on *E. faecalis* expansion was observed as early as day 1 in morphine treated animals and sustained in all days tested post treatment. The effect of morphine on *E. faecalis* abundance in the gut microbiome was antagonized by naltrexone treatment. This results suggest that expansion of *E. faecalis* can potentially act as a biomarker of gut dysbiosis following morphine treatment.

High risk of bacterial infection in hospitalized patients is a significant concern especially in those patients that are on opioids for pain management. *C. rodentium* is a natural mouse pathogen that models intestinal infection by E. coli (Enterohaemorrhagic Escherichia coli, EHEC) in human and animal hosts(Yi and Goldberg 2009). While establishing colonization, *C. rodentium* delivers a variety of virulence factors, such as translocated intimin receptor (Tir), into epithelial cells by means of the type III secretion

system in order to facilitate their adherence and invasion(Kamada et al. 2012). EspFU directly binds and activates N-WASP, leading to actin polymerization events. The expression of most virulence genes in *C. rodentium* is controlled by a regulator, Ler protein, a member of the histone-like nucleoid structuring (H-NS) protein family. Thus, Ler is a virulence factor that regulates *C. rodentium* virulence. It has been known that wild type mice are able to clear C. rodentium infection(Kamada et al. 2012). We next asked the question whether morphine can increase *C. rodentium* growth in intestine contents and modulate gut microbiome in the context of *C. rodentium* infection. The results show that morphine increases *C. rodentium* load in fecal matter at day 5 post infection. Furthermore, to determine *C. rodentium* bacterial adherence to intestines, we conducted Fluorescent *in situ* hybridization (FISH) on tissue sections by using specific profess to detect *C. rodentium*. The FISH results reveal that morphine treatment increases *C. rodentium* adherence to small intestines and colon, when compared to placebo treatment.

Pathogen colonization in the intestine has been shown to be controlled by bacterial virulence and through competition with gut commensal microbiota. To investigate if morphine treatment increases *C. rodentium* virulence, we determined virulence factors, ler and tir, mRNA levels by qPCR in fecal pellets of mice infected with *C. rodentium* at day 5 post infection. Expression was normalized to that of the 16S rRNA genes. Expression of virulence factors, Ler and Tir, were significantly increased in the morphine treatment group when compared to placebo. Same effects were seen in fecal samples of small intestines, cecum and colon.

At day 5 post *C. rodentium* infection, when compared to placebo treatment, mice receiving morphine revealed an increased number of colonies growing on the antibiotic selective LB agar plates, indicating systemic dissemination of *C. rodentium* into mesenteric lymph nodes, spleen, liver and blood circulation.

Beta diversity analysis results revealed morphine treatment shifts gut microbiome in the context of *C. rodentium* infection, indicating that the relative bacterial abundance and composition in gut were changed. The Principle coordinate plot reveals that primary clustering is by *C. rodentium* infection. Furthermore, UniFrac measure shows that morphine treatment induces distinct gut microbiome in the context of *C. rodentium* infection.

To begin to understand early events that occur in morphine-treated mice that can precipitate increased *C. rodentium* infection, we evaluated the extent of epithelial damage at day 5 post infection. Histologic evaluation revealed that morphine treatment disrupts morphological structure of mucosal surface of intestines. Histological evaluation of the tight junction protein ZO-1 on sections of jelly-rolled small intestines reveals that morphine treatment decreases tight-junction organization between intestinal epithelial cells(Meng et al. 2013; H. Wang et al. 2008). However, *C. rodentium* infection alone does not result in histological damages in intestinal mucosal epithelial integrity and barrier function at day 5 post infection.

In the early stage of infection pathogenic bacteria require virulence factor-mediated adhesion(Minsoo Kim et al. 2009; Hew, Korakli, and Vogel 2007). In the late phase, virulence factor is downregulated and healthy intestinal microbiota would outcompete the pathogen. Our study shows that morphine treatment induces microbial

dysbiosis, gut barrier dysfunction, and dysregulated immune response. In a mouse model of *C. rodentium* infection, morphine treatment results in 1) increase of virulence factors expression, 2) increase of pathogen outgrowth and colonization in gut, 3) Alteration of gut microbiome, 4) damaged integrity of gut epithelial barrier function, and 5) the promotion of *C. rodentium* systemic dissemination.

Microbial dysbiosis leading to a disruption of host-microbes homeostasis is not only alteration of microbial composition, but more importantly disruption of functional consequence of the microbiota. Previous studies have been well demonstrated that gut metabolites play an important link between gut microbes and host biological functions(Nicholson et al. 2012). For example, bile acids can mediate resistance to C. difficile infection. Bacterial metabolites regulate GI barrier function via Xenobiotic Sensor PXR and TLR4(Venkatesh et al. 2014; Hagey and Krasowski 2013). However, it is still unknown how morphine treatment can modulate composition and abundance of gut metabolites. Gut bacteria play an important role in bile acid and morphine metabolism in gut. Primary bile acids and M3G (Morphine-3-glucuronide) are produced in live and excreted into intestine through bile duct(Klepstad et al. 2003; Oliveira et al. 2014). In gut, deconjugating bacteria facilitate their metabolism(Van Crugten et al. 1991). It is unknown whether morphine-induced microbial dysbiosis can modulate bile acids and morphine metabolism in gut. Morphine is conjugated to morphine-3-glucuronide (M3G) in liver and excreted to gut via biliary tract. Intestinal deconjugating bacteria transform M3G to morphine, which is reabsorbed back to systemic circulation.

To determine the effect of morphine treatment on gut metabolome, the same samples previously used for gut microbiome analysis, are used for metabolomic analysis.

LC-MS based metabolomics analysis are able to determine composition and abundance of gut metabolites(Shi, Yao, and Chen 2012; Yao et al. 2013). In this study, morphine treatment induces distinct gut metabolomics profile when compared to placebo at day 3 post treatment. To identify metabolites contributing to distinct metabolome following morphine or placebo treatments, we performed a loading plot of principal components analysis model in which each dot represents a single molecular metabolite. It has been shown through the loading plot that the abundance of certain metabolites are increased by morphine treatment while certain metabolites are decreased by morphine treatment.

In a longitudinal study, fecal matters were taken from mice at prior to and day 1, day 2, day 3 post morphine or placebo treatment. Metabolic profile following morphine treatment was determined by analyzing molecular metabolites extracted from these fecal matters. Our results show that morphine treatment resulted in a gradual and differential shift in metabolites in a time dependent manner. Bile acids decreased gradually, while phosphatidylethanolamines (PEs) and saturated fatty acids increased as a consequence of morphine treatment.

We used an opioid receptor antagonist, naltrexone, to determine whether it can reverse effect of morphine on gut metabolomics profile. The result shows that naltrexone can alleviate morphine induced gut metabolomics shift and reverse the effect of morphine on bile acid metabolism. This figure shows that, the abundance of secondary bile acid, deoxycholic acid significantly decreased following morphine treatment and morphine induced decrease was antagonized by naltrexone. The abundance of phospholipid, PE, which is a major component of cell membrane, was increased by morphine treatment, indicating increased cell injury, but morphine induced increased was inhibited by

naltrexone treatment. This result indicates that deoxycholic acid (DCA) levels and phosphatidylethanolamines (PE) can be used as biomarkers to indicate morphine-modulation of gut metabolome.

Morphine is conjugated to morphine-3-glucuronide (M3G) in liver and excreted to gut via biliary tract. Intestinal de-conjugating bacteria transform M3G to morphine, which is reabsorbed back to systemic circulation. We found that the ratio of M3G/MS serum concentration increases in a time course study. The slow-released morphine from pallet implantation and morphine from reabsorption can make a stable serum concentration of morphine. The decrease of morphine serum concentration indicates disruption of morphine biotransformation and reabsorption in gut. The ratio of M3G/MS also increases in feces between day 1 and day 2 post morphine treatment, indicating decreased M3G deconjugation in the gut.

To summarize effects of morphine treatment on gut metabolome, morphine treatment when compared to placebo, 1) results in dramatic changes in the fecal metabolome; 2) differentially and gradually alters fatty acids and bile acids, 3) increases PE levels indicating significant cell injury. It is worthy to note that morphine induced dysbiosis disrupts morphine metabolism and its enterohepatic recirculation.

My study reveals opioids-induced distinct alteration of gut microbiome and metabolome, may contribute to opioids-induced pathogenesis and morphine pharmacokinetics. This is the first study to demonstrate that morphine promotes pathogen dissemination in the context of intestinal *C. rodentium* infection, indicating morphine modulates virulence factor-mediated adhesion of pathogenic bacteria and induces disruption of mucosal host defense during *C. rodentium* intestinal infection in mice.

Potential Problems and Future Directions

Gut microbiome analysis

Rapid progress in next generation sequencing has provided a new tool to investigate the gut microbiome and helped us complete mission that was impossible to do so. However, technology limitation still remains as major obstacles.

Firstly, current 16S rRNA genes analysis can only provide limited approaches. There are conserved regions and variable regions on 16S rRNA genes. We identify and classify organisms by gene sequence variations. Carl Woese and George E. Fox were the first to investigate bacterial phylogeny by using 16S rRNA genes(Woese and Fox 1977). Because no primer pair is completely universal, primer specificity leads to an acceptable bias (Tremblay et al. 2015). It is possible that certain amount of organisms can't be identified when using primers against V4 regions of 16S rRNA sequences. It has been shown, MiSeq V4 exhibits the highest similarity to reach the expected taxonomic distribution and shotgun libraries, and results in highest quality, by comparing primer (V4, V7-8, and V6-V8) and platform (454 pyrosequencing and Illumina MiSeq) effects (Tremblay et al. 2015). A variety of studies comparing amplicons from different hypervariable regions all revealed that V4 amplicons exhibit the greatest similarity to shotgun sequencing bacterial community profiles(Kumar et al. 2011; Ward et al. 2012; Minseok Kim, Morrison, and Yu 2011; He et al. 2015). Thus, in this study we use primers for V4 regions to amplify 16S rRNA genes.

Secondly, reference database *per se* has been limited. Operational taxonomic unit (OTU) is an operational definition of a species or group of species. It's a unique number

for each group of species and differentiated by the sequence similarity. OTU ID is assigned to the center sequence of the nested cluster, and all the other sequences share the same OTU ID share the same OTU ID at the same level. Normally, we use 97% of similarity level. The taxonomic database we are currently using is GreenGenes (v13_5). It covers *Archaea* and *Bacteria* including 1,262,986 sequences. When we use 99% cut off value, we limit the reference base to 203,452 OTUs, while when we use 97% cut off value, we limit the reference base to 99,322 OTUs. OTUs are not identically equal to bacterial strains; chimeric and miss-annotated OTU picking may contribute to a higher errors; taxonomy updates lags years behind, indicating many unclassified. Furthermore, the GreenGene (v13_5) overlooks intestinal fungi which can only be identified by 18S rRNA genes.

Thirdly, OTU picking methods is limited. In this study, we use closed-reference OTU picking. Any reads that don't hit a reference sequence are discarded. We are also able to use *de novo* clustering, or open-reference OTU picking. In *de novo* clustering method, reads are clustered based on similarity to one another. Open-reference OTU picking considers any reads which don't hit a reference sequence are clustered de novo.

Fourthly, 16S rRNA sequences analysis can't tell you information on metabolic profiles. Microbiome information obtained from 16S rRNA sequencing tells us what taxa are in the habitat unit (who's there), while information on biological functions can only be indirectly obtained from 16S rRNA sequences. We can predict function based on taxonomy using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). In this study, we use BugBase packages, which relies on PICRUSt and KEGG database to predict biological functions based on 16S rRNA genes

sequencing(Langille et al. 2013). Alternatively, whole genome shotgun sequencing and RNASeq may provide better approaches to investigate biological functions. As technology progresses it may help decrease cost in genes sequencing, thus provide access to a better understanding of gut microbiome and their biological functions.

Fifthly, it is not clear how the results of our study about discovery of morphine-induced gut microbiome and metabolome alteration can be correlated with previously well established study on effects of morphine on host phenotype, behavior, immune response. It would be meaningful in terms of diagnostics or therapeutics to identify which determinants account for morphine-induced microbiome alteration and how the host genotypes, diet, metabolic and immune factors, as well as pathologic outcome, can affect gut microbiome. A genetic method, multitaxon Insertion Sequencing (INSeq) has been developed to determine precision microbiota manipulation and identify determinants that affect microbiota(Goodman, Wu, and Gordon 2011). By using INSeq technology, Wu, et al generated unique mutants of bacteria with a single insertion of isogenic transposon to each strain to identify genetic and metabolic factors that account for change of gut microbiome through analyzing relationship between mutation and bacterial abundance differences(M. Wu et al. 2015). This INSeq method can be applied in our future study to identify major determinants that involve in the morphine-induced microbial dysbiosis.

Host-microbes interaction and diseases

Alteration of gut microbiome is associated with many diseases, such as obesity, diabetes, autoimmune diseases, inflammatory bowel disease, colon cancer, and higher

risk of infections. Our study suggests morphine-induced gut microbiome alteration is associated with higher susceptibility to bacterial infections. The distinctness of morphine-modulated gut microbiome and metabolome provide potential diagnostic strategies. However, it is still unclear how clinical intervention alleviates morphine-induced adverse effects. After understanding mechanism that gut commensal microbiota outcompete pathogens we may take good advantage of clinical intervention by using commensal species to limit pathogen colonization and invasion. Fecal microbiota transplantation has shown to be promising therapeutic intervention clinically in curing recurrent *C. difficile* infection (CDI)(Weingarden et al. 2014; Cammarota, Ianiro, and Gasbarrini 2014). Also, the nutrition resource plays an important role in modulating the gut microbiota. Literature shows that commensals and pathogenic bacteria may grow on structurally similar nutrition(Kamada et al. 2012). Thus, nutrition competition plays a crucial role in clearing pathogenic bacteria from the gut. It would be a potential therapeutic strategy for prevention or intervention of intestinal infection in terms of modulation of gut microbiota during pain management using morphine.

In this longitudinal study, morphine treatment induces microbial dysbiosis and functional consequences following microbial dysbiosis exhibit a time-course dependent fashion. However, it is still unknown how long the microbial dysbiosis and its effect may remain. The morphine serum levels remained elevated at least within 144 hours following pellet implantation(Patrick et al. 1975). It would be worthy to investigate effect of morphine on gut homeostasis in the longer term study and under different drug administration methods, such as i.p. injection or intravenous infusion pump. It is interesting to reveal whether and if so, how long it takes to reach the recovery of gut

homeostasis or remain vicious loop of microbial dysbiosis. To be noted, morphine withdrawal-induced immunosuppression and high risk of bacterial infection has been well investigated(Das et al. 2011; Feng et al. 2005; Sabita Roy et al. 2011), however, effects of morphine withdrawal on gut microbiome and their relationship with pathological outcome is still unknown.

It has been shown that morphine-induced microbial dysbiosis exhibits a signature of distinctness. However, it is unclear the direct relationship between gut microbiome disruption and pathological outcomes. Gnotobiotic mouse model has been widely used in determine the direct relationship in host-microbes interaction(Reyes et al. 2013). Some concerns, such as unmatured immune system and abnormal physiological phenotypes, limit their use(Chung et al. 2012; Kau et al. 2011; Erny et al. 2015). Our laboratory's study of fecal transplantation in SPF mice has shown that transplantation of morphine modulated fecal matters to non-treated mouse can lead to pathological consequence similar to that of morphine treated mice, indicating direct effect of microbial dysbiosis on pathological consequence in the host (unpublished data). However, it is not known which bacteria can determine the pathological outcomes, such as immune response, physiological development or behavior changes. Through precision antibiotic manipulation of gut microbiome, the mechanism under which gut bacteria account for morphine-induced pathological consequence can be better determined in future study.

A common concern of mouse model is its limitation, especially on immune systems and gut microbiome composition, when compared to human subjects. Humanized NSG™ (hu-NSG™) mouse models is a mouse model with humanized immune systems. By generating humanized gut microbiome within gastrointestinal tract

of humanized NSG mouse models may provide a powerful tool to expand the impact of study on mice.

Better understanding mechanism of vulnerability to infection in patients under pain management and drug abusers can help develop therapeutic strategy following our study. After elucidating the mechanism by which morphine modulates host immune system and gut microbiota, we may conduct intervention of cytokines, such as IL-17A, or antibodies, such as IgA, antimicrobial peptides (AMPs) to reverse side effects of morphine.

Our previous study showed that IL17A neutralization can rescue opioid exacerbation of gram positive sepsis induced by cecal ligation and puncture (CLP)(Meng et al. 2015). Meng et al, demonstrated that IL17A neutralization act as anti-inflammatory effects, which improves the survival rate and sustains inflammation in CLP mice treated with morphine. Furthermore, high levels of IL-17A compromise gut epithelial barrier function and increase gut permeability in morphine treated CLP animals. Thus, IL-17A seems to play a negative role. However, in present study, we found morphine treatment suppresses *C. rodentium*-induced IL17A immune response, inhibits *C. rodentium* clearance and promotes systemic dissemination. It has been well demonstrated that IL-17 and IL-22 enhance innate barrier defenses against *C. rodentium*, and induce antimicrobial peptide production and neutrophil recruitment, indicating a protective role(Geddes et al. 2011; Rubino, Geddes, and Girardin 2012; Z. Wang et al. 2014). The role that IL-17A plays in these two scenarios brings up a paradox about inflammation and pathogenesis. Pro-inflammatory response to pathogen infection improves bacterial clearance and is part of self-protection mechanism of host defense. However, inflammation may also lead to

overreaction or dysregulated immune response that disrupts homeostasis and damage host physiological function. Similarly, gut commensal microbiota, which is characterized by adaptability and resilience against perturbation, play an important role in maintain gut homeostasis and outcompete invading pathogens. However, highly virulent pathogens are capable of colonization in gut, adherence to mucosal epithelium, and translocation to systemic circulation. During this process, invading pathogens results in microbial dysbiosis, which therefore leads to dysregulated immune response and intestinal barrier dysfunction. Thus, the thought of pathogenesis seems beyond infection and disease, but is focused on host-microbes interaction. Here the question is not about whether infection induces disease. The question is whether host-microbes interaction damages host. In this way, we may better understand pathogenesis and find an appropriate way to cure diseases.

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