Infectious Colitis is Exacerbated by Prolonged Stressor Exposure: Implications for Probiotic Intervention

DISSERTATION

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Abstract

Many illnesses, including gastrointestinal diseases, are exacerbated by inflammation contributed by inflammatory monocytes that are recruited to help clear pathogens. However, an increased accumulation of inflammatory monocytes can lead to excessive inflammation and tissue damage. As a result, the recruitment of inflammatory monocytes is tightly regulated. Psychological stressors are known to exacerbate inflammatory diseases, but whether these stressor-induced exacerbations involve inflammatory monocytes is unknown. Recently it has been demonstrated that exposure of mice to the stressor, social disruption (SDR), can significantly enhance *Citrobacter rodentium*-induced infectious colitis which includes enhanced colonic monocyte/macrophage accumulation, all of which can be significantly reduced by treatment with the probiotic *Lactobacillus reuteri*. Thus, this study was designed to determine the involvement of inflammatory monocytes in stressor-enhanced infectious colitis, as well as the corollary hypothesis of how probiotic intervention can reduce stressor-enhanced infectious colitis.

Exposure of mice to SDR at the onset of *C. rodentium* challenge causes significant increases in pathogen-induced colonic histopathology, colonic inflammatory mediator gene expression (i.e., tumor necrosis factor-α, CCL2, inducible nitric oxide synthase), colonic monocyte/macrophage accumulation, and pathogen translocation from...
the colon to the spleen, all of which are reduced by *L. reuteri* treatment. The reduction of SDR-enhanced, pathogen-induced CCL2 is of importance as CCL2 is responsible for the recruitment of inflammatory monocytes which are heavily involved in the induction of colitis. In order to test the hypothesis that SDR-enhanced infectious colitis is due to a CCL2-mediated recruitment of inflammatory monocytes, CCL2-deficient mice, which have defective monocyte recruitment, were utilized. Exposing CCL2-deficient mice to SDR during *C. rodentium* challenge caused an increase in colonic pathogen levels however the resulting pathogen-induced infectious colitis is negated. Also, the addition of monocytes from naïve mice to *C. rodentium*-infected mice alone was not enough to enhance infectious colitis. In determining the source of colonic CCL2 during SDR-exposure *in vitro* evidence has demonstrated that bacterial byproducts of *L. reuteri* can significantly reduce pathogen-induced CCL2 and TNF-α gene expression in colonic epithelial cells, but not macrophages. And the infection of mice that are unable to activate the transcription factor NF-κB in either intestinal epithelial cells or myeloid-derived cells (i.e., monocytes and macrophages) with *C. rodentium* has demonstrated that myeloid-derived, NF-κB-induced upregulation of proinflammatory cytokines is responsible for pathogen-induced colitis.

Lastly, to retest our overarching hypothesis of the efficacy of probiotic intervention on stressor-enhanced infectious colitis, mice were exposed to a different psychological stressor, prolonged physical restraint (RST), at the inception of bacterial challenge with *C. rodentium*, with and without *Lactobacillus reuteri* treatment. Probiotic intervention with *L. reuteri* was able to reduce RST-induced disease severity as
evidenced by reductions of pathogen translocation from the colon to the spleen possibly through the fortification of the colonic barrier. However, *L. reuteri* was unable to reduce RST-enhanced colonic pathogen colonization, colonic histopathology, or colonic inflammatory mediator mRNA expression. Taken together, probiotic intervention with *L. reuteri* can reduce stressor-exacerbated infectious colitis however the degree to which colitis is reduced is dependent on the severity of colitis.
This manuscript is dedicated to my parents, Doug and Sara Hufnagle, to my husband, Brian, and to my son, William.

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CHAPTER 1: Introduction

Colonic inflammation, or colitis, affects millions of individuals worldwide with the two main causes being infectious (i.e., bacterial or viral) and autoimmune (i.e., inflammatory bowel diseases) in nature. Signs and symptoms vary between individuals and are related to the cause of colitis. The most common symptoms are pain, loss of appetite, bloating, diarrhea, and cramping all of which can be exacerbated during stressful life events [1-3]. It is not fully understood how psychological stressors can exacerbate gastrointestinal (GI) disease, however there is emerging evidence for the involvement of the indigenous microbiota found within the GI tract [2, 4-13].

Recently our laboratory has demonstrated that two different murine stressors have the ability to significantly alter the intestinal microbiota, which is largely attributed to reductions in the abundance of beneficial lactobacilli [8]. When stressor-exposed mice are challenged with the GI pathogen *Citrobacter rodentium* the resulting infectious colitis that occurs is exacerbated over that of non-stressed, infected control mice [8, 14]. It is hypothesized that stressor-induced changes in the microbiota contributes heavily to stressor-enhanced, *C. rodentium*-induced infectious colitis due to the fact that restoring beneficial lactobacillus species can reduce stressor-exacerbated infectious colitis [14]. While the involvement of the colonic microbiota has been demonstrated in stressor-exacerbated, *C. rodentium*-induced infectious colitis, it is unclear how the immune
response is altered during this paradigm. Thus, this study was proposed to determine the involvement of a known contributor to colitis i.e., the inflammatory monocyte. This study was also designed to determine the actions of *Lactobacillus reuteri*, the beneficial microbe which reduces stressor-enhanced infectious colitis, in the colon.

**The Stress Response**

The term stress is often defined as a state of threatened homeostasis [15, 16]. This threat, be it real or perceived, evokes a stress response which has been conserved over time and across species in order to prepare the organism to confront or flee from its stressor in the so called “flight or fight” response. When faced with a stressor, multiple neuroendocrine pathways of the stress response are rapidly activated, including the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) which culminate with the production of glucocorticoids and catecholamines, respectively [17, 18]. While the activation of these evolutionary conserved responses help the organism adapt to its ever changing environment it can have adverse behavioral and immunological effects.

**Hypothalamic-Pituitary-Adrenal Axis**

As the name implies, the three main glands of the endocrine system involved in the activation of the HPA axis are the hypothalamus, pituitary gland, and the adrenal glands. The hypothalamus receives information regarding potentially harmful entities from the amygdala via the limbic system. When an organism encounters something that
is deemed a stressor the paraventricular nucleus (PVN) of the hypothalamus secretes corticotropin-releasing hormone (CRH) which travels the short distance from the hypothalamus to the anterior lobe of the pituitary gland. The action of CRH on the anterior pituitary gland will cause the production and release of adrenocorticotropic hormone (ACTH) into the bloodstream which acts upon the cortex of the adrenal glands to stimulate the production of glucocorticoids (GCs). Upon release from the adrenal cortex, GCs enter the circulation and serve as a negative feedback on the hypothalamus in order to ensure the stress response does not perpetuate without the presence of the stressor. In addition to providing a negative feedback for the stress response, GCs have two main functions in the body: metabolic and immune. Metabolic functions include the induction of gluconeogenesis and catabolism of fat from adipose tissue in order to provide additional energy to confront stressful stimuli while immunologic functions include immunosuppression and a blunting of the inflammatory response. Regardless of the function of GCs, the resulting actions are due to the interaction of GC with the steroid receptor aptly termed the glucocorticoid receptor (GCR), a receptor which is found in virtually every cell of the body.

Glucocorticoids belong to a family of steroid hormones which derive their main structure from cholesterol. These small, lipophilic molecules readily diffuse through the plasma membrane and bind to GCRs, which are sequestered in the cytoplasm. The immunosuppression that is associated with the stress response is partially attributed to the induction of apoptosis of immune cells by the GC-GCR interaction. Similarly, the actions of the GC-GCR can modulate gene expression related to pro- and anti-
inflammatory cytokine production, chemokine expression, and adhesion molecule
expression all of which are involved in the inflammatory response. The anti-
inflammatory effects of the interaction of GC with GCR can be distilled down to two
different phenomena: transactivation and transrepression, both of which can interfere
with the transcription factor NF-κB. Transactivation occurs by the direct binding of GC-
bound GCR to glucocorticoid response elements (GREs) in the promoter region of anti-
inflammatory target genes such as the cytokine, interleukin (IL)-10 or the inhibitor of
NF-κB, IκB. The induction of gene transcription by the binding of GCR to DNA can also
interfere with proinflammatory gene transcription by the competition for cofactors of
transcription. Proinflammatory gene transcription can also be reduced through
transrepression which can be achieved either directly or indirectly. Direct transrepression
occurs when the GCR binds directly to a transcription factor, such as NF-κB, in order to
interfere with its ability to transcribe proinflammatory genes such as TNF-α, IL-1β, and
IL-6. Indirectly, the GCR can bind to sections of DNA, termed non-glucocorticoid
response elements, which causes a conformational change in the DNA which disallows
the binding of NF-κB to DNA.

**Sympathetic Nervous System**

The SNS, together with the parasympathetic nervous system, comprise the
autonomic nervous system. Like the HPA axis, the SNS is activated when the organism
is confronted with a stressful stimulus culminating in the quick release of catecholamines
(i.e., epinephrine and norepinephrine) which are the archetypical hormones involved in
the fight or flight response. Catecholamines are responsible for pupil dilation, vasodilatation, increased heart rate and respiration, enhanced sweat production, as well as a decrease in digestion all of which aid in preparing the organism to confront its stressor. The sympathetic nervous system is made up of a two nerve system; pre- and postganglionic. The preganglionic nerves originate within the spinal cord, extending only a short distance. At the synapse, the much longer postganglionic nerve extends throughout the body, innervating most tissues of the body. When activated, the preganglionic nerve will release acetylcholine into the synapse to activate nicotinic acetylcholine receptors found on the postganglionic nerve which stimulates the release of norepinephrine. Norepinephrine is also released by chromaffin cells of the adrenal medulla, but it is to a much lesser extent than that of the postganglionic nerves. Epinephrine, however, is made in large quantities by adrenal medulla chromaffin cells.

In addition to the effect of preparing the organism for confrontation with a stressor, catecholamines also modulate the immune response, including immune trafficking, antibody production, and the inflammatory response, through their actions on adrenergic receptors. There are two types of adrenergic receptors; α and β with each receptor having several subtypes. These G protein-coupled receptors can be found on cells of the immune system, including neutrophils and macrophages. Depending on which receptor is ligated, a different response will occur through a signaling cascade which culminates with changes in cyclic AMP (cAMP). For example, the ligation of the α-adrenergic receptor can enhance proinflammatory cytokine secretion from macrophages [19], however activation of a β-adrenergic receptor on macrophages can
reduce its production of proinflammatory cytokines as well as reduce its phagocytic proclivities [20, 21].

**Stressor Effects on the GI Tract**

The GI tract and the brain are in constant contact with each other with a large amount of the communication originating in the gut, usually pertaining to digestion. This communication takes place via the 100 million neurons present within the gut, collectively known as the enteric nervous system, which rivals the amount present within the spinal cord and is why the gut is often referred to as our “second brain”. Under normal, non-stressed conditions, the parasympathetic nervous system is active and contributes to the “rest and digest” state of the gut. When the organism encounters a stressor, the SNS is activated which shuts down the parasympathetic nervous system in order to divert resources from the energy-intensive process of digestion to the potentially physical encounter with the stressful stimulus. Because of this, stressor exposure can cause changes in GI motility, pH, intestinal permeability, mucus production, antimicrobial peptide production, and composition of the intestinal microbiota, all of which can contribute to the exacerbation of GI diseases [1, 2, 22-24].

Currently the mechanism by which life stressors are able to enhance colitis is unknown, however emerging evidence suggests that the initial immunosuppression which is observed during stressor exposure can lead to disruptions in the intestinal microbiota which, in turn, may enhance intestinal inflammation [25, 26]. Circulating GCs are often to blame with regard to stressor-induced immunosuppression due to their ability to reduce
inflammation as well as cause immune cells to die via apoptosis. Research has demonstrated that removing the adrenal glands or the use of GC receptor antagonists can reverse the initial immunosuppression which is observed in the intestine during stressor exposure [25, 27]. It is theorized that this initial immunosuppression in the intestine can lead to the overgrowth of commensal microbes, however catecholamines can also contribute to the overgrowth of the indigenous microbiota, especially Gram negative bacteria [28]. For example, culturing bacteria, such as *Escherichia coli*, with either epinephrine or norepinephrine can cause a significant increase in bacterial growth [24, 29]. It is theorized that this phenomenon might occur because of the ability of these hormones to make iron more bioavailable for the bacteria [30]. Iron can induce virulence factors in pathogenic bacteria, such as enhance expression of adhesion pili, induce Type III secretion systems, as well as enhance motility [31, 32].

In addition to their immunosuppressive actions, GCs have also been attributed to a leaky gut barrier, as treatment of non-stressed mice with exogenous GCs (i.e., dexamethasone) can cause an increase in intestinal permeability [33, 34]. The enhanced permeability of the gut epithelium is due to the loosening of tight junctions found between epithelial cells which can allow commensal bacteria, food antigen or other noxious substances to penetrate from the lumen to the lamina propria. Glucocorticoids are not the only hormone within the gut to enhance permeability. Enteroendocrine cells of the small and large intestine can produce CRH which is heavily involved in modulating inflammation, increases gut permeability, changes in gut motility, and visceral hypersensitivity [35-38]. Major responders to CRH in the GI tract are mast cells
which will degranulate and release cytokines, chemokines, histamine, and other secreted substances [39, 40]. Degranulation of histamine is of importance to GI barrier function because histamine can also loosen tight junctions of the intestinal epithelium to enhance permeability [41, 42].

Taken together, the current theory of the contribution of stress hormones on the induction of colitis is as follows. Corticotropin-releasing hormone can cause the release of mucus from goblet cells which along with its actions on motility, allows for the initial quick movement of the luminal contents through the intestine [43]. Catecholamines enhance the growth, and potential virulence, of Gram negative commensal bacteria. Glucocorticoids will cause an initial reduction in inflammation as well as suppress the local immune system which can also contribute to the growth of the microbiota. After the initial release of mucus, goblet cells will remain depleted which causes a thinning on the mucus layer and consequently allows the commensal microbes to come into closer contact with the intestinal epithelial cells [44, 45]. The actions of glucocorticoids and CRH-induced mast cell degranulation can lead to the loosening of tight junctions, thereby allowing the contents of the lumen to penetrate through the intestinal epithelium into the lamina propria where remaining immune cells, epithelial cells, and stromal cells will react, resulting in enhanced inflammation.

The Microbiota

Vertebrates and bacteria have coevolved over the millennia with some bacteria learning to better evade the vertebrate immune response and others have evolved to have
a mutually beneficial relationship with their hosts. The human body harbors approximately 100 trillion microbes, collectively called the microbiota, with the highest concentration found within the GI tract, or more specifically the distal portion of the colon. We have coevolved over time to not only tolerate, but rely on these microbes as they aid in the development of our immune system, provide nutrients, break down potentially noxious substances, and act as a barrier against potentially pathogenic organisms [9, 11, 13, 46, 47]. To demonstrate the importance of the colonic microbiota in particular, mice which are raised in germ-free conditions (i.e., they have no prior exposure to bacteria, viruses or parasites) have a blunted immune response, which is characterized by a reduction of secretory IgA, reductions in the size of Peyer's patches, lymphoid aggregates, and resident tissue immune cells, as well as underdeveloped intestinal villi [48, 49].

The use of germ-free mice has led to numerous breakthrough discoveries of the involvement of the intestinal microbiota in conditions such as obesity, inflammatory bowel disease (IBD), and their effects on cognition and behavior [4, 12, 50-52]. It is known that stress hormones can alter the intestinal microbiota in a condition known as dysbiosis [8, 9, 53-55]. Microbial shifts, such as the reduction of the relative abundance of the protective genera *Lactobacillus* and increases in the abundance of Gram negative genera are thought to contribute to the stressor-enhanced pathologies of the GI tract through an as of yet undetermined mechanism [8]. The overgrowth of Gram negative bacteria can be detrimental as many opportunistic pathogens, e.g. *E. coli*, belong to this group of bacteria and may contribute to pathology associated with IBD [56-59]. Recent
studies have demonstrated that adherent *E. coli* can be found adhered to lesions within the intestinal mucosa in patients with active Crohn’s disease, while neighboring healthy tissue was generally devoid of bacterial adherence [60-62].

The reduction of the relative abundance of beneficial species can be detrimental to the host as these protective bacteria help fortify the intestinal barrier, aid in the stimulation of antimicrobial peptides and IgA, and compete for nutrients and space. Protective members of the commensal microbiota are also believed to contribute to oral tolerance through the induction of thymic stromal lymphopoietin (TSLP), TGF-β, and IL-10 production by intestinal epithelial cells [11, 63-65]. These cytokines can act on CD103+ dendritic cells which in turn can convert naïve T cells into FOXP3+ T regulatory cells [66, 67]. Because of this, it is possible that reductions in the relative abundance of beneficial microbes may lead to the loss of tolerance to the intestinal luminal contents.

*Lactobacillus reuteri*

*Lactobacillus reuteri* is a Gram positive bacterium which inhabits the gastrointestinal tract of mammals such as humans, rodents, dogs, sheep, cattle and birds, however the incidence to which *L. reuteri* is found within these mammals varies [68]. In rodents *L. reuteri* is one of the most prevalent species present within the GI tract while it is estimated that as little as 4% of present day humans are the host to *L. reuteri* [69]. While the natural niche of *L. reuteri* has not been described in humans, in rodents it has been shown to directly adhere to the stratified squamous epithelium of the forestomach where it can be found in a biofilm [70]. Although natural colonization of humans by *L.
*L. reuteri* has declined in the last 5 decades, its use through supplementation is well tolerated in infants, children, the elderly, and immunocomprised individuals, making it an ideal candidate as a probiotic organism.

The beneficial effects of *L. reuteri* are numerous and currently the wealth of knowledge of the effects of *L. reuteri* on health comes from animal research, however there have been clinical trials involving humans. Perhaps one of the more prominent findings with regard to *L. reuteri* is its ability to reduce infantile colic [71-74]. The etiology for infantile colic is as of yet, undefined, however it is marked by excessive crying in infants for more than 3 hours a day for at least 3 days per week [75, 76]. When colicky infants were treated with *L. reuteri* upwards of 95% of patients responded with a significant reduction in crying time [77-79]. In addition to the effects of *L. reuteri* on colicky infants, treatment with these beneficial bacteria has also been demonstrated to contribute to overall gut health, including the reduction of diarrheal illnesses in children and adults [80-83].

Much of the mechanisms of how *L. reuteri* supplementation is able to effect gut health are being investigated in mammals other than humans. For example, the administration of *L. reuteri* to mice was able to enhance intestinal resistance to the protozoal parasite *Cryptosporidium parvum* [84], decrease colonization of *Salmonella Enteriditis* in chickens [85], and even inhibit the growth of clinical isolates of methicillin-resistant *Staphylococcus aureus* [86, 87]. The ability of *L. reuteri* to interfere with the growth of other microorganisms has been attributed to both the production of lactic acid as well as the antimicrobial substance reuterin as well as to the phenomenon of
In addition to its effects on bacterial and parasitic growth, *L. reuteri* is also able to modulate host immunity. The treatment of rodents with *L. reuteri* reduced experimental colitis [14, 90-95]. It is theorized that the ability of *L. reuteri* to reduce experimental colitis stems from its ability to reduce proinflammatory cytokine (i.e., TNF-α) and chemokine (i.e., CCL2) production by dendritic cells and macrophages [96, 97]. Current evidence has revealed that *L. reuteri* is able to produce a substance, possibly histamine, which is able to interfere with the activation of the proinflammatory cytokines and chemokines via the MAP kinase pathway [97, 98].

**Probiotics**

Probiotics are live, naturally occurring microorganisms which when administered in appropriate amounts can have beneficial effects on the host with little to no side effects. The use of probiotics to ameliorate gastrointestinal distress was first published almost 100 years ago when a medic noticed that many soldiers had severe diarrheal illness, however, one soldier did not. An *Escherichia coli* strain, later named *E. coli Nissle 1917*, was isolated and given to the sick soldiers and they recovered quickly from their illness [99, 100]. In fact to this day the amelioration of diarrheal disease is the most recognized use of probiotics and has been demonstrated to reduce the incidence and duration of diarrhea due to *Rotavirus* enteritis [64, 101] as well as non-diarrheal disease producing infections such as *Clostridium difficile* [102] and *Helicobacter pylori* [103].

The idea behind the use of probiotics to combat GI damage stems from the idea that the GI tract of the affected individual is in a state of dysbiosis. Common probiotic species
are from the genera *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*, but even some strains of *E. coli* have been shown to have positive effects on GI health. Most probiotic bacteria can also be present in the GI tract as commensal bacteria and it is interesting to note that species of the *Lactobacillus* genus are highly susceptible to stressor exposure [55]. It is possible that the loss of these commensal/probiotic bacteria during exposure to stress contributes to stressor-exacerbated colitis.

Exactly how probiotics are able to ameliorate GI damage is still under investigation, but a key idea is that probiotic species can help restore the indigenous microbiota to its proper balance [11, 104-106]. The indigenous microbiota that is found in our GI tract is comprised of hundreds of different species that live together in a relatively stable state. Simple things, such as changes in diet, changes in sleep, antibiotic consumption, and exposure to stress can all change the relative composition of the microbiota. One way probiotics are thought to help heal GI damage is by restoring the natural balance of the indigenous microbiota potentially by competing for nutrients, changing the pH, or even via the production of their own antimicrobial peptides. In addition to restoring the microbiota to its natural balance, probiotics can also stabilize the tight junctions that are found between intestinal epithelial cells which can reduce mucosal permeability as well as increase mucus production which provides an excellent barrier between the microbiota and the epithelial barrier [91, 107-109]. Along with the increase in mucus production, probiotics can also increase mucin, β-defensin, and IgA production, all of which have been shown to control overgrowth of the microbiota [11, 63, 64].
Many of the aforementioned effects of probiotics may be more prevalent if the probiotic is taken prior to mucosal damage. Healing damage that has already occurred may need more than enhanced mucus and tight junction stabilization. However, probiotics have been shown to help heal pre-existing mucosal damage in patients with Crohn’s disease and ulcerative colitis, as well as in animal models. For example, the multistrain probiotic mixture, VSL#3, can repair the epithelial barrier in the spontaneous colitis model in IL-10−/− mice [110]. Patients with mild to moderate ulcerative colitis who were supplemented with VSL#3 in addition to their regular treatment had a reduction in symptoms and were protected from relapse for up to one year [111, 112]. Some of the healing effects of probiotics have been attributed to their ability to significantly reduce proinflammatory cytokine expression which is likely due to their ability to reduce the activation of transcription factors such as NF-κB and AP-1 [97, 113, 114]. Probiotics can also reduce the accumulation of inflammatory cells in the colon which can be attributed to different mechanisms: they can reduce chemokine expression as well as enhance FOXP3+ T regulatory cells in the colon [115-117].

*Citrobacter rodentium*

*Citrobacter rodentium*, a Gram-negative, non-motile rod, is a naturally occurring murine pathogen which belongs to the family of pathogens collectively known as attaching and effacing (A/E) bacteria which upon colonization induce an immune response that is similar to both murine and human inflammatory bowel disease [118, 119]. Other members of the A/E family include enteropathogenic and enterohemorrhagic
*Escherichia coli* (EPEC and EHEC, respectively). Not all mice infected with *C. rodentium* will develop full diarrheal disease, however many will have loose stools and increased water retention within the lumen of the colon. In highly susceptible strains of mice, this non-invasive pathogen can be found outside of the colon in sites such as the spleen and liver and in severe cases lesions can develop in the colon and, while rare, rectal prolapse or even death can occur [118, 120, 121].

Within hours of challenge, either by the natural fecal-oral route or experimental inoculation, *C. rodentium* can be found in the lymphoid tissue of the cecum. Two to three days post oral challenge the pathogen can be found at its permanent site of colonization, the distal portion of the colon, with the peak of colonization occurring between days 10-14 post oral challenge and full clearance by day 24 post-challenge in wild type mice. *Citrobacter rodentium* colonizes all strains of mice, however the level to which colonization occurs is strain-dependent [120, 122]. Disease progression parallels pathogen burden in the colon which can vary from subclinical symptoms to fatal. Mice that are susceptible to infection by *C. rodentium* will develop severe colitis due to an overzealous host T\textsubscript{H}1 immune response, characterized by inflammatory monocyte/macrophage accumulation, CD4\textsuperscript{+} T\textsubscript{H}1 cells, and IgG\textsubscript{1}-producing plasma cells [118-120]. Because of the immune response that is elicited by infection, *C. rodentium* can be utilized as a tool to study inflammatory bowel disease, such as Crohn’s disease and ulcerative colitis, in addition to it being a murine model for EPEC and EHEC infection.
Along with its A/E family members, *C. rodentium* must form intimate attachments with the host colonic epithelial cells in order to cause disease. This interaction allows the pathogen to inject effector proteins into the host epithelial cell via a type III secretion system (TTSS). The TTSS, along with other effector proteins, is encoded within the locus of enterocyte effacement pathogenicity island. These effector proteins are involved in the adherence to and the effacement of the colonic epithelium, as well as the formation of the actin-rich pedestals which are unique features of the A/E pathogen family. The main characteristic of *C. rodentium* infection is the development of colonic hyperplasia, or megacolon, which can be observed as early as 5 days post-challenge with the peak of hyperplasia occurring around Day 12 post-challenge [118, 123]. As the pathogen begins to be cleared, the colonic hyperplasia begins to resolve itself, however colonic hyperplasia can still be observed up to 10 days post-pathogen clearance (i.e., Day 34 post-challenge). Of importance to this study, *C. rodentium*-induced hyperplasia is generally devoid of injury or substantial inflammation in outbred mouse strains [122].

**Intestinal Epithelial Cells**

There is only a single cell layer of epithelial cells that protects the host from the contents of the gastrointestinal tract, which includes food antigen as well as a significant burden of microorganisms. These epithelial cells are responsible for not only providing a structural barrier from these potentially harmful substances, but also provide the initial immune response to gastrointestinal pathogens through the production of antimicrobial
peptides, reactive nitrogen and oxygen species, proinflammatory cytokines, and chemokines in order to initiate a full immune response [124]. In the small intestine and colon, epithelial cells arise from stem cells found at the base of the intestinal crypt. The initial division of the stem cell gives rise to an immature epithelial cell. As the epithelial cell migrates along the intestinal villus it will differentiate into one of 4 different epithelial cells; Paneth cells, goblet cells, enteroendocrine cells, or absorptive epithelial cells. Paneth cells are found only in the small intestine where they are responsible for the production of antimicrobial peptides. Goblet cells are responsible for the production of mucins which is important in maintaining the protective mucus layer that lines the digestive tract. Enteroendocrine cells produce hormones which can help regulate digestion of food as well as modulate motility of the luminal contents through the colon. Absorptive epithelial cells, or enterocytes, have multiple functions in the intestine. In the small intestine they aid in the absorption of nutrients from the luminal contents while in the colon they are absorb mainly water from the luminal contents. These cells are also the prime producers of cytokines, chemokines, as well as antimicrobial peptides. With the exception of Paneth cells, intestinal epithelial cells are short lived. As they migrate along the intestinal villus they differentiate into their mature form, but once they reach the tip of the villus they die via apoptosis and are sloughed off into the lumen. This enhanced turnover in the intestinal epithelial layer is the reason for the quick and self-limiting nature of gastrointestinal infections.

The primary site for *C. rodentium* colonization is the apical surface of the absorptive intestinal epithelial cell in the distal portion of the colon. Because of the
intimate contact that must be made with the epithelial cell coupled with the non-invasive nature of *C. rodentium*, the immune response to this pathogen begins with the epithelial cell. Currently much of the focus on the pathology induced by *C. rodentium* has been focused on the actions of immune cells, i.e., inflammatory monocytes, neutrophils, and T cells [125-127]. Although minimal investigation has occurred with regard to the contribution of the intestinal epithelium to the inflammatory response during *C. rodentium* challenge, it is known that epithelial cells are a primary source of nitric oxide through the upregulation of iNOS *in vivo* at the peak of colonization [128]. In addition, the activation of the proinflammatory transcription factors, NF-κB and AP-1 have been shown to be upregulated during *C. rodentium* challenge, which means it is possible the intestinal epithelial cell is a contributor to the inflammatory response to *C. rodentium* [129-131]

**The Inflammatory Response**

The initial immune response to infection and physical tissue damage is the inflammatory response which is characterized by swelling, pain, heat, redness, and even loss of function. During infection, the central goal of the inflammatory response is to recruit cells of the innate and adaptive immune system to the site of infection in order to eliminate the invading pathogen. This evolutionary conserved response commences when resident tissue cells, such as epithelial cells, macrophages, and dendritic cells, use pattern recognition receptors (PRRs) to recognize unique microbial byproducts known as pathogen-associated molecular patterns (PAMPs). Ligation of PRRs by their respective
ligand activates a signaling cascade which concludes with the activation of proinflammatory cytokines, chemokines, and adhesion molecules in order to create an environment capable of recruiting immune cells to destroy the infectious agent to bring and restore the tissue to homeostasis. The resulting inflammation that is induced by the inflammatory response is indispensable for the clearance of pathogen, however it is also just as crucial to be able to ensure that the inflammatory response is not overly robust and does not perpetuate beyond the presence of pathogen as often the tissue damage that is associated with infection is due to the host response.

Pathogen Recognition Receptors and Pathogen-Associated Molecular Patterns

Pathogen recognition receptors are the first line of defense during pathogen infection and without them we would be highly vulnerable to bacterial, viral, and parasitic invaders. Conversely, over-activation of these receptors either by an enhanced pathogen load or a dysregulation in cell signaling can lead to an overzealous immune response which can actually enhance pathology. Toll-like receptors belong to a family of membrane-bound PRR that recognize PAMPs such as peptidoglycan (PG), lipopolysaccharide (LPS), flagellin, double stranded RNA, and unmethylated DNA. Upon activation a conserved signaling cascade is set in motion which coalesces with the activation of the mitogen-activated protein (MAP) kinase signaling pathway and the transcription factor Nuclear Factor-κB (NF-κB). Together, activation of these pathways is responsible for the transcription of proinflammatory cytokines, chemokines, and other effector molecules necessary for inducing the inflammatory response to fight infection.
As it pertains to *C. rodentium* recognition there are two important TLRs: TLR2 and TLR4. Toll-like receptor 2 recognizes PG, a component of both Gram positive and Gram negative bacteria. It has been previously shown that TLR2 signaling in the resident tissue cells during *C. rodentium* challenge is protective while the ligation of TLR4, which recognizes LPS an outer membrane component of Gram negative bacteria, in newly recruited immune cells is detrimental [125, 126].

Members of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family are intracellular PRRs with similar functions as the aforementioned TLRs. The NLR family member Nod1 and Nod2 recognize meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP) subunits of peptidoglycan, respectively. The subunit DAP is found almost exclusively in Gram negative bacteria while MDP is found in both Gram positive and Gram negative bacteria. Similar to that of TLRs, activation of NLRs will induce a signaling cascade which results in the activation of the NF-κB and MAP kinase pathways. *Citrobacter rodentium*, being a Gram negative bacterium, has been shown to activate both Nod1 and Nod2 with differing results [132, 133]. Activation of both receptors was shown to be necessary to elicit the much needed T\(_\text{H}17\) immune response that is involved in clearing pathogen. Nod2 receptor activation in the colonic stromal cells, and potentially the colonic epithelial cells, is necessary to produce the chemokine CCL2 which is responsible for the recruitment of inflammatory monocytes to the colon during *C. rodentium* infection [132]. Without these intracellular receptors mice have enhanced susceptibility to *C. rodentium*.
NF-κB Signaling

The transcription factor NF-κB is ubiquitous to all mammalian cells and its activation can occur via two distinct pathways: classical and non-classical. Traditionally, NF-κB activation via the classical pathway, i.e. dependent on I kappa kinase (IKK) signaling, can lead to the transcription of a plethora of genes involved in apoptosis, cell cycle regulation, inflammation, as well as inhibition of its own signaling (negative feedback). The genes that are transcribed are dependent on which subunits of NF-κB that are activated as well as on other signaling cascades, such as MAP kinases, which can interact with NF-κB to change its phosphorylation and/or acetylation state. During classical activation, the subunits of NF-κB (i.e., p65 and p50) are held in a relatively inactive state within the cytoplasm by the inhibitor of NF-κB (IκB). When the cell is activated, for example by ligation of PRR, there is a signaling cascade which results in the activation of I kappa kinase, IKK. This kinase is comprised of three subunits: IKKα, IKKβ, and IKKγ, with IKKα and IKKβ serving as catalytic subunits involved in the degradation of IκB. During classical activation the IKKβ subunit phosphorylates IκB, causing a conformational change which allows it to release NF-κB. The newly free NF-κB subunits can now move to the nucleus where it can bind to NF-κB-specific response elements in the promoter regions of various target genes including proinflammatory cytokines, e.g. TNF-α, IFN-γ, and IL-1β, chemokines, e.g. CCL2, CXCL1, CX3CL1, and effector molecules, e.g. iNOS and β-defensin 2.
Immune Cell Recruitment

The rapid recruitment of leukocytes capable of killing invading pathogens to the site of infection is vital to early containment of the pathogenic organism. There are tissue resident immune cells found throughout the body which are able to respond to an infectious onslaught both directly and by recruiting additional immune cells to aid in the elimination of pathogen. Upon the recognition of PAMPs by PRRs, proinflammatory cytokines, such as TNF-α and IL-1β, are produced largely through NF-κB activation. The main action of these cytokines during the inflammatory response is to cause nearby blood vessels to become more permeable and upregulate the expression of adhesion molecules, such as P- and E-selectin, ICAM-1 and MadCAM-1. These surface adhesion molecules aid in slowing down circulating immune cells in a process termed tethering and when enough contacts are made through adhesion molecules the circulating immune cell will slow to a rolling pace. In addition to proinflammatory cytokines, chemokines, which are small chemoattractant molecules, will also be produced. The initial chemokines produced include CXCL1 and CCL2 which attract neutrophils and monocytes, respectively. Now that the leukocyte is slowing down on the reactive endothelium the chemokines that are produced can interact with the chemokine receptors found on the leukocyte (i.e., CXCL1-CXCR2, CCL2-CCR2). This interaction causes the integrin receptors, e.g. LFA-1, found on leukocyte to become high affinity receptors which allows for a more firm adhesion of the leukocyte with the vascular endothelium. This firm adhesion can completely stop the leukocyte from moving. The number of contacts between the leukocyte and endothelial cell is numerous and this causes the
leukocyte to flatten along the endothelium. Next there are rearrangements in the actin of the leukocyte as well as the loosening of the tight junctions between the endothelial cells so the leukocyte can move out of the circulation and into the tissue. Once the leukocyte is through the blood vessel and in the tissue it follows a concentration gradient moving to higher and higher concentrations of chemokines and adhesion molecules until it finally reaches the site of infection where it will come in contact with the organism that triggered the inflammatory response.

Neutrophils

Neutrophils are the most abundant immune cell found in the blood. Two distinct features of neutrophils are their multi-lobed nucleus as well as the presence of granules. These granulocytic cells originate from a myeloid cell precursor in the bone marrow. Upon release from the bone marrow, neutrophils enter the circulation where they can live for up to 5-6 days. Because of their abundance in the blood, neutrophils are able to respond to an inflammatory stimulus within minutes which makes them the first responders to most infections. Once a neutrophil enters a tissue it becomes activated and will only live for approximately 2 days. The main chemokine involved in the recruitment of neutrophils is CXCL1, a murine homolog of human IL-8, which interacts with the neutrophil receptor CXCR2. Previous studies have shown that lamina propria CD11b+ dendritic cells are among the primary producers of CXCL1 during C. rodentium challenge [134]. As neutrophils have been shown to produce minimal amounts of proinflammatory cytokines, they mainly contribute to the eradication of pathogen through
either phagocytosis or through the degranulation of preformed vesicles which contain antimicrobial substances such as myeloperoxidase, defensins, and lysozyme. Mice with defective neutrophil recruitment, i.e., CXCR2-deficient mice, have enhanced C. _rodentium_ colonization and prolonged clearance which was coupled with a deeper penetration of pathogen in the colonic crypts [135].

**Monocytes and Macrophages**

Similar to neutrophils, monocytes are derived from myeloid precursor cells within the bone marrow. These effector cells can be found in the circulation or the spleen, or they can migrate to tissues and differentiate into resident tissue macrophages to function as sentinels. Monocytes enter the circulation as effector cells fully capable of phagocytosis, antigen presentation, and cytokine production. In addition to their effector activities, they also serve as precursor cells to macrophages. Resident tissue macrophages are replenished by circulating monocytes because resident tissue cells of the intestine do not proliferate [136]. During normal conditions (i.e., without infection), intestinal epithelial cells and stromal cells will produce the chemokine CXCL1 and cytokine transforming growth factor-β (TGF-β) in order to recruit monocytes from the circulation to the intestinal lamina propria [136, 137]. Once monocytes enter the intestine they will begin to terminally differentiate into resident macrophages, which in the case of the intestine are non-inflammatory, phagocytic cells during normal homeostasis conditions. The reason for the inflammation-anergic nature of intestinal macrophages is because of the large amount of bacterial antigen that is present within the
intestine. If intestinal macrophages retained their inflammatory properties during normal homeostasis the gut would be constantly inflamed. Epithelial and stromal cell-produced TGF-β and IL-10 are largely responsible for the anti-inflammatory nature of resident tissue macrophages which is due to their ability to inhibit NF-κB activation [138, 139].

During a gastrointestinal infection, such as with *C. rodentium*, inflammatory monocytes are recruited from the circulation, spleen and bone marrow through the production of the chemokine CCL2 [132, 140]. This chemokine interacts with its receptor, CCR2, which is found on monocytes that tend to be more inflammatory in nature. Once inflammatory monocytes reach the infected tissue through the interactions with the reactive endothelium of neighboring blood vessels they will produce ample amounts of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 as a result of phagocytosing and killing the invading pathogen. The recruitment of these cells is necessary for the elimination of the bacterial pathogen, as a delay in the recruitment of inflammatory monocytes can allow the pathogen to perpetuate to high levels that may prove difficult for the resident intestinal immune cells to clear alone [132]. A delicate balance must be struck, however, as the over recruitment of inflammatory monocytes, to not only intestinal sites of infection, but all sites of infection, can lead to an enhanced inflammatory state which can result in unwanted tissue damage that can be detrimental to the host organism. In fact, recent research has shown that patients with active Crohn's disease have a significant accumulation of macrophages in the areas of inflamed tissue which, due to the non-chemotactic nature of terminally differentiated macrophages, is
believed to be due to the enhanced recruitment of inflammatory blood monocytes [141-143].

Lymphocytes

The innate immune system is characterized by a non-specific response to an invading organism. Specialized cells of the innate immune response, (i.e., macrophages and dendritic cells) are able to present antigen to cells of the adaptive immune system (i.e. lymphocytes) in order to mount a specific response to the pathogen which includes the production of antigen-specific antibodies as well as the development of immunological memory to protect the organism from a subsequent attack by the pathogen. Lymphocytes are the main cell-type of the adaptive immune response which include T cells and B cells both of which are derived from hematopoietic lymphoid progenitor cells in the bone marrow. Immature T cells will migrate from the bone marrow to the thymus to finish their maturation whereas B cells will remain within the bone marrow. Prior to their encountering antigen, naive B and T cells will enter the circulation and home to secondary lymphoid organs, such as lymph nodes and the spleen. It is here where cells of the innate immune system will present antigen in order to activate antigen-specific T and B cell responses.

Both T and B cell responses are necessary for the clearance of *C. rodentium* from the murine colon. Antibody production by B cells, specifically IgG1, is necessary for the opsonization of *C. rodentium* which is essential for phagocytosis by neutrophils and macrophages [144, 145]. A subclass of T cells, the CD4+ T_H1 helper T cell is also
required for the effective clearance of pathogen [145-147]. These helper T cells will produce the cytokine interferon-γ (IFN-γ) which also helps macrophages by enhancing their bactericidal activity.

Purpose of Study:

Clinicians and patients alike have known for decades that exposure to psychological stressors can exacerbate colitis; however the mechanism by which this occurs remains elusive. Current theories indicate the involvement of the microbiota in the intensification of colitis as the use of antibiotics is a current part of the pharmacological regimen against colitis [148]. In addition to antibiotics, anti-inflammatory drugs, i.e., synthetic glucocorticoids and TNF-α inhibitors, and immunosuppressive drugs are also prescribed to patients in order to relieve colonic inflammation to allow the mucosa to heal. While the current pharmacological strategies do aid in the treatment of disease, they can carry serious side effects of which enhanced susceptibility to infection is the most pertinent. This has led to the investigational use of probiotics as a therapy for patients with colitis.

Current studies within our lab have demonstrated that probiotic intervention with L. reuteri during exposure to social disruption (SDR), a murine stressor, can significantly reduce C. rodentium-induced infectious colitis. The infectious colitis that is enhanced during SDR-exposure is mild; therefore we set out to determine if probiotic intervention could modulate a more severe form of colitis. Chapter 2 investigates the effects of probiotic intervention with L. reuteri on stressor-exacerbated, C. rodentium-induced
infectious colitis. In this chapter the murine stressor, prolonged restraint, is used. Prolonged restraint alone does not induce colitis, however this stressor can exacerbate \textit{C. rodentium}-induced infectious colitis to moderate to severe levels. Chapters 3 and 4 delve deeper to tease apart the mechanism for stressor-enhanced infectious colitis. In Chapter 3, the murine stressor, social disruption, is used to explore the role of newly recruited inflammatory monocytes in stressor-exacerbated infectious colitis by use of genetically modified mice as well as through the transfer of monocytes to naïve recipients. The importance of classical NF-κB signaling in epithelial and myeloid-derived cells of the colon during \textit{C. rodentium} challenge is explored in Chapter 4 along with the effects of \textit{L. reuteri} treatment prior to \textit{C. rodentium} challenge on intestinal epithelial cells and macrophages \textit{in vitro}.

Overall, we have demonstrated that probiotic intervention with \textit{L. reuteri} can reduce stressor-enhanced infectious colitis, however the extent of the reductions were contingent on the severity of colitis. The evidence provided here within demonstrates that stressor-induced changes in the colon, potentially through the reduction of protective species such as \textit{L. reuteri}, can lead to the heightened recruitment of inflammatory monocytes and macrophages which will perpetuate the inflammatory response to \textit{C. rodentium} potentially through an NF-κB-related mechanism.
CHAPTER 2: Probiotic Lactobacillus reuteri attenuates the stressor-enhanced severity of Citrobacter rodentium infection

Introduction

Citrobacter rodentium, along with enteropathogenic and enterohemorrhagic Escherichia coli (EPEC and EHEC, respectively), belongs to the group of non-invasive pathogens collectively known as attaching and effacing (A/E) bacteria. In order to colonize its host, C. rodentium, along with its human homologs, must intimately attach to the intestinal epithelium. This interaction allows the pathogen to inject effector proteins into the host epithelial cell via a type III secretion system. These proteins, which include intimin, Tir, EspF, and Map, are located on the locus of enterocyte effacement pathogenicity island and are involved in the adherence to and the effacement of the colonic epithelium, as well as the formation of actin-rich pedestals beneath the pathogen [118]. Upon colonization of the colonic epithelium, A/E pathogens induce an increase in leukocyte infiltration, inflammatory cytokine production, and structural changes to colonic tissue that is similar to EPEC and EHEC infection as well as both murine and human inflammatory bowel disease [118, 119, 149]. Because mice are not naturally susceptible to intestinal EPEC or EHEC, murine infection with C. rodentium has become a widely used model of intestinal E. coli infection. C. rodentium does not normally cause disease in humans, but has been shown to colonize all strains of mice. The disease can be either subclinical or fatal.
depending on the host mouse strain [120]. Strains that only develop subclinical symptoms, such as CD-1 and C57BL/6, are considered resistant to \textit{C. rodentium}-induced infectious colitis while mice that succumb to infection by \textit{C. rodentium}, such as FVB/N and C3H/HeJ, are deemed susceptible [118, 120, 122]. While it is recognized that host genetics contribute to enhanced susceptibility [120, 122], recent data indicate that the colonic microbiota also modulate host susceptibility to \textit{C. rodentium} in part by altering mucosal immunity [150-152]. Antibiotic treatment can disrupt the natural balance of the microbiota which can leave mice more susceptible to \textit{C. rodentium} infection [153]. Conversely, supplementation with beneficial bacteria can reduce susceptibility to \textit{C. rodentium} infection [154-157].

Animal stressors are useful for our understanding of the impact of the host stress response on infectious diseases. Our laboratory has shown that exposure to laboratory stressors, including the most commonly used experimental stressor in biomedical and biobehavioral research, namely physical restraint [158], significantly changes the structure of the colonic microbiota and reduces the levels of beneficial members of the genus \textit{Lactobacillus} [8, 55, 159, 160]. Because the lactobacilli can help to regulate mucosal inflammation, and mediate resistance/susceptibility to \textit{C. rodentium} [113, 134, 154-157], we hypothesized that exposing resistant CD-1 mice to a prolonged restraint stressor during challenge with \textit{C. rodentium} would significantly increase disease pathology.

It has been long known that exposure to psychological stressors can exacerbate both infectious and noninfectious diseases. And, gastrointestinal (GI) diseases, such as
Crohn’s disease, ulcerative colitis, and enteric infectious disease are often exacerbated during stressful periods [3, 161]. While the mechanisms by which stressor-exposure exacerbates GI diseases are still under investigation, disruptions of gut epithelial permeability are likely involved. The GI tract is lined by a single layer of epithelial cells that keeps the interior of the body separated from the contents of the gut. During stressful life events the gut can become inflamed [162] and the tight junctions that help control what is transported across the epithelial layer are loosened leading to a “leaky gut.” The loosening of tight junctions, chiefly by the proinflammatory cytokine tumor necrosis factor alpha (i.e. TNF-α), allows the contents of the gut, including commensal microbes, to pass through the epithelial layer and enter into the lamina propria.

The penetration of microbes into the lamina propria results in a stronger inflammatory response, in part through activation of mucosal immune cells [10]. Thus, strategies to reinforce the epithelial barrier and suppress mucosal immune activity could be beneficial in preventing stressor-induced exacerbations of GI diseases. One potentially useful strategy involves the administration of probiotic microbes, such as *Lactobacillus reuteri*. The Gram-positive bacterium *L. reuteri* can be found in both humans and laboratory animals, including mice. Many strains of *L. reuteri* are currently being used as probiotics, because research has shown that *L. reuteri* can suppress inflammation and immune cell activity [68, 89, 97, 98]. Moreover, *L. reuteri* has been shown to reduce experimental colitis and prevent experiment-induced intestinal permeability in rats [90, 91]. Because stressor exposure reduces the levels of commensal *L. reuteri* found in the murine colon (Galley and Bailey, Unpublished Observations), this
study tested the hypothesis that stressor exposure would increase the severity of *C. rodentium*-induced colonic pathology in a resistant mouse strain. This study also tested the corollary hypothesis that treating the mice with *L. reuteri* would prevent any stressor-induced increases in disease severity.

**Materials and Methods**

**Mice.** Outbred, male CD-1 mice aged 6-8 weeks were purchased from Charles River Laboratories (Wilmington, MA) and housed 3 mice per cage, unless otherwise indicated, in ventilated polycarbonate cages (32 cm x 18 cm x 15 cm). For all experiments, mice were allowed to acclimate to their surroundings for 1 week prior to experimentation. Lights were maintained on a 12 hr on, 12 hr off cycle (lights on at 0600). Mice were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility and had free access to food and water except during experimental procedures. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at the Ohio State University (2009A0235).

**Prolonged Restraint.** Prolonged restraint was performed as previously described [8, 163]. Briefly, mice were divided into three groups, home cage control (HCC), food and water-deprived (FWD), and prolonged restraint (RST). Restrained mice were placed in well-ventilated 50 ml conical tubes at the beginning of their active cycle (i.e., at 1800) and were removed from the tube the following morning at 0900. Because the stressor-exposed mice do not have access to food or water at the time of restraint, FWD mice had their food and water removed from their cages at the time of restraint but were allowed to freely move about their cage. Both RST and FWD mice were allowed free access to their
food and water from 0900-1800. Mice were restrained or deprived of food and water each night for seven consecutive nights. Control mice were left undisturbed in their cages for the duration of the experiment.

**Infection and bacterial enumeration.** *Citrobacter rodentium* strain DBS120(pCRP1::Tn5) [164] was grown overnight in Difco™ LB Broth, Lennox at 37°C. Cultures were centrifuged and the resulting pellet was resuspended in sterile PBS to give a final concentration of 3 x 10⁹ colony forming units (CFU)/ml. Mice were then challenged at 0900 on the day following the first night of restraint via oral gavage with 100 μl containing 3 x 10⁸ CFU and were food and water deprived for 2 hours post-challenge. Mice in the RST group were restrained during the first 6 days post-challenge. The day of infection is referred to as Day 0 and all data collected will be referenced as days post-challenge. Fecal shedding of *C. rodentium* was monitored on days 1, 3, 6, 12, and 24 post-challenge. Fresh stool was collected from individual mice by placing them in a sterile cage where 1-2 stool pellets were collected per mouse. The stool was then weighed, homogenized manually in 3 ml sterile PBS, and serially diluted at a ratio of 1:20. In order to enumerate *C. rodentium* only, each dilution was grown via pour plate method in MacConkey agar supplemented with the antibiotic kanamycin (40 μg/ml) overnight at 37°C. At the time of sacrifice, spleens were flash frozen in liquid nitrogen. Spleens were then thawed, weighed and suspended in 5ml sterile PBS. Spleens were homogenized using a stomacher (medium speed for 1 min) and then serially diluted and plated in the same fashion as stool samples.
Experiment 1 design. The first set of experiments was designed to test the hypothesis that prolonged stressor exposure would exacerbate C. rodentium-induced infectious colitis. To test the effects of prolonged stressor exposure on the severity of C. rodentium infection, two replicate time course experiments were performed. In the first time course experiment, all mice were housed 3/cage/group (i.e., HCC, FWD, or RST). One cage from each group was sacrificed on Days 1, 3, 6, 12, and 24 post-challenge. In the replicate time course experiment, all mice were housed 3/cage/group with the exception of mice to be sacrificed on Day 12 post-challenge, which were housed 5/cage/group. One cage from each group was sacrificed on Days 0, 1, 3, 6, 12, and 24 post-challenge for a grand total of 105 mice between the two time course experiments. There were no differences in any of the measurements taken from mice that were housed either 3 or 5 per cage on Day 12 post-challenge (analysis not shown). For each experiment all mice were infected with C. rodentium immediately following the first night of stressor exposure, with the exception of mice sacrificed on Day 0, as these mice were used as non-infected baseline controls for colonic gene expression analyses. Stressor exposure continued for 6 more days, after which mice were left alone in their cage throughout the remainder of the experiment. Each time point reflects the day post-challenge. Mice were sacrificed at their designated time point by CO₂ asphyxiation and tissue samples were collected. Because there were no differences between the HCC and FWD control groups for any of the measures assessed, these groups were combined as one control group (CON).
**Probiotic Administration.** *Lactobacillus reuteri* strain 23272 (American Type Culture Collection, ATCC) was grown overnight in Difco™ Lactobacilli MRS Broth at 37°C with 5% CO₂. Cultures were centrifuged and the resulting pellet was resuspended in sterile PBS vehicle (VEH). The bacteria were then added to the drinking water to give a final concentration of $5 \times 10^7$ CFU/ml of drinking water. Mice received either probiotic or VEH in their water for 1 day prior to the first day of stressor exposure until the mice were sacrificed on Day 12 post-challenge. Water supplemented with *L. reuteri* was monitored throughout the duration of the experiment to ensure viability. One ml of water was removed via the lixit and serially diluted 1:20. Each dilution was grown in Difco™ Lactobacilli MRS Agar at 37°C with 5% CO₂. Mice typically consumed 3 ml of the fluid per day, corresponding to approximately $1.5 \times 10^8$ CFU/ml.

**Experiment 2 design.** The second set of experiments was designed to test the hypothesis that probiotic intervention could prevent stressor-enhanced disease severity associated with *C. rodentium* challenge. Because our initial set of experiments demonstrated that the peak of *C. rodentium*-induced infectious colitis was on Day 12 post-challenge, our second set of experiments was focused on Day 12 post-challenge. Our hypothesis was tested over 3 replicate experiments involving 6 experimental groups (i.e., HCC/VEH, HCC/*L. reuteri*, FWD/VEH, FWD/*L. reuteri*, RST/VEH, RST/*L. reuteri*). Mice were housed 3/cage/group for a total of 54 infected mice. Non-infected control mice were used as baseline control samples for Real Time PCR analysis (4 cages of non-infected control mice housed at 3 mice/cage for a total of 12 non-infected control mice). Stressor exposure and *C. rodentium* challenge were the same as the first set of
experiments, but mice were given probiotic \textit{L. reuteri} in their water beginning 1 day prior to stressor exposure and continuing through Day 12 post-challenge. Mice were sacrificed on Day 12 post-challenge by \textit{CO₂} asphyxiation and tissue samples were collected. Because there were no differences between the HCC and FWD mice within treatment groups for any of the measures assessed in the second set of experiments, these groups were combined based on treatment condition (CON/VEH and CON/\textit{L. reuteri}).

\textbf{Histopathology.} On days 0, 1, 3, 6, 12, and 24 post-challenge colons were excised and cut in half longitudinally where one section was used for further analysis of colonic cytokines by Real Time PCR and the other was fixed in 10\% formalin-buffered phosphate for 24-48 hrs. Samples were embedded in paraffin and 5 μm-thick sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation by a board-certified veterinary pathologist (N.M.A.P.) who was blinded to treatment groups. The severity of lesions in the colon was scored, according to previously defined criteria \cite{165}. Briefly, each colon was scored on 5 different categories; inflammation, dysplasia, hyperplasia, edema, and epithelial defects. Each category received a score of 0 to 4 in 0.5 increments based on the degree of lesion severity: 0 (absent), 1 (mild), 2 (moderate), 3 (marked), and 4 (severe). All 5 categories were added together to garner a total histologic colitis index score with a maximum value of 20.

\textbf{Semiquantitative Real-Time PCR.} Colons were harvested and total RNA was isolated using a standard single-step isolation protocol (TRI-zol, Invitrogen). After isolation, total RNA was spectrophotometrically quantified. A reverse transcription reaction was performed to synthesize cDNA using the Avian Myeloblastosis Virus (AMV) Reverse
Transcriptase enzyme kit (Promega Corporation, Madison, WI). These samples then underwent Real-Time Polymerase Chain Reaction (PCR), in order to amplify the nucleotide sequences of interest. In all cases, 18S rRNA was used as the housekeeping gene. The sequences of primers and probes used can be found in Table 1. SYBR green was used in place of a labeled probe sequence for β-defensin 3, Claudin 1, and Claudin 5. The PCR reaction took place in the Prism 7000 Sequence Detection System and began with 2 min at 50°C, 10 min at 95°C, and then 40 amplification cycles of 15 sec at 95°C, and 1 min at 60°C. The relative amount of mRNA was determined using the comparative cycle threshold method (Ct) as previously described [8, 163]. The baseline control samples for Experiment 1 were control samples from Day 0 and the Day 12 non-infected control samples for Experiment 2. Each control sample was set at a value of 1 and was used as a reference for all other experimental samples.

**Sickness Assessment.** On Day 12 post-challenge mice were subjected to behavioral testing on the open field. The open field consists of a 30 cm x 30 cm x 25 cm plexiglass box. A 6 x 6 grid was drawn on the floor in order to test locomotion. Each mouse was placed in the open field separately and video recorded for 5 minutes. This test is designed to take advantage of the natural exploratory behaviors of mice. Mice are naturally curious about new environments and will explore, unless this desire to explore is overridden by illness [166, 167]. Sickness behavior was assessed by counting the total number of lines that were crossed by all 4 paws of each mouse within the 5 minute time period. Open spaces are avoided by mice due to the perceived risk of predation, however their curious nature will cause them to eventually explore the open space of the center of
the open field. The time spent in the center of the open field was recorded. Mice with more anxiety-like behaviors will spend less time in the center of the open field [168].

**Cytokine Analysis.** Blood was collected by cardiac puncture immediately following sacrifice. Serum was collected by centrifuging samples at 1500g for 10 minutes at 4°C where it was stored at -80°C. Serum was diluted 4-fold with serum dilution buffer (BioRad). A commercially available multiplex bead-based cytokine immunoassay (BioPlex, BioRad Laboratories Inc., Hercules, CA) was used to measure the content of interleukin-1β (IL-1β), IL-2, IL-6, IL-10, IL-12(p70 subunit), interferon gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) according to manufacturer’s protocol. Results were expressed as picograms/mL recovered from serum. The lower limits of detection were 14.1 pg/mL IL-1β, 0.4 pg/mL for IL-2, 0.5 pg/mL for IL-6, 1.4 pg/mL for IL-10, 0.5 pg/mL for IL-12(p70), 0.1 pg/mL for IFN-γ, and 6.0 pg/mL for TNF-α.

**Statistical Analysis.** Stressor-induced changes in *C. rodentium* colonization, gene expression, and serum cytokine levels were analyzed by a two-factor analysis of variance (ANOVA), with group (CON vs RST) and day as between factors for the first set of experiments and stressor exposure (CON vs RST) and probiotic treatment (VEH vs *L. reuteri*) as between factors for the second set of experiments. Analyses were conducted using IBM SPSS for Windows (SPSS, Chicago, IL). Post-hoc analysis comprised of two-tailed Student’s t-test with Bonferroni correction applied. Chi-squared tests were performed on pathogen prevalence in the spleen. P values of less than 0.05 were considered significant.
Results

Prolonged restraint enhances pathogen colonization and colonic histopathology.

Exposure to RST changed *C. rodentium* levels throughout the entire 24 day experiment as compared to their control counterparts \[F(4, 84) = 3.57, P=0.01; \text{Figure 1A}\]. This effect was mainly due to significant differences in colonization in the RST group on Days 1, 6, and 12 post-challenge (P<0.005). While the peak of colonization was on Day 6, we did not observe any significant colonic pathological changes until Day 12 post-challenge. Increased colon mass due to epithelial cell hyperplasia, a hallmark of *C. rodentium* infection, was significantly increased on Day 12 post-challenge in the stressor-exposed group \[F(1,13) = 38.1, P<0.001; \text{Fig. 1B}\]. Total colonic histopathology, which is the combination of the scores given to inflammation, edema, hyperplasia, dysplasia, and epithelial defects, was also significantly increased by prolonged restraint \[F(2, 45) = 8.2, P=0.001; \text{Fig. 1C}\]. Control colons had a relatively normal appearance with small numbers of lymphocytes infiltrating the mucosa (Fig. 1D). However, stressor-increased histopathology was evidenced by mucosal thickening, regional ulcerations in the surface epithelium, as well as significant inflammatory cell infiltrate that extends from the mucosa to the edematous submucosa (Fig. 1E).

Prolonged restraint enhances inflammatory mediator gene expression in the colons of infected mice. Colonic inflammatory mediator mRNA expression (i.e., CCL2, TNF-α, and iNOS) was assessed using Real-Time PCR. Colonic CCL2 mRNA expression was significantly increased by stressor-exposure throughout the 24 day experiment as
compared to their control counterparts \[F(5, 90) = 4.5, P=0.001; \text{Figure } 2A\]. Mice exposed to prolonged restraint also had enhanced colonic TNF-\(\alpha\) mRNA expression over non-stressed controls \[F(5,93) = 9.2, P<0.001; \text{Fig } 2B\]. Similar to CCL2 and TNF-\(\alpha\), colonic iNOS gene expression was significantly increased throughout the 24 day experiment as compared to colons of control mice \[F(5,93) = 45.3, P<0.0001; \text{Fig } 2C\]. The most evident increase in colonic CCL2, TNF-\(\alpha\), and iNOS mRNA expression occurred on Day 12 post-challenge.

*Prolonged restraint causes significant changes in spleens of infected animals.* One of the main characteristics of prolonged restraint is the reduction in spleen mass \[169\] which was observed on days 1, 3, and 6 post-challenge (i.e. after 2, 5, and 7 days of restraint) in stressor-exposed mice (Fig. 3A). But, this reduction was not evident throughout the experiment; there were significant changes in spleen mass across the 24 day experiment \[F(4, 76) = 20.1, P<0.001\]. To determine whether the unexpected increase in spleen mass on Day 12 post-challenge in stressor-exposed mice was associated with an increased escape of *C. rodentium* from the colon and translocation to the spleen, we set out to determine if *C. rodentium* could be cultured from the spleen. On Days 0, 1, 3, 6, and 24 post-challenge there were no countable colonies in the CON or RST groups (data not shown). However, on Day 12 post-challenge there was an increase in the likelihood (88% vs. 25%) of observing *C. rodentium* in the spleens of stressor-exposed mice \[\chi^2(1) = 8.21, P<0.005; \text{Fig. } 3B\]. Not only was there an increase in the likelihood of detecting *C. rodentium* in the spleens of stressor exposed mice, there was also enhanced pathogen
burden in those mice found to have *C. rodentium* in the spleen \(F(1, 22) = 10.0, P<0.01\); Fig. 3C].

*Probiotic intervention does not reduce RST-enhanced pathogen colonization or colonic histopathology.* As with the previous experiment, stressor exposure significantly increased fecal *C. rodentium* levels on Day 12 post-challenge \(F(1, 50) = 17.2, P<0.0001\); Figure 4A]. There were no changes in colonization between vehicle (VEH)-treated and *L. reuteri*-treated groups. Treatment with *L. reuteri* also had no effect on the stressor-induced increase in colonic histopathology. Colon mass on Day 12 post-challenge was significantly increased in both the VEH and *L. reuteri* treated stressor-exposed mice when compared to control mice \(F(1, 49) = 24, P<0.001\); Fig. 4B]. Total colonic histopathology was also increased in the RST mice, compared to the CON mice \(F(1, 28) = 9.4, P=0.005\); Fig. 4C]. In no case did treatment with *L. reuteri* impact colonic histopathology; histopathology was similar in VEH-treated and *L. reuteri*-treated mice. No lesions were observed in mice from either control group (Fig. 4D, 4E). Stressor-enhanced histopathology was evidenced by lesions containing inflammation, epithelial defects, hyperplasia, and dysplasia. Neutrophilic inflammation extended from the mucosa to the submucosa and was frequently associated with epithelial erosion and ulceration (Fig. 4F, 4G).

*Probiotic intervention reduces stressor-enhanced colonic gene expression.* Colonic inflammatory mediator gene expression was previously shown to be increased on Day 12
post *C. rodentium* challenge in the RST group as compared to their control counterparts. Stressor-exposure caused a significant increase in colonic CCL2 mRNA gene expression on Day 12 post-challenge [F(1, 49) = 22.4, P<0.001; Figure 5A]. While *L. reuteri* treatment reduced the mean level of CCL2 mRNA, the *L. reuteri*-induced reduction was not large enough to be considered statistically significant. Similarly, colonic TNF-α gene expression also experienced a significant increase in stressor-exposed mice as compared to non-stressed, infected controls [F(1, 50) = 25.3, P<0.001; Fig. 5B] with a non-significant reduction in *L-reuteri*-treated, stressor-exposed mice. Stressor-exposure also caused a significant increase in colonic iNOS gene expression [F(1, 50) = 35.2, P<0.001; Fig. 5C]. This stressor-enhanced increase of colonic iNOS mRNA was unchanged by *L. reuteri* treatment. The antimicrobial peptide β-defensin 3 was significantly reduced in the colons of *C. rodentium*-challenged, stressor-exposed mice as compared to infected, non-stressed controls [F(1, 30) = 6.5, P<0.05; Fig. 5D]. This stressor-enhanced 5-fold decrease in β-defensin 3 gene expression in colons of VEH-treated mice was reversed by *L. reuteri* treatment. There was a significant reduction in colonic claudin 1 mRNA expression in stressor-exposed mice as compared to non-stressed controls ([F(1, 30) = 15.0, P=0.001; Fig. 5E], an effect which was unchanged by *L. reuteri* treatment. Claudin 5 was also significantly reduced in stressor-exposed mice as compared to non-stressed controls [F(1, 29) = 6.0, P=0.01; Fig 5F]). The observed stressor-enhanced decrease in VEH-treated mice was reversed by *L. reuteri* treatment.
Probiotic intervention reduces stressor-enhanced pathogen dissemination to the spleen.

On Day 12 post-challenge spleen mass was significantly changed by *L. reuteri*-supplementation \([F(1, 50) = 7.6, P<0.01; \text{Figure } 6A]\). There was a significant increase in Day 12 spleen mass in stressor-exposed, VEH-treated mice which was prevented by *L. reuteri* treatment. On Day 12 post-challenge, there was a significant increase in the likelihood of discovering *C. rodentium* in stressor-exposed mice as compared to non-stressed mice \(\chi^2(1) = 4.75, P<0.05; \text{Fig. } 6B\). While there were no significant changes in pathogen dissemination in VEH-treated and *L. reuteri*-treated RST groups, there was a 24% drop in pathogen prevalence in spleens from *L. reuteri*-treated, stressor-exposed mice (78% vs. 54%). Pathogen burden was also significantly increased in stressor-exposed mice as compared to non-stressed control mice \([F(1, 47) = 6.1, P<0.05; \text{Fig. } 6C]\). This effect was diminished in *L. reuteri*-fed, stressor exposed mice.

Probiotic intervention reduces anxiety-like behaviors and systemic IL-6. Exposure to prolonged restraint during *C. rodentium* challenge did not impact locomotion in a novel environment (Figure 7A). However, there was a trend for an interaction between stressor exposure and probiotic treatment (i.e., *L. reuteri*) in that mice that were fed *L. reuteri* had a reduction in the amount of time spent in the center of the open field as compared to stressor-exposed, VEH-treated mice \([F(1, 49) = 3.30, p=0.075; \text{Fig. } 7B]\). Even though this interaction was not quite statistically significant using a two factor ANOVA, we tested our *a priori* hypothesis that VEH-treated mice exposed to the stressor would spend less time in the center of the open field compared to VEH-treated controls. As predicted,
stressor exposure significantly reduced the time in the center of the open field in VEH-treated, stressor-exposed mice as compared to VEH-treated control mice [t(25) = 2.31, p<0.05; Fig. 7B] an effect which was not observed in stressor-exposed mice treated with L. reuteri.

Serum IL-1β levels were significantly reduced in stressor-exposed mice as compared to control groups [F(1, 34) = 10.2, P<0.01; Fig. 7C]. Lactobacillus reuteri treatment did not change serum IL-1β levels in either control or stressor-exposed groups. However, there were changes in serum levels of IL-6 [F(1, 36) = 7.3, P=0.01; Fig. 7D]. On Day 12 post-challenge there was a significant increase in circulating IL-6 in VEH-treated, stressor-exposed mice as compared to VEH-treated control mice. This stressor-enhanced increase in circulating IL-6 was blocked in stressor-exposed mice that were treated with L. reuteri. There were no significant changes in serum levels of IL-12p70, IFN-γ, TNF-α, IL-10, or IL-2 in between CON or RST mice in either treatment group (Table 2).

Discussion

The results of this study confirm previous reports that outbred CD-1 mice do not develop severe colitis when challenged with C. rodentium [118, 121, 123]. Low levels of the pathogen were evident in the stool of non-stressed control mice orally challenged with 300 million CFUs of C. rodentium, and these control mice developed very low levels of colitis. However, simply exposing these mice to a prolonged restraint stressor during pathogen challenge was sufficient to significantly increase disease severity. Stressor exposure significantly increased C. rodentium levels in the colon, and
significantly increased pathogen-induced chemokines and cytokines. These inflammatory mediators aid in the recruitment of immune cells to help fight infection and also contribute to the eradication of pathogen. However, overexpression of these genes can contribute to colonic tissue damage and immunopathology. Even though all mice were orally challenged with *C. rodentium*, chemokine and cytokine mRNA levels in the colons of mice exposed to the stressor during challenge with *C. rodentium* were 5-250 times higher than the levels found in non-stressed control mice. One of these chemokines, namely CCL2, is known to recruit inflammatory monocytes to the colon [170, 171], and inflammatory monocytes are prolific producers of TNF-α and iNOS [171]. Thus, it is possible that overexpression of CCL2 recruits a larger number of inflammatory monocytes to the colon, ultimately leading to higher TNF-α and iNOS levels. While this hypothesis has not yet been completely tested, the colonic histopathology is consistent with this rationale. The total colitis index, which incorporated a scoring of the infiltration of leukocytes, ranged from 0.5 - 7 in the control mice to 10 - 15 in the stressor exposed mice. This colitis, which in stressor-exposed mice included epithelial erosion and ulceration, further demonstrates the impact that stressor exposure can have on even “non-susceptible” hosts.

Susceptibility and resistance to *C. rodentium* is largely dependent upon host genetics, but studies have shown that intestinal microbes, including probiotics, can also impact disease severity. In a previous study we demonstrated that exposure to the prolonged restraint stressor was sufficient to significantly alter microbial communities in the cecum [8]. More recent data also indicate that colonic tissue-associated microbiota
can be affected in mice exposed to prolonged restraint, with a reduction in *L. reuteri* often being evident in the stressor-exposed mice (data not shown). *Lactobacillus reuteri* has been shown to reduce colitis in germfree mice challenged with the closely related A/E pathogen EHEC [88]. Thus, it was predicted that treating mice with *L. reuteri* would attenuate the stressor-induced increase in colonic pathology in *C. rodentium*-challenged mice.

Treating mice with *L. reuteri* had mild effects on *C. rodentium*-induced colitis. Treatment with *L. reuteri* did reduce the mean colonic levels of CCL2, TNF-α, and iNOS mRNA in mice exposed to the stressor during challenge with *C. rodentium*. In addition, the mean colonic histopathology score was reduced in *L. reuteri*-treated mice exposed to the stressor. But in general these differences were not quite large enough to be statistically significant which may be due to the cage-level influences of supplementation of *L. reuteri* in the drinking water. Along with the inflammatory mediators TNF-α and iNOS, antimicrobial peptides, such as β-defensins, also contribute to the eradication of pathogens [172]. Stressor-exposure during challenge with *C. rodentium* caused a significant reduction in β-defensin 3 in VEH-treated mice which was reversed with *L. reuteri* treatment.

Throughout the duration of the experiment it was consistently noted that stressor-exposed mice that were treated with *L. reuteri* exhibited fewer signs of illness (e.g., cleaner fur and more active) compared to VEH-treated, stressor-exposed mice. Because overt illness behaviors can signify that the pathogen has escaped the colon and translocated to systemic sites, such as the spleen, we further investigated whether *C.
rodentium could be detected at systemic sites and whether systemic signs of illness were evident. Exposure to the stressor during challenge with *C. rodentium* resulted in a significant increase in the likelihood of detecting *C. rodentium* in the spleen. This is important, because unlike invasive gastrointestinal pathogens, such as *Salmonella* species, *C. rodentium* does not have mechanisms to invade its host [118, 173].

*Citrobacter rodentium* and its attaching and effacing *E. coli* relatives stay within the digestive tract attached to the apical surface of the colonic epithelium during infections in an immunocompetent host [118, 173]. However, during infection, *C. rodentium* can disrupt the tight junctions found between intestinal epithelial cells via the injection of effector proteins through a Type III secretion system into the host epithelial cell. Among the inserted proteins are *E. coli* secreted protein F (EspF) and mitochondria associated protein (Map), which have been implicated in the disruption of tight junctions via changes in the expression and location of two integral proteins involved in the formation of the tight junction, namely claudin 5 and zonula occludins-1 [125, 174]. The non-invasive nature of *C. rodentium*, coupled with the fact that the tight junctions between intestinal epithelial cells help to regulate what can and cannot pass through the intestinal epithelial layer, led us to hypothesize that our stressor-enhanced translocation of pathogen from the colon to the spleen may have been due to the loosening of the epithelial tight junctions. In our model, we observed significant reductions in gene expression of both claudin 1 and claudin 5 in the colons of stressor-exposed mice. Importantly, treatment with *L. reuteri* did not change *C. rodentium* levels in the colon; however it was able to ameliorate the stressor-enhanced reduction in claudin 5 which we
believe contributed to the reduction of pathogen translocation from the colon to the spleen.

The ability of *L. reuteri* to reduce the translocation of *C. rodentium* from the colon to the spleen, potentially through stabilization of tight junction proteins, suggested that *L. reuteri* treatment would prevent the systemic manifestations of stressor-exposure during *C. rodentium* challenge. Indeed, mice exposed to the stressor during *C. rodentium* challenge had significantly higher levels of circulating IL-6, but lower levels of IL-1β. Pathogen challenge often leads to behavioral changes in mice, in large part through the actions of circulating cytokines on brain biology. Previous studies have shown that elevations in circulating IL-6 can lead to the development of illness behavior and anxiety-like behavior in mice [175]. In our study, treating mice with *L. reuteri* ameliorated the stressor-enhanced reduction in the amount of time mice spent in the center of the open field, suggesting that *L. reuteri* reduces anxiety-like behavior in mice exposed to the prolonged restraint stressor during *C. rodentium* challenge. When taken together, our data indicate that treatment with *L. reuteri* can attenuate the stressor-induced increases in pathogen translocation from the colon to the spleen, elevations in circulating IL-6, and anxiety-like behavior. Future studies will determine whether these factors are causally related and will determine whether stabilization of the epithelial tight junctions are necessary for the beneficial effects of *L. reuteri*.

Our study demonstrates the adverse effects of prolonged stressor exposure on gastrointestinal infection and disease progression, and suggests that probiotics may be beneficial for some, but not all, of the stressor effects. This may be particularly important
for diseases like post-infectious irritable bowel syndrome (PI-IBS) where infection
severity and stressor exposure are both known to be predisposing factors [22].
Understanding the mechanisms by which probiotics can impact gastrointestinal diseases,
and the conditions under which they have beneficial effects, will facilitate that rationale
design and use of probiotic microbes.
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Primers were purchased from Invitrogen (Carlsbad, CA), and the probes were purchased from Applied Biosystems (Foster City, CA).

Table 1. Sequences of primers and probes used for Real Time PCR.
Figure 1. Stressor exposure enhances *C. rodentium*-induced infectious colitis.

(A) To determine the extent of colonization by *C. rodentium*, stool samples were collected on days 0, 1, 3, 6, 12, and 24 post-challenge. Exposure to restraint stress significantly increased *C. rodentium* colonization as compared to CON mice. * indicates p<.005 RST vs. CON on each day post-challenge. † indicates p=.07 RST vs. CON on Day 3 post-challenge. Sample sizes are: Day 1, n = 11 CON and n = 5 RST; Days 3, 6, and 24, n = 12 CON and n = 5 RST; Day 12, n = 16 CON and n = 8 RST. (B) Colons were removed on Day 12 and weighed without contents. Exposure to prolonged restraint significantly increased the weight of the colonic tissue as compared to CON mice. * indicates p<0.0001 CON vs. RST. Sample sizes are: n = 10 CON and n = 5 RST. (C) On days 6, 12, and 24 post-challenge, colons were removed, fixed in formalin, and then subsequently embedded in paraffin. Colons were sectioned and stained with hematoxylin and eosin in order to visualize and score the pathology present in each sample. Stressor exposure significantly increased total pathology scores. * indicates p<.0001 between RST and CON on Day 12 post-challenge. Sample sizes are: Day 6, n = 6 CON, n = 3 RST; Day 12, n = 16 CON, n = 8 RST; Day 24, n = 12 CON, n = 6 RST. (D) Representative image of H&E stained colon from a CON mouse on Day 12 post-challenge. (E) Representative image of H&E stained colon from an RST mouse on Day 12 post-challenge. * marks a region with significant inflammatory cell infiltration, whereas X marks regional ulcerations in the surface epithelium. Data are the mean ± standard error.
Figure 2. Stressor exposure during infection with *C. rodentium* enhances colonic inflammatory mediator gene expression.

Mice were restrained for 1 day prior and 6 days post oral challenge with *C. rodentium*. Mice were sacrificed on days 0, 1, 3, 6, 12, and 24 post-challenge. Colons were processed in order to quantify gene expression by Real Time PCR. Stressor exposure significantly increased gene expression of (A) CCL2 on Day 12 post-challenge. * indicates p<0.0005 between RST and CON on Day 12 post-challenge. Sample sizes are: Day 0 *n* = 6 CON, *n* = 3 RST; Day 1 *n* = 11 CON, *n* = 5 RST; Days 3 and 6 *n* = 12 CON, *n* = 6 RST; Day 12 *n* = 16 CON, *n* = 8 RST; Day 24 *n* = 11 CON, *n* = 6 RST. (B) Prolonged restraint significantly increased TNF-α gene expression on Days 6 and 12 post-challenge. † indicates p<0.06 between RST and CON on Day 6 post-challenge. * indicates p<0.0005 between RST and CON on Day 12 post-challenge. Sample sizes are: Day 0 *n* = 6 CON, *n* = 3 RST; Days 1, 3, 6, and 24 *n* = 12 CON, *n* = 6 RST; Day 12 *n* = 16 CON, *n* = 8 RST. (C) Exposure to prolonged restraint also significantly increased iNOS mRNA expression on Days 3, 6, and 12 post-challenge. * indicates p<0.005 between RST and CON on each day post-challenge. Sample sizes are: Day 0 *n* = 6 CON, *n* = 3 RST; Days 1, 3, 6, and 24 *n* = 12 CON, *n* = 6 RST; Day 12 *n* = 16 CON, *n* = 8 RST. Data are the mean ± standard error.
Figure 3. Stressor exposure enhances the translocation of *C. rodentium* from the colon to the spleen.

Mice were restrained for 1 day prior to oral challenge with *C. rodentium* and then 6 days post-challenge. Spleens were removed, weighed, and processed in order to enumerate *C. rodentium* by pour plate method. (A) Stressor exposure significantly increased the size of spleens on Day 12 post-challenge as compared to CON mice. * indicates p<0.00001 between CON and RST on Day 12 post-challenge. Sample sizes are: Day 1 *n* = 6 CON, *n* = 3 RST; Days 3 and 6 *n* = 12 CON, *n* = 6 RST; Day 12 *n* = 16 CON, *n* = 8 RST; Day 24 *n* = 11 CON, *n* = 6 RST. (B) Stressor exposure increased the likelihood of pathogen translocation from the colon to spleen on Day 12 post-challenge. * indicates p<0.005 between CON and RST as assessed by chi-squared (χ²) analysis. Sample sizes are: *n* = 16 CON, *n* = 8 RST. (C) Stressor exposure also significantly increased the pathogen burden in the spleen. * indicates p<0.01 between CON and RST. Sample sizes are: *n* = 16 CON, *n* = 8 RST. Horizontal line in panel C represents *C. rodentium* detection limit. Data are the mean ± standard error.
Mice were restrained for 1 day then orally challenged with *C. rodentium*. Restraint continued for 6 days post-challenge during which the drinking water was either supplemented with PBS vehicle (VEH) or *L. reuteri* beginning 1 day prior to RST. (A) To determine the extent of colonization by *C. rodentium*, stool samples were collected on Day 12 post-challenge. Exposure to restraint stress significantly increased *C. rodentium* colonization as compared to CON mice regardless of treatment. * indicates an ANOVA main effect for group (CON < RST, p<0.0001). Sample sizes are: *n* = 18 CON/VEH, *n* = 18 CON/ *L. reuteri*; *n* = 9 RST/VEH, *n* = 9 RST/ *L. reuteri*. (B) Colonic tissue was removed on Day 12 post-challenge and weighed without contents. Exposure to prolonged restraint significantly increased the weight of the colonic tissue as compared to CON mice regardless of probiotic intervention. * indicates an ANOVA main effect for group (CON < RST, p<0.0001). Sample sizes are: *n* = 18 CON/VEH, *n* = 17 CON/ *L. reuteri*; *n* = 9 RST/VEH, *n* = 9 RST/ *L. reuteri*. On Day 12 post challenge, colons were removed, fixed in formalin, and embedded in paraffin. Colons were sectioned and stained with hematoxylin and eosin in order to visualize and score the pathology present in each sample. (C) Stressor-exposure enhanced colonic histopathology in both VEH and *L. reuteri*-treated mice. * indicates an ANOVA main effect for group (CON < RST, p=0.005). Sample sizes are: *n* = 11 CON/VEH, *n* = 12 CON/ *L. reuteri*; *n* = 5 RST/VEH, *n* = 4 RST/ *L. reuteri*. (D) Representative image of H&E stained colon from a CON/VEH mouse. (E) Representative image of H&E stained colon from a CON/ *L. reuteri* mouse. (F) Representative image of H&E-stained colon from a RST/VEH mouse. (G) Representative image of H&E-stained colon from a RST/ *L. reuteri* mouse. * marks an area with significant inflammatory cell infiltrate, X marks an area with surface epithelial erosions, and M marks dysplasia due to disorganized mucosal glands. Data are the mean ± standard error.
Figure 5. Probiotic intervention reduces stressor-enhanced colonic gene expression during the peak of infection.

(A) Stressor exposure significantly increased gene expression of CCL2 regardless of treatment. * indicates an ANOVA main effect for group (CON < RST, p<0.001). Sample sizes are: n = 17 CON/VEH, n = 18 CON/L. reuteri; n = 9 RST/VEH, n = 9 RST/L. reuteri. (B) Stressor exposure also significantly enhanced TNF-α gene expression in both VEH and L. reuteri-treated mice. * indicates an ANOVA main effect for group (CON < RST, p<0.001). Sample sizes are: n = 18 CON/VEH, n = 18 CON/L. reuteri; n = 9 RST/VEH, n = 9 RST/L. reuteri. (C) Stressor exposure significantly increased colonic iNOS gene expression in both VEH and L. reuteri-treated mice. * indicates an ANOVA main effect for group (CON < RST, p=0.001). Sample sizes are: n = 12 CON/VEH, n = 12 CON/L. reuteri; n = 6 RST/VEH, n = 4 RST/L. reuteri. (D) Stressor-exposure significantly reduced β-defensin 3 gene expression only in VEH-treated mice. * indicates p=0.05 between CON/VEH and RST/VEH as assessed by Student’s t-test with Bonferroni correction factor as a post-hoc test. Sample sizes are: n = 12 CON/VEH, n = 12 CON/L. reuteri; n = 6 RST/VEH, n = 4 RST/L. reuteri. (E) Claudin 1 was also significantly reduced in RST mice regardless of the treatment group. * indicates an ANOVA main effect for group (CON < RST, p<0.001). Sample sizes are: n = 12 CON/VEH, n = 12 CON/L. reuteri; n = 6 RST/VEH, n = 4 RST/L. reuteri. (F) Claudin 5 was significantly reduced by RST only in VEH-treated mice. * indicates p<0.05 between CON/VEH and RST/VEH as assessed by Student’s t-test with Bonferroni correction factor as a post-hoc test. Sample sizes are: n = 12 CON/VEH, n = 12 CON/L. reuteri; n = 5 RST/VEH, n = 4 RST/L. reuteri.

Data are the mean ± standard error.
Figure 6. Probiotic intervention reduces stressor-enhanced pathogen translocation to the spleen.

Mice were restrained for 1 day then orally challenged with *C. rodentium*. Restraint continued for 6 days post-challenge during which the drinking water was either supplemented with VEH or *L. reuteri* beginning 1 day prior to RST. Spleens were removed, weighed, and processed in order to enumerate *C. rodentium* by pour plate method. (A) Stressor exposure significantly increased the size of spleens on Day 12 post-challenge in VEH-treated mice as compared to CON/VEH mice. This effect was blocked in stressor-exposed mice that received *L. reuteri*. ** indicates p<0.00001 between CON/VEH and RST/VEH. * indicates p<0.01 between RST/VEH and RST/L. reuteri. Sample sizes are: n = 18 CON/VEH, n = 18 CON/L. reuteri; n = 9 RST/VEH, n = 9 RST/L. reuteri. (B) Stressor exposure significantly enhanced the likelihood of detecting pathogen in the spleens in both VEH and *L. reuteri*-treated mice. * indicates p<0.05 between CON and RST as assessed by chi-squared ($\chi^2$) analysis. Sample sizes are: n = 17 CON/VEH, n = 17 CON/L. reuteri; n = 8 RST/VEH, n = 9 RST/L. reuteri. (C) Stressor exposure significantly increased pathogen load in the spleens of mice regardless of treatment. * indicates an ANOVA main effect for group (CON < RST, p<0.05). Sample sizes are: n = 17 CON/VEH, n = 17 CON/L. reuteri; n = 8 RST/VEH, n = 9 RST/L. reuteri. The horizontal line in panel C represents *C. rodentium* detection limit. Data are the mean ± standard error.
Figure 7. Probiotic intervention reduces stressor-enhanced anxiety-like behavior and significantly reduces circulating IL-6 at the peak of infection.

(A) There were no changes in the total number of lines crossed in either stressor-exposed group. Sample sizes are: n = 12 CON/VEH, n = 12 CON/L. reuteri; n = 6 RST/VEH, n = 4 RST/L. reuteri. (B) There was an increase in anxiety-like behavior as evidenced by a reduction in the time spent in the center of the open field in VEH-treated, stressor-exposed mice, which was restored with L. reuteri treatment. * indicates p<0.05 between CON/VEH and RST/VEH. Sample sizes are: n = 18 CON/VEH, n = 18 CON/L. reuteri; n = 9 RST/VEH, n = 9 RST/L. reuteri. Cytokine levels were determined by Bio-Plex® assay. (C) Circulating IL-1β was reduced by stressor-exposure on Day 12 post challenge and L. reuteri failed to modulate this reduction. * indicates an ANOVA main effect for group (CON > RST, p=0.01). Sample sizes are: n = 13 CON/VEH, n = 11 CON/L. reuteri; n = 7 RST/VEH, n = 7 RST/L. reuteri. (D) Circulating IL-6 was significantly increased by stressor-exposure in VEH-treated mice on Day 12 post challenge. Probiotic intervention with L. reuteri reduced stressor-enhanced circulating IL-6 levels. * indicates p<0.001 between CON/VEH and RST/VEH. Sample sizes are: n = 14 CON/VEH, n = 13 CON/L. reuteri; n = 7 RST/VEH, n = 6 RST/L. reuteri. Data are the mean ± standard error.
Table 2. Serum Cytokine Levels.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CON-VEH</th>
<th>CON-<em>L. reuteri</em></th>
<th>RST-VEH</th>
<th>RST-<em>L. reuteri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12 p70</td>
<td>25.0 +/- 2.9</td>
<td>21.5 +/- 4.1</td>
<td>21.7 +/- 6.8</td>
<td>14.0 +/- 4.0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>9.6 +/- 1.4</td>
<td>9.7 +/- 2.4</td>
<td>4.7 +/- 1.6</td>
<td>6.6 +/- 2.4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>65.6 +/- 12.5</td>
<td>71.1 +/- 12.5</td>
<td>46.2 +/- 14.3</td>
<td>44.8 +/- 10.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>140.5 +/- 18.1</td>
<td>126.2 +/- 23.4</td>
<td>140.6 +/- 42.6</td>
<td>84.6 +/- 20.7</td>
</tr>
<tr>
<td>IL-2</td>
<td>4.6 +/- 0.7</td>
<td>6.8 +/- 1.7</td>
<td>3.3 +/- 0.9</td>
<td>3.8 +/- 1.1</td>
</tr>
</tbody>
</table>

Cytokine levels were determined by Bio-Plex® Assay. Data are the mean +/- standard error.
The exposure of mice to prolonged restraint enhances *C. rodentium*-induced infectious colitis to such an extent that the relatively resistant CD-1 mouse becomes fairly susceptible to pathogen colonization and subsequent pathogen-associated tissue destruction [14]. The ultimate goal of our research is to determine how exposure to psychological stressors can exacerbate *C. rodentium*-induced infectious colitis. In order to achieve this we wanted to make use of genetically modified mice which were bred on the C57BL/6 parent strain. This inbred mouse strain is among the strains that are considered resistant to *C. rodentium* infection, however in our experience the C57BL/6 mouse is more readily colonized than the CD-1 mouse. Prior to our employing genetically modified mice to test our hypotheses, we determined that exposing the C57BL/6 mouse strain to prolonged restraint at the onset of *C. rodentium* challenge was too much for this mouse strain to handle, as they all succumbed to infection even when a significantly lower infectious dose was used (i.e., $3 \times 10^6$ CFU vs $3 \times 10^8$ CFU) and a reduction of the prolonged restraint itself (i.e., 12 hrs vs 16 hrs) (data not shown). It quickly became evident that a different stressor would be necessary to further elucidate the mechanism of stressor-enhanced infectious colitis. Because of this, the next chapter will focus on the effects of the social disruption stressor on *C. rodentium*-induced infectious colitis in C57BL/6 male mice.
CHAPTER 3: Stressor-enhanced infectious colitis is exacerbated by enhanced monocyte recruitment

Introduction

The gastrointestinal tract harbors one of the largest communities of bacterial organisms within the body, collectively called the microbiota, with upwards of $10^{12}$ microorganisms found within the distal portion of the colon [13, 176, 177]. Because of the constant exposure to foreign antigen, the immune cells of the gastrointestinal tract are for the most part immunologically inert and produce little to no inflammation upon stimulation [178, 179]. These cells are in place chiefly for the surveillance of pathogenic organisms [136, 137]. When a pathogen is detected the cells of the gastrointestinal tract, such as intestinal epithelial cells and resident macrophages, must produce chemokines (i.e., CCL2, CCL3, and CXCL1) as well as proinflammatory cytokines (i.e., tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6) in order to recruit immune cells of both the innate and adaptive immune system to eradicate the pathogen and restore homeostasis. These newly recruited immune cells contribute to the production of proinflammatory cytokines and inflammatory mediators, such as nitric oxide produced via the enzyme inducible nitric oxide synthase (iNOS), in order to eliminate the pathogen. As it is in other tissues, production of these inflammatory mediators must be
tightly regulated as an overzealous immune response can lead to enhanced tissue pathology and can worsen the prognosis of the infection [124, 180-182].

Inflammatory monocytes are among the first responders to pathogen challenge in general, but also to the murine gastrointestinal pathogen *Citrobacter rodentium* [132, 183-185]. These mononuclear phagocytes are recruited to the site of infection from the blood and bone marrow predominantly by the chemokine CCL2 [132, 140, 141, 186]. Once they enter from the circulation to the infected tissue, inflammatory monocytes will differentiate into macrophages which are capable of producing copious amounts of TNF-α, iNOS, and a plethora of other inflammatory mediators [137, 171]. These cells will remain in the tissue as long as there is a pathogenic stimulus. Once pathogen is cleared, the inflammatory cells must also be removed from the lesion through a process termed resolution in order to reduce the risk of further tissue destruction [187].

Recent evidence has demonstrated that exposure of mice to a psychosocial stressor termed social disruption (SDR) can enhance the accumulation of CD11b+ cells, which include both neutrophils and monocytes, in both the circulation as well as the spleen [188-191]. Social disruption not only enhances the egress of these cells from the bone marrow to the spleen, but the monocytes in particular produce significantly more TNF-α, IL-1β, and IL-6 when stimulated with LPS *in vitro* [189, 192, 193]. Stressor-primed monocytes also have enhanced *in vitro* killing of *Escherichia coli* which is attributed to enhanced iNOS and peroxynitrite production [53, 194, 195]. These same CD11b+ monocytes from SDR-exposed mice exhibit a primed phenotype with enhanced expression of Toll-like receptors 2 and 4, as well as CD86 [195]. While the SDR-
enhanced priming of monocytes may aid in the rapid clearance of pathogen, this phenomenon can also be deleterious to the host. For example, mice subjected to SDR have enhanced lung inflammation even in the absence of antigenic stimulation [190]. In addition, when endotoxic shock is induced by an intraperitoneal LPS injection, mice exposed to SDR succumb more readily than non-stressed, control mice [196].

Stressor exposure has long been known to contribute to the severity of both infectious and non-infectious diseases including gastrointestinal (GI) diseases, such as Crohn’s disease, ulcerative colitis, and enteric infectious disease [3, 161]. While the mechanisms by which stressor exposure exacerbates GI diseases are still under investigation, it is likely that enhanced recruitment of inflammatory monocytes help perpetuate disease severity [170, 171]. Previously, we have shown that pathogen colonization, as well as the resulting infectious colitis exacerbated in mice exposed to SDR (Figure 8A and 8B). This stressor-enhanced severity of infectious colitis can be prevented with the administration of the probiotic *Lactobacillus reuteri* during stressor exposure (Fig. 8C). The main aspects of SDR-enhanced infectious colitis that are reduced by probiotic intervention are chemokine production (e.g. CCL2, data not shown), proinflammatory cytokine production (e.g. TNF-α, data not shown), and colonic macrophage accumulation (Fig. 8D and 8E). Inflammatory monocytes, i.e. CCR2⁺ CD11b⁺ Ly6C⁺ cells, are recruited to the colon in response to CCL2, and are major producers of tissue-damaging TNF-α [170, 171, 197]. Due to the evidence of SDR-enhanced monocyte accumulation in both the spleen and circulation we postulate that the enhanced *C. rodentium*-induced infectious colitis during exposure to SDR is due to the
heightened recruitment of monocytes to the colon during *C. rodentium* challenge. We will also be testing the corollary hypothesis that the primed nature of monocytes from SDR-exposed mice also contribute to the intensification of *C. rodentium*-induced infectious colitis.

**Materials and Methods**

**Mice.** Inbred, male C57BL/6 mice aged 6-8 weeks were purchased from Charles River Laboratories (Wilmington, MA) and housed 3 mice per cage in ventilated polycarbonate cages (32 cm x 18 cm x 15 cm). CCL2-deficient mice were obtained from Tim Eubank, Ph.D at The Ohio State University. For all experiments, mice were allowed to acclimate to their surroundings for 1 week prior to experimentation. Lights were maintained on a 12 hr on, 12 hr off cycle (lights on at 0600). Mice were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility and had free access to food and water except during experimental procedures. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at the Ohio State University (2009A0235).

**Social Disruption.** Social disruption (SDR) is a psychosocial stressor which takes advantage of intermale aggression in mice and involves the repeated social defeat of resident experimental mice [189]. Social disruption began with the addition of an aggressive intruder mouse at the beginning of their active cycle (i.e., at 1700). The intruder mouse will repeatedly attack and defeat the resident cage mice for 2 hours after which the intruder is removed from the cage. Mice exposed to SDR will receive 6
consecutive nights of stressor exposure after which they will be left alone in their cages. Control mice (HCC) are left undisturbed in their cages.

**Adoptive Transfer.** Naive, male, C57BL/6 donor mice were exposed to social disruption as previously described with control mice being left undisturbed in their cages. Donor mice remained uninfected throughout the duration of SDR. The morning following the last night of SDR, mice were sacrificed by CO₂ asphyxiation and spleens were removed. Splenocytes were removed as previously described [189, 194, 198] and CD11b⁺ splenocytes were isolated using magnetic bead separation (Miltenyi Biotec, Inc). CD11b⁺ splenocytes were pooled by donor group (i.e., HCC vs SDR) at a concentration of 5 x 10⁷ cells/ml of 1X PBS. Naïve, male, C57BL/6 recipient mice were injected with 100 μl, (i.e., 5 x 10⁶ donor cells/mouse) of either HCC or SDR donor cells via the tail vein. Recipient mice were subjected to social disruption immediately following the adoptive transfer and challenged with *C. rodentium* immediately following the first night of SDR. A second non-stressed control recipient group was also infected along with the stressor-exposed mice. Adoptive transfer groups were set up as outlined in Table 3.

**Infection and Bacterial Enumeration.** *Citrobacter rodentium* strain DBS120(pCRP1::Tn5) [164] was grown overnight in Difco™ LB Broth, Lennox at 37°C. Cultures were centrifuged and the resulting pellet was resuspended in sterile PBS to give a final concentration of 3 x 10⁷ colony forming units (CFU)/ml. Mice were then challenged immediately following the first night of SDR at 1900 via oral gavage with 100 μl containing 3 x 10⁶ CFU and were food and water deprived for 2 hours post-challenge. Mice in the SDR group were exposed to the stressor for 5 days post-challenge. The day
of infection is referred to as Day 0 and all data collected will be referenced as days post-challenge. Fecal shedding of *C. rodentium* was monitored on days 0, 1, 3, 6, and 12 post-challenge. Fresh stool was collected from individual mice by placing them in a sterile cage where at least 1-2 stool pellets were collected per mouse. The stool was then weighed, homogenized manually in 3 ml sterile PBS, and serially diluted at a ratio of 1:20. In order to enumerate *C. rodentium* only, each dilution was grown via pour plate method in MacConkey agar supplemented with the antibiotic kanamycin (40 μg/ml) overnight at 37°C. At the time of sacrifice, spleens were flash frozen in liquid nitrogen. Spleens were then thawed, weighed and suspended in 5ml sterile PBS. Spleens were homogenized by stomacher and then serially diluted and plated in the same fashion as stool samples.

**Histopathology.** On Day 12 post-challenge colons were excised and cut in half longitudinally where one section was used for further analysis of colonic cytokines by Real Time PCR and the other was fixed in 10% formalin-buffered phosphate for 24-48 hrs. Samples were embedded in paraffin and 5 μm-thick sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation by a board-certified veterinary pathologist (N.M.A.P.) who was blinded to treatment groups. The severity of lesions in the colon was scored, according to previously defined criteria [165]. Briefly, each colon was scored on 5 different categories: inflammation, dysplasia, hyperplasia, edema, and epithelial defects. Each category received a score of 0 to 4 in 0.5 increments based on the degree of lesion severity: 0 (absent), 1 (mild), 2 (moderate), 3 (marked), and
4 (severe). All 5 categories were added together to garner a total pathology score with a maximum value of 20.

**Semiquantitative Real-Time PCR.** Colons were harvested and total RNA was isolated using a standard single-step isolation protocol (TRI-zol, Invitrogen). After isolation, total RNA was spectrophotometrically quantified. A reverse transcription reaction was performed to synthesize cDNA using the Avian Myeloblastosis Virus (AMV) Reverse Transcriptase enzyme kit (Promega Corporation, Madison, WI). These samples then underwent Real-Time Polymerase Chain Reaction (PCR), in order to amplify the nucleotide sequences of interest. In all cases, 18S rRNA was used as the housekeeping gene. The sequences of primers and probes used can be found in Table 1. SYBR green was used in place of a labeled probe sequence for CXCL1. The PCR reaction took place in the Prism 7000 Sequence Detection System and began with 2 min at 50°C, 10 min at 95°C, and then 40 amplification cycles of 15 sec at 95°C, and 1 min at 60°C. The relative amount of mRNA was determined using the comparative cycle threshold method (Ct) as previously described [8, 163].

**Immunohistochemistry.** Colons were sliced longitudinally at the time of sacrifice and fixed in 10% formalin-buffered phosphate for 24-48 hours. Samples were embedded in paraffin and subsequently sliced in 5 µm sections. In order to visualize macrophages in the colon, sections adjacent to samples stained by H&E were stained with an antibody specific for the macrophage marker F4/80 as previously described [199]. Cells positive for F4/80 were quantified digitally as previously described by analyzing every 5th image of the entire length of the colon by Adobe Photoshop [199].
Statistical Analysis. Stressor-induced changes in \textit{C. rodentium} colonization, gene expression, and serum cytokine levels were analyzed by a two-factor ANOVA, with group (WT vs CCL2\(^{-/-}\)) and stressor-exposed status (HCC vs SDR) as between factors for the experiments involving CCL2-deficient mice, a two-factor ANOVA with donor status (HCC vs SDR) and recipient status (HCC vs SDR) as between factors for the adoptive transfer experiments using IBM SPSS Statistics for Windows, Version 19.0 (SPSS, Chicago, IL). Post-hoc analysis comprised of two-tailed Student’s t-test with Bonferroni correction applied. Chi-squared tests were performed on pathogen prevalence in the spleen. P values of less than 0.05 were considered significant.

Results

Stressor-exposed, CCL2-deficient mice have reduced colonic histopathology despite increases in pathogen burden. Both C57BL/6 wild type (WT) and CCL2-deficient (CCL2\(^{-/-}\)) mice were exposed to the SDR stressor and challenged with \textit{C. rodentium} in the same manner as previously described. As previously demonstrated, there was a significant increase in pathogen burden in WT mice exposed to SDR [F(3, 95) = 5.8, P<0.05; Figure 9A]. There was also an increase in the colonization of \textit{C. rodentium} in colons of SDR-exposed, CCL2\(^{-/-}\) mice; however it was not significant as compared to non-stressed CCL2\(^{-/-}\) mice. Despite this, there were still near-similar levels of \textit{C. rodentium} colonization in WT and CCL2\(^{-/-}\) mice exposed to SDR on Day 12 post-challenge. Colonic histopathology was significantly increased by SDR exposure on Day 12 post-challenge [F(1, 23) = 4.3, P<0.05; Fig. 9B], however this increase was not
dependent on the strain of the mouse \( F(1, 23) = 3.2, P=0.08; \) Fig. 9B]. Previous reports have demonstrated that stressor exposure can significantly enhance \( C. \ rodentium \)-induced colonic pathology which led us to test the \textit{a priori} hypothesis that SDR-exposure would significantly enhance colonic pathology on Day 12 post-challenge [8, 14]. As predicted, SDR exposure significantly increased Day 12 \( C. \ rodentium \)-induced colonic pathology in WT mice \( t(26) = 4.0, p<0.001; \) Fig. 9B] an effect which was not observed in SDR-exposed, CCL2\(^{-/-}\) mice despite near similar pathogen levels of \( C. \ rodentium \). Control colons from WT and CCL2\(^{-/-}\) mice had a relatively normal appearance with small numbers of lymphocytes infiltrating the mucosa (Fig. 9C and E) as did colons from SDR-exposed, CCL2\(^{-/-}\) mice (Fig. 9F). Stressor-increased histopathology was evidenced by mucosal thickening, mild mucosal damage as well as significant inflammatory cell infiltrate only in colons from WT mice (Fig. 9D).

\textit{Stressor-enhanced, \( C. \ rodentium \)-induced colonic inflammatory mediator gene expression is negated in CCL2\(^{-/-}\) mice.} Colonic inflammatory mediator gene expression was previously shown to be increased on Day 12 post \( C. \ rodentium \) challenge in SDR-exposed mice as compared to their control counterparts. On Day 12 post-challenge there was a significant increase in CCL2 mRNA expression in SDR-exposed WT mice \( F(1, 7) = 33.0, P=0.001; \) Figure 10A]. Colonic CCL2 gene expression was not detected in CCL2\(^{-/-}\) mice. Colonic TNF-\( \alpha \) mRNA expression was also significantly increased on Day 12 post-challenge in both SDR-exposed groups \( F(1, 15) = 17.5, P=0.001; \) Fig. 10B]. There was also a significant increase in colonic TNF-\( \alpha \) expression in WT mice as
compared to CCL2\(^{–/–}\) mice \([F(1, 15) = 14.7, P<0.005; \text{Fig. 10B}]\). Similar to previous data, iNOS mRNA expression was also significantly increased in SDR-exposed WT mice on Day 12 post-challenge \([F(1,15) = 9.5, P<0.01; \text{Fig. 10C}]\). This stressor-enhanced increase in colonic iNOS expression was diminished in SDR-exposed, CCL2\(^{–/–}\) mice. Another chemokine, which is involved in neutrophil recruitment; CXCL1, was also significantly increased by SDR-exposure on Day 12 post-challenge as compared to their WT control counterparts \([F(1, 15) = 4.8, P<0.05; \text{Fig. 10D}]\). This Day 12 SDR-enhanced increase in CXCL1 was negated in \(C.\) rodentium-challenged, CCL2\(^{–/–}\) mice.

\textit{Stressor-enhanced, \(C.\) rodentium-induced colonic macrophage accumulation is reduced in CCL2\(^{–/–}\) mice.} The colons of SDR-exposed, WT mice had a significant increase in macrophage accumulation on Day 12 post-challenge over that of WT control mice as visualized by F4/80 staining via immunohistochemistry \([F(1, 14) = 5.2, P<0.05; \text{Figure 11A}]\). This stressor-enhanced effect on colonic macrophage accumulation was significantly reduced in CCL2\(^{–/–}\) mice exposed to the SDR stressor.

\textit{Stressor-enhanced pathogen translocation to the spleen is diminished in CCL2\(^{–/–}\) mice.} Stressor exposure has been shown to enhance the translocation of the non-invasive \(C.\) rodentium from the lumen of the colon to the spleen at the peak of infection, i.e. Day 12 post-challenge \([14]\). Wild type mice that were exposed to SDR at the onset of \(C.\) rodentium challenge had a significant increase in spleen mass \([F(1, 18) = 4.8, P<0.05; \text{Figure 12A}]\), a phenomenon which was negated in SDR-exposed CCL2\(^{–/–}\) mice. The
significant increase in spleen mass in SDR-exposed WT mice coincided with a significant increase in the likelihood of detecting *C. rodentium* in the spleen [$\chi^2 (1) = 4.52, P<0.05$; Fig. 12B]. Stressor exposure failed to increase *C. rodentium* translocation in CCL2$^{-/-}$ mice; however this reduction did not quite reach significance (Fig. 12C).

*The transfer of SDR-primed CD11b$^+$ splenocytes does not enhance* *C. rodentium colonization or resulting tissue pathology.* In order to determine if CD11b$^+$ splenocytes from SDR-exposed mice are able to enhance *C. rodentium*-induced infectious colitis, cells were transferred from non-infected control and SDR-exposed mice into infected mice as outlined in Table 3. The addition of cells, regardless of donor (HCC vs SDR), caused a significant increase in colonization in control recipient animals [$F(2, 168) = 6.4, P<0.005$; Fig. 13A] which was most evident on Day 12 post-challenge. Exposure of recipient mice to SDR caused significant changes in pathogen colonization as compared to recipient mice which served as controls [$F(1, 168) = 14.9, P<0.001$; Fig. 13B]. The development of megacolon, as observed by an increase in colon mass, was evident in mice challenged with *C. rodentium* on Day 12 post-challenge, however there were no significant changes based on either donor or recipient status. There was, however, a significant increase in Day 12 post-challenge colonic histopathology in recipient mice which were exposed to social disruption [$F(1, 24) = 5.56, P<0.05$; Fig. 13D] regardless of the type of cells which were transferred. No lesions were observed in HCC mice which did not receive cells (Fig. 13E). Control mice that received cells from either HCC or SDR-exposed mice has mild pathology which manifested as enhanced immune cell
infiltrate as well as a slight thickening of the colonic villi (Fig. 13 F and G). Colons from stressor-exposed mice had significant immune cell infiltration, lengthening of the villi, as well as focal epithelial erosions regardless of the cell type which was received (Fig. 13 H, I, and J).

*Colonic gene expression is not altered by the transfer of stressor-primed CD11b* superscript*+* splenocytes. Day 12 post-challenge has consistently been shown to be the peak of stressor-enhanced, *C. rodentium*-induced colonic gene expression. On Day 12 post-challenge there was a trend towards a significant increase in colonic CCL2 mRNA expression in recipient mice which were exposed to SDR as compared to control recipient animals [F(1, 42) = 3.6, P=0.065; Fig. 14A]. There was also a donor effect [F(2, 42) = 3.9, P<0.05; Fig. 14A] which was primarily due to increases in CCL2 expression in both HCC recipients which received additional splenocytes. The proinflammatory cytokine TNF-α was significantly increased in SDR-exposed recipient mice on day 12 post-challenge as compared to control recipient mice [F(1, 42) = 4.8, P<0.05; Fig. 14B]. There was also a significant increase in TNF-α expression in both control recipient groups which received cells [F(2, 42) = 3.6, P<0.05; Fig. 14B]. Colonic iNOS mRNA expression was significantly increased on Day 12 post-challenge in SDR-exposed recipient mice over that of control recipient mice [F(1, 42) = 6.4, P<0.05; Fig. 13C] with a similar donor effect [F(2, 42) = P=0.05; Fig. 14C] in that control recipient mice that received cells from either control or SDR-exposed mice had an increase in
expression over that of control recipients which received no cells. The neutrophil chemoattractant protein CXCL1 was not significantly altered on Day 12 post-challenge.

*Stressor-enhanced colonic monocyte/macrophage accumulation is unchanged by the transfer of CD11b⁺ splenocytes.* The colons of recipient mice were stained to visualize the monocyte/macrophage marker F4/80 on Day 12 post-challenge. In recipient mice which did not receive cells at the time of pathogen challenge, there was a stressor-induced increase in colonic F4/80⁺ cells. Control mice which received cells had an increase in the amount of F4/80⁺ cells which was unchanged in stressor-exposed recipient mice (Figure 15A).

*The transfer of CD11b⁺ splenocytes alters spleen mass, but not pathogenic translocation from the colon to the spleen.* Just as Day 12 post-challenge is the peak of *C. rodentium* colonization and pathology, it is also when pathogen translocation from the colon to the spleen can be detected. On Day 12 post-challenge there was a significant increase in spleen mass which, in this instance, was dependent on donor status \[F(2, 42) = 3.3, P<0.05; \text{Fig. 16A}\]. This enhanced splenomegaly was most evident in control recipient mice which received splenocytes from either control or SDR-exposed donor animals (p<0.05). This increase in spleen mass of control recipient mice did not translate to an enhanced likelihood for detecting pathogen in the spleen as only recipient mice exposed to social disruption had a significant increase in the likelihood of detecting *C. rodentium* in the spleen on Day 12 post-challenge \(\chi^2 (1) = 13.5, P<0.001; \text{Fig. 16B}\) as compared to
infected, control recipient mice. This effect was only observed within the recipient mice as the addition of naive or SDR-primed splenocytes did not alter the effect of pathogen translocation. While there was a significant increase in the likelihood of detecting pathogen in the spleen, the pathogenic burden within the spleens of both the control and recipient mice were not significantly different.

**Discussion**

The results of this study confirm that the exposure of mice to the SDR stressor exacerbates *C. rodentium*-induced infectious colitis, as evidenced by the enhanced colonic pathogen colonization as well as the infectious colitis that is attributed to *C. rodentium*. Exposure of mice to SDR at the onset of pathogenic challenge caused significant increases in colonic *C. rodentium* colonization, total colonic histopathology, colonic inflammatory mediator gene expression, and pathogen translocation from the colon to the spleen as compared to infected, non-stressed wild type control mice at the peak of infection, i.e., Day 12 post-challenge. Of the inflammatory mediators tested, the chemokine CCL2, which aids in the recruitment of monocytes and macrophages, as well as neutrophils, dendritic cells, and T cells, had a 6-fold increase in mRNA expression on Day 12 post-challenge in SDR-exposed, wild type mice over that of infected, wild type control mice. This increase in colonic CCL2 mRNA expression correlated with a similar 6 fold increase in F4/80+ macrophages in the colons of SDR-exposed wild type mice on Day 12 post-challenge, however the F4/80 marker does not distinguish between newly recruited inflammatory monocytes and resident tissue macrophages [200]. It has been
established that tissue resident macrophages produce little to no inflammatory mediators in the colon, therefore it was hypothesized that the stressor enhanced increases in infectious colitis are due to an overabundance of inflammatory monocytes in the colon. To test this hypothesis CCL2−/− mice were exposed to SDR during C. rodentium challenge in order to assess the importance of CCL2-dependent recruitment of inflammatory monocytes during stressor exposure.

Similar to that of wild type mice, exposing CCL2−/− mice to SDR at the onset of C. rodentium challenge resulted in an increase in Day 12 post-challenge colonic colonization to levels comparable to SDR-exposed wild type mice. In spite of the similar levels of colonization in both stressor-exposed groups, there was no increase in Day 12 colonic histopathology, colonic inflammatory mediator gene expression, or pathogen translocation from the colon to the spleen in CCL2−/− mice as compared to stressor-exposed WT control mice. These reductions in stressor-exacerbated infectious colitis may be attributed to reduced colonic macrophage accumulation. There has been extensive research with regard to the F4/80+ populations within the colon at rest and during inflammation (reviewed in [137]). At rest, F4/80+ cells in the colon are TLR− CCR2− CX3CR1+ and will not produce inflammatory mediators in response to stimuli. During experimental colitis, there is a large influx of inflammation-producing F4/80+ cells, which are TLR+ CCR2+ CX3CR1− Ly6C^hi and are believed to arrive in the colon in response to the chemokine CCL2 [171]. Additionally, if the recruitment of this population of inflammatory F4/80+ cells to the colon can be reduced, for example through the use of CCR2−/− mice or CCL2-neutralizing antibodies, there is a reduction in
experimental colitis [142, 143, 184]. Hence, it is possible that the reduced accumulation of colonic F4/80+ cells in SDR-exposed CCL2−/− mice as compared with SDR-exposed WT mice contribute to the observed decreases in stressor-induced infectious colitis.

Exposure of mice to the SDR stressor without infection can lead to a primed phenotype in splenic CD11b+ monocytes, in which these cells are better able to produce inflammatory mediators during stimulation with LPS or nonpathogenic E. coli [194, 195]. Thus, the corollary hypothesis that SDR-primed macrophages alone will enhance C. rodentium-induced infectious colitis was tested through the transfer of splenic CD11b+ cells from non-infected, stressor-exposed mice into mice immediately prior to exposure to SDR and pathogen challenge. It was hypothesized that the addition of SDR-primed CD11b+ splenocytes at the onset of C. rodentium challenge would exacerbate the pathogen-induced colitis. Interestingly, the transfer of CD11b+ splenocytes enhanced pathogen colonization in HCC recipient mice regardless of the donor status (i.e., HCC donor vs SDR donor). This increase in C. rodentium colonization in HCC recipient mice was to similar levels observed in SDR-exposed mice that did not receive cells. The transfer of additional CD11b+ splenocytes to HCC recipient mice caused increases in colonic CCL2, TNF-α, and iNOS, as well as colonic histopathology; however these increases did not reach statistical significance. There was a significant increase in colonic histopathology as well as inflammatory mediators on Day 12 post-challenge, however this could be attributed to the exposure of the recipient mice to SDR and not dependent on the transfer of additional monocytes/macrophages. Pathogen translocation
from the colon to the spleen was also only evident in SDR-exposed recipient mice, which was unchanged by the transfer of CD11b\(^+\) splenocytes.

These results, coupled with that of the CCL2\(^{-/-}\) data, led to the conclusion that the recruitment of monocytes/macrophages is necessary for the development of SDR-enhanced infectious colitis. Interestingly, the data presented here within also led to the conclusion that the priming of splenocytes that occurs during social disruption did not have an effect on infectious colitis, in that mice that received cells from SDR-exposed mice did not have a difference in infectious colitis as compared with mice that received cells from control animals. While this could have been due to the current design of the study, we believe that the changes that occur in the colon during stressor exposure creates an environment which permits a more inflammatory state so when a monocyte, regardless of donor status, enters this unique microenvironment it will contribute significantly to \(C.\) \textit{rodentium}-induced infectious colitis.

Many changes can occur in the gastrointestinal tract during exposure to psychological stressors which include changes in motility of the digestive contents, fluctuations in mucus production, and alterations to the indigenous microbiota \([2, 4, 5, 8, 9, 23, 24, 37]\). While it isn’t fully understood with what regard these changes can influence gastrointestinal diseases, such as enteric infectious disease, there is increasing evidence which points to the importance of the microbiota that reside within the gastrointestinal tract \([2, 8, 9, 53, 107, 201]\). Evidence to support this theory stemmed from the fact that patients infected with the enteric pathogen \textit{Clostridium difficile} often have a significant reduction in bacterial diversity in their gastrointestinal tract and
restoring the intestinal microbiota via fecal transplantation has had tremendous success in preventing further infection in patients who are susceptible to recurrent *C. difficile* infection [202-205]. It is possible that the shift in the microbiota of the gastrointestinal tract in the stressed individual favors a more inflammatory environment that can make the stressed individual more susceptible to developing colitis.
Figure 8. Probiotic intervention with Lactobacillus reuteri significantly reduces SDR-enhanced infectious colitis.

Mice were subjected to SDR on the day of and for 5 days post-challenge with C. rodentium. Probiotic intervention with L. reuteri took place immediately following each cycle of SDR. (A) Stressor-exposure significantly enhances C. rodentium colonization in both vehicle and probiotic-treated mice. (B) Colonic histopathology is significantly enhanced by SDR-exposure on Days 6 and 12 post-challenge. (C) Stressor-enhanced colonic histopathology is reduced by L. reuteri probiotic intervention. (D) Colonic macrophage accumulation, as assessed by the quantification of F4/80+ cells, is significantly enhanced by stressor exposure on Day 12 post-challenge. (E) Social disruption-enhanced monocyte/macrophage accumulation is reduced by treatment with L. reuteri on Day 12 post-challenge, however there was a significant increase in accumulation on Day 24 post-challenge. Data are the mean ± standard error.
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Table 3. Adoptive Transfer groups.
Figure 9. Stressor-enhanced, C. rodentium-induced infectious colitis is negated in CCL2-deficient mice.

Both wild type and CCL2−/− mice were infected with 3 x 10^6 CFU of C. rodentium immediately following the first night of SDR. Social disruption continued for 5 days post infection. (A) Samples were collected on days 0, 1, 3, 6, and 12 post-challenge. Pathogen was enumerated via pour plate method. Exposure to SDR significantly increased C. rodentium colonization in mice with intact CCL2. *p<0.05 SDR WT vs HCC WT on Days 6 and 12. (B) On day 12 post-challenge, colons were removed, fixed in formalin, and embedded in paraffin. Colons were sectioned and stained with hematoxylin and eosin in order to visualize and score the pathology present in each sample. Stressor exposure significantly increased total pathology (as measured by hyperplasia, dysplasia, edema, inflammation and epithelial defects) 12 days post infection. *p<0.05 SDR WT vs HCC WT. Images are taken from slides representative of a (C) WT control colon, (D) WT SDR colon, (E) CCL2−/− control colon, and (F) CCL2−/− SDR colon. n = 3 HCC WT, n = 6 SDR WT, n = 8 HCC CCL2−/−, n = 11 SDR CCL2−/−. Data are the mean ± standard error.
Figure 10. Social disruption enhances *C. rodentium*-induced colonic gene expression in mice with intact CCL2.

Mice were subjected to SDR on the day of and for 5 days post-challenge with *C. rodentium*. Mice were sacrificed on day 12 post-challenge. Colons were processed in order to quantify gene expression by Real Time PCR. Stressor exposure significantly increased colonic mRNA expression of (A) CCL2, (B) TNF-α, (C) iNOS, and (D) CXCL1 as compared to controls on day 12 post-challenge which was ablated in CCL2-deficient mice. ND = Not Detectable. *p<0.01 SDR WT vs HCC WT. In TNF-α figure, a = HCC vs SDR, p<0.001. n = 3 HCC WT, n = 6 SDR WT, n = 8 HCC CCL2<sup>−/−</sup>, n = 11 SDR CCL2<sup>−/−</sup>. Data are the mean ± standard error.
Figure 11. Stressor exposure enhances colonic macrophage accumulation in wild type mice.

Mice were subjected to the SDR stressor on the day of and for 5 days post-challenge with *C. rodentium*. Colons were removed from mice on day 12 post-challenge and subsequently fixed in formalin and embedded in paraffin. Colons were sectioned and stained with an antibody for the surface marker F4/80 in order to visualize macrophages. Individual pixels were counted and averaged per colon in order to give the number of F4/80⁺ pixels/field. (A) Wild type mice exposed to social disruption had a significant increase in cells positive for F4/80 on day 12 post-challenge. This effect was reduced in CCL2-deficient, stressor exposed mice. Images are taken from slides representative of a (B) WT control, (C) WT SDR, (D) CCL2⁻/⁻ control, and (E) CCL2⁻/⁻ SDR colon. *p<0.05 SDR WT vs HCC WT. n = 3 HCC WT, n = 6 SDR WT, n = 6 HCC CCL2⁻/⁻, n = 6 SDR CCL2⁻/⁻. Data are the mean ± standard error.
Figure 12. Stressor exposure enhances the translocation of *C. rodentium* from the colon to the spleen in a CCL2-dependent manner.

Mice were infected with *C. rodentium* immediately after the first night of SDR. Stressor exposure continued for 5 more nights post-challenge. On day 12 post-challenge, spleens were removed and processed in order to enumerate *C. rodentium* by pour plate method. (A) Splenomegaly was only observed in wild type, stressor-exposed mice. (B) Spleens from wild type, stressor-exposed mice also had an increase in the likelihood of pathogen dissemination (C) and an increased bacterial load (horizontal line represents detection limit). *p<0.05 SDR WT vs HCC WT. n = 3 HCC WT, n = 6 SDR WT, n = 8 HCC CCL2−/−, n = 11 SDR CCL2−/−. Data are the mean ± standard error.
Figure 13. Social disruption-primed splenocytes do not enhance *C. rodentium* colonization or resulting infectious colitis.

Donor mice were either subjected to 6 cycles of SDR or left alone to serve as controls. CD11b$^+$ splenocytes from each donor was transferred into recipient mice immediately prior to SDR-exposure and subsequent pathogen challenge. Data presented within are from recipient mice. (A) Samples were collected on days 0, 1, 3, 6, and 12 post-challenge. Pathogen was enumerated via pour plate method. The addition of splenocytes from either control of SDR-exposed mice had a significant increase in pathogen colonization on Day 12 post-challenge as compared with mice which did not receive cells. *p*<0.05 HCC and SDR Donor vs No Donor. (B) Recipient mice exposed to SDR had a significant increase in colonization over that of HCC recipients presented in panel A. (C) Colon mass was increased by *C. rodentium* on Day 12 post-challenge, however it was unaffected by the transfer of splenocytes. (D) On Day 12 post-challenge, colons were removed, fixed in formalin, and embedded in paraffin. Colons were sectioned and stained with hematoxylin and eosin in order to visualize and score the pathology present in each sample. Stressor exposure significantly increased total pathology (as measured by hyperplasia, dysplasia, edema, inflammation and epithelial defects) on Day 12 post-challenge. This increase in pathology was unaffected by the addition of splenocytes to recipient mice. *p*<0.05 SDR recipients vs HCC recipients. Images are taken from slides representative of HCC recipients that received (E) no cells, (F) HCC cells, and (G) SDR cells, and of SDR recipients that received (H) no cells, (I) HCC cells, and (J) SDR cells. $n$ = 6 HCC/No Donor, $n$ = 9 HCC/HCC Donor, $n$ = 9 HCC/SDR Donor, $n$ = 6 SDR/No Donor, $n$ = 9 SDR/HCC Donor, $n$ = 9 SDR/SDR Donor. Data are the mean ± standard error.
Figure 14. The addition of SDR-primed splenocytes does not alter *C. rodentium*-induced colonic gene expression.
Recipient mice were subjected to SDR immediately following the transfer of splenocytes from naïve or stressor-exposed mice. Recipient mice were challenged with *C. rodentium* after the first cycle of social disruption and sacrificed on Day 12 post-challenge. Colons were processed in order to quantify gene expression by Real Time PCR. (A) There was a significant increase in CCL2 expression in HCC recipient mice which received either cells from HCC or SDR-exposed donor mice. There was also a trend for significance in the increase of CCL2 in recipient mice as compared with control recipient mice. Stressor exposure significantly increased colonic mRNA expression of (B) TNF-α and (C) iNOS, but only in recipient mice. The receipt of cells from either control or SDR-exposed mice also significantly increased TNF-α and iNOS gene expression in HCC recipient mice. (D) The chemokine CXCL1 was unaffected by stressor exposure. †=0.065 HCC recipients vs SDR recipients, *p<0.05 for HCC recipients vs SDR recipients. \( n = 6 \) HCC/No Donor, \( n = 9 \) HCC/HCC Donor, \( n = 9 \) HCC/SDR Donor, \( n = 6 \) SDR/No Donor, \( n = 9 \) SDR/HCC Donor, \( n = 9 \) SDR/SDR Donor. Data are the mean ± standard error.
Figure 15. Colonic monocyte/macrophage accumulation is unaffected by the transfer of CD11b$^+$ splenocytes.

Recipient mice were subjected to SDR immediately following the transfer CD11b$^+$ splenocytes from non-infected naïve or stressor-exposed mice. Recipient mice were challenged with *C. rodentium* after the first cycle of social disruption and sacrificed on Day 12 post-challenge. Colons were removed from mice on day 12 post-challenge and subsequently fixed in formalin and embedded in paraffin. Colons were sectioned and stained with an antibody for the surface marker F4/80 in order to visualize macrophages. Individual pixels were counted and averaged per colon in order to give the number of F4/80$^+$ pixels/field.

(A) Stressor-enhanced F4/80$^+$ cell accumulation was unchanged in mice which received donor cells. Images are taken from slides representative of HCC recipient mice (B) No Donor, (C) HCC Donor, (D) SDR Donor, and SDR-exposed recipient mice (E) No Donor, (F) HCC Donor, and (G) SDR Donor.  $n = 1$ for each group.
Recipient mice were subjected to SDR immediately following the transfer of splenocytes from naïve or stressor-exposed mice. Recipient mice were challenged with *C. rodentium* after the first cycle of social disruption and sacrificed on Day 12 post-challenge. Spleens were removed, weighed, and processed in order to enumerate *C. rodentium*. (A) The transfer of cells from either HCC or SDR-exposed mice caused a significant increase in spleen mass in recipient mice which served as controls. *p<0.05* HCC/HCC Donor and HCC/SDR Donor vs HCC/No Donor (B) There was a significant increase in the ability to detect pathogen in the spleens of recipient mice as compared to control recipient animals, *p<0.05* HCC recipients vs SDR recipients, (C) however there were no changes in the abundance of pathogen found in the spleen (horizontal line represents the limit of detection). *n* = 6 HCC/No Donor, *n* = 9 HCC/HCC Donor, *n* = 9 HCC/SDR Donor, *n* = 6 SDR/No Donor, *n* = 9 SDR/HCC Donor, *n* = 9 SDR/SDR Donor. Data are the mean ± standard error.
SDR-enhanced infectious colitis is mediated by a CCL2-dependent recruitment of inflammatory monocytes. In addition, *L. reuteri* treatment can also reduce SDR-enhanced colonic CCL2 mRNA expression and colonic monocyte/macrophage accumulation, however it is unclear as to which cells within the colon are bring modulated by *L. reuteri* treatment. We hypothesize that intestinal epithelial cells and non-tissue resident macrophages are possible contributors of CCL2. The use of *in vitro* cell culture techniques will be utilized to determine the effects of *L. reuteri* pretreatment on *C. rodentium*-induced gene expression. Within Chapter 4, we also set out to determine the importance of epithelial cell and myeloid-derived NF-κB signaling during *C. rodentium* challenge through the use of knockout mice.
CHAPTER 4: Differential signaling by intestinal epithelial cells and macrophages during 
*C. rodentium* challenge

**Introduction**

Approximately 70% of the immune cells within our body can be found inside the gastrointestinal tract, which are in place to keep the trillions of commensal organisms found in the lumen of the gut in check. A delicate balance must be maintained in order to tolerate this large community of commensal organisms as well as the great burden of food antigen without constantly mounting an immune response. This can occur because the resident cells of the intestinal lamina propria are immunologically inert [48, 137, 206, 207]. Lamina propria resident macrophages, in particular, retain their phagocytic abilities yet will not produce proinflammatory cytokines or chemokines even after an encounter with a pathogenic organism [137]. This phenomenon is of the utmost importance in order to maintain homeostasis, and is believed to be achieved by the inactivation of NF-κB signaling in resident tissue macrophages which is at least in part attributable to the presence of the anti-inflammatory cytokines IL-10 and TGF-β within the lamina propria [171]. Because of this, the initiation of immune responses within the colon is the responsibility of cells such as intestinal epithelial cells and colonic stromal cells.
The intestinal epithelial cell is a key player in the intestinal immune response, including the response to *C. rodentium* [208]. The epithelial cell is often the first line of defense against gastrointestinal pathogens because of its location between the luminal contents and the rest of the body. In the case of *C. rodentium* infection, the pathogen can actually be found adhered to the epithelial cell, which is where the initial immune response to this murine pathogen begins [118]. While much of the research pertaining to *C. rodentium* and its A/E family members has focused on the response of immune cells to these pathogens, there is evidence that intestinal epithelial cells are significant contributors to inflammation and immune cell recruitment in response to pathogen challenge [209, 210]. Additionally, the proinflammatory cytokines, e.g. TNF-α and IL-1β, and chemokines, e.g. CCL2 and CXCL1, and enzymes, e.g. iNOS, have been shown to be upregulated in epithelial cells, stromal cells, and newly recruited immune cells as a result of signaling cascades which coalesce with the activation of the transcription factor NF-κB [126, 129, 133, 211]. During *C. rodentium* challenge, the activation of NF-κB begins with the ligation of pattern recognition receptors, e.g. Toll-like receptors and Nod-like receptors, which recognize conserved bacterial motifs such as LPS and peptidoglycan. The signaling cascades that result from the activation of these receptors lead to the activation of the inhibitor of κB kinase (IKK) which leads to the phosphorylation, and subsequent degradation of the inhibitor of NF-κB, IκB. Once NF-κB is free of its inhibitor it is able to move to the nucleus where it can bind to κB binding sites within promoter sequences in order to activate the transcription of proinflammatory cytokines and chemokines. NF-κB activation is necessary for bacterial clearance;
however uncontrolled activation can lead to an overzealous host response, including the excessive production of proinflammatory cytokines as well as enhanced immune cell recruitment due to an overabundance of chemokines which can contribute to tissue damage [211].

One such example of the detrimental effects of enhanced immune cell recruitment during gastrointestinal infection is demonstrated in our model of stressor-exacerbated infectious colitis. *Citrobacter rodentium*-induced infectious colitis is exacerbated in mice exposed to either prolonged restraint or social disruption, which is revealed through enhanced bacterial colonization, exacerbated colonic histopathology, increases in colonic inflammatory mediator gene expression, and even enhanced pathogen translocation from the colon to the spleen in infected, stressor-exposed mice [8, 14] [Chapter 3]. Interestingly, the administration of the probiotic *Lactobacillus reuteri* during stressor-exposure is enough to reduce the stressor-exacerbated infectious colitis [14] [Chapter 3]. The ability of *L. reuteri* to reduce stressor-enhanced infectious colitis is relative to the severity of the colitis that is induced; severe colitis that arises during exposure to prolonged restraint is minimally affected, while the milder infectious colitis that occurs following exposure to social disruption is virtually negated [14] [Chapter 3]. Currently the mechanism by which *L. reuteri* is able to reduce stressor-enhanced infectious colitis is unknown; however it is hypothesized that probiotic intervention during stressor exposure can modulate the immune response to *C. rodentium* rather than the infection itself, owing to the fact that *L. reuteri* does not change pathogen levels but it can reduce the resulting *C. rodentium*-induced pathology.
Through the use of CCL2-deficient (CCL2−/−) mice, which are unable to recruit inflammatory monocytes to infection sites, we have verified that the amplified infectious colitis due to stressor-exposure is reliant on the enhanced recruitment of inflammatory monocytes in that stressor-exposed, CCL2−/− mice do not develop enhanced colonic histopathology despite having similar levels of pathogen as compared to their wild type counterparts. Similarly, the addition of CD11b+ splenocytes, which include monocytes, to wild type mice at the onset of C. rodentium challenge can also exacerbate pathogen-induced infectious colitis. While it has become increasingly clear that newly recruited inflammatory monocytes are key to stressor-exacerbated infectious colitis, it is unclear as to which cell type is responsible for the heightened production of CCL2 to draw in a superfluous amount of inflammatory monocytes. There is evidence, however, that CCL2 production during C. rodentium challenge is through a NF-κB-dependent mechanism via NOD2 activation [132]. While this study attributed the enhanced CCL2 production to colonic stromal cells, we believe that the intestinal epithelial cell may also be a heavy contributor to stressor-enhanced CCL2 production via activation of NF-κB, and thereby contribute to enhanced inflammatory monocyte accumulation. Thus, this study was designed to test the hypothesis that NF-κB-derived gene expression from intestinal epithelial cells is necessary for the exacerbation of colitis via the recruitment of inflammatory monocytes, as well as the corollary hypothesis that inflammatory monocytes contribute to infectious colitis also via NF-κB activation. To test this hypothesis, two different strains of mice were challenged with C. rodentium in which classical NF-κB activation is defective via the deletion of IKKβ in either intestinal
epithelial cells (IKKβΔIEC) or myeloid-derived cells (IKKβΔMY). Due to the ability of *L. reuteri* to significantly reduce stressor-exacerbated infectious colitis, a secondary study was designed in order to test the hypothesis that *C. rodentium*-induced gene expression in intestinal epithelial cells, and not macrophages, will be modulated by pretreatment with *L. reuteri*.

**Materials and methods**

**Mice.** Inbred, male C57BL/6 mice aged 6-8 weeks served as wild type (WT) controls and were purchased from Charles River Laboratories (Wilmington, MA) and housed 3 mice per cage in ventilated polycarbonate cages (32 cm x 18 cm x 15 cm). Two different strains of inhibitor of κB kinase beta (IKKβ)-deficient mice were obtained from Prosper Boyaka, Ph.D at The Ohio State University. The first strain had an IKKβ deficiency in all myeloid-derived cells (IKKβΔMY) and the second has an IKKβ deficiency in only intestinal epithelial cells (IKKβΔIEC). Both knockout strains were on a C57BL/6 background. For all experiments, mice were allowed to acclimate to their surroundings for 1 week prior to experimentation. Lights were maintained on a 12 hr on, 12 hr off cycle (lights on at 0600). Mice were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility and had free access to food and water. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at the Ohio State University (2009A0235R-1).
**Infection and Bacterial Enumeration.** *Citrobacter rodentium* strain DBS120(pCRP1::Tn5) [164] was grown overnight in Difco™ LB Broth, Lennox at 37°C. Cultures were centrifuged and the resulting pellet was resuspended in sterile PBS to give a final concentration of $3 \times 10^9$ colony forming units (CFU)/ml. Mice were then challenged via oral gavage with 100 μl containing $3 \times 10^8$ CFU and were food and water deprived for 2 hours post-challenge. The day of infection is referred to as Day 0 and all data collected will be referenced as days post-challenge. Fecal shedding of *C. rodentium* was monitored on days 0, 1, 3, 6, and 12 post-challenge. Fresh stool was collected from individual mice by placing them in a sterile cage where at least 1-2 stool pellets were collected per mouse. The stool was then weighed, homogenized manually in 3 ml sterile PBS, and serially diluted at a ratio of 1:20. In order to enumerate *C. rodentium* only, each dilution was grown via pour plate method in MacConkey agar supplemented with the antibiotic kanamycin (40 μg/ml) overnight at 37°C. At the time of sacrifice, spleens were flash frozen in liquid nitrogen. Spleens were then thawed, weighed and suspended in 3ml sterile PBS. Spleens were homogenized then serially diluted and plated in the same fashion as stool samples. *Lactobacillus reuteri* strain 23272 (American Type Culture Collection, ATCC) was grown overnight in Difco™ Lactobacilli MRS Broth at 37°C with 5% CO₂. Cultures were centrifuged and the resulting pellet was resuspended in sterile PBS vehicle (VEH).

**Histopathology.** Colons were excised and cut in half longitudinally on Day 12 post-challenge where one section was used for inflammatory mediator gene expression analysis by Real Time PCR and the other was fixed in 10% formalin-buffered phosphate
for 24-48 hrs. Samples were embedded in paraffin and 5 μm-thick sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation by a board-certified veterinary pathologist (N.M.A.P.) who was blinded to treatment groups. The severity of lesions in the colon was scored, according to previously defined criteria [165]. Each colon was scored on 6 different categories; inflammation, dysplasia, hyperplasia, edema, crypt defects, and epithelial defects. Each category received a score of 0 to 4 in 0.5 increments based on the degree of lesion severity: 0 (absent), 1 (mild), 2 (moderate), 3 (marked), and 4 (severe). All 6 categories were added together to garner a total pathology score with a maximum value of 24.

**Immunohistochemistry.** Colons were sliced longitudinally at the time of sacrifice and fixed in 10% formalin-buffered phosphate for 24-48 hours. Samples were embedded in paraffin and subsequently sliced in 5 μm sections. In order to visualize macrophages in the colon, sections adjacent to samples stained by H&E were stained with an antibody specific for the macrophage marker F4/80 as previously described [199]. Cells positive for F4/80 were quantified digitally as previously described by analyzing every 5th image of the entire length of the colon by Adobe Photoshop [199].

**Cell Culture.** The murine colonic epithelial cell line, CMT-93 (CCL-223, American Type Culture Collection, ATCC), as well as the murine macrophage cell line RAW 264.7 (TIB-71, ATCC) were used to determine the effects of *L. reuteri* on *C. rodentium*-induced gene transcription. CMT-93 cells were cultured in complete high glucose Dulbecco’s Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 1.5 mM L-glutamine, 10 mM Hepes buffer, 1 M sodium pyruvate,
1.4X non-essential amino acids, and 0.0035% β-mercaptoethanol at 37°C with 10% CO₂. RAW 264.7 cells were maintained in complete Roswell Park Memorial Institute 1640 Medium (RPMI) supplemented with 10% FBS, 0.075% sodium bicarbonate, 10 mM Hepes buffer, 1.5mM L-glutamine, and 0.0035% β-mercaptoethanol at 37°C with 5% CO₂. Each cell line was cultured separately in 6-well tissue culture treated plates at 1 x 10⁶ cells/well. Cells were allowed to adhere overnight prior to bacterial stimulation. To test the ability of L. reuteri or its byproducts to modulate C. rodentium-induced gene expression 2 separate experiments were performed in each cell line. The initial experiment utilized lived, intact L. reuteri, in which a concentration of 3 x 10⁸ cells/well (i.e., 300:1 of L. reuteri:in vitro cell line) was added to the monolayer of cells for 1 hour prior to challenge with C. rodentium. In the second set of experiments, conditioned supernatants of L. reuteri were pH-adjusted to 7.0 and filter-sterilized in order to ensure only bacterial byproducts and not whole bacteria were present in solution. These bacterial byproducts were added for 1 hour prior to C. rodentium challenge at a concentration of 5% (v/v). After the 1 hour pre-stimulation, cells were challenged with C. rodentium at a concentration of 1 x 10⁸ cells/well (i.e., 100:1 of C. rodentium:in vitro cell line) for 2 hours. At the end of C. rodentium challenge, supernatants were removed and total RNA was harvested for mRNA analysis by semi-quantitative Real Time PCR.

**Semiquantitative Real-Time PCR.** Colons and in vitro cell cultures were harvested and total RNA was isolated using a standard single-step isolation protocol (TRI-zol, Invitrogen). After isolation, total RNA was spectrophotometrically quantified. A reverse transcription reaction was performed to synthesize cDNA using the Avian Myeloblastosis
Virus (AMV) Reverse Transcriptase enzyme kit (Promega Corporation, Madison, WI). These samples then underwent Real-Time Polymerase Chain Reaction (PCR), in order to amplify the nucleotide sequences of interest. In all cases, 18S rRNA was used as the housekeeping gene. The sequences of primers and probes used can be found in Table 1. SYBR green was used in place of a labeled probe sequence for CXCL1. The PCR reaction took place in the Prism 7000 Sequence Detection System and began with 2 min at 50°C, 10 min at 95°C, and then 40 amplification cycles of 15 sec at 95°C, and 1 min at 60°C. The relative amount of mRNA was determined using the comparative cycle threshold method (Ct) as previously described [8, 163].

**Statistical Analysis.** Changes in *C. rodentium* colonization, gene expression, and serum cytokine levels were analyzed by a two-factor analysis of variance (ANOVA) with groups as between factors using IBM SPSS Statistics for Windows, Version 19.0 (SPSS, Chicago, IL). Post-hoc analysis comprised of two-tailed Student’s t-test with Bonferroni correction applied. Chi-squared test was performed on pathogen prevalence in the spleen. P values of less than 0.05 were considered significant.

**Results**

Citrobacter rodentium colonization and resulting pathology is significantly reduced in mice with altered NF-κB signaling. Colonic *C. rodentium* colonization was significantly reduced in both strains of IKKβ-deficient mice throughout the 12 day experiment as compared to wild type (WT) control mice [F(2, 116) = 14.0, P<0.001; Figure 17A] with the most evident reduction being in IKKβ^{ΔMY} mice on Days 3, 6, and 12 post-challenge. As previously demonstrated, colon mass on Day 12 post-challenge is significantly
increased in WT mice as compared to uninfected, WT controls. This pathogen-induced increase was significantly reduced in both IKKβ^{AMY} and IKKβ^{AIEC} mice as compared to infected WT controls on Day 12 post-challenge [F(3, 28) = 19.1, P<0.001; Fig. 17B]. The total colitis index, as assessed by increases in colonic hyperplasia, dysplasia, inflammation, edema, and epithelial and crypt defects, was significantly reduced in each IKKβ-deficient strain on Day 12 post-challenge as compared to WT controls [F(2, 28) = 13.4, P<0.001; Fig. 17C]. The increase in colonic pathology presented as increases in significant immune infiltrates along with mucosal damage and thickening of the colonic villus, as well as focal epithelial ulcerations (Fig. 17D). The colons from IKKβ^{AIEC} mice also had a thickening of the mucosa along with enhanced inflammatory infiltrates however it was to a lesser extent as compared to colons of WT mice (Fig. 17E). Colons from IKKβ^{AMY} mice had a relatively normal appearance (Fig. 17F).

*Colonic inflammatory mediator gene expression is significantly reduced in mice with classical NF-κB signaling deficiencies.* The peak of colonic gene expression due to *C. rodentium* challenge often coincides with the peak of pathogen colonization. On Day 12 post-challenge there was a negligible increase in colonic CCL2 mRNA expression in infected WT mice as compared to non-infected WT mice and while there was a reduction in colonic CCL2 mRNA expression in both IKKβ^{AMY} and IKKβ^{AIEC} on Day 12 post-challenge as compared to infected WT control mice, the reduction approached, but did not reach statistical significance [F(2, 29) = 2.8, P=0.08 Figure 18A]. On Day 12 post-challenge there was a four-fold increase in colonic TNF-α mRNA expression in infected
WT control mice which was significantly reduced in both IKKβ<sup>AMY</sup> and IKKβ<sup>IEC</sup> mice [F(2, 29) = 3.8, P<0.05; Fig. 18B]. As previously demonstrated, iNOS mRNA expression is increase by *C. rodentium* on Day 12 post-challenge in WT mice, an increase which was reduced in mice with alterations in NF-κB signaling [F(2, 29) = 12.4, P<0.001; Fig. 18C]. This reduction in colonic iNOS expression was most evident in IKKβ<sup>AMY</sup> mice. The chemokine CXCL1 is increased in infected, WT mice on Day 12 post-challenge which was blocked in mice with defective NF-κB signaling in both myeloid cells and intestinal epithelial cells [F(2, 30) = 12.8, P<0.001; Fig. 18D].

*Monocyte/macrophage accumulation is reduced in IKKβ-deficient mice.* There was a decrease in monocyte/macrophage accumulation in IKKβ-deficient mice as compared to WT mice on Day 12 post-challenge as visualized by F4/80 staining via immunohistochemistry. This reduction, however, approached, but did not reach statistical significance [F(2, 8) = 2.5, P=0.1; Figure 19A].

*Spleen mass, but not splenic pathogen burden and prevalence, is reduced in mice with altered NF-κB signaling.* Similar to colon mass, spleen mass is increased in WT mice on Day 12 post-challenge. This *C. rodentium*-induced increase in spleen mass is negated in both strains of mice with NF-κB signaling deficiencies [F(2, 27) = 8.7, P=0.001; Figure 20A]. While *C. rodentium* is a non-invasive pathogen, it can be sometimes be found in spleen, particularly during a severe infection. There were no significant changes in the likelihood of detecting the pathogen in the spleen of WT or either IKKβ-deficient strain
on Day 12 post-challenge (Fig. 20B). Similarly, there was no change in the pathogen burden in either IKKβ-deficient strain as compared to that which was detected in infected, WT control mice (Fig 20C).

*Pretreatment with live* L. reuteri *can reduce C. rodentium-enhanced gene expression in CMT-93 colonic epithelial cells in vitro.* Colonic epithelial cell mRNA expression was determined after 3 hours of stimulation (i.e., 1 hour of pretreatment, 2 hours of challenge with *C. rodentium* or vehicle) by Real Time PCR. There were significant changes in CCL2 gene expression by CMT-93 cells depending on the condition in which they were treated \[F(3, 27) = 8.1, P\leq 0.001; \text{Figure 21A}\]. Colonic epithelial cells stimulated with live *L. reuteri* alone did not produce a significant amount of CCL2 as compared to vehicle-treated control cells. There was, however, a significant increase in CCL2 mRNA expression in CMT-93 cells challenged with *C. rodentium*. This *C. rodentium*-induced expression of CCL2 was significantly reduced by pretreatment of CMT-93 cells with live *L. reuteri*. There were also significant changes in mRNA expression in the chemokine CXCL1 \[F(3, 29) = 30.2, P<0.0005; \text{Fig. 21B}\]. Stimulation with live *L. reuteri* was enough to cause a 5-fold significant increase in CXCL1 gene expression in CMT-93 cells. Stimulation with *C. rodentium* also caused a significant increase in CXCL1 gene expression to a much greater extent (i.e., a 14-fold increase) over vehicle-treated control samples, which was significantly reduced by a 1 hour pretreatment with *L. reuteri*. Tumor necrosis factor-α mRNA expression in CMT-93 cells stimulated with either live *L. reuteri* or *C. rodentium* was also significantly altered after 3 hours of treatment \[F(3, 29)\]
Live *L. reuteri* stimulation caused a 3-fold increase in TNF-α mRNA expression in CMT-93 cells, while stimulation with *C. rodentium* caused a 14-fold increase in TNF-α mRNA expression. When CMT-93 cells were pretreated with *L. reuteri* prior to challenge with *C. rodentium*, pathogen-induced TNF-α gene expression was significantly reduced to that of *L. reuteri* stimulation only.

*Stimulation of CMT-93 cells with bacterial byproducts of *L. reuteri* prior to challenge with *C. rodentium* reduces pathogen-induced gene expression.* Cell-free, pH-adjusted supernatants from overnight cultures of *L. reuteri* were used to modulate *C. rodentium*-induced gene expression. Epithelial cell CCL2 mRNA gene expression was significantly altered by *L. reuteri* supernatant pretreatment [F(3, 29) = 5.7, P<0.005; Figure 22A]. The conditioned supernatants of *L. reuteri* did not change CCL2 gene expression over that of the vehicle-treated control cells, however pretreatment with these bacterial byproducts were able to significantly reduce *C. rodentium*-enhanced CCL2 mRNA expression. Similarly, CXCL1 mRNA expression was also significantly changed by *L. reuteri* byproduct stimulation in CMT-93 cells [F(3, 29) = 6.1, P<0.005; Fig. 22B]. Unlike stimulation with live *L. reuteri*, the conditioned supernatants of *L. reuteri* did not cause an increase in CXCL1 mRNA expression; however this pretreatment was still able to significantly reduce *C. rodentium*-enhanced CXCL1 expression. Lastly, TNF-α mRNA expression by CMT-93 cells was significantly changed by the bacterial byproducts of *L. reuteri* [F(3, 28) = 24.1, P<0.0001; Fig. 22C]. Similar to CXCL1, TNF-α mRNA expression was not changed by conditioned supernatants of *L. reuteri* over that of...
vehicle-treated CMT-93 cells. The pretreatment of CMT-93 cells with the bacteria byproducts of \textit{L. reuteri} significantly reduced \textit{C. rodentium}-enhanced TNF-\(\alpha\) mRNA gene expression to near vehicle-treated cells.

\textit{Citrobacter rodentium-induced mRNA expression is not attenuated by pretreatment with live L. reuteri in RAW 264.7 macrophages.} The ability of live \textit{L. reuteri} pretreatment to modulate \textit{C. rodentium}-induced inflammatory mediator gene expression in RAW 264.7 macrophages was also assessed by Real Time PCR. Macrophage production of CCL2 mRNA was significantly changed by 3 hours of stimulation [F(3, 20) = 8.1, \(P \leq 0.001\); Figure 23A]. Stimulation with either live \textit{L. reuteri} or \textit{C. rodentium} caused a significant increase in CCL2 gene expression and pretreatment of RAW 264.7 cells with live \textit{L. reuteri} prior to challenge with \textit{C. rodentium} failed to reduce pathogen-induced CCL2 expression. Tumor necrosis factor-\(\alpha\) mRNA expression was also significantly altered after 3 hours of stimulation as compared to vehicle-treated control macrophages [F(3, 28) = 8.3, \(P < 0.0001\); Fig. 23B]. Similar to CCL2 expression, TNF-\(\alpha\) mRNA expression was significantly enhanced by stimulation with live \textit{L. reuteri} and \textit{C. rodentium}. There was no change in TNF-\(\alpha\) gene expression when \textit{C. rodentium}-challenged macrophages were pretreated with live \textit{L. reuteri}. Expression of iNOS was also significantly changed as compared to vehicle-treated control cells [F(3, 28) = 3.1, \(P < 0.05\); Fig. 23C]. Similar to both CCL2 and TNF-\(\alpha\), iNOS mRNA expression was enhanced to near similar levels by either live \textit{L. reuteri} or \textit{C. rodentium}. There was no change in \textit{C. rodentium}-induced iNOS expression after pretreatment with live \textit{L. reuteri}
Pathogen-induced macrophage mRNA expression is unchanged by pretreatment with L. reuteri byproducts. Gene expression of the chemokine CCL2 was significantly changed at 3 hours post-stimulation [F(3, 20) = 57.0, P<0.0001; Figure 24A]. Treatment with the bacterial byproducts of L. reuteri alone was enough to significantly enhance CCL2 mRNA expression in RAW 264.7 macrophages to near similar levels as pathogen stimulation. Pretreatment of macrophages with L. reuteri byproducts prior to challenge with C. rodentium did not reduce CCL2 gene expression as it did in CMT-93 cells. The expression of TNF-α in macrophages was also significantly changed as compared to vehicle-stimulated controls [F(3, 28) = 313.9, P<0.0001; Fig. 24B]. There was a significant increase in TNF-α mRNA gene expression in macrophages treated with L. reuteri byproducts only, however this increase was significantly lower than that of pathogen-induced TNF-α expression. There was no change in C. rodentium-induced TNF-α mRNA expression when macrophages were pretreated with conditioned supernatants of L. reuteri. The enzyme iNOS was significantly changed after 3 hours of stimulation as compared to vehicle-stimulated controls [F(3, 28) = 57.2, P<0.0001; Fig. 24C]. Stimulation with either L. reuteri supernatants or C. rodentium caused a significant increase in iNOS mRNA gene expression as compared to control samples. Pathogen-induced macrophage iNOS expression was unchanged by pretreatment with conditioned L. reuteri supernatants.
Discussion

The results of this study highlight the influences that intestinal epithelial cells and myeloid cells can have during *C. rodentium* challenge. Mice that lack the ability to classically activate NF-κB in intestinal epithelial cells have a reduction in *C. rodentium*-induced colon mass, colonic histopathology, and colonic inflammatory mediator gene expression as compared to wild type controls. Interestingly, there is an even further reduction in infection-induced colon mass, histopathology, and colonic gene expression in mice with defective NF-κB signaling within myeloid-derived cells. While these results are striking, a confound presents itself in which there is a significant reduction in colonic pathogen colonization in both strains of IKKβ-deficient mice. The pathologic changes of the colon during *C. rodentium* colonization parallels that of the pathogenic burden which is present within the colon, therefore it is possible the reductions in colonic pathology is simply due to a reduction in pathogen burden [118]. While this may be likely, it must also be noted that all mice within this study were inoculated with $3 \times 10^8$ CFU of *C. rodentium*; however the colonization rates vary drastically between different strains. Colonic pathogen burden was the heaviest within the wild type mice with maximum colonization reaching $1 \times 10^5$ CFU/g of colonic contents. There was a near 10-fold reduction in *C. rodentium* colonization in IKKβ^ΔIEC^ mice compared to wild type mice. The reduction of colonic colonization of *C. rodentium* was even more prominent within IKKβ^ΔMY^ mice with regard to the colonization of wild type mice in that there was a 100-fold reduction in pathogen prevalence which coincided with only 50% of IKKβ^ΔMY^ mice becoming colonized. The cause of these reductions in colonic *C. rodentium* colonization
in IKKβ-deficient mice is currently under investigation with the hypothesis that reductions in NF-κB-derived inflammation as the main culprit.

There is gaining evidence within the realm of mucosal immunity research that suggests that the colonization of pathogens on mucosal surfaces must be met with a certain degree of inflammation [212]. One theory as to why mucosal inflammation contributes to the colonization of pathogens is that mucosal inflammation causes a shift in the commensal microbiota which pathogens can take advantage of to increase their own colonization. Evidence to support this theory was revealed in a study performed by Lupp et.al., in which it was shown that exposure to *C. rodentium* significantly alters the intestinal microbiota, the kinetics of which paralleled the pathogen-induced inflammatory response; as the inflammatory response to pathogen increased, the colonic bacterial diversity decreased, which may have allowed for the increased *C. rodentium* colonization. Interestingly, when mice were challenged with *Campylobacter jejuni*, a non-natural murine gastrointestinal pathogen which does not induce inflammation, there were no significant changes in either pathogen colonization or changes in the microbiota. In order to ensure these changes were not only associated with pathogen-induced inflammation, two additional forms of experimentally induced colitis, dextran sodium sulfate and IL-10-deficiency, were used. Dextran sodium sulfate (DSS), which induces colitis through an unknown mechanism, enhances colonic inflammation and also causes shifts in the microbiota. Challenging DSS-exposed mice with a nonpathogenic strain of *E. coli* caused a significant increase in its colonization as compared to control mice. A similar enhancement of nonpathogenic *E. coli* colonization was found to occur in IL-10-
deficient mice, which develop spontaneous colitis [212]. It has been long known that the microbiota contribute to the development of spontaneous colitis in IL-10-deficient mice as raising them under germ-free conditions can prevent colitis from occurring.

Similar to DSS-exposure as well as IL-10−/− mice, exposure to murine psychological stressors can alter the intestinal microbiota which can lead to enhanced bacterial colonization [8]. For example, stressor-exposure alone prior to challenge with *C. rodentium* is enough to significantly enhance pathogen burden, a phenomenon which was linked to disturbances in the microbiota [8, 14]. Interestingly, the most susceptible of the commensal bacteria are generally thought of as beneficial, probiotic bacteria, such as members of the *Lactobacillus* genus. One such member, *Lactobacillus reuteri*, can be reduced during stressor-exposure and when this probiotic strain is fed to mice during stressor exposure, the stressor-enhanced effects on infectious colitis are diminished [14]. Because it is unknown how *L. reuteri* is able to counteract the negative effects of stressor-exposure on *C. rodentium*-induced colitis without changing pathogen colonization, we hypothesized that these beneficial bacteria were able to modulate cell signaling within the colon. Intestinal epithelial cells and macrophages became our main targets due to the nature of infection, i.e., *C. rodentium* attaches directly to epithelial cells and inflammatory monocytes/macrophages contribute heavily to stressor-exacerbated colitis. In our study, pretreatment of CMT-93 epithelial cells with live *L. reuteri* prior to challenge with *C. rodentium* was enough to reduce pathogen-enhanced CCL2, TNF-α, and CXCL1 gene expression; however, the *L. reuteri* treatment alone also mildly enhanced gene expression in the CMT-93 cells. It should be noted that, in the colon,
commensal bacteria generally do not come into contact with intestinal epithelial cells due to the thick mucus layer [10, 178]. Because of this, we also tested the ability of cell-free bacterial byproducts of \textit{L. reuteri} to modulate \textit{C. rodentium}-induced gene expression. When CMT-93 epithelial cells were treated with these cell-free supernatants, there were no basal increases in CCL2, TNF-\textalpha, or CXCL1 gene expression, and pretreatment prior to \textit{C. rodentium} challenge nearly negated the pathogen-induced increases. \textit{Citrobacter rodentium}-induced gene expression in macrophages, on the other hand, was not modulated by pretreatment with either live \textit{L. reuteri} or its cell-free bacterial byproducts. In fact, \textit{L. reuteri} alone and its cell-free supernatants enhanced macrophage CCL2, TNF-\textalpha, and iNOS gene expression.

The ability of the probiotic \textit{L. reuteri} to modulate \textit{C. rodentium}-induced gene expression in intestinal epithelial cells, but not macrophages has led to the idea that the presence of beneficial bacterial species, such as \textit{L. reuteri}, help stabilize gene production by intestinal epithelial cells through the production of an unknown substance. When these beneficial species are reduced, such as by stressor-exposure, there is a reduction in the availability of the beneficial substance which is able to reduce pathogen-enhanced gene expression and there will be a heightened production of inflammatory mediators by the intestinal epithelial cell. It is possible that the enhanced inflammatory mediator gene production which is seen during stressor exposure is due to increased NF-\textkappaB activation within the epithelial cell as there is a reduction in \textit{C. rodentium}-induced pathology as well as colonization in \textit{IKKp}^{\text{IEC}} mice. Previous studies have also shown that \textit{L. reuteri} is able to reduce NF-\textkappaB activation by stabilizing I\kappaB [213, 214]. Because of this it is
also possible that \textit{L. reuteri} is able to reduce stressor enhanced gene expression \textit{in vivo} and epithelial cell gene expression \textit{in vitro} by reducing NF-κB activation.
Figure 17. *Citrobacter rodentium* colonization and resulting infectious colitis is reduced in IKKβ-deficient mice.

Wild type, IKKβ\textsuperscript{ΔIEC}, and IKKβ\textsuperscript{ΔMY} mice were challenged with 3 x 10\textsuperscript{8} CFU of *C. rodentium*. (A) Colonic pathogen colonization was followed throughout the 12 day experiment. Stool samples were collected on Days 0, 1, 3, 6, and 12 post-challenge and pathogen was enumerated via pour plate method. *Citrobacter rodentium* was significantly reduced in both IKKβ\textsuperscript{ΔIEC} and IKKβ\textsuperscript{ΔMY} throughout the 12 day experiment as compared to WT mice. *p*<0.001 IKKβ\textsuperscript{ΔIEC} or IKKβ\textsuperscript{ΔMY} vs WT on Days 3, 6, and 12. (B) On day 12 post-challenge, colons were removed, fixed in formalin, and embedded in paraffin. Prior to fixation, colons were weighed. Wild type mice presented with a thickening of the colon on Day 12 post-challenge which was evident by enhanced mass, however there was a significant reduction in colon mass in both IKKβ\textsuperscript{ΔIEC} and IKKβ\textsuperscript{ΔMY} mice on Day 12 post-challenge. a = WT vs. uninfected WT *p*<0.005. * = IKKβ\textsuperscript{ΔIEC} or IKKβ\textsuperscript{ΔMY} vs. infected WT *p*<0.005. (C) Paraffin-embedded colons were sectioned and stained with hematoxylin and eosin in order to visualize and score the pathology present in each sample. There was a significant reduction in the total colitis index in IKKβ\textsuperscript{ΔIEC} and IKKβ\textsuperscript{ΔMY} mice as compared to WT. Colitis scores were also significantly reduced in IKKβ\textsuperscript{ΔMY} as compared to IKKβ\textsuperscript{ΔIEC} colons. *p*<0.0001 IKKβ\textsuperscript{ΔIEC} or IKKβ\textsuperscript{ΔMY} vs WT. Images are taken from slides representative of a (D) WT control colon, (E) IKKβ\textsuperscript{ΔIEC} colon, and (F) IKKβ\textsuperscript{ΔMY} colon. *n* = 12 WT, *n* = 13 IKKβ\textsuperscript{ΔIEC}, *n* = 6 IKKβ\textsuperscript{ΔMY}. Data are the mean ± standard error.
Colonic *Citrobacter rodentium* colonization

Main effect of strain $F(2, 116) = 14.0, P<0.001$; WT>IEC>MY

Limit of detection

Histologic Colitis Index (max = 24)

Colon Mass (g)

WT

IKKβ^ΔIEC^

IKKβ^ΔMY^

Day 12 Post-Challenge

Uninfected WT WT IKKβ^ΔIEC^ IKKβ^ΔMY^

Day 12 Post-Challenge

Histologic Colitis Index (max = 24)

Colon Mass (g)

WT IKKβ^ΔIEC^ IKKβ^ΔMY^
Figure 18. Pathogen-induced colonic mRNA expression is reduced in IKKβ-deficient mice.

Wild type, IKKβΔIEC, and IKKβΔMY mice were challenged with 3 x 10^8 CFU of C. rodentium. On Day 12 post-challenge, colons were removed and processed in order to quantify mRNA expression by Real Time PCR. (A) There was a non-significant reduction in colonic CCL2 mRNA in both IKKβΔIEC and IKKβΔMY on Day 12 post-challenge as compared with WT colons. (B) There was a trend towards a significant reduction in colonic TNF-α mRNA expression in IKKβΔIEC as compared to WT controls, while colonic TNF-α was significantly reduced in IKKβΔMY colons as compared to colons of WT mice. *p<0.05 IKKβΔMY vs WT, †<0.06 IKKβΔIEC vs WT. (C) There was a non-significant reduction in iNOS gene expression in IKKβΔIEC colons as compared to colons of WT mice. iNOS gene expression was significantly reduced in IKKβΔMY colons. *p<0.001 IKKβΔMY vs WT. (D) CXCL1 mRNA expression was significantly reduced in colons from both IKKβΔIEC and IKKβΔMY mice as compared with colons from WT controls. *p<0.001 IKKβΔIEC or IKKβΔMY vs WT. n = 12 WT, n = 13 IKKβΔIEC, n = 6 IKKβΔMY. Data are the mean ± standard error.
Figure 19. Colonic monocyte/macrophage accumulation is reduced in IKKβ-deficient mice.

Colons were removed from mice on day 12 post-challenge and subsequently fixed in formalin, embedded in paraffin, then sectioned and stained with an antibody for the surface marker F4/80 in order to visualize macrophages. Individual pixels were counted via Photoshop and averaged per colon in order to give the number of F4/80+ pixels/field. (A) Colonic monocyte/macrophage accumulation was reduced in IKKβ-deficient mice, however this reduction was not significant. Images are taken from slides representative of a (B) WT control, (C) IKKβΔIEC, and (D) IKKβΔMY. n = 3 WT, n = 4 IKKβΔIEC, n = 3 IKKβΔMY. Data are the mean ± standard error.
Figure 20. Spleen mass, but not splenic pathogen burden, is modulated in IKKβ-deficient mice.

Wild type, IKKβ^{ΔIEC}, and IKKβ^{ΔMY} mice were challenged with 3 x 10^8 CFU of *C. rodentium*. On Day 12 post-challenge, spleens were removed, weighed, and plated in order to enumerate *C. rodentium*. (A) On Day 12 post-challenge the spleens of WT mice exhibited an increase in mass as compared to non-infected WT mice. There was a significant reduction in spleen mass in both IKKβ^{ΔIEC} and IKKβ^{ΔMY} as compared to spleens from infected, WT mice. * IKKβ^{ΔIEC} or IKKβ^{ΔMY} vs. infected WT, * p<0.01 IKKβ^{ΔIEC} or IKKβ^{ΔMY} vs Infected WT. (B) There was no change in the likelihood of detecting the translocation of *C. rodentium* from the colon to the spleen in either IKKβ^{ΔIEC} or IKKβ^{ΔMY} as compared with the WT control spleens. (C) There also was no significant changes in the pathogenic burden found within the spleens in either IKKβ-deficient strain. * n = 12 WT, n = 13 IKKβ^{ΔIEC}, n = 6 IKKβ^{ΔMY}. Data are the mean ± standard error.
Figure 21. *Citrobacter rodentium*-induced inflammatory mediator gene expression is modulated by pretreatment with *L. reuteri* in intestinal epithelial cells *in vitro*.

The colonic epithelial cell line, CMT-93, were pretreated with live, intact *L. reuteri* for 1 hour prior to challenge with *C. rodentium*. At the end of the 2 hour pathogen challenge, total RNA was isolated in order to quantify mRNA expression by Real Time PCR. (A) CCL2 gene expression was significantly increased by 2 hours of *C. rodentium* challenge as compared to vehicle (PBS) control samples which pretreatment with *L. reuteri* significantly reduced. (B) CXCL1 mRNA expression was significantly enhanced by stimulation with *L. reuteri* or *C. rodentium* alone, however pretreatment with *L. reuteri* prior to pathogen challenge significantly reduces pathogen induced gene expression. (C) TNF-α mRNA expression was also significantly increased by *L. reuteri* and more so by *C. rodentium* alone. Similar to CCL2 and CXCL1 expression, pathogen-induced TNF-α gene expression was significantly reduced by *L. reuteri* pretreatment. a = treatment vs. control, p<0.005. b = LR/CR vs CR, p<0.005. LR, *L. reuteri* treatment alone. CR, *C. rodentium* treatment alone. LR/CR, *L. reuteri* pretreatment prior to *C. rodentium* challenge. n = 8/treatment. Data are the mean ± standard error.
Figure 22. Pathogen-induced inflammatory mediator mRNA expression is significantly reduced by *L. reuteri* byproducts.

Cell-free supernatants from overnight cultures of *L. reuteri* were added to CMT-93 cells for 1 hour prior to a 2 hour challenge with *C. rodentium*. Messenger RNA expression was quantified by Real Time PCR. (A) Pathogen-induced gene expression was significantly reduced by *L. reuteri* bacterial byproducts. (B) *Citrobacter rodentium*-induced CXCL1 mRNA expression was significantly reduced by pretreatment with cell-free *L. reuteri* bacterial byproducts. (C) Pathogen-induced TNF-α mRNA expression is also significantly to near baseline levels by pretreatment with *L. reuteri* bacteria byproducts. a = treatment vs. control, p<0.05. b = LR Supe./CR vs CR, p<0.005. LR Supe., *L. reuteri* byproduct treatment alone. CR, *C. rodentium* treatment alone. LR Supe./CR, *L. reuteri* byproduct pretreatment prior to *C. rodentium* challenge. n = 8/treatment. Data are the mean ± standard error.
Figure 23. Inflammatory mediator gene expression is significantly enhanced in RAW 264.7 macrophages regardless of treatment.

The murine macrophage cell line, RAW 264.7, were pretreated with live, intact *L. reuteri* for 1 hour prior to a 2 hour challenge with *C. rodentium*. Total RNA was isolated and inflammatory mediator mRNA expression was quantified by Real Time PCR. (A) CCL2 mRNA expression was significantly enhanced by *L. reuteri* alone, *C. rodentium* alone, and by probiotic pretreatment prior to *C. rodentium* challenge as compared to vehicle control samples. p<0.05. (B) Macrophage TNF-α mRNA expression was also significantly enhanced in all groups stimulated with either *L. reuteri* or *C. rodentium* as compared to non-challenged controls. p<0.001. (C) iNOS gene expression was significantly enhanced in both groups stimulated with *L. reuteri* with a trend towards significance within the group challenged with *C. rodentium* alone. a, p<0.05 individual treatment vs. control. †p=0.06 treatment vs control. LR, *L. reuteri* treatment alone. CR, *C. rodentium* treatment alone. LR/CR, *L. reuteri* pretreatment prior to *C. rodentium* challenge. n = 8/treatment. Data are the mean ± standard error.
Figure 24. Inflammatory mediator gene expression is significantly enhanced in RAW 264.7 macrophages regardless of treatment.

RAW 264.7 cells were pretreated with cell-free supernatants from overnight cultures of *L. reuteri* for 1 hour prior to a 2 hour challenge with *C. rodentium*. Total RNA was isolated and inflammatory mediator mRNA expression was quantified by Real Time PCR. (A) CCL2 mRNA expression was significantly enhanced by treatment with cell-free supernatants from *L. reuteri* alone, *C. rodentium* alone, and by probiotic byproduct pretreatment prior to *C. rodentium* challenge as compared to vehicle control samples. (B) Macrophage TNF-α mRNA expression was also significantly enhanced in all groups stimulated with either *L. reuteri* supernatants or *C. rodentium* as compared to non-challenged controls. (C) iNOS gene expression was significantly increased in macrophages stimulated with *L. reuteri* byproducts, *C. rodentium*, or the pretreatment of *C. rodentium*-challenged cells with *L. reuteri* byproducts. a= individual treatment vs. control. p<0.0001. LR Supe., *L. reuteri* byproduct treatment alone. CR, *C. rodentium* treatment alone. LR Supe./CR, *L. reuteri* byproduct pretreatment prior to *C. rodentium* challenge. n = 8/treatment. Data are the mean ± standard error.
CHAPTER 5: Discussion

Patients with inflammatory diseases, such as periodontal disease, cardiovascular disease, and rheumatoid arthritis often report a worsening of symptoms following a period of psychological stress [215-218]. The same is true for patients with IBD. However, the added stigma of gastrointestinal distress can perpetuate the stress response beyond the initial stressor leading to a vicious cycle of flares and relapses [1, 2, 23, 180]. The mechanism by which psychological stressors exacerbate colitis has remained elusive, but recent evidence has demonstrated that stressor-induced changes in the intestinal microbiota may contribute to exacerbated colitis [5-8, 10, 23, 27, 201]. Current therapies for IBD patients include antibiotics, anti-inflammatories, and immunosuppressant drugs, all of which can lead to serious side effects as well as contribute to the changes in the microbiota [23]. Probiotics are live microorganisms which can confer health benefits to the host. These beneficial bacteria have been demonstrated to be able to reduce proinflammatory cytokine and chemokine production, as well as restore balance to the indigenous microbiota [64, 87, 96-98, 103, 109, 113]. Because of this, the use of probiotics as a therapy for IBD patients is under investigation [6, 11, 23, 64, 80, 81, 83, 88, 92, 109, 111, 219]. However, despite the wealth of research which suggests that probiotics are able to reduce inflammation, clinical trials testing the efficacy of probiotics as a therapy for IBD patients have had mixed results. It is possible that the failure of
many of these trials is due to poor patient compliance or flaws in the study design, or that probiotic strains have been selected for their reported effects on macrophages and not epithelial cells. Perhaps the most difficult decision in testing the efficacy of probiotic intervention in resolving colitis is choosing the proper probiotic bacteria as there are dozens of beneficial bacteria available and it is possible that what may work for one individual may not work for another. Because of this, it is important to elucidate the mechanism by which stressor exposure can lead to flares of colitis.

*Exposure to prolonged restraint renders the resistant CD-1 mouse susceptible to C. rodentium-induced colitis*

All known mouse strains become colonized upon challenge with the colonic pathogen *C. rodentium* which causes disease symptoms ranging from subclinical to fatal, largely dependent on the host mouse strain [120]. The outbred CD-1 mouse is among the strains which are considered relatively resistant to *C. rodentium*-induced pathology, in that it does become colonized, however there is minimal pathogen-associated pathology even at the peak of colonization. Bailey et al. (2010) demonstrated that exposure to prolonged restraint (RST) can alter the intestinal microbiota which left the resistant CD-1 mouse vulnerable to colonization by *C. rodentium*. In this study, mice were exposed to RST prior to challenge with *C. rodentium*. While exposure to RST prior to pathogen challenge did significantly increase the colonization of *C. rodentium*, the resulting pathogen-induced infectious colitis was mild. Therefore, the first study presented within this manuscript was the assessment of the consequences of RST exposure at the onset of
*C. rodentium* challenge. As predicted, when CD-1 mice were exposed to RST during the first week of *C. rodentium* challenge, there was a significant increase in pathogen burden over non-stressed controls. The resulting RST-induced infectious colitis was more severe than the aforementioned pathology which was induced by exposing mice to RST prior to *C. rodentium* challenge. The moderate to severe RST-induced infectious colitis was demonstrated by significant increases in colonic histopathology, colonic CCL2, TNF-α, and iNOS mRNA expression, as well as increases in the abundance and likelihood of pathogen translocation from the colon to the spleen. Next, we set out to determine if probiotic intervention with *L. reuteri* could reduce RST-enhanced, *C. rodentium*-induced infectious colitis.

*Stressor-exacerbated disease severity is reduced by probiotic intervention with L. reuteri*

In order to test the hypothesis that probiotic intervention can reduce stressor-exacerbated infectious colitis, careful consideration was made with regard to which probiotic bacteria was used. Bailey et al, (1999) demonstrated that the maternal separation stressor could significantly reduce the fecal shedding of lactobacilli in infant rhesus monkeys. Colonic murine lactobacilli are also susceptible to exposure to either prolonged restraint or social disruption (Galley and Bailey, Unpublished Observation). In the mouse colon the most prevalent species of the *Lactobacillus* genus are *L. reuteri*, *L.intestinalis*, and *L. animalis*, all of which are thought to be significantly reduced during stressor exposure. Due to its probiotic properties, coupled with the evidence that it is
reduced in the colon during stressor exposure, *L. reuteri* was chosen to test our hypothesis that probiotic intervention can reduce stressor-enhanced infectious colitis.

Treatment of mice with *L. reuteri* did not modulate *C. rodentium* colonization in either stressor-exposed or non-stressed control mice. This observation was important because it indicated that changes in infectious colitis are due to the effects of *L. reuteri* that are independent of pathogen levels. Stressor-enhanced colon mass and overt colonic histopathology was not reduced by *L. reuteri* treatment and while the overall mean of RST-enhanced colonic CCL2 and TNF-α mRNA expression was reduced, there was no significant change as compared to their vehicle-treated counterparts. There were changes, however in colonic barrier integrity with regard to probiotic intervention. *Lactobacillus reuteri* treatment was able to restore RST-reduced colonic β-defensin 3 and claudin 5 mRNA expression which likely contributed to the reductions in stressor-enhanced pathogen escape from the colon to the spleen in *L. reuteri*-treated mice. In addition to the effects on barrier integrity, RST-exposed mice which received *L. reuteri* also had reductions in sickness behaviors. While not all aspects of RST-exacerbated infectious colitis were prevented by probiotic intervention, overall disease severity was reduced. It is possible that prolonged restraint enhanced colitis to a degree that was too severe to be overcome with probiotic intervention alone. Thus, a stressor which induces a less severe form of infectious colitis (namely SDR), was utilized to further assess the effects of *L. reuteri* on stressor-induced exacerbation of infectious colitis.
SDR-enhanced infectious colitis is dependent on CCL2-mediated immune recruitment

Similar to prolonged restraint, exposure to social disruption (SDR) enhances *C. rodentium*-induced infectious colitis although the resulting colitis is less severe. Interestingly, probiotic intervention with *L. reuteri* can prevent SDR-enhanced colonic histopathology, colonic inflammatory mediator mRNA expression, and colonic monocyte/macrophage recruitment without changing *C. rodentium* burden in the colon. Because inflammatory monocytes are often the chief producers of inflammatory mediators and therefore, among the main contributors of colitis the involvement of inflammatory monocyte recruitment during stressor-exacerbated infectious colitis was assessed through the use of CCL2-deficient (*CCL2<sup>−/−</sup>*) mice. Without the ability to recruit monocytes to the colon, stressor exacerbated infectious colitis was significantly attenuated as evaluated by reductions in colonic histopathology, colonic inflammatory mediator gene production, macrophage recruitment in the colon, and pathogen translocation from the colon to the spleen.

Social disruption is a unique stressor in that CD11b<sup>+</sup> splenocytes from mice exposed to this psychosocial stressor exhibit a primed phenotype and when stimulated with LPS *ex vivo* these splenocytes will produce significantly more proinflammatory cytokines than LPS-stimulated splenocytes from control mice [160, 198]. In order to determine if the primed nature of splenocytes, which include monocytes, from SDR-exposed mice contributes to stressor-exacerbated infectious colitis, CD11b<sup>+</sup> splenocytes from non-infected, stressor-exposed mice were transferred to naïve mice prior to *C. rodentium* challenge. While the transfer of cells to control recipient mice did enhance
pathogen burden within the colon, it did not change the outcome of stressor-enhanced infectious colitis. Therefore we have concluded that stressor-induced changes in the colon override the primed status of SDR-primed monocytes with regard to enhanced *C. rodentium*-induced infectious colitis.

*Probiotic* L. *reuteri blocks* *C. rodentium*-induced gene expression in intestinal epithelial cells, *but not macrophages*

The newly recruited inflammatory monocyte contributes to the exacerbation of infectious colitis by SDR exposure through a CCL2-dependent mechanism, however it is not known which cells within the colon are responsible for producing CCL2 during stressor exposure. Recent evidence suggests the involvement of colonic stromal cells which reside within the colonic lamina propria in the production of CCL2 in response to *C. rodentium*, while evidence for the involvement of colonic epithelial cells is lacking due to the difficulties associated with epithelial cell removal [132]. However, given the intimate contact made with epithelial cells during *C. rodentium* challenge it is hypothesized that these cells are heavily involved in the initiation of the colonic inflammatory response to *C. rodentium*. In fact, stimulating colonic epithelial cells with pathogen *in vitro* caused a significant increase in both CCL2 and TNF-α mRNA expression both of which can be significantly reduced by pretreatment with either live *L. reuteri* or its bacterial byproducts. Similar to the epithelial cells, *C. rodentium*-stimulated macrophages also had significant increases in CCL2 and TNF-α as compared to vehicle treated cells *in vitro*, however these pathogen-induced increases were not reduced by live
L. reuteri or L. reuteri byproduct pretreatment. With these results taken into consideration, we now believe that probiotic intervention reduces stressor-enhanced, CCL2-dependent monocyte recruitment through its interactions with colonic epithelial cells.

**NF-κB signaling in intestinal epithelial cells and myeloid cells contribute to C. rodentium-induced pathology**

How probiotic microorganisms exert their beneficial effects in the colon is currently unknown. Current evidence supports the theory that the anti-inflammatory properties of probiotics may be attributed to the ability of beneficial microbes to interfere with the transcriptional activation, i.e., NF-κB and AP-1, of inflammatory mediators [63, 97, 113]. The ability to reduce the activation of these transcription factors has been attributed to nonviable components of probiotic microbes which include secreted proteins, e.g. histamine [98], or structural components, e.g. muramyl dipeptide and CpG DNA [108, 110, 220]. Due to the experimental design of the aforementioned in vitro data, we hypothesize that L. reuteri produces a substance which can interfere with C. rodentium-induced transcriptional activation. It is commonly known that C. rodentium can activate NF-κB in colonic epithelial cells as well as macrophages [113, 126, 129-131, 211]. In order to determine the differential effects of epithelial-derived and monocyte/macrophage-derived NF-κB signaling, two different strains of mice were used in which classical NF-κB signaling was interrupted: the intestinal epithelial cell (IKKβ^{IEC}) and myeloid-derived cells (IKKβ^{AMY}). Mice with defective NF-κB signaling
had reductions in colon mass, colonic histopathology, colonic inflammatory mediator mRNA expression, and colonic $F4/80^+$ cell accumulation as compared to wild type controls. There was a consistent trend in which $C.\ textit{rodentium}$-induced changes in $\text{IKK}\beta^{\text{AIEC}}$ and $\text{IKK}\beta^{\text{AMY}}$ were reduced as compared to wild type mice. While this is likely due to reductions in pathogen colonization in each IKK\(\beta\)-deficient strain, we believe that NF-\(\kappa\)B-induced inflammation is necessary for colonic pathogen colonization.

Conclusions

The tolls that psychological stressors can take on the body are of growing concern in our modern world where exposures to life stressors have become part of our day to day lives. The effects of stressor exposure on the gut are of particular interest considering the increasing prevalence of patients with inflammatory bowel disease and irritable bowel syndrome and determining a mechanism would help define better treatment options for patients. Our goal of the work presented within this manuscript was to begin to understand the mechanisms by which psychological stressors can exacerbate infectious colitis and determine the potentials for probiotic intervention. Given the data which was collected, our working model is as follows: $\text{Lactobacillus reuteri}$, and possibly other beneficial bacteria, are able to produce substances which can interfere with inflammatory mediator gene expression in intestinal epithelial cells. Stressor exposure reduces the presence of members of the $\text{Lactobacillus}$ genus, including the reduction of the beneficial species $L.\ reuteri$. The stressor-induced changes in the commensal microbiota allows for an increase in $C.\ textit{rodentium}$ colonization. The decreased bioavailability of the substance
produced by *L. reuteri* allows for the enhanced production of *C. rodentium*-induced chemokines and proinflammatory cytokines by intestinal epithelial cells, potentially through an NF-κB-related mechanism. Enhanced CCL2 production by intestinal epithelial cells leads to the heightened recruitment of inflammatory monocytes to the colon which contribute to the stressor-exacerbated colonic pathology through an NF-κB-related mechanism. Depending on the severity of the colitis that is induced by stressor exposure, probiotic intervention with *L. reuteri* is an effective treatment option for the resulting stressor-induced pathology during an enteric infection.
References

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