INNATE IMMUNE ACTIVATION OF SWINE GASTROINTESTINAL EPITHELIAL CELLS AND TISSUES IN RESPONSE TO MICROBIAL EXPOSURE

by

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B.S., University of Wisconsin – LaCrosse, 1994
M.S., Kansas State University, 2001

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Animal Science and Industry
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ABSTRACT

The three experiments described below offer support of immune function by the swine gastrointestinal epithelium. Experiment one evaluated mediators that regulate the movement of macrophages (macrophage migration inhibitory factor; MIF), neutrophils (interleukin 8; IL8), dendritic cells (CC chemokine ligand 20; CCL20) and epithelial remodeling (osteopontin; OPN) in pigs challenged with *Salmonella enterica* serovar *Typhimurium* (ST) or *Choleraesuis* (SC). The proximal ileum had greater IL8 expression than the distal ileum ($P < 0.05$), and ST increased CCL20 ($P < 0.05$). In vitro, MIF, IL8, CCL20 and OPN mRNA expression induced by lipopolysaccharide (LPS), ST or SC using pig jejunal epithelial cells (IPEC-J2) resulted in increased IL8 secretion, and increased IL8 and CCL20 mRNA by ST and SC ($P < 0.05$). Experiment two evaluated how *Lactobacillus reuteri* (LR) and *Bacillus licheniformis* (BL) differed from ST or SC in their ability to regulate, stimulate, or modify IL8, CCL20, and tumor necrosis factor α (TNFα) in IPEC-J2 cells. ST stimulated an increase in IL8 secretion, with increases in IL8 mRNA ($P < 0.05$). BL increased IL8 mRNA ($P < 0.0001$). CCL20 mRNA was upregulated by ST ($P < 0.05$) and BL ($P < 0.05$). Only ST increased TNFα mRNA ($P < 0.05$). Another objective evaluated whether pre-exposure of IPEC-J2 cells to LR or BL modified ST induced IL8 secretion. IL8 secretion was increased by ST ($P < 0.0001$), and reduced by LR ($P < 0.05$). Only the BL/ST co-treated wells blunted basolateral IL8 secretion ($P < 0.0001$). Experiment three characterized the swine CCL20 mRNA sequence and evaluated tissue expression. Cloning of CCL20 from the porcine jejunum predicted a 97 amino acid peptide. All healthy tissues expressed CCL20 mRNA. In animals challenged with *Salmonella* spp., SC increased spleen and liver CCL20 expression. The data demonstrate that invasive bacterial pathogens in the pig gastrointestinal tract trigger upregulation of selected proinflammatory mediators; *Salmonella* spp. elicited differing patterns of activation in vitro and in vivo; IPEC-J2 cells increased IL-8 secretion in response to ST and BL, but not LR, while ST stimulated secretion was inhibited basolaterally by BL pre-exposure; and numerous porcine tissues are prominent sources CCL20.

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DEDICATION

This dissertation is dedicated to “Bailey” Skjolaas; she’s seen me through it all.

CHAPTER 1 - Literature Review: Summary of the intestinal mucosal immune system and microbial pathogenesis

INTRODUCTION

The aim of achieving a greater understanding to the fundamental mechanisms of bacterial pathogenesis and host immunity relates to the numerous bacterial infections that contribute to public health risks and economic losses. Initially, the bacterial model systems investigating inflammation and infection in domestic livestock or laboratory animals were conducted utilizing bacterial lipopolysaccharide (LPS, the cell wall component of gram negative bacteria). This model system results in strong proinflammatory responses in mice and swine, but at least in swine, LPS does not mimic the inflammatory response elicited by live enteric bacteria. Therefore a greater understanding of the mechanisms involved in live enteroinvasive bacterial pathogenesis is warranted.

Traditionally, the gastrointestinal epithelial monolayer has been identified as the site of nutrient absorption which also provides physical barrier protection against luminal pathogens and toxins. More recently, however, it has become well accepted that intestinal epithelial cells (IEC) are also active participants of the gastrointestinal immune system. IEC are now regarded as participants of antigen presentation, and sources of both chemokine and cytokine mediators that assist in the gastrointestinal immune defense. Therefore, IEC are a key intermediary in the crosstalk between the pathogen status of the gut lumen and underlying gastrointestinal immune tissues.

Low dose dietary antibiotics, used as growth promotants, have been widely incorporated into livestock production since the 1950s, and the advantages for weanling pigs include improvement in average daily weight gain and feed efficiency. However, such use of low dose dietary antibiotics has been questioned in recent years because of concerns that their use in livestock feed may lead to antibiotic resistant strains of pathogens that pose both a risk to livestock and (or) a public health risk. Direct-fed microbials, including those containing *Lactobacillus* and *Bacillus* spp., are potential alternatives to low dose in-feed antibiotics.

The following literature review will focus on gut associated mucosal immunology and the role of gastrointestinal epithelial cells in the protection against enteropathogenic bacteria. Attention will also be given to the expression of various cytokine and chemokine mediators known to regulate the movement of macrophages (macrophage migration inhibitory factor; MIF), neutrophils (interleukin 8, IL8), dendritic cells (CC chemokine ligand 20, CCL20) and epithelial remodeling (osteopontin; OPN) and their role in conferring protection against bacterial invasion. The final three chapters detail a series of in vitro and in vivo experiments providing new information on the response of swine and (or) isolated gastrointestinal epithelial cells to the invasive swine enteropathogens *Salmonella enterica* serovar *Typhimurium* (ST) or *Choleraesuis* (SC).

ORGANIZATION OF THE INNATE INTESTINAL IMMUNE SYSTEM

The intestinal epithelium represents the largest surface area of the body and performs multiple functions, which include those necessary for digestion, absorption and immune defense. The gastrointestinal tract (GIT) is home to roughly 500 to 1,000 species of bacteria with a biodiversity that includes Archaea, Eukarya, viruses, and bacteriophages, establishing a community of approximately 10^{12} resident microorganisms (Sonnenburg et al., 2004). This makes GIT immune defense and host protection of utmost importance. It is essential that the gastrointestinal system differentiate between pathogenic and commensal microbes in order to avoid over stimulation of the immune system yet maintain a disease free status. The GIT provides barrier protection against commensal and pathogenic invaders, mediates crosstalk to more conventional immune cells, and is generally considered the host's first line of defense against pathogenic microbes (Eckmann et al., 1995). The intestinal immunological barrier is composed of the gut associated lymphoid tissue, and 25% of the intestinal mucosa is considered lymphoid tissue housing 70 to 80% of all immunogenic secreting cells (Langkamp-Henken et al., 1992). Immune cell compartments and cells associated with the gut mucosa include: villus and crypt intestinal epithelial cells, paneth cells, the follicle associated epithelium (FAE) overlying the Peyer's patches (PP) including both intestinal epithelial cells (IEC) and specialized epithelial microfold (M) cells; there is also the isolated lymphoid tissue, mesenteric lymph nodes (MLN), and the lamina propria (LP) which contains intraepithelial lymphocytes (IEL) including T and B

lymphocytes, monocytes, and dendritic cells (Brandtzaeg and Pabst, 2004; Dotan and Mayer, 2003; Kagnoff, 1993; Muller et al., 2005).

These mucosal associated immune components assist in both the innate and adaptive immune responses. The function of the innate immune system is to quickly identify and respond to foreign microorganisms, to incapacitate pathogens, and act as an adjuvant for the acquired immune system (Yuan and Walker, 2004). Essential components of the intestinal innate immune system include the epithelial cell monolayer (a mechanical barrier), secretory IgA, antimicrobial peptides and proteins, commensal intestinal microbes, and biochemicals such as gastric acid, biliary and pancreatic secretions and mucins (Yuan and Walker, 2004). Active antigen sampling occurs at the mucosal surface followed by delivery to the lymph node tissues, whereupon systemic immune activation occurs (Brandtzaeg and Pabst, 2004). The M cells found in the FAE of the PP are considered the primary site of antigen sampling in the gut lumen, but both the villus epithelium and the PP of the small intestine are also participants of antigen sampling (Neutra et al., 1999).

Generally, residents of the jejunal PP include populations of lymphocytes, dendritic cells, macrophages, villous epithelium, and the specialized follicle-associated epithelium, which along with M cells, are considered the main site of antigen uptake (Beier and Gebert, 1998). The jejunal PP within the pig populate both the jejunum and proximal ileum, are small, persist throughout life and number between 25 to 35 PP (Stokes et al., 1994). The ileal PP in the pig is a large single ileocecal patch which extends along the terminal ileum (2.5 m in length) but involutes with age (Stokes et al., 1994). T and B cell populations residing in each PP location vary with age and infection (Stokes et al., 1994).

Gut associated participation in the innate immune response is mediated through pattern recognition receptors which are conserved germ line encoded receptors and molecules that recognize conserved pathogen associated molecular patterns. Toll like receptors (TLRs) are one such pattern recognition receptor whose expression has been identified on intestinal epithelial cells (Abreu et al., 2003). The function of these TLRs is to convert the gut recognition of pathogen associated molecular patterns into signals for enhanced immune function, including the increase in anti-microbial peptide expression, barrier fortification, and proliferation of epithelial cells (Abreu et al., 2005).

In addition to the TLRs, the intracellular receptor for endotoxin, nucleotide oligomerization domain (NOD) 1 and NOD 2, are also pattern recognition receptors which assist in monitoring the cytoplasm of cells for pathogen associated molecular patterns of invasive enteric pathogens (Abreu et al., 2005; Yuan and Walker, 2004). Together with TLRs, NOD1 (also known as CARD4; recognizing gram negative peptidoglycan) and NOD2 (CARD15; which recognizes both gram positive and negative peptidoglycan) contribute to the induction of the innate and acquired immune system (Collier-Hyams and Neish, 2005; Inohara et al., 2004). Aside from the above mentioned ligands, NOD1 and NOD2 also recognize specific motifs in bacterial LPS, peptidoglycan, and bacterial muramyl dipeptide, which lead to the induction of proinflammatory mediators (Inohara et al., 2004; Eckmann, 2004). TLRs and NOD proteins may act cooperatively in immune responses against bacteria. MDP has been shown to synergize with LPS resulting in the production of cytokines, and the cross induction of signaling components needed by the NOD and TLR mediated pathways may account for the synergistic effect (Inohara et al., 2004). For example, LPS up-regulates the expression of NOD2, and RICK, while MDP up-regulates MyD88, in TLR signaling (Inohara et al., 2004).

Intestinal epithelial cells constitutively express NOD1 (Kim et al., 2004) and proinflammatory cytokines are produced in response to peptidoglycan in gram-negative bacteria (Eckmann, 2004). The signaling pathways involved in NOD1 and NOD2, and therefore the effects on pro-inflammatory cytokine production, include the caspase activation and recruitment domain which functions as an effecter domain and associates with RICK (a protein kinase) by a homophillic CARD-CARD interaction (Inohara and Nunez, 2003). RICK in turn activates IκB kinase and the transcription factor NFκB through the assembly of large multi-protein complexes as a result of oligomerization at the centrally located NOD domain (Girardin et al., 2003; Inohara and Nunez, 2003). In various human colonic epithelial cell lines, bacterial pathogens (such as *Escherichia coli*, *Salmonella enterica* and *Shigella flexneri*) that bypassed the TLR activation pathway demonstrated activation of NFκB and NFκB target genes through NOD1 signaling, and therefore are a possible backup mechanism for activating the innate immune system during infection (Kim et al., 2004). In comparison, NOD2 is present in macrophages, dendritic and paneth cells, and are inducible in enterocytes (Eckmann, 2004).

Peroxisome proliferator-activated receptors (PPARs) have also been identified as mediators in the control of gastrointestinal inflammation. Specifically, PPARs are ligand

inducible transcription factors belonging to the nuclear hormone receptor family (Kostadinova et al., 2005). Their anti-inflammatory action is mediated by: 1) the inhibition of pro-inflammatory cytokines, adhesion molecules and extracellular matrix proteins, 2) stimulation of anti-inflammatory molecules, and 3) modulation of the proliferation, differentiation and survival of immune cells (including macrophages, B cells and T cells) (Kostadinova et al., 2005). There are three isoforms of PPAR which include alpha, beta/delta, and gamma; and all three isoforms have been found in the GIT of humans and the rat (Wang et al., 2005). Specifically, as determined by RT-PCR, the rat jejunum most abundantly expressed the alpha isoform, while the ileum expressed the beta form, and gamma was found in the duodenum, jejunum, cecum and colon; likewise, the human GIT ubiquitously expressed mRNA for all three isoforms along the entire tract (Bagga et al., 2003). PPARγ is mainly expressed in epithelial cells, stellate cells, monocytes/macrophages, kupffer cells, dendritic cells, and B and T lymphocytes; with high levels documented in adipose tissue, large intestine (colon epithelium), and hematopoietic cells, followed by intermediate levels in the kidney, liver, stomach, small intestine, and pancreas (reviewed in Auwerx, 2002; Debril et al., 2001; Wu, 2003). Natural ligands that are weak activators of PPARγ include polyunsaturated fatty acids, oxidized low density lipoprotein, some eicosanoids, and 15 deoxy-Δ12,14-prostaglandin (PG)J_2 (15d-PGJ2) (Wu, 2003). The PPARγ has been associated with inflammatory bowel disease in that patients with ulcerative colitis have reduced levels of the gamma receptors, yet many studies on this topic seem to have produced contradictory results (reviewed in Wu, 2003). Gastrointestinal inflammation may, in part, be mediated by the cyclopentenone prostanoid ligand, 15d-PGJ2; which leads to the reduced expression of the pro-inflammatory cytokine, IL8, by inhibition of NFκB via inhibition of IκBα degradation (Su et al., 1999). This mechanism may assist in attenuating the ability of pathogenic microorganisms to cause damage to the GIT (Gupta et al., 2001).

Intestinal epithelial and paneth cells also release gene encoded antibiotic peptides and proinflammatory cytokines that contribute to the innate immune response by blocking pathogenic microbes and microbial colonization. Antimicrobial peptides found within the small intestine reside in paneth and epithelial cells that populate the crypts and include defensins, cathelicidins, bactericidal / permeability increasing protein, the chemokine macrophage inflammatory protein 3 alpha / CCL20, as well as the bacteriolytic enzymes phospholipase A2 and lysozyme (reviewed in Muller et al., 2005; Ouellette, 1999). These peptides confer their

antimicrobial action through the formation of pores, activation of bacterial autolysins, disruption of outer and inner bacterial membranes, and degradation of bacterial lipids and peptidoglycan (Muller et al., 2005).

Gastrointestinal defense against Salmonellosis

In the United States an estimated 1.4 million persons are infected annually with *Salmonella* spp., a gram negative bacteria, which mostly results in a self-limiting diarrhea, vomiting, abdominal cramps, and fever, but also contributes to approximately 15,000 hospitalizations and roughly 500 deaths (Mead et al., 1999). Among the poultry, swine, and feedlot cattle industries within the United States, 50% or more of the flocks and herds have been reported positive for *Salmonella* at various points within the production cycle (Hurd, 2004). Disease is usually seen with 10^8 to 10^{11} CFU of *Salmonella* via the oral route of infection and pigs can shed up to 10^6 CFU of SC or 10^7 CFU of ST per gram of feces (Fedorka-Cray et al., 2000). SC (as well as many other *Salmonella* spp.) has the ability to survive, remain viable and maintain infectivity for long periods in the environment (Gray and Fedorka-Cray, 2001). Young pigs exposed to ST can be carriers for up to 28 wk after exposure (Wood et al., 1989) and sub-clinically infected hosts increase the risk for infecting others and contaminating the food chain. A farm to fork mathematical model estimates the human health costs associated with salmonellosis in pork to be $81.53 million (90% confidence interval ranging from $18.75 to $197.44 million) (Miller et al., 2005). Costs that account for human illness and lost production within the United States are estimated to be as high as 2.3 billion dollars annually (Hurd, 2004).

Public health risks and economic losses associated with bacterial infections have led to the aim of understanding the mechanisms of bacterial pathogenesis and host immunity throughout the various stages of infection. Initially, bacterial model systems that investigated inflammation and infection in domestic livestock or laboratory animals were conducted utilizing bacterial lipopolysaccharide (LPS, the cell wall component of gram negative bacteria), which initiated strong proinflammatory responses. In pigs, this LPS model system produces rapid febrile responses; early transient increases followed by prolonged suppression in plasma levels of prostaglandin E_2 (PGE_2) (a pyrogenic mediator); reduced feed intake; and inactivity (Johnson and von Borell, 1994; Wright et al., 2000). Peripherally administrated LPS in pigs also resulted in increased plasma proinflammatory cytokines (tumor necrosis factor α, (TNFα) and interleukin

1(IL1)), and plasma cortisol (an indicator of hypothalamic-pituitary-adrenal (HPA) axis activation); and reduced insulin-like growth factor (IGF-1) and increased growth hormone (GH) (two components of the somatotropic axis) (Webel et al., 1997; Wright et al., 2000).

In contrast to the classical LPS model, administration of live Salmonellae via oral or intranasal routes has demonstrated a much different host response profile. In pigs, a single oral dose of ST induced a strong febrile response, reduced feed intake, elevated plasma cortisol and reduced IGF-1; however, it did not affect plasma TNFα or PGE_2 levels (evaluated over 5 d) (Balaji et al., 2000; Turner et al., 2002). In germ free piglets, the systemic response to both nonvirulent and virulent strains of ST (evaluated after 24 h) resulted in an increased plasma IFNγ, but levels of TNFα, IL18 (induces IFNγ), IL1β, and IL10 were not different from control piglets (Splichal et al., 2002). Localized within the ileum, however, the virulent ST strain (evaluated 24 h post exposure) significantly increased local IFNγ, TNFα, and IL1β (Splichal et al., 2002). This is not to say that serum TNFα has not been reported in pigs exposed to *Salmonella,* but significant increases are generally seen in the weeks after challenge; such that intranasal challenge with 10^4 ST induced significant increases after 6 wk, and intranasal challenge with 10^6 ST increased TNFα during wk 2 to 4 post challenge (Stabel et al., 1995). In that these data were collected from germ free pigs, they may not completely represent the natural gastrointestinal environment of pigs whose GIT have been colonized with commensal bacteria, yet the lack of a systemic cytokine response matches those of the weaned piglets previously described above, and suggests that ST induces a more localized immune response as opposed to the systemic LPS model.

In vitro cultures of intestinal epithelial cells also differ in their responses after exposure to LPS, ST or SC which further suggests that LPS is not a completely accurate model of bacterial infection. The swine jejunal epithelial cell line, IPEC-J2 (Rhoads et al., 1994) readily produced IL8, and increased the gene expression of IL8 and CCL20 after ST and to a much lesser extent SC exposure, but failed to respond to LPS (Skjolaas et al., 2006). Several, but not all, human intestinal epithelial cell lines (which expressed low levels of TLR4) including Caco-2, T84, HT-29, and differentiated HT-29/MTX have also demonstrated a similar pattern of hyporesponsiveness to LPS even in the presence of soluble CD14 and (or) LPS binding protein (Abreu et al., 2001; Bocker et al., 2003; Fierer et al., 1993), but when exposed to various pathogenic *Salmonella* strains, readily secreted several proinflammatory mediators including

PGE_2, $PGF_2\alpha$, IL6, IL8 and CCL20 (Yang et al., 1997; Fierer et al., 1993; Stadnyk, 2002; Weinstein et al., 1997). Comparing across numerous intestinal epithelial lines exposed to various *Salmonella* spp., several cytokines have been noted to increase in either gene expression or secretion profiles including those from the CXC chemokine classification (IL8, GROα, GROβ, GROγ, IP-10, Mig, I-TAC) the CC chemokines (MCP-1, MIP-1β, MIP-3α, RANTES) and cytokines (G-CSF, GM-CSF, IL6 and TNFα) (reviewed in Eckmann and Kagnoff, 2001).

Salmonella spp. serve as a good model for studying bacterial pathogenesis, yet not all serovars of *Salmonella* elicit the same proinflammatory response in the host or within in vitro challenges. For example, most clinical cases of salmonellosis in swine are caused by host adapted SC and non-host adapted ST, both of which are also human pathogens (Schwartz, 1999; Taylor, 1999). The swine adapted SC is capable of causing systemic typhoid like disease and septicemia, while ST is not host adapted and the infection generally results in localized enterocolitis (Schwartz, 1999; Meyerholz et al., 2002). Evaluation of immune gene expression from the MLN of 8 wk old pigs exposed to either ST or SC demonstrated a profile whereby SC induced a intense and extended up-regulation of immune genes, while ST exposure resulted in both a transient up-regulation of IFNγ, SOCS1, STAT1 and a down-regulation of IL4, IL6, IL1β, and TLR4 (Uthe et al., 2005). The two strains also demonstrated differing patterns in pigs undergoing intranasal challenge in that ST increased TNFα in pigs several weeks after exposure, and an increase in TNFα was never observed in SC challenged pigs which exhibited clear signs of endotoxic shock (Stabel et al., 1995). When investigating early invasion events, both ST and SC were able to invade the pig ileum without cell specificity as early as 10 min after inoculation; however, the two serovars demonstrated differences in M cell invasion, the formation of filopopodia and lamellipodia, and swelling patterns (Meyerholz and Stabel, 2003). In addition, Meyerholz and Stabel, (2003) also observed that ST entered M cells in smaller numbers and SC induced more filopodia and lamellipodia but less swelling.

The gut epithelium is not the only cell that mediates protection against *Salmonellae* infections. Immune cells that play a key role in controlling and clearing *Salmonella* include monocytes/macrophages, neutrophils, and dendritic cells. Cytokines released from monocytes and macrophages after exposure to *Salmonella* include those associated with inflammation (IL1, IL6, TNFα), chemoattraction (MIP1-α, MIP1-β, MIP-2), the induction of acute phase reactions, proliferation, and differentiation (IL6, GM-CSF, IL18, IL12p70); which ultimately leads to the

further induction of both the innate and adaptive immune responses (reviewed in Eckmann and Kagnoff, 2001).

Cytokines produced by animal models against *Salmonella* include the classical proinflammatory cytokines IL1 and TNFα, the "IFNγ axis" cytokines (IFNγ, and the IFNγ inducing cytokines IL12 and IL18), as well as the anti-inflammatory cytokines IL4 (lack of is protective) and IL10 (strongly increased); all of which aid in increasing survival (reviewed in Eckmann and Kagnoff, 2001).

The involvement of probiotics in gastrointestinal immunity

As mentioned above, the intestinal mucosa functions to serve as an active barrier in the defense against the continuous challenge of food antigens and pathogenic microorganisms which are continually entering the intestinal tract. Aside from immune system protection, harmful agents are also cleared from the gut by the actions of gastric acid, peristalsis, mucus, intestinal proteolysis, and the intestinal biota (reviewed in Nava et al., 2005). The hypothesis that beneficial bacterial communities in the gut lead to positive effects on host health has led to the development of therapeutics that are based on the consumption of beneficial bacterial cultures (Nava et al., 2005) that are marketed for both human and animal health benefits. In addition to the above mentioned probiotics associated health benefit hypothesis, the use of probiotics in human and animal health has increased in recent years due to additional concerns about the failure to identify and generate new antibiotics, the impending ban on the use of low dose antibiotics as growth promotants in animal feed, and the emergence of pathogenic microbes with antibiotic resistance genes (Hong et al., 2005). The mechanisms by which probiotic bacteria could mediate changes in the GIT are not well understood. However, Nava et al., (2005) suggested that probiotics may mediate their action by competing for intestinal epithelium adhesion receptors, and by the production of antibacterial substances (e.g., bacteriocins or colicins) which modulate immune responses.

Bacterial spore formers, including those in the genus *Bacillus*, are one type of probiotic commonly seen in use today (Hong et al., 2005). These probiotics are primarily used in their spore form, and have demonstrated beneficial effects in the prevention of gastrointestinal disorders (Hong et al., 2005). Yet, interactions of *Bacillus* spp. in the gastrointestinal system are complex and not well understood. Several studies have been completed with pigs using the

BioPlus® 2B supplement (containing *Bacillus licheniformis* and *Bacillus subtilis* spores) which contributed to improved sow and piglet performance (Alexopoulos et al., 2004). Several *Bacillus* spp. have also been suggested to serve as a probiotic in broiler chickens. Barbosa et al., (2005) isolated several *Bacillus* spp. from the chicken gut, and all strains examined demonstrated the ability to sporulate efficiently in the laboratory setting, to tolerated simulated gastrointestinal conditions, and to exhibit antimicrobial activity against a broad spectrum of bacteria, including: *Clostridium perfringens*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Barbosa et al., 2005).

Lactic acid bacteria (LAB) are Gram positive bacteria with immunostimulatory properties (Meydani and Ha, 2000) that are also widely used as probiotics with reported health benefits against gastrointestinal disorders (diarrhea, inflammatory bowel disease, lactose intolerance, *Salmonella* or *Shigella* infections) (Goldin, 1998; Madsen et al., 2001). The immunostimulatory effect of LAB are generally due to changes to the gastrointestinal microecology which can lead to stimulation of the gut associated lymphoid tissue resulting in cytokine and antibody production, phagocytic activity, and increased T cell and NK cell activity (reviewed in Meydani and Ha, 2000). Among the LAB, *Lactobacillus* and *Bifidobacterium* are normal occupants of a healthy human intestinal microflora; however, not all LAB strains offer the same degree of immune function enhancement (Goldin, 1998; Meydani and Ha, 2000).

Lactobacillus reuteri (LR) has been used in several studies to investigate its use as a probiotic. Isolates of LR from pig feces have been proposed to be a strong probiotic choice in that LR has the ability to produce a broad spectrum antimicrobial compound (reuterin), which survives under gastrointestinal conditions (such as low pH) and has the capacity to colonize the intestinal tract (Rodriguez et al., 2003). LR has also demonstrated direct anti-inflammatory activity in human epithelial cells when live (but not heat-killed, or gamma-irradiated) LR inhibited TNFα and ST induced IL8 expression and NFkB translocation in T84 and HT29 cells (Ma et al., 2004). Oral ingestion of LR by healthy adult subjects also significantly increased CD4 T cell populations in the ileum which may assist in maintaining gut health (Valeur et al., 2004).

Two other mechanisms by which *Lactobacillus* spp. offer immune enhancement and host protection against infection with ST are via bactericidal activities as well as competition for attachment sites. Examples of two intestinal isolates that offer such protection include

Lactobacillus reuteri JCM 1081 and *Lactobacillus crispatus* JCM 8779, both of which are highly adhesive to Caco-2 cells, are able to reduce adhesion of pathogenic *Escherichia coli* and ST, and offer bactericidal substances (Todoriki et al., 2001). *Lactobaillus rhamnosus* also demonstrated increased host protection within BALB/c mice challenged with either a single oral dose or repeated daily doses (5 d) of ST, whereby probiotic fed mice exhibited higher health scores, higher serum and intestinal tract anti-*Salmonella* antibody titers, decreased pathogen burdens in visceral organs, and higher overall survival rates (Gill et al., 2001).

The cellular and molecular mechanisms by which non-pathogenic bacteria directly influence the intestinal epithelium in order to limit the activation of the immune system are not fully understood. Pre-exposure of intestinal epithelial cells lines (T84, HT-29, and HeLa) to an avirluent strain of *Salmonella* abrogated the wild type ST or TNFα stimulated production of inflammatory cytokines, which was a result of the stabilization of IκBα (Neish et al., 2000). As mentioned earlier, pathogenic strains of bacteria activate the NFkB directed transcription of proinflammatory cytokines (Elewaut et al., 1999), but the avirulent *Salmonella* strain inhibited IκBα ubiquitination and degradation, thereby blocking NFkB translocation to the nucleus and gene activation (Neish et al., 2000). *Lactobacillus* spp. also produces nonbacteriocin molecules whose actions have yet to be fully understood. Cell free culture supernatants from *Lactobacillus acidophilus* have demonstrated that these non-lactic acid nonbacteriocin molecules have direct antagonistic activity on ST cells, which results in the rapid killing of ST via decreased intracellular ATP, increased membrane permeability with LPS release, and increased sensitivity to lytic enzymes (Coconnier-Polter et al., 2005). Coconnier et al., (2000) also demonstrated that cell-free culture supernatants from *Lactobacillus acidophilus* could affect intracellular *Salmonella* residing in Caco-2 or TC7 cells, which included such effects as: decreased numbers of apical ST-induced F-actin rearrangements, decreased transcellular passage, inhibition of intracellular growth, and inhibited adhesion-dependent ST-induced IL8.

Bacteroides thetaiotaomicron is also a common anaerobic resident of the human intestine which has been documented to participate in decreasing proinflammatory cytokine expression by modulating the NFkB and PPARγ pathways (Kelly et al., 2004). Kelly et al., (2004) demonstrated that Caco-2 cells stimulated with *S. enteritidis*, *E. coli*, Phorbol myristate acetate (PMA), or flagellin enhanced nuclear translocation of the transcription factor NFkB subunit RelA to the nucleus, but when cells where co-incubated with the above mentioned stimulants and

B. thetaiotaomicron the NFkB subunit RelA was exported back out of the nucleus into the cytoplasm while other transcription factors (AP1, JunD) as well as phosphorylation and degradation of IkBα were not affected. Likewise, PPARγ accumulates in the nucleus in Caco-2 cells exposed to *S. enteritidis*, and co-culture with *B. thetaiotaomicron* also resulted in it is redistribution back to the cytosol, and the two events (NFkB, RelA, and PPARγ redistribution) appeared to be linked and essential for the nuclear export process (Kelly et al., 2004).

Gastrointestinal epithelial cells induce cytokine / chemokine production

Intestinal epithelial cells are considered prominent sources of chemokines and cytokines which lead to the recruitment of macrophages, lymphocytes, and polymorphonuclear leukocytes and can therefore further initiate both the innate and adaptive immune responses (Jung et al., 1995; Maaser and Kagnoff, 2002). Interestingly, it is the pathogenic bacteria, but not commensal, that are the trigger of such proinflammatory responses (Eckmann et al., 1997). Jung et al., (1995) demonstrated that after challenge with invasive (but not non-invasive) bacterial strains, human colon epithelial cell lines (T84, HT29, and Caco-2) expressed the proinflammatory cytokines IL8, monocyte chemotactic protein –1 (MCP1), GM-CSF, and TNFα. Cyclooxygenase-2 (a rate limiting enzyme for prostaglandin production) and NO synthase (controls NO production) are also upregulated after infection with invasive bacteria (reviewed in Elewaut et al., 1999). The porcine intestinal epithelial cell line IPEC-J2 expresses mRNA for IL1α, IL6, IL7, IL8, IL12p40, IL18, TNFα, GM-CSF, CCL20, MIF, and OPN; and are also a prominent source of secreted IL8 (Schierack et al., 2005; Skjolaas et al., 2006). The central regulator involved in the IEC response to enteroinvasive bacterial pathogens is the signal transduction pathway that includes Iκκ intermediates and the downstream activation of NFκB (Elewaut et al., 1999; Gewirtz et al., 2000).

A closer look at selected chemokines/cytokines

The modulation of gastrointestinal inflammation is multi-factorial as previously discussed. Here we will look a bit more closely at the role of two chemokines and two cytokines which function as assistants in the protection and repair of the GIT.

Interleukin 8

This cytokine is now classified as CXC chemokine ligand 8 (CXCL8) , and was first identified as a neutrophil activating factor (Thelen, 2001). Receptors that IL8 is known to bind with high affinity include CXCR1 and CXCR2, (Holmes et al., 1991; Murphy, 1997). Various types of bacteria have been shown to stimulate the release of IL8. These bacteria include those that remain inside phagosomal vacuoles (*Salmonella* spp.) and those that enter the cytoplasm (*Listeria monocytogenes*) (Eckmann et al., 1993b). However, not all bacteria or bacterial products elicit an IL8 response, for example, LPS or noninvasive bacteria including *E. coli* and *Enteroccocus faecium* were found to be unable to induced an IL8 response (Eckmann et al., 1993b). Functionally, IL8 is considered to have an important role in the initiation of inflammatory immune responses, and the induction of IL8 by inflammatory stimuli (e.g., LPS, TNFα or IL1β) aids in recruitment and activation of other immune cells (Harada et al., 1996; Miller and Krangel, 1992). IL8 secreted from IEC may also be involved in mucosal healing as IL8 demonstrated colonic epithelial cell migration (Caco-2 and HT-29 cells) by CXCR1 mediated pathways, without induction of cell proliferation (Sturm et al., 2005).

In regards to IL8 produced from IEC, Eckmann et al., (1993b) have shown that epithelial cells from the intestine and cervix function to serve as an early signaling system to the host immune system by releasing IL8 after bacterial invasion. The production of IL8 by polarized model intestinal epithelial lines in response to ST is predominantly toward the basolateral compartment and was observed to readily induce polymorphonuclear transepithelial migration (Eckmann et al., 1993a; McCormick et al., 1993). This induction of IL8 secretion as a result of ST is mediated by increases in intracellular Ca^{2+} and the transcription factor NFκB (Gewirtz et al., 2000). In conjunction with increased IL8 secretion, IL8 mRNA expression was also increased as soon as 60 minutes post *S. dublin* entry (Fierer et al., 1993). IL8 mRNA expression increased in IPEC-J2 cells treated with ST or SC (10^8/well), but not LPS (5 ng/well) (Skjolaas et al., 2006), which is consistent with those observations taken from human gastrointestinal epithelial cell lines. The predominantly basolateral secretion of IL8 has also been documented in the IPEC-J2 cell line post ST and SC exposure, but SC stimulated significantly less IL8 release (Schierack et al., 2005; Skjolaas et al., 2006). The implication of IL8 within the GIT has further been noted in patients with the gastrointestinal inflammatory disorder, ulcerative colitis, who may have greater sensitivity to IL8 in that they demonstrated a strong upregulation in the

expression of CXCR1 receptors within colonic epithelial cells, which contrasts with normal mucosal tissues that demonstrate minimal expression of CXCR1 and no expression of CXCR2 (Williams et al., 2000).

CC chemokine ligand 20

This chemokine is also termed macrophage inflammatory protein 3α (MIP3α) (Hieshima et al., 1997), or liver and activation regulated chemokine (LARC) (Hieshima et al., 1997), or Exudos-1 (Hromas et al., 1997). Structurally, CCL20 is a 799 basepair sequence containing 4 exons and three introns and encodes for a 96 amino acid protein (for an excellent review see Schutyser et al., 2003). The functional and highly specific receptor for CCL20 is CCR6 (Baba et al., 1997; Liao et al., 1997). Functionally, CCL20 is a chemoattractant for CD34+, CD11b+ immature myeloid derived dendritic cells (Iwasaki and Kelsall, 2000) and CD45RO+ memory T cells, both of which contribute to the adaptive immune response. Although not all dendritic cells demonstrated the same migratory response to CCL20 in that murine lymphoid CD8αα dendritic cells that do not express CCR6 fail to migrate toward CCL20, and myeloid dendritic cells that reside in the spleen and express CCR6 also fail to migrate toward CCL20 (Iwasaki and Kelsall, 2000).

The expression of CCL20 has been documented within the normal human liver, lung, appendix, tonsil, lymph nodes, peripheral blood leukocytes, thymus, fetal liver, fetal lung, small intestine, colonic epithelial cells, stomach, bladder, pancreas, testis, cervical epithelial cells, placenta, and skin (reviewed in Schutyser et al., 2003). Human cell types that express CCL20 in vitro include normal peripheral blood mononuclear cells (PBMCs), monocytes, macrophages, T lymphocytes, dendritic cells, neutrophils, mast cells, eosinophils, colonic epithelial cells, lower airway epithelial cells, keratinocytes, melanocytes, human umbilical vein endothelial cells (HUVEC), dermal microvascular endothelial cells, dermal fibroblast, and endometrial stromal cells (Schutyser et al., 2003) .

Major expression of CCL20 has also been documented throughout the GIT, including the colon, small intestine, and appendix. In vivo, inflamed tissue from the human colon was a major site of CCL20 mRNA expression; however, the normal colonic tissue maintains minimal CCL20 expression (Izadpanah et al., 2001). In BALB/c mice CCL20 expression was constitutive in the colon and appendix and upon LPS stimulation this expression was reduced (Tanaka et al., 1999). Yet, within the small intestine of BALB/c mice there was not a strong

constitutive expression of CCL20, but LPS stimulation was able to induce a strong increase in the mRNA expression (Tanaka et al., 1999). In contrast, C57BL/6 mice have shown constitutive expression of CCL20 in the small intestine; but it is important to note that regardless of these mice strain differences, it is widely accepted that the intestine, colon, and appendix appear to be major sites of CCL20 expression in mice (Tanaka et al., 1999).

Therefore, it seems that CCL20 plays a predominant role in the GIT. In vitro, several human IEC lines have been shown to express CCL20 mRNA and to produce the secreted CCL20 protein, and both have been found to be increased post stimulation with either TNFα, IL1α, or exposure to *Salmonella* or *E. coli* (Izadpanah et al., 2001). Various human IEC lines express constitutive (HT-29, Caco-2, LS174T, and I-407), and regulated (HT29 and Caco-2) expression of CCL20 mRNA levels (Izadpanah et al., 2001). Polarized epithelial cells mainly secrete CCL20 protein in the basolateral direction, and functions through the NFkB target gene pathway (Fujiie et al., 2001; Izadpanah et al., 2001). Human IEC lines predominantly express CCR6 on the apical surface of polarized T84, Caco-2 and HCA-7 cell lines and CCL20 stimulation of the IEC CCR6 receptor results in tyrosine phosphorylation of the scaffolding adaptor protein p130cas, which is a key part of the cell migration focal adhesion kinase pathway (Yang et al., 2005). CCL20 was also able to reduce forskolin stimulated increases of cAMP levels and chloride secretion in T84, Caco-2 and HCA-7 cells; indicating CCL20 as a player in down regulation of ion secretion by IEC during bacterial infection and barrier compromise (Yang et al., 2005).

Macrophage migration inhibitory factor

This cytokine is a 12.5 kDa, 115 amino acid protein, first discovered in 1966 as a T cell derived cytokine. MIF was named for its ability to inhibit the random migration of macrophages in vitro, and is associated with the delayed type hypersensitivity response (reviewed in (Bernhagen et al., 1998; Bucala, 1996; Metz and Bucala, 1997; Nishihira, 1998). The MIF gene is less than 1 kB and consists of three exons that are separated by two introns (Bernhagen et al., 1998). Among the various species studied so far, it has been found that the rat, mouse, bovine, human, chicken, and porcine MIF all share close sequence homology (Abraham et al., 1998); (Nishihira, 1998).

Physiological roles of MIF in vivo are not fully understood, but it is known that MIF can prevent the inhibitory effects of glucocorticoids, increase antibody production, activate T cells,

regulate insulin release from pancreatic β cells, activate the growth promotion signaling pathway ERK-1/2, and inhibit apoptosis (reviewed in Maaser and Kagnoff, 2002). It has been suggested that MIF may serve a protective role in ST infections, such that $MIF^{-/-}$ mice challenged with ST failed to control the infection, failed to induce a protective Th1 response (as represented by serum IFNγ, IL12, and TNFα) and had higher levels of IL1β, NO, and corticosterone levels (Koebernick et al., 2002). Caco-2 cells challenged with *S. dublin* have also been reported to increase MIF secretion, but not mRNA levels (which remained unchanged) and bacterial entry was confirmed to be the trigger for MIF release as noninvasive mutants of *S. dublin* did not show the same significant MIF release (Maaser et al., 2002). In contrast, splenocytes isolated from young pigs and stimulated with concanavalin A upregulated the expression of MIF mRNA as compared to unstimulated splenocytes as determined by northern blot analysis (Skjolaas et al., 2002).

Maaser et al., (2002) have explored the expression and regulation of MIF in human intestinal tissue samples as well as the human IEC lines HT-29, LS174T, Caco-2, 1407, and HCA-7 to attempt to characterize MIF function in the gastrointestinal system. Biologically active MIF (verified to arrest macrophage migration) was found to be expressed constitutively and at high levels both in vitro and in vivo. Human IEC expression of MIF as determined by immunoblot analysis for protein expression from non-inflamed gastric, duodenal, and colon mucosa cell lysates, and mRNA expression from colon and gastric epithelial cells (amplified by reverse transcription PCR), all demonstrated strong expression (Maaser et al., 2002). Immunoblot analysis of confluent monolayers of Caco-2, HT-29, and T84 cell lines also demonstrated the same strong expression (Maaser et al., 2002). The constitutive expression of MIF mRNA is also evident in IPEC-J2 cells, and MIF mRNA was not affected by treatments of LPS, ST, or SC (Skjolaas-Wilson et al., 2003). The polarity of MIF secretion within IEC differs from that of other inflammatory associated cytokines such as IL8 and CCL20 in that MIF release was 70-80% into the apical direction of a transwell insert, and human colon lavage fluid also confirmed the apical release of MIF from epithelial cells (Maaser et al., 2002).

Osteopontin

This cytokine is also known as early T lymphocyte activation 1 (Eta-1), was discovered in 1979, and is a 60 kDa protein of approximately 300 amino acid residues (Mazzali et al., 2002; O'Regan and Berman, 2000). This protein has been found to be multifunctional and is expressed in many cell types including: bone, macrophages, endothelial cells, smooth muscle cells, and epithelial cells (Mazzali et al., 2002). Within the bone and at epithelial surfaces OPN is constitutively expressed, however, it can be upregulated in endothelial, smooth muscle, and inflammatory mononuclear cells (O'Regan and Berman, 2000).

There is considerable variation in the forms of OPN due to alternate splicing, post-translational phosphorylation and glycoslyated modification and has binding sequences for calcium, heparin, and arginine-glycine-aspartic acid (RGD sequence) (Mazzali et al., 2002; O'Regan and Berman, 2000). OPN is also classified as an extracellular matrix protein due to the presence of the RGD sequence, which is known to be an integrin-binding motif. The promoter contains motifs for a purine rich sequence, and ETS-like sequence, along with glucocorticoid and vitamin D response elements (Mazzali et al., 2002). Receptors for OPN include integrins (that are RGD dependent) as well as the receptor CD44 (Weber et al., 2002). OPN function is also controlled by cleavage at the RGD domain by thrombin (O'Regan and Berman, 2000). The C terminal domain of OPN interacting with CD44 induces macrophage chemotaxis whereas β_3 integrin receptors by interaction with the N terminal of OPN induces cell spreading and activation (Weber et al., 2002).

The role of OPN in inflammation is that of a chemoattractant and inducer of T helper (Th) cells to secrete a panel of cytokines consistent with a Th1 response. As a chemoattractant OPN has been documented to support adhesion of human and murine T cells and macrophages in vitro (O'Regan and Berman, 2000). The accumulation of macrophages at sites of subcutaneous injection of OPN has been observed in vivo, along with high expression of OPN by macrophages post stimulation with N formyl methionyl leucyl phenylalanine (O'Regan and Berman, 2000).

Th cells are a division of T lymphocytes identified as CD4+ cells that are MHC II restricted. Th cells are divided into two functional groups classified as Th1 and Th2. The Th subsets are believed to arise from a common precursor cell that becomes differentiated upon exposure to various factors (reviewed in Pellegrini et al., 2000). As a modulator of cell mediated immunity, OPN has been shown to be an augmenting factor for the Th1 profiles. Th1 cells

primarily secrete TNFβ, IL2 and IFNγ, and induce the classical cell mediated immune response aiding in the clearance of intracellular pathogens (Allen and Maizels, 1997). IL12 secretion by activated monocytes, macrophages, and dendritic cells plays a critical role in the development of the Th1 response and cell-mediated immunity in that it causes the secretion of IFNγ from NK cells, and T cells, while enhancing the cytolytic functions of CD8+ T cells (Franchimont et al., 2000). NK cells and Th1 cells perpetuate the Th1 pathway by secreting IFNγ, which feeds back on macrophages and dendritic cells to increase IL-12 release, producing a positive feedback loop (Almawi et al., 1999). On the other hand, Th2 cells secrete IL4, IL5, IL6, IL10, and IL13 and direct the immune system toward antibody production, providing help to the B lymphocytes, and assisting in the clearance of extracellular pathogens and parasites (Allen and Maizels, 1997; MacPhee et al., 2000). Some factors that induce Th2 differentiation include: IL4, IL10, B cells as antigen presenting cells, a high dose of antigen, and the absence of cytotoxic T lymphocytes (reviewed by Fitch et al., 1993). OPN has also been implicated in the increase of CD3 mediated T cell production of IFNγ (O'Regan et al., 2000). In murine macrophages stimulated with LPS, OPN also directly induced production of IL12 while inhibiting IL10 expression thus suggesting a shift toward a Th1 profile (O'Regan et al., 2000). However, OPN also increases CD40 ligand which augments T cell dependent IL12 production by human monocytes which may be the reason why OPN has the ability to also induce B cell proliferation and antibody production events (O'Regan et al., 2000).

The GIT is also a site of prominent OPN expression. Brown et al., (1992) demonstrated by immunohistochemical studies that OPN is prominently deposited at the luminal surfaces of epithelial cells including those associated with the GIT, gall bladder, pancreas, urinary and reproductive tracts, lung, breast, salivary glands, and sweat glands. This group further suggested that epithelial secreted OPN binds to integrins on luminal surfaces and serves an important role in assisting in the communication between epithelial surfaces and the external environment (Brown et al., 1992). Illeal samples taken from the normal human intestine and subjected to immuno-histochemistry also revealed expression of OPN protein within IEC, CD38 positive plasma cells, and macrophages from the lamina propria; with complementary OPN mRNA expression in the IEC as well (Gassler et al., 2002). Within our laboratory, we have observed consistent expression of OPN mRNA by polarized IPEC-J2 cells, with increased expression after ST exposure (Skjolaas et al., 2006).

There appears to be an association between OPN and gastrointestinal health especially in regards to the generation of a Th1 cytokine profile. In Crohn's disease the local immune response tends toward the Th1 profile, and OPN has been associated with both normal and diseased GIT tissue (Gassler et al., 2002; Qu and Dvorak, 1997). Relative to gastrointestinal disorders, the health status of the GIT seems to alter the normal pattern of OPN expression. Within actively inflamed ileal biopsies taken from Crohn's disease patients a loss of IEC OPN protein and mRNA expression was observed with a corresponding increase in the density of GIT immune cells expressing OPN protein and mRNA (Gassler et al., 2002). OPN is likely to also play a role in phagocytic and secretory cell functions in the health and disease states of the GIT. Tissue biopsies from patients with Crohn's disease, Whipple's disease and colitis have revealed OPN expression (as detected by immunogold labeling) in the duodenum, ileum, jejunum, and colon; and localized within lysosomes, mucigen granules, phagolysosomes, and golgi, of such cell types as macrophages, absorptive, mucous, Paneth, and epithelial cells (Qu and Dvorak, 1997).

SUMMARY AND CONCLUSIONS

In regards to understanding the immune and inflammatory functions and regulation of the gastrointestinal epithelium in domestic farm animal species, there is still a great deal to be elucidated. In light of the fact that a healthy gut mucosal epithelium is generally free of any ongoing inflammation, gaining a greater understanding of how this system protects against invading pathogens can assist in the understanding of the enteroinvasive pathogen/gastrointestinal disease state, or autoimmune diseases associated with imbalanced microbial populations. Ongoing concerns about the generation of antibiotic resistant strains of bacteria as a result of the use of antibiotics as growth promotants in the livestock industry has facilitated a push for growth promotant alternatives. The selection of future non-antibiotic candidate feed additives, such as probiotics or commensal bacteria, should offer a means by which bacterial adhesion and invasion is reduced thereby limiting the stimulation or over-stimulation of the gastrointestinal immune system. With an increased understanding of the physiology of the GIT, the development of potential feed additives or oral vaccine adjuvants could be generated that exploit M cell function, or TLRs, or enterocytes, or signaling pathways

involving PPARs and NOD, or take advantage epithelial chemokine or cytokine regulation to enhance growth or vaccine efficacy.

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CHAPTER 2 - Effects of *Salmonella enterica* serovars *Typhimurium* (ST) and *Choleraesuis* (SC) on chemokine and cytokine expression in swine ileum and jejunal epithelial cells

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ABSTRACT

The gastrointestinal epithelium represents a barrier to potentially invasive enteric pathogens, maintains a role in innate immune surveillance, and is a source of both chemokine and cytokine chemotactic mediators in response to bacterial invasion. In the current study, we evaluated cytokine and chemokine mediators known to regulate movement of macrophages (macrophage migration inhibitory factor; MIF), neutrophils (IL8), dendritic cells (CCL20) and epithelial remodeling (osteopontin; OPN) in response to invasive swine enteropathogens *Salmonella enterica* serovar *Typhimurium* (ST) or *Choleraesuis* (SC). For the in vivo experiment, weaned pigs served as uninfected controls (0 h) or were given 3 x 10^9 CFU ST orally. Pigs were sacrificed at 8, 24, 48, and 144 h after inoculation and total RNA was extracted from defined segments of proximal (PI) and distal (DI) ileum. Relative expression of MIF and OPN were not affected by ST. IL8 expression was increased numerically ($P = 0.17$ for the interaction term) at 24 and 144 h in the PI and these increases accounted for greater expression in the PI relative to the DI ($P < 0.05$). Relative expression of CCL20 was increased at 24 h after ST ($P < 0.05$). Next, we evaluated the time course of MIF, IL8, CCL20 and OPN mRNA expression induced by application of lipopolysaccharide (LPS), ST or SC in vitro using pig jejunal epithelial cells (IPEC-J2). Cells were grown to confluency on permeable membranes, and treated apically with LPS (10 ng/ml), ST or SC (10^8/well). After 1 h, cells were washed to remove LPS or extracellular bacteria, and media containing gentamicin was added to kill remaining extracellular bacteria. Media and RNA were collected at 1.5, 3, and 6 h after treatment. MIF mRNA was not affected by LPS or bacterial treatment. Similarly, IL8 expression was not affected by LPS, but was increased by ST and SC relative to controls at 1.5 and 3 h post exposure ($P < 0.05$ for all

comparisons). Treatment with SC increased CCL20 mRNA relative to controls at 3 h ($P < 0.05$), while ST increased CCL20 at 1.5, 3 and 6 h with maximal expression at 6 h ($P < 0.05$ for all comparisons). ST and SC increased polarized IL8 secretion. Our data demonstrate that invasive bacterial pathogens in the pig gastrointestinal tract trigger upregulation of selected cytokine and chemokine mediators, but serovars of Salmonella elicited differing patterns of activation in vitro.

1. INTRODUCTION

A dynamic population of immune cells is maintained in the lamina propria in close association with the gastrointestinal epithelium in healthy pigs under production conditions. This population of cells is thought to be influenced by the commensal microbiota resulting in chemotactic signals that recruit and maintain appropriate cell types in the lamina propria. It has long been accepted that the presence of invasive enteric pathogens provokes increased migration of cells into the lamina propria and subepithelium, and salmonellae organisms are swine enteric pathogens that have long been associated with neutrophil infiltration into affected mucosal sites (Rothkotter et al., 1999). Although chemoattractive signals are undoubtedly emitted from sites colonized and penetrated by Salmonella spp., the time course of salmonellae-induced chemotactic signals in the pig gastrointestinal tract have not been thoroughly characterized.

Microfold cells, the so-called M cells present in Peyer's patches of gut associated lymphoid tissue, have been traditionally considered key in luminal antigen sampling, but their specific role in emitting cell trafficking signals is not known. However, there is now compelling evidence that enterocytes are key in generating chemotactic signals in response to enteric pathogens, including *Salmonella enterica* serovar *Typhimurium* (ST) (Eckmann et al., 1997). That enterocytes substantially outnumber M cells in the epithelium (Eckmann, Kagnoff 2001), even in Peyer's patches (Gebert et al., 1996), suggests they could normally serve important roles in cell recruitment in response to salmonellae organisms.

Here we report in vitro and in vivo experiments designed to determine dynamic changes in putative chemotactic signals in the pig gastrointestinal tract. Our working hypothesis was that the presence of an invasive bacterial pathogen would trigger upregulation of selected cytokine and chemokine mediators. We evaluated IL8 (also known as CXC chemokine ligand 8, CXCL8) as it is involved in neutrophil chemotaxis and is secreted by intestinal epithelial cells after

invasion by various bacteria (Eckmann et al., 1993b; Thelen 2001). CC chemokine ligand 20 (CCL20), also termed macrophage inflammatory protein 3α (MIP3α) (Hieshima et al., 1997) was also evaluated as it is a chemoattractant for CD34+ immature dendritic cells and CD45RO+ memory T cells and was increased after stimulation with either TNFα, IL1α, or exposure to bacteria including Salmonella (Izadpanah et al., 2001). Finally, macrophage migration inhibitory factor (MIF) and osteopontin (OPN) were also evaluated as MIF is known to affect macrophage movement and was rapidly increased after Salmonella dublin challenge of Caco-2 cells (Maaser et al., 2002) and OPN is a key mediator of recruitment and retention of macrophages and T cells to sites of inflammation (Mazzali et al., 2002).

2. MATERIALS AND METHODS

2.1 In vivo challenge with Salmonella enterica serovar Typhimurium

Twenty crossbred barrows, typical of commercial pigs and approximately 5 wk of age were used and the experimental protocol was approved by the Kansas State University Institutional Animal Care and Use Committee. These pigs showed no clinical signs or laboratory evidence of salmonellosis or any other enteric diseases. Pigs were penned in an environmentally controlled isolation facility at 25°C and under constant light with ad libitum access to feed and water. After a period of 7 d in the facility, pigs were challenged orally with 3 x 10^9 CFU of ST or sterile medium (time 0 control). The ST was a primary isolate from a clinical case of salmonellosis in pigs and was confirmed to be ST at the National Veterinary Services Laboratory, Ames, IA, USA. Samples of the entire gut wall were obtained at two locations that included the continuous Peyer's patch. A sample containing the entire ileum was excised from the ileocecal fold back to the ileocolic junction and the digesta flushed with ice cold sterile phosphate buffered saline (PBS). Samples were obtained from the anterior and posterior ends of this excised, flushed segment and were labeled proximal ileum (PI) and distal ileum (DI), respectively. Gut samples were obtained at 0, 8, 24, 48, and 144 h after oral ST challenge (n = four pigs/sacrifice time). Tissue samples were placed in cold RNAlater® (Ambion, Inc.) until RNA extraction.

2.2 Culture of jejunal epithelial cells

The neonatal jejunal epithelial cell line IPEC-J2 was derived from a single animal less than 12 h old (Rhoads et al., 1994). These cells were a gift from Dr. Bruce Schultz, Anatomy and Physiology, Kansas State University. Cell growth was maintained on 50% DMEM - 50% F12 medium with fetal bovine serum (5%), insulin/ transferrin/ Na-selenite media supplement (1%), epidermal growth factor (5 ng/ml), and streptomycin/ penicillin (1%) (Invitrogen™ Life Technologies, cell culture products). For experimentation, cells were seeded onto six-well Costar Snapwells (Corning Inc, Corning, NY) at 2.5 x 10^5 to 4.0 x 10^5 per well in a 0.5 ml volume and allowed to adhere for 24 h before being washed and re-fed every other day for 7 d of growth to allow for confluency and tight junction formation. In this in vitro system, average cell density was of 2.5 x 10^5 per well and transepithelial resistance typically was 4000 ohm*cm^2. Twenty four hours before experimentation, the cells were washed and replacement media was as above but devoid of antibiotics.

2.3 Bacteria for cell culture challenge

Salmonella enterica serovars *Typhimurium* (ST) and *Choleraesuis* (SC) were from primary isolates from swine clinical cases (obtained from Dr. Jerome Nietfeld, Diagnostic Medicine/Pathobiology, Kansas State University). Identification of Salmonella serotypes was verified by National Veterinary Services Laboratory, Ames Iowa, USA. Both strains were grown in Luria Bertani medium at 37°C, for 24 h, at which point bacterial populations were estimated by spectrophotometry at 600 nm optical density. Bacteria was then pelleted and resuspended in DMEM/F12 growth media devoid of FBS and antibiotics.

2.4 Exposure of jejunal epithelial cells to LPS and bacteria

Treatments included control (uninfected cells), LPS (catalogue number L-6529 from E. coli 055:B5; Sigma Chemical Co., St. Louis, MO; 10 ng/ml), SC, and ST (both added at 1.0 x 10^8 bacteria/well). Confluent IPEC-J2 cells, as described above, were washed twice with PBS and 0.5 ml of media alone (control), LPS, or bacteria were added to the top (apical) wells, while 2.5 ml of media was added to the bottom (basolateral) wells and plates were further incubated at 37°C, 5% CO_2 for 1 h. Then, cells from all treatments were washed and both apical and basolateral media were replaced with fresh media containing 50 µg/ml gentamicin. Media were removed from both the apical and basolateral compartments at 1.5, 3, and 6 h after LPS or

bacteria exposure for determination of IL8 secretion (swine specific IL8 sandwich ELISA catalog KSC0181, Biosource International, Camarillo, CA USA), and cells were subjected to RNA extraction procedures. This experiment was replicated three times with cells from two wells per treatment contributing data to each treatment x time mean.

2.5 Real time PCR analysis

Total RNA was extracted with TRI Reagent® (Sigma-Aldrich Co., St. Louis, MO) using the manufacturer's protocol. Following total RNA isolation, the DNA-free™ (Ambion Inc., Austin, TX) kit was used to ensure removal of contaminating DNA from all RNA samples. Samples were frozen in 50 µl of nuclease-free water (Ambion Inc., Austin, TX). RNA quality was verified by agarose gel electrophoresis and visualization of the 28S and 18S ribosomal RNA. RNA quantity was determined by spectrophotometry using an optical density of 260 nm. Reverse transcription was carried out using TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA). Briefly, reverse transcription was carried out in a 50 µl final volume that included 25 mM $MgCl_2$, 500 µM dNTP's, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, 50 U/µl MultiScribe reverse transcriptase, and TaqMan RT buffer. The reverse transcription mixture was incubated at 25°C for 10 min, heated to 37°C for 60 min, and inactivated at 95°C for 5 min. The resultant cDNA was stored (-20°C) until used. Real-time quantitative PCR was utilized to quantify the products of interest (IL8, CCL20, MIF, and OPN) relative to the quantity of 18S rRNA in total RNA isolated from cultured IPEC-J2 cells and isolated porcine ileal samples. The PCR reactions were carried out in 96-well plates with the appropriate forward and reverse primers (900nM), the appropriate TaqMan TAMRA probe (200 nM), PCR Mastermix (Applied Biosystems, Foster City, CA), and 3.5 µl of the cDNA sample. The porcine specific cytokine primers and detection probes (Table 1) were synthesized from published GenBank sequences using Primer Express® software (Applied Biosystems, Foster City, CA). Commercially available eukaryotic 18S rRNA (Applied Biosystems, Foster City, CA) primers and probe were used as an endogenous control. Assays using non-template controls and samples were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Thermal cycling parameters were utilized according to manufacturer recommendations and included 50 cycles of 15 s at 95°C and 1 min at 60°C.

2.6 Statistical analyses

Relative abundance of chemokines and cytokines in ileal samples were determined using the ΔΔCT method using the average pre-inoculation ΔCT (0 h) as the reference expression (n = tissue from four pigs at each time point). These ΔΔCT values were analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with time, tissue location (PI and DI), and their interaction term (time*tissue) in the model. Real-time PCR data from cultured jejunal enterocytes were handled similarly except that ΔΔCT values were calculated relative to the average ΔCT of cells from control wells. Data bars in Figure 2 represent six observations per treatment obtained from duplicate wells of cells from three independent in vitro experiments. The model included effects of treatment, time and their interaction (treatment*hour). Polarized secretion of IL8 from cultured cells were analyzed with effects of treatment, time and location (apical or basolateral) in the model. Secretion of IL8 was expressed as pg/well to account for the considerably greater volume of media present in the basolateral compartment (2.5 ml) compared to the apical compartment (0.5 ml).

3. RESULTS

3.1 In vivo challenge with Salmonella enterica serovar Typhimurium

This experiment was a time dependent study designed to evaluate the effect of oral ST challenge on MIF, IL8, CCL20, and OPN mRNA expression in ileal gut wall samples obtained from two distinct sites that contained the continuous Peyer's patch (Figure 1). The expression of MIF did not change significantly in response to oral ST, although there was a modestly greater abundance of MIF in the DI ($P < 0.05$). There was a tendency for a time x tissue location interaction with respect to IL8 ($P = 0.17$) with numerically elevated expression at 24 and 144 h after ST of approximately 8- and 14-fold, respectively. These numeric increases in IL8 contributed to significantly greater expression of IL8 in the PI ($P < 0.05$). For CCL20, there was a tendency for a time x tissue location interaction ($P = 0.14$) as CCL20 was increased about four-fold at 24 h post-ST relative to time 0. This increase in CCL20 was largely accounted for by the increase in CCL20 expression in the PI. The expression of OPN was not affected by time post-ST or ileal sampling location.

3.2 In vitro challenge of swine gut epithelium with LPS and Salmonella enterica serovars Typhimurium and Choleraesuis

Because our evaluation of changes in selected cytokines and chemokines in the pig ileum after oral ST included cells within the entire width of the gut wall and not just the epithelial layer, we next sought to evaluate this same battery of chemoattractive mediators in gastrointestinal epithelial cells. We turned to an in vitro setting in subsequent experiments to specifically evaluate the responses of swine gut epithelial cells to direct bacterial challenge. The IPEC-J2 cell line was used as a model gut epithelium to evaluate the effects of LPS, and two salmonella serovars ST, and SC on MIF, IL8, CCL20, and OPN mRNA expression (Figure 2) and polarized secretion of IL8 into the apical and basolateral media (Figure 3).

Relative levels of MIF mRNA were not affected by either LPS or bacterial treatment. LPS did not affect IL8 mRNA, but IL8 mRNA was increased by about threefold at 1.5 h (Figure 2B; $P < 0.05$) and about twofold at 3 h after SC ($P < 0.05$) relative to control. Similarly, IL8 mRNA was increased by about threefold at both 1.5 and 3 h post ST relative to control wells (Figure 2B; $P < 0.05$ for both comparisons). Both salmonellae serovars affected expression of CCL20 relative to untreated wells (Figure 2C). Treatment with SC increased CCL20 mRNA by about 200-fold at 3 h ($P < 0.05$), whereas ST increased CCL20 by over 200-fold ($P < 0.05$), 600-fold ($P < 0.05$), and about 150-fold ($P < 0.05$) at 1.5, 3, and 6 h, respectively. Only ST treatment affected OPN mRNA with just over a twofold increase relative to control wells at 6 h after bacterial treatment (Figure 2D; $P < 0.05$).

Media from both the apical and basolateral compartments were collected at 1.5, 3.0 and 6.0 h after treatment and the concentration of IL8 was determined using a swine-specific ELISA (Figure 3). The concentration in each compartment was adjusted to reflect the greater volume in the basolateral compartment and expressed as picograms/well. Treatment with ST increased IL8 secretion in the basolateral direction (636.6 ± 132.0 pg/well; $P < 0.05$) above that of the control basolateral compartment (143.51 ± 132.0 pg/well) at 3 h. At 6 h post-ST, IL8 secretion was increased in both the apical (1592.0 ± 132.0 pg/well; $P < 0.0001$) and the basolateral (2975.2 ± 132.0 pg/well; $P < 0.0001$) directions relative to controls (205.2 ± 132.0 pg/well apical and 123.9 ± 132.0 pg/well basolateral). At 6 h after treatment, ST-stimulated IL8 secretion was strongly, though not completely, polarized towards the basolateral compartment (apical, 1592.0 ± 132.0 pg/well; basolateral, 2975.2 ± 132.0 pg/well; $P < 0.0001$). Treatment with SC stimulated IL8

secretion, but only after 6 h and only in the apical direction (546.6 ± 132.0 pg/well; P = 0.06) as compared to control (205.2 ± 132.0 pg/well).

4. DISCUSSION

Salmonella enterica serovar *Typhimurium* is an economically important swine enteropathogen and we have extensively characterized the pathophysiological consequences of oral challenge with this particular isolate, the same isolate used in the current studies. In earlier work, a single oral dose of ST produced transient enteric disease, including fever, inappetence, slowed growth (Balaji et al., 2000; Turner et al., 2002b; Turner et al., 2002a; Burkey et al., 2004), activation of the endocrine stress axis (Balaji et al., 2000), and disruption of the endocrine growth axis (Balaji et al., 2000; Jenkins et al., 2004; Davis et al., 2005). Despite these unmistakable pathophysiologic changes, most of these effects resolved within approximately the first week following oral inoculation, and remarkably, these changes occurred completely in the absence of ST-induced elevations of systemic TNFα (Balaji et al., 2000; Fraser et al., 2005), IL1β (Fraser et al., 2005), or IL6 (Burkey et al., 2004). Collectively, these observations suggested to us that the inflammatory sequelae provoked by ST in the pig were effectively confined by the gut mucosal immune system. In the current experiments, we used this same swine model but focused on candidate molecules that could affect trafficking of immune cells into the subepithelial and lamina propria compartments in the pig gut mucosa in response to ST.

Of many possible chemoattractant molecules, our initial focus was on a select group of cytokines and chemokines known to regulate movement of macrophages (MIF; Calandra, Roger 2003; Lolis, Bucala 2003), chemotaxis of neutrophils (IL8; Eckmann et al., 1993b; Follin et al., 1991; Fierer et al., 1993) trafficking of dendritic cells (CCL20; Dieu-Nosjean et al., 2000; Sierro et al., 2001), and epithelial remodeling in response to bacteria (OPN; Denhardt et al., 2001). We chose to evaluate expression of these inflammatory markers in defined locations within the ileum, without regard to the presence or absence of overt inflammation; as the pig ileum is reported to be a site for rapid epithelial invasion of ST (Meyerholz et al., 2002).

In vivo, the expression of MIF and OPN in the current study was largely unaffected by ST through 144 h post-inoculation. However, based upon the Ct values from the real-time PCR assay for both of these cytokines (data not shown) both appeared to be expressed constitutively

at relatively high levels. Although MIF is a product of both epithelial cells (Arndt et al., 2002; Imamura et al., 1996; Maaser et al., 2002) and a variety of immune cells (Calandra et al., 1994; Calandra, Roger 2003), our previous studies in pig splenocyte cultures, presumably devoid of epithelial cells, indicated that MIF could not be detected by Northern blot analysis unless the cells were activated by mitogen (Skjolaas et al., 2002). Similarly, cells in our in vitro cultures of pig jejunal epithelial cells were also largely unresponsive to direct stimulation by 10^8 ST or SC. Yet, like the gut wall, these epithelial cells constitutively expressed high levels of both MIF and OPN (again based upon the Ct values in the real-time PCR assays), and this may suggest MIF and OPN expression in ileal gut wall samples may have been primarily from the epithelial layer. Taken together, our results, both in vivo and in vitro suggest neither MIF nor OPN are acutely regulated by ST in the pig small bowel wall.

A statistically significant time post-ST or tissue by time interaction was not observed for IL8 mRNA in the pig ileum, and this precluded comparisons back to 0 h. Although relative expression of IL8 appeared to be increased at 24 and 48 h, the response at those times was highly variable among animals. There was a significant effect of location within the ileum, and we interpreted this effect to be accounted for by the high levels of IL8 expression at 24 and 48 h in the proximal ileum. At both times, those numerical elevations could be accounted for by relatively high fold changes by two of the animals at each time point. Salmonellosis in pigs has long been associated with neutrophilic invasion in the gut subepithelium (Grondahl et al., 1998; Lee et al., 2000; Lesser et al., 2000) and IL8 is a potent neutrophil chemoattractant (Eckmann et al., 1993b; Follin et al., 1991). However it is clear from our study that IL8 is not uniformly upregulated along the pig ileal gut wall following a single oral dose of ST, even though this bacterial challenge results in a host of pathophysiological sequelae (Balaji et al., 2000). Thus it seems likely that IL8 may be upregulated only in focal segments of gut actively contending with bacterial invasion and destined to be involved with recruitment of inflammatory cells.

Similar to IL8, no tissue by time interaction was detected for CCL20, but there was a significant increase in CCL20 at 24 h after ST inoculation. It would appear that the majority of this increase was in the PI. The putative function of CCL20 in the pig ileum would be to recruit dendritic cells and perhaps T cells into inflamed gut wall (Izadpanah et al., 2001). However, like IL8, it is clear that CCL20 is upregulated transiently and not uniformly in the swine gut following a dose of ST that induces enteric disease in pigs.

It has become apparent within approximately the past decade that gastrointestinal epithelial cells not only serve a role in nutrient absorption, but also serve important functions in innate immune surveillance as well as provide a barrier to potentially invasive enteric pathogens. In terms of immune surveillance, considerable data have been gathered in model human epithelial systems utilizing a variety of cell lines. In general, these cell lines demonstrate transient upregulation of IL8 mRNA (Eckmann et al., 1993a; Eckmann et al., 1993b) and polarized secretion of IL8 favoring the basolateral direction (McCormick et al., 1993; Eckmann et al., 1993a). Our in vitro observations of both IL8 mRNA in response to SC and ST, and the highly polarized secretion of IL8, at least in response to ST (differences between the serovars discussed in more detail below), is consistent with published results from human cell lines and points to a role of the swine intestinal epithelium in recruiting neutrophils to focal areas of inflammatory damage.

Like IL8, CCL20 too was upregulated by both SC and ST. The response to SC was much diminished relative to ST which stimulated a several hundred fold increase in CCL20 relative expression by 3 h. Even so, the response to SC, though more short-lived, was not trivial as the bacteria stimulated CCL20 mRNA by over 200-fold. These results follow the same trend as Caco-2 cells challenged with Salmonella *Typhimurium* ATCC14028 as reported by Sierro et al. (2001), whereby CCL20 mRNA expressed maximal transcription approximately 3 h after challenge. However, IPEC-J2 cells seemed to have an increased magnitude of CCL20 mRNA expression following ST and SC challenge as compared to approximately a 27 fold increase in Caco-2 cells after ST challenge (Sierro et al., 2001).

The lack of response of swine epithelial cells in vitro to LPS, both in terms of IL8 mRNA expression and secretion, is consistent with hyporesponsiveness observed in other model epithelial systems (Schuerer-Maly et al., 1994). The lack of response to LPS may have important physiological relevance in preventing unnecessary inflammatory responses to non-invasive bacteria or LPS that is normally present in the digesta at relatively high concentrations.

Relative to the comparisons among the swine salmonellae servoars in our in vitro experiments, we were surprised to find ST generally more potent than SC in stimulating both IL8 and CCL20 mRNA, and in stimulating IL8 secretion in swine epithelial cells. However, these observations may not be entirely unexpected for two reasons. First, SC is generally more likely to cause systemic disease in pigs than ST (Reed et al., 1986). So, in general, if SC has adopted

strategies that provoke reduced chemotactic responses from gut epithelial cells, this may provide the bacteria greater success in breaching epithelial barriers because it would generate a less robust innate immune response to epithelial detection. Or, secondly, it is possible that SC is less invasive to the intestinal epithelium and is more likely to gain infective access in field settings via oral or nasal epithelium. Stabel et al. (1995) observed a much reduced TNFα response to intranasal SC compared to ST, and this led them to suggest that serovar differences in immune responsiveness in pigs may underlie pathogenicity. In our IPEC-J2 in vitro setting, SC was able to induce an IL8 secretory response, however, as compared to ST, the response was less robust, occurred later (6 h post SC challenge) and only in the apical direction. This generally supports the hypothesis that SC may avoid provoking gut associated inflammatory signals and this may contribute to its host-adapted nature.

For our in vitro salmonella culture experiments, 10^8 bacteria were added to the apical chamber of the model intestinal epithelium. Although this number of bacteria is commonly used for challenge of a variety of epithelial cell lines (Eckmann et al., 2000; Gewirtz et al., 2000; Sierro et al., 2001), the activation is designed to stimulate maximal chemokine responses from epithelial cells (Eckmann et al., 1997). In our system, this number equates roughly to 400 bacteria per enterocyte, and it is difficult to accurately assess the physiological relevance of this bacterial load per unit area within the in vivo context of the swine small intestinal environment. However, what is clear from our in vivo studies is that, despite the variability among pigs, in individual animals in which ileal IL8 was upregulated, particularly at 144 h after oral inoculation, the magnitude of the upregulation exceeded that observed in vitro by several fold. This may indicate that, once breach of the epithelium occurs and inflammatory processes are initiated in focused regions of the gut, additional cell types within the gut wall are recruited for more sustained upregulation of IL8 than can be provided by the epithelium alone.

In conclusion, we have shown that Salmonella serovars in the pig gastrointestinal tract triggered variable upregulation of IL8 and CCL20, with expression levels varying with either time and (or) location within the ileum. In contrast, MIF and OPN were largely unaffected by Salmonella serovars either in the ileum or in vitro. The use of a model swine gut epithelium also demonstrated changes in IL8 and CCL20 expression by enterocytes that was provoked by ST and SC, but ST appeared to elicit greater polarized secretion of IL8 and expression of CCL20. These

differences may partially underlie differences in disease pathogenesis in swine between the two Salmonella serovars.

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Figure 2.1 Relative abundance of macrophage migration inhibitory factor (MIF; panel A time*tissue means; panel B tissue means), interleukin 8 (IL8; panel C time*tissue means; panel D tissue means), macrophage inflammatory protein (MIP)3α/CC chemokine ligand-20 (CCL20; panel E time*tissue means; panel F time means), and osteopontin (OPN; panel G) mRNA from porcine proximal (PI) and distal ileum (DI) isolated at 0, 8, 24, 48, and 144 h after 10^9 CFU oral *Salmonella enterica* serovar *Typhimurium* challenge. Each time*tissue bar in panels A, C, E, and G represents least square means of tissue obtained from four pigs at each time.

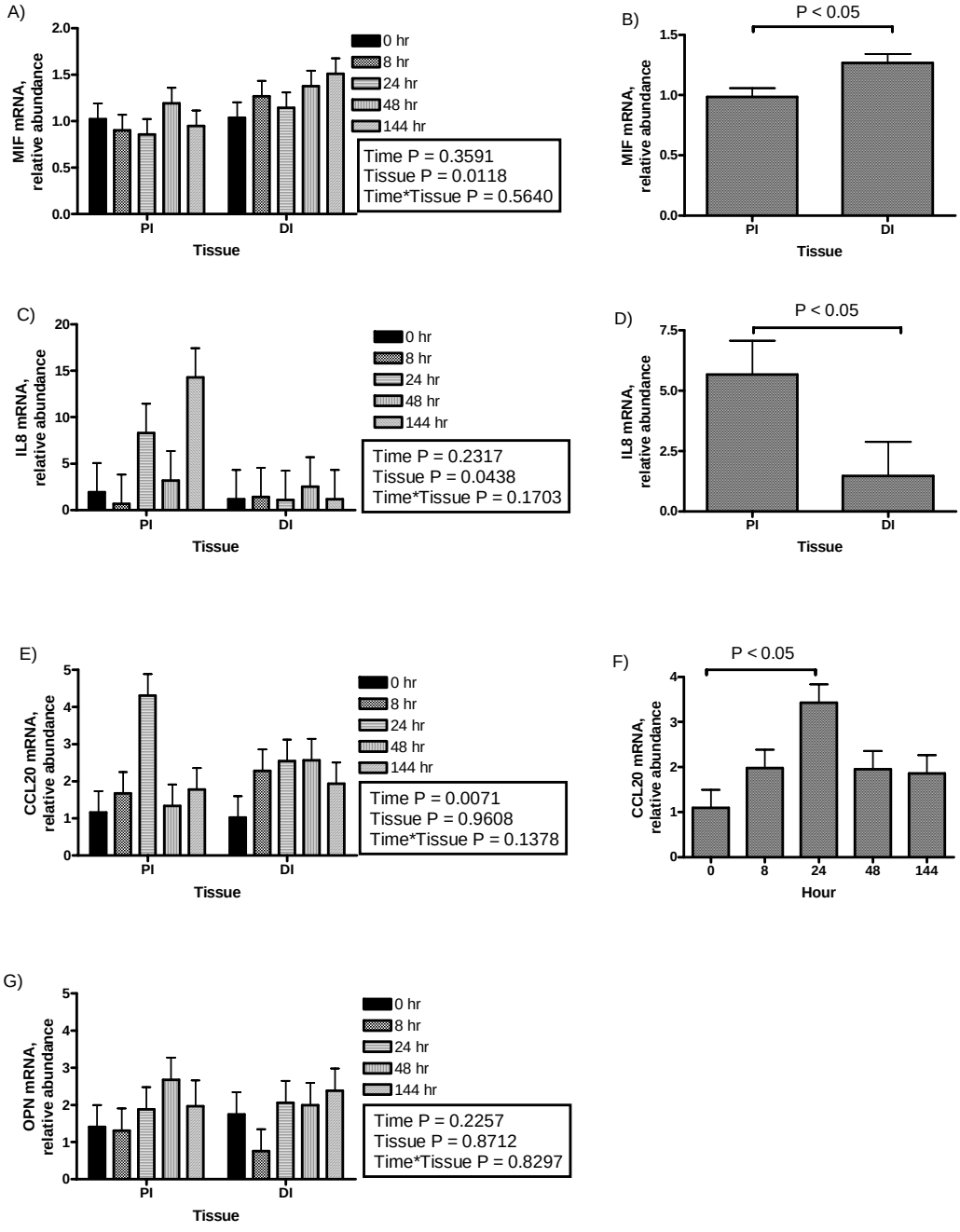
A)
MIF mRNA, relative abundance
0 hr
8 hr
24 hr
48 hr
144 hr
Time P = 0.3591
Tissue P = 0.0118
Time*Tissue P = 0.5640
PI
DI
Tissue
B)
P < 0.05
MIF mRNA, relative abundance
PI
DI
Tissue
C)
IL8 mRNA, relative abundance
0 hr
8 hr
24 hr
48 hr
144 hr
Time P = 0.2317
Tissue P = 0.0438
Time*Tissue P = 0.1703
PI
DI
Tissue
D)
P < 0.05
IL8 mRNA, relative abundance
PI
DI
Tissue
E)
CCL20 mRNA, relative abundance
0 hr
8 hr
24 hr
48 hr
144 hr
Time P = 0.0071
Tissue P = 0.9608
Time*Tissue P = 0.1378
PI
DI
Tissue
F)
P < 0.05
CCL20 mRNA, relative abundance
0
8
24
48
144
Hour
G)
OPN mRNA, relative abundance
0 hr
8 hr
24 hr
48 hr
144 hr
Time P = 0.2257
Tissue P = 0.8712
Time*Tissue P = 0.8297
PI
DI
Tissue

Figure 2.2 Relative abundance of macrophage migration inhibitory factor (MIF; panel A), interleukin 8 (IL8; panel B), macrophage inflammatory protein (MIP)3α/CC chemokine ligand-20 (CCL20; panel C) and osteopontin (OPN; panel D) mRNA from cultured porcine jejunal epithelial cells (IPEC-J2) treated with media alone (control), 10 ng/ml lipopolysaccharide (LPS), 10^8 CFU/well *Salmonella enterica* serovar *Choleraesuis* (SC), or 10^8 CFU/well *Salmonella enterica* serovar *Typhimurium* (ST). Total RNA extracted at 1.5, 3.0 and 6.0 h post treatment. Each treatment*hour bar represents the least square mean (± SEM) of six observations. Asterisks denote significant differences between control and the treated wells.

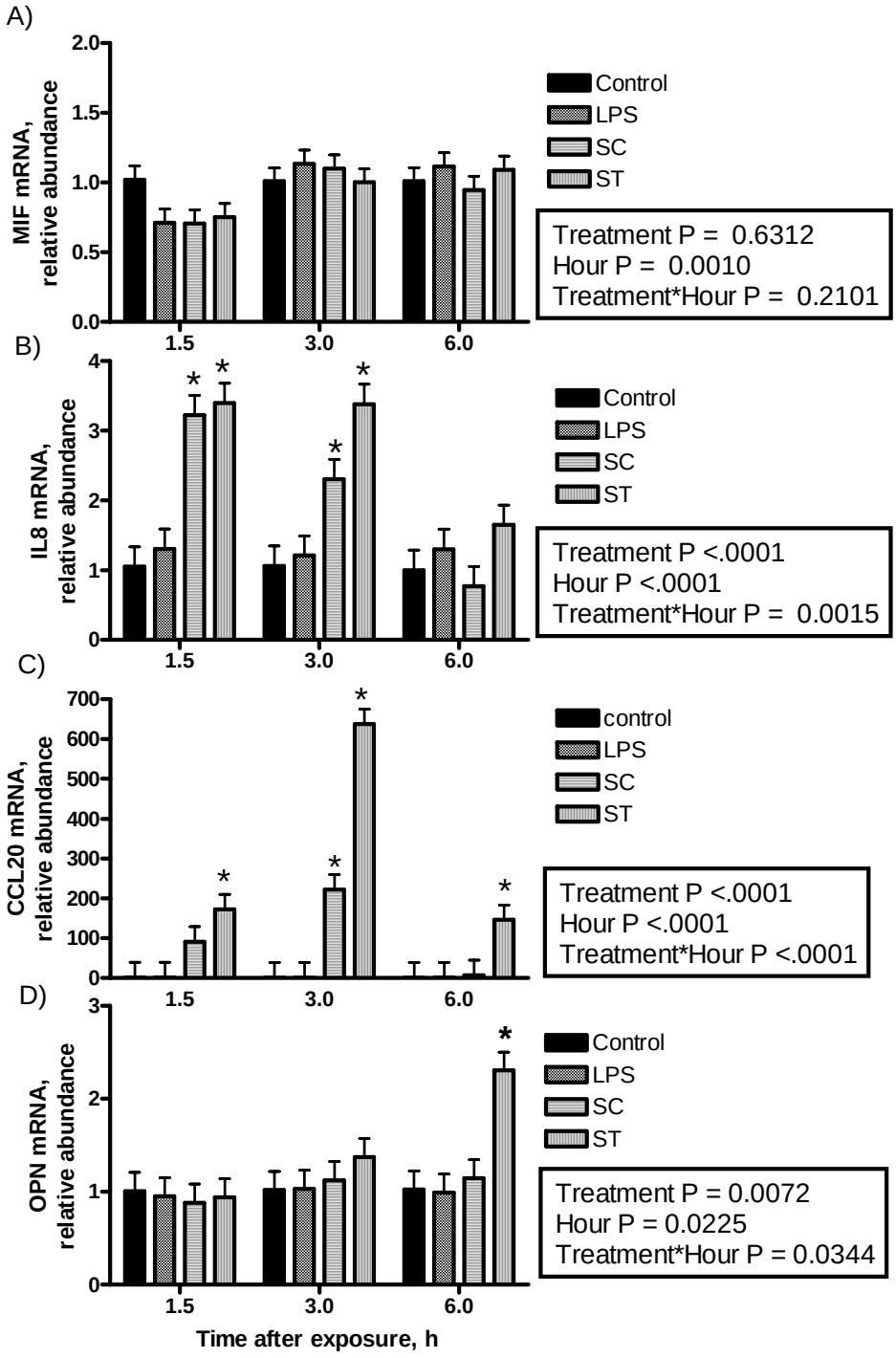
A)
MIF mRNA, relative abundance
2.0
1.5
1.0
0.5
0.0
Control
LPS
SC
ST
Treatment P = 0.6312
Hour P = 0.0010
Treatment*Hour P = 0.2101
1.5
3.0
6.0
B)
IL8 mRNA, relative abundance
4
3
2
1
0
Control
LPS
SC
ST
Treatment P <.0001
Hour P <.0001
Treatment*Hour P = 0.0015
C)
CCL20 mRNA, relative abundance
700
600
500
400
300
200
100
0
control
LPS
SC
ST
Treatment P <.0001
Hour P <.0001
Treatment*Hour P <.0001
D)
OPN mRNA, relative abundance
3
2
1
0
Control
LPS
SC
ST
Treatment P = 0.0072
Hour P = 0.0225
Treatment*Hour P = 0.0344
Time after exposure, h

Figure 2.3 Polarized interleukin 8 (IL8) secretion by confluent porcine jejunal epithelial cells (IPEC-J2) monolayers treated with media alone (control; CON), 5 ng/well (10 ng/ml) lipopolysaccharide (LPS), 10^8 CFU/well *Salmonella enterica* serovar *Choleraesuis* (SC), or 10^8 CFU/well *Typhimurium* (ST) in the apical (AP) compartment for 1 h. Treatment media were removed and replaced with media containing gentamicin. Media from the AP and basolateral (BL) compartments were collected and assayed for cytokines at 1.5, 3.0, and 6.0 h after the onset of LPS or bacterial treatment. Each bar represents the least square mean (± SEM) of four observations. Significant accumulation of IL8 was observed at 6 h only in wells treated with ST, with greater secretion in the BL.

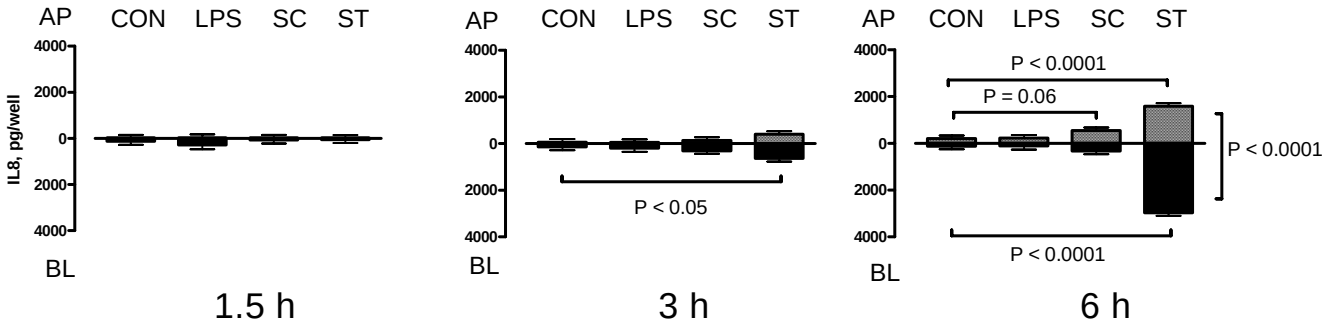

Table 2.1 Primer and probe sequences used for cytokine and chemokine quantification by real-time RT-PCR

Primer set	Product length	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Probe sequence (5'-3')[1]
Microphage migration inhibitory factor (MIF)	75	ATG CCG ATG TTC GTG GTA AAC	CTG AGT CAG CTC GGA GAG GAA	TCC CCG CGC CTC TGT GCC
Interleukin 8 (IL8)	73	GAG CTC TCC ATC CAG CAC AAG	TCT CTT CCT GGC TCT GGT CCT A	CCC CTG GGA CAG CAG AGC TCA CA
Osteopontin (OPN)	81	CCC AAG GCC ATC CTC GTT	ACT CGT CTC CTG ACT GTC CTT CTC	CAG CGC CTG CAC GTG GCT
CCL20	76	GCT CCT GGC TGC TTT GAT G	CAG TCA AAG TTG CTT GCT TCT GA	CGG TGC TGC TCT ACC TCT GCA

[1]All probes were synthesized with 6-FAM as the 5' fluorophore and TAMRA as the 3' quencher dye.

CHAPTER 3 - Effects of *Salmonella enterica* serovar *Typhimurium*, or serovar *Choleraesuis*, *Lactobacillus reuteri* and *Bacillus licheniformis* on chemokine and cytokine expression in the swine jejunal epithelial cell line, IPEC-J2

ABSTRACT

Direct-fed microbials, including *Lactobacillus* and *Bacillus* spp., are potential replacements for low dose in-feed antibiotics for swine and other livestock. To understand the function of these microbes in the gut, the current study used pig jejunal epithelial cells (IPEC-J2) to evaluate how *Lactobacillus reuteri* (LR) and *Bacillus licheniformis* (BL) differed from *Salmonella enterica* serovars Typhimurium (ST) or Choleraesuis (SC) in their ability to regulate, stimulate, or modify the proinflammatory mediators, interleukin 8 (IL8), CC chemokine 20 (CCL20), and tumor necrosis factor α (TNFα). To optimize the positive control to drive IL8 secretion by IPEC-J2 cells, cells were treated apically with various concentrations of ST (vs. control (CTL)) for 1 h, followed by a wash. Media containing gentamicin was added and collected at 6 h post treatment. Compared to CTL, 10^8 ST produced maximal IL8 secretion in both the apical and basolateral directions, with significant basolateral polarization ($P < 0.0001$). We next evaluated the time course of IL8 secretion, and IL8, CCL20, and TNFα mRNA expression by IPEC-J2 cells treated apically with 10^8 ST, SC, LR, and BL vs. CTL. Media and RNA were collected at 1.5, 3.0 and 6.0 h post treatment. Only ST stimulated an increase in IL8 secretion at any time point, with increases in IL8 mRNA at both 3 and 6 h ($P < 0.05$). However, BL increased IL8 mRNA at 1.5 h ($P < 0.0001$). Neither LR nor SC affected IL8 mRNA expression. CCL20 mRNA was strongly upregulated by ST ($P < 0.05$) and BL (1.5 and 3.0 h; $P < 0.05$), but not LR or SC. Only ST increased TNFα mRNA relative to CTL ($P < 0.05$). Two experiments were conducted to determine if pre-exposure of IPEC-J2 cells to LR or BL modified ST induced IL8 secretion. Confluent cells were treated apically overnight with various levels of LR or BL (in separate experiments) followed by ST challenge. Media were collected at 4 (LR experiment) or 5 h (BL experiment) post ST. In the LR study, IL8 secretion was increased by ST

as compared to CTL ($P < 0.0001$), reduced by LR ($P < 0.05$), and LR+ST co-treatments failed to alter ST stimulated secretion. In the BL experiment, secretion of IL8 was increased by ST ($P < 0.0001$), but blunted basolaterally in BL+ST co-treated wells. The data demonstrate that IPEC-J2 cells increase IL8 secretion in response to ST, and IL8 mRNA in response to ST and BL, but not LR. Furthermore, ST stimulated secretion of IL8 is inhibited basolaterally in the presence of BL.

1. INTRODUCTION

Low dose dietary antibiotics, used as growth promotants, have been widely incorporated into livestock production since the 1950s. The advantages of low-dose antimicrobials (particularly those included in diets for weanling pigs) have been well documented and include improvement in average daily weight gain and feed efficiency (Hays, 1978; Dritz et al., 2002; Gaskins et al., 2002; Zimmerman, 1986). A definitive link between antibiotic use and gut pathogen load has not been established, yet several hypotheses and documented physiological, nutritional and metabolic effects on growth have been reported. Some of these effects include: 1) nutrient protection from microbial breakdown which increases their availability to the host, 2) thinning of the small intestinal barrier thereby allowing greater nutritional absorption, 3) decreased microbial toxins, 4) reduced sub-clinical intestinal infections, and 5) antimicrobial altered interactions between the gut and potential pathogens (Butaye et al., 2003; Gaskins et al., 2002). However, use of low dose dietary antibiotics has been questioned in recent years because of concerns that their use in feed for the growth promotion of livestock may lead to strains of pathogens that are resistant to antibiotic therapy (Bach Knudsen, 2001). Because of these concerns, there is an ongoing search for alternatives to the various antibiotics that are commonly used in livestock diets. Researchers have investigated many different natural alternatives including mineral supplements, acidifiers, botanicals, enzymes, and probiotics (Verstegen and Williams, 2002).

Lactic acid bacteria are widely used as probiotics in humans with reported health benefits against gastrointestinal disorders including diarrhea, inflammatory bowel disease, lactose intolerance, and *salmonella* or *shigella* infections (Madsen, 2001). *Lactobacillus* spp. are also found as probiotic applications in both companion and farm animal species. *Lactobacilli reuteri* (LR) isolates from pig feces have been shown to produce a broad spectrum antimicrobial

compound (reuterin), and survive under low pH gastrointestinal conditions (Rodriguez et al., 2003). Rodriguez et al. (2003) also noted bile salt hydrolase activity which further confirmed the capacity of LR to colonize the intestinal tract, and suggests the benefit of LR as a probiotic. LR has also demonstrated direct anti-inflammatory activity in human epithelial cells in that live, but not heat-killed, or gamma-irradiated LR was able to inhibit TNFα and *Salmonella enterica* serovar Typhimurium induced IL-8 secretion and NFκB translocation in T84 and HT29 cells (Ma et al., 2004). Various *Bacillus* species are also in use as probiotic products for humans and animals (Hong et al., 2005), but their mechanism of action has yet to be fully understood. Several studies have been completed in pigs using the BioPlus® 2B supplement (containing *Bacillus licheniformis* and *Bacillus subtilis* spores), which demonstrated improved sow and piglet performance (Alexopoulos et al., 2004).

Here we report in vitro experiments designed to determine dynamic changes in putative chemotactic and cytokine signals in the representative pig gastrointestinal epithelial cell line (IPEC-J2; Rhoads et al., 1994) after exposure to two pathogenic (*Salmonella enterica* serovar Typhimurium (ST) and *Salmonella enterica* serovar Choleraesuis (SC)), or two prototypical probiotic bacterial strains which have been classified as probiotics (*Lactobacillus reuteri* (LR) and *Bacillus licheniformis* (BL)). In addition, we evaluated how pretreatment of IPEC-J2 cells with the probiotic microbes (LR or BL) would affect ST mediated changes in the chemotactic signal, IL8. Our working hypothesis was that the presence of invasive bacterial pathogens, but not the commensal microbes, would trigger upregulation of selected cytokine and chemokine mediators, and that pre-exposure of IPEC-J2 cells to LR and BL would downregulate the proinflammatory IL8 secretory response induced by ST. We evaluated components of the epithelial inflammatory gene and protein network, including the chemokines IL8 (neutrophil attractant), and CC chemokine ligand CCL20 (CCL20, dendritic cell attractant), as well as the proinflammatory cytokine TNFα.

2. MATERIALS AND METHODS

2.1 Cell culture

The neonatal jejunal epithelial cell line IPEC-J2 was derived from a single animal less than 12 h old (Rhoads et al., 1994). These cells were a gift from Dr. Bruce Schultz, Department of Anatomy and Physiology, Kansas State University. Cell growth was maintained on 50%

DMEM - 50% F12 medium (Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (Hyclone, Logan, UT, USA), 1% insulin/ transferrin/ Na Selenite media supplement (Sigma, St. Louis, MO, USA), 5 ng/ml epidermal growth factor (Invitrogen, Carlsbad, CA, USA) and 1% streptomycin/penicillin (Invitrogen, Carlsbad, CA, USA). For experimentation cells were seeded onto 12 mm, six-well Costar Snapwells (Corning Inc, Corning, NY, USA) at 2.5 x 10^5 to 4.0 x 10^5 per well in a 0.5 ml volume and allowed to adhere for 24 h before being washed and re-fed every other day to allow for confluency and tight junction formation. Cells were used for experimentation within 14 d from seeding. In this in vitro system, average cell density was based on hemacytometer counts of six individual trypsinized wells and was determined to be approximately 2.5 x 10^5 cells per well. Transepithelial resistance was typically 4000 ohm cm^2 (which is comparable to findings by Schierack et al., 2005)). Twenty four hours before experimentation, the cells were washed and replacement media was as above but devoid of antibiotics.

2.2 Bacteria for cell culture challenge.

Salmonella enterica serovars Typhimurium (ST) and Choleraesuis (SC) were primary isolates from swine clinical cases (obtained from Dr. Jerome Nietfeld, Associate Professor, Diagnostic Medicine / Pathobiology, Kansas State University Veterinary College). Identification of *Salmonella* serotypes were verified by National Veterinary Services Laboratory, Ames, Iowa, USA. Both salmonella strains were grown in Luria Bertani (LB) medium at 37°C, for 24 h, at which point bacterial populations were estimated by spectrophotometry at 600 nm optical density. *Lactobacillus reuteri* (LR, ATCC no. 53608, swine intestine isolate) and *Bacillus licheniformis* (BL, Weigmann) Chester, ATCC no. 10716) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The LR bacterial strain was propagated overnight at 37°C, 5% CO_2 in Lactobacilli MRS broth, while BL was cultured in LB at 37°C. Growth curves were established for both microbes and bacterial populations after overnight growth were estimated from the appropriate growth curve. All bacteria were then individually pelleted and resuspended in DMEM/F12 growth media (but devoid of antibiotics) for use in IPEC-J2 challenge.

2.3 *Salmonella enterica* serovar *Typhimurium* induced IL8 upregulation in IPEC-J2 cells.

Treatments included control (uninfected cells), or ST at 10^4, 10^5, 10^6, 10^7, or 10^8 bacteria per well. Confluent IPEC-J2 cells were as described above except that they were seeded into 24 mm six-well Costar Transwells (Corning Inc, Corning, NY, USA) which allowed for a cell density of 1.5 x 10^6 cells per well after confluency. Average cell density was based on hemacytometer counts of six individual trypsinized wells. The application of 1.0 ml of media alone (control), or diluted bacteria were added to the top chamber, while 2.5 ml of media were added to the bottom chamber and plates were further incubated at 37°C, 5% CO_2 for 1 h to allow bacterial entry. After removal of the extracellular bacteria, the cultures were incubated for 6 h in the presence of 50 μg/ml of gentamicin (Gibco, Grand Island, NY, USA) to kill the remaining extracellular bacteria, and media were removed from both the apical and basolateral compartments for later IL8 cytokine secretion analysis.

2.4 Exposure of IPEC-J2 cells to pathogenic or commensal bacteria

Treatments included control (uninfected cells), SC, ST, LR, and BL (10^8 bacteria/well). Confluent IPEC-J2 cells, as described above for 12 mm Costar Snapwells, were washed twice with PBS and 0.5 ml of media alone (control), or diluted bacteria were added to the top chamber, while 2.5 ml of media were added to the bottom chamber and plates were further incubated at 37°C, 5% CO_2 for one hour to allow bacterial entry. After removal of the extracellular bacteria, the cultures were incubated for 1.5, 3.0, and 6.0 h in the presence of 50 μg/ml of gentamicin (Gibco, Grand Island, NY). At indicated times, media were removed from both the apical and basolateral compartments for later IL8 secretion (six replicates) and cells subjected to RNA extraction for later evaluation of chemokine or cytokine gene expression (three replicates).

2.5 Overnight exposure of IPEC-J2 cells to Lactobacillus reuteri or Bacillus licheniformis followed by challenge with Salmonella enterica serovar Typhimurium

Cells were handled as indicated above for 24 mm six-well Costar Transwells. The LR pre-exposure experimental design included treatments of control (no bacterial treatment, but appropriate wash steps), ST alone (10^8 bacteria/well), LR alone (1.5 x 10^8; about 100 LR/enterocyte) and three levels of LR pretreated cells: 1.5 x 10^6 (about one LR/enterocyte; designated LR6ST), 1.5 x 10^7 (about 10 LR/enterocyte; designated LR7ST), and 1.5 x 10^8 (about 100 LR/enterocyte; designated LR8ST), followed by ST (10^8 bacteria/well) challenge. The BL

pre-exposure experimental design was similar and included treatments of control (no bacterial treatment, but appropriate wash steps), ST alone (10^8 bacteria/well), BL alone (10^8; about 66 BL/enterocyte) and three levels of BL pretreated cells: 10^6 (< 1 BL/enterocyte; designated BL6ST), 10^7 (about six BL/enterocyte; designated BL7ST), and 10^8 (about 66 BL/enterocyte; designated BL8ST) followed by ST (10^8 bacteria/well) challenge. Initially, confluent IPEC-J2 cells were washed with PBS and 1.0 ml of media alone (control), or diluted LR (or BL) were added to the top chamber, while 2.5 ml of media was added to the bottom chamber and plates were further incubated at 37°C, 5% CO_2 overnight to allow LR or BL pre-exposure before ST challenge. ST was then added directly to the top (apical) wells the following morning and plates were further incubated at 37°C, 5% CO_2 for 1 h. Cells from all treatments were then washed and both apical and basolateral media were replaced with fresh media containing 50 μg/ml gentamicin and incubated at 37°C, 5% CO_2 for 4 h for the LR experiment and 5 h for the BL experiment. Then, media were removed from both the apical and basolateral compartments. Experiments were replicated four times.

2.6 RNA extraction and reverse transcription (RT) PCR

Total RNA was extracted at the indicated time points using TRI® Reagent (Sigma-Aldrich Co., St. Louis, MO) RNA isolation reagent as per the manufacturer's protocol. Following total RNA isolation, the DNA-*free*™ (Ambion Inc., Austin, TX, USA) kit was used to ensure removal of contaminating genomic DNA from all RNA samples. Samples were frozen in 50 μl of Nuclease-Free Water (Ambion Inc., Austin, TX, USA). RNA was quantified by spectrophotometry using an optical density of 260 nm. Reverse transcription was carried out using TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). Briefly, reverse transcription was carried out in a 50 μl final volume that included 25 mM $MgCl_2$, 500 μM dNTP's, 2.5 μM random hexamers, 0.4 U/μL Rnase inhibitor, 50 U/μL MultiScribe reverse transcriptase, and TaqMan RT buffer. The reverse transcription mixture was incubated at 25°C for 10 min, heated to 37°C for 60 min, and inactivated at 95°C for 5 min. The resultant cDNA was stored (-20°C) until used.

2.7 Real time PCR analysis for IL8, CCL20, and TNFα gene expression

Real-time quantitative PCR was utilized to quantify the products of interest (IL8, CCL20, TNFα) relative to the quantity of 18S rRNA in total RNA isolated from cultured IPEC-J2 cells. The PCR reactions were carried out in 96-well plates with the appropriate forward and reverse primers (900nM), the appropriate TaqMan TAMRA probe (200 nM), PCR Mastermix (Applied Biosystems, Foster City, CA), and 3.5 μL of the cDNA sample. The sequences for IL8 and CCL20 primer-probe sets were as previously published (Skjolaas et al., 2006). The porcine specific TNFα primers and detection probe (forward primer, 5'-3': TCC AAT GGC AGA GTG GGT ATG; reverse primer, 5'-3': AGC TGG TTG TCT TTC AGC TTC AC; and probe: 5'-3': TGC CCT CCT GGC CAA CGG) were synthesized from a published sequence (GenBank Accession: NM_214022) using Primer Express® software (Applied Biosystems, Foster City, CA.). The TNFα probe was synthesized with 6-FAM as the 5' fluorophore and TAMRA as the 3' quencher dye. Commercially available eukaryotic 18S rRNA (Applied Biosystems, Foster City, CA) primers and probe were used as an endogenous control. Assays using non-template controls and samples were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Thermal cycling parameters were utilized according to manufacturer recommendations and included 50 cycles of 15 s at 95°C and 1 min at 60°C. Relative expressions of the genes of interest were normalized with the 18S rRNA endogenous control.

2.8 ELISA for IL8

IL8 was quantified in the apical and basolateral supernatants by a porcine specific sandwich ELISA (DuoSet® Porcine IL8, catalog number DY535, R&D systems, Minneapolis, MN, USA) as per the manufacturer's instruction.

2.9 Statistical analysis

Relative abundance of chemokines and cytokine gene expression were determined from real-time PCR data of cultured jejunal enterocytes by the ΔΔCT method using the average ΔCT of cells from control wells as the reference expression. Polarized secretion of IL8 from cultured cells were analyzed with effects of treatment, time and location (apical or basolateral) in the model for Figure 2, but because Figures 1 and 4 consisted of one time point they were analyzed with effects of treatment, location (apical or basolateral) and their interaction in the model.

Secretion of IL8 was expressed as pg/well to account for the considerably greater volume of media present in the basolateral compartment compared to the apical compartment. Data bars in Figures 1, 3 and 5 represent three observations per treatment obtained from independent in vitro experiments. Data bars in Figure 2 represent six observations per treatment obtained from independent in vitro experiments; and Figure 4 represents four observations per treatment obtained from independent in vitro experiments.

3. RESULTS

3.1 In vitro challenge of swine gut epithelial (IPEC-J2) cells with increasing concentrations of Salmonella enterica serovar Typimurium

This experiment was conducted to evaluate a range of apical ST that would drive strong apical and basolateral IL8 secretion (Figure 1). Cells demonstrated a significant difference in IL8 secretion in both the apical and basolateral direction when exposed to 10^6, 10^7, and 10^8 ST per well. At 10^6 ST per well, IL8 was increased above control wells in the apical ($P < 0.01$) and basolateral ($P< 0.0001$) directions and demonstrated polarized secretion into the basolateral compartment ($P < 0.001$). Both the 10^7 and 10^8 ST treatments generated maximal IL8 responses with highly significant increases in IL8 secretion as compared to controls ($P < 0.0001$ in both the apical and basolateral directions) and both concentrations generated highly significant polarized secretion into the basolateral direction ($P < 0.0001$). Therefore the use of 10^8 ST per well for proinflammatory stimulation of IPEC-J2 cells was selected for use in future experiments.

3.2 In vitro challenge of IPEC-J2 cells with Salmonella enterica serovars Typhimurium or Choleraesuis; or commensal microbes, Lactobacillus reuteri or Bacillus licheniformis

The aim of this experiment was to individually expose the swine jejunal epithelial cell line (IPEC-J2) to two pathogenic bacteria: ST, and SC, as well as two commensal bacterial strains LR and BL; and compare the pathogenic and commensal microbe mediated responses. To determine whether challenged IPEC-J2 cells demonstrated differential signaling during a proinflammatory response, protein secretion of IL8 was evaluated by collection of culture media from both the apical and basolateral compartments of Costar Snapwells plates at 1.5, 3.0 and 6.0 h after bacterial challenge and assayed by ELISA (Figure 2). The concentration in each compartment was adjusted to reflect the greater volume in the basolateral compartment and

expressed as pg/well. Treatment with ST demonstrated an initial trend toward an increase in IL8 secretion in the basolateral direction (Figure 2B; P =.12) above that of the control basolateral compartment at 3 h. At 6 h post ST (Figure 2C), IL8 secretion was increased in both the apical ($P < 0.0001$) and the basolateral ($P < 0.0001$) directions relative to controls, and was also strongly, though not completely, polarized towards the basolateral compartment ($P < 0.0001$). All other treatments did not affect IL8 secretion at any time point.

In addition to protein secretion, real time PCR was performed to determine whether the above mentioned bacterial strains affected IL8, CCL20, and TNFα, mRNA expression (Figure 3). Relative levels of IL8 mRNA were most affected by the ST treatment with increased mRNA expression by about ten-fold at 1.5 h and approximately five-fold by both 3 h and 6 h post ST relative to control wells (Figure 3A; $P < 0.0001$ and $P < 0.05$ respectively). The BL treatment also increased IL8 mRNA about seven-fold relative to controls, but only within the first 1.5 h post exposure (Figure 3A; $P<0.0001$). Neither LR nor SC affected the expression of IL8 mRNA. CCL20 mRNA expression exhibited a similar pattern as IL8 demonstrating a strong up-regulation as a result of ST, BL, and a slight up-regulation by SC. Treatment with ST increased CCL20 by over 150-fold (Figure 3B; $P < 0.05$), 350-fold (Figure 3B; $P < 0.0001$), and about 175-fold (Figure 3B; $P < 0.05$) at 1.5, 3, and 6 h, respectively. While treatment with BL increased CCL20 by 200-fold at 1.5 h and approximately 125 fold after 3.0 h (Figure 3B; $P < 0.05$ for both observations), it did not demonstrated a difference from untreated controls at the 6.0 h time point. SC demonstrated a trend toward an increase in CCL20 mRNA by about 75-fold at 1.5 and 3 h (Figure 3B; $P = 0.166$ and $P = 0.197$ respectively). Similarly to IL8 mRNA expression, CCL20 was unchanged relative to control by the LR treatment. ST was the only bacteria that generated a significant increase in TNFα mRNA relative to control wells, with approximately 60, 150, and 80-fold increases at 1.5, 3.0, and 6.0 h respectively (Figure 3C; $P = .057$, $P < 0.0001$, and $P < 0.05$, respectively). The LR, BL, and SC treatments did not affect TNFα expression particularly at 3.0 and 6.0 h post treatment. It may be noteworthy to mention that SC and BL demonstrated trends toward an increase in TNFα gene expression at the 1.5 h time point with approximately 34 and 45-fold increases respectively (Figure 3C; $P = .261$ and $P = .141$ respectively).

3.3 In vitro challenge of swine gut epithelium with Lactobacillus reuteri or Bacillus licheniformis followed by challenge with Salmonella enterica serovar Typhimurium

Because *Lactobacillus* and *Bacillus* spp. are commensal microbes used as a probiotics in both domestic animal species and humans, we chose to evaluate whether LR or BL had anti-inflammatory effects on IPEC-J2 cells challenged with ST. Cells were initially exposed to various amounts of LR or BL overnight followed by ST challenge for 1 h; fresh media were collected from both the apical and basolateral compartments at 4 h (LR experiment) or 5 h (BL experiment) post ST infection. As in previous experiments, we evaluated the inflammatory response by measuring the IL8 secretion profile (Figures 4 and 5).

In the experiment involving LR pre-exposure to IPEC-J2 cells, treatment with ST increased IL8 in both the apical (Figure 4; $P < 0.0001$) and the basolateral (Figure 4; $P < 0.0001$) directions relative to controls, and was strongly, though not completely, polarized towards the basolateral compartment ($P < 0.0001$). Pretreatment with LR (at all concentrations) followed by ST challenge failed to change the ST stimulated IL8 secretion profile and all LR+ST co-treatment wells exhibited the same profile as wells receiving ST alone (Figure 4). Of particular note, the wells that received only LR demonstrated a significantly reduced IL8 secretion profile in both the apical and basolateral directions as compared to controls (Figure 4 insert; $P < 0.05$ for both directions).

Cells pre-exposed to BL and co-cultured with ST demonstrated evaluated IL8 secretion (Figures 5). The ST alone treatment stimulated an increase in both the apical and basolateral compartments (Figure 5; $P<0.0001$ for both), with a polarized release in the basolateral direction (Figure 5; $P<0.05$). Interestingly, and unlike LR, the BL+ST co-treatments (at all levels of BL) inhibited ST stimulated basolateral secretion of IL8, and were not different from controls (Figure 5). In contrast, the BL alone treatment significantly increased IL8 secretion into the apical direction above that of control (Figure 5; $P < 0.05$). Likewise, the BL+ST co-treatments, BL6ST and BL7ST, also stimulated a secretory response into the apical direction with significant differences from control (Figure 5; $P < 0.001$ for BL6ST and BL7ST) and from BL alone (Figure 5; $P<0.05$). Exposure of cells to all BL + ST co-treatments reduced ST induced apical secretion of IL8, with the greatest reduction by the BL8ST treatment (Figure 5, $P < 0.05$).

4. DISCUSSION

As a result of the pursuit to find alternatives to in-feed antibiotics for growth promotion in farm animal production a push for a greater understanding of how probiotics may modify the gastrointestinal environment and (or) nutritional status has emerged. Our objective was to determine whether two common dietary probiotic microbes (LR and BL) could modulate proinflammatory responses in swine jejunal epithelial cells. We challenged IPEC-J2 cells with LR or BL as previously described and included ST and SC as representative pathogenic treatments for adequate comparisons of proinflammatory stimulation. As representative proinflammatory mediators, we chose to focus on two chemokines known to regulate the chemotaxis of neutrophils (IL8; Eckmann et al., 1993; Fierer et al., 1993; Follin et al., 1991), and the trafficking of dendritic cells (CCL20; Dieu-Nosjean et al., 2000; Sierro et al., 2001), along with the well known pleiotropic inflammatory cytokine TNFα (Tracey and Cerami, 1994).

Salmonella was chosen as a prototypic invasive pathogen because of its economic importance as a swine enteropathogen that has been extensively characterized in vivo. Our group has shown that single dose oral challenges of young pigs with ST resulted in transient enteric disease, fever, inappetence, slowed growth (Balaji et al., 2000; Burkey et al., 2004; Turner et al., 2002a; Turner et al., 2002b), activation of the endocrine stress axis (Balaji et al., 2000) and disruption of the growth axis (Balaji et al., 2000; Jenkins et al., 2004). In some of our experiments we included SC for pathogenic comparison since it is also an important swine enteropathogen, but seems to have differing inflammatory properties in vitro (Schierack et al., 2005; Skjolaas et al., 2006) and in vivo (Reed et al., 1986).

Lactobacillus and *Bacillus* species are already in use as probiotics for various domestic animal species, and therefore selected as representative commensal bacteria. Several *Lactobacillus* species isolated from pigs and poultry have been shown to be bile tolerant, able to adhere to intestinal cells, inhibit the growth of *Salmonella* and block *Salmonella* invasion in cultured human cell lines (Casey et al., 2004; Tsai et al., 2005). Reuterin produced by *Lactobacillus reuteri* spp. also demonstrates antimicrobial activity and has been suggested as having a strong probiotic potential in pigs (Rodriguez et al., 2003). The *Bacillus* spp. have also been evaluated as a probiotic for use in pigs; demonstrating improved health, fertility, and weight gain in sows and piglets (Alexopoulos et al., 2004; Zani et al., 1998).

We have previously demonstrated that ST is a potent activator of proinflammatory signals in IPEC-J2 cells when challenged with 10^8 CFU/well for 1 h followed by a wash and additional incubation in the presence of gentamicin (Skjolaas et al., 2006; Burkey et al., submitted). In the current study, we have further demonstrated that 10^6 CFU/well is sufficient at stimulating a significant IL8 secretory profile with polarized secretion toward the basolateral direction; but that maximal secretion occurred with 10^7 and 10^8 CFU/well. Although there was no difference in IL8 secretion in either the apical or basolateral compartments between the 10^7 as compared to 10^8 ST treatments, we chose 10^8 ST as positive control for all other future experiments since this number of bacteria is commonly used for challenge of a variety of epithelial cell lines (Eckmann et al., 2000; Gewirtz et al., 2000; Sierro et al., 2001), and the activation is designed to stimulate maximal chemokine responses from epithelial cells (Eckmann et al., 1997), which seems to be the case for the IPEC-J2 cell line as well. Schierack et al., (2005) challenged IPEC-J2 cells with *Salmonella* multiplicity of infection of 100:1 (*Salmonella*: enterocyte) for 1 h for evaluation of IL8 secretion. In the current study, our multiplicity of infection was approximately 66:1, but IL8 concentrations, both basal and stimulated secretion, are comparable to the Schierack study. Therefore, the use of 10^8 CFU/well for all challenge experiments ensured maximal secretion of IL8 for comparison with the commensal microbes.

In evaluating whether the commensal strains could affect inflammatory signals when cultured with IPEC-J2 cells, we found that neither the LR nor the BL strain elicited a secretory IL8 response at any time point evaluated. However, BL was able to induce upregulation of IL8, CCL20, and TNFα mRNA expression, albeit an early response that was extinguished by 3 and 6 h. Previously, ST was found to be more potent than SC in stimulating both IL8 and CCL20 mRNA, and in stimulating IL8 secretion in swine epithelial cells (Skjolaas et al., 2006). The data presented here support our previous finding. Consistent with this observation, Schierack et al., (2005) observed reduced invasion efficiency by SC as compared to ST into the IPEC-J2 cell line. The lack of an inflammatory response by LR or BL suggests that these microbes co-exist with the gastrointestinal epithelium without triggering proinflammatory alarm responses.

Since BL and LR did not stimulate an inflammatory response, we next evaluated whether they could affect the inflammatory response to ST. Numerous studies have provided evidence for the beneficial protective effects of *Lactobacillus* species against pathogenic bacteria, which include blocking entry and inhibiting growth (Tsai et al., 2005). Our data suggest that LR

offered no protection against ST induced IL8 secretion in IPEC-J2 cells since all wells co-treated with LR and ST exhibited significant increases in IL8 secretion, similar to wells treated with ST alone. The co-treatment of LR with ST contrasts results obtained where LR (at 10 bacteria/cell) reduced 10^7 ST stimulated IL8 response from T84 human enterocytes (Ma et al., 2004). The contrast of their results to ours may be related to the particular *Lactobacillus reuteri* strain, since not all strains have demonstrated equal adhesion ability to Caco-2 cells (Todoriki et al., 2001). The inability of LR in the current study to affect ST mediated IL8 secretion may also be due to culture conditions required by IPEC-J2 cells, as DMEM has been shown to delay the killing activity of lactic acid. Since inhibition of ST growth depends on the pH lowering effect of *Lactobacillus* spp. (Fayol-Messaoudi et al., 2005), our pH conditions may not have been optimal for LR. However, in our experiment, LR may have promoted an enhanced anti-inflammatory condition in our model epithelium as LR exposure of IPEC-J2 cells resulted in IL8 accumulation in the media that was significantly lower than control wells not exposed to any bacteria.

When we evaluated whether BL offered any protection against ST mediated IL8 secretion by IPEC-J2 cells, we found that BL pre-treatment followed by BL and ST co-culture resulted in a diminished IL8 secretion in the basolateral direction. Therefore, BL may inhibit ST mediated stimulation of inflammatory signals, but further investigation is warranted to determine if this was due to BL blocking ST adhesion and entry, or by an antibiotic mediated inhibition of ST. The latter seems plausible since ATCC 10716 BL, employed in our experiment, is known to produce bacitracin (Konz et al., 1997), and overnight cultures in LB media have produced as much as 30.0 ± 3.9 units/ml of antibiotics (Ishihara et al., 2002).

The contrasting effects of BL treatment on IL8 secretion comparing those in Figure 2 (at 6 h) and Figure 5 is almost certainly attributable to the more prolonged incubation of the cells with BL (in Figure 5). In that regard, treatment of IPEC-J2 cells with BL alone increased IL8 secretion, but only in the apical direction. This raises the intriguing question of whether BL within the gut luminal contents could impose a persistent stimulation of IL8 towards the gut lumen. Such an effect would be presumed to generally not be inflammatory, and likely ineffective in stimulating neutrophil invasion.

In conclusion, in a model swine gut epithelium, ST stimulated increased IL8 secretion, as well as IL8, CCL20, and TNFα mRNA, with levels varying according to time. In contrast, IL8 secretion and IL8, CCL20, and TNFα gene expression were unaffected by LR or BL.

Enterocytes pre-treated with either BL or LR and co-cultured with ST demonstrated that BL, but not LR, inhibited ST stimulated IL8 secretion in the basolateral direction. These observations shed new light on the potential interaction of probiotic bacteria with intestinal epithelium and their potential to modulate inflammatory responses of enterocytes to invasive enteropathogens.

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Figure 3.1 Polarized interleukin 8 (IL8) secretion by confluent porcine jejunal epithelial cell (IPEC-J2) monolayers treated with media alone (control; CTL), 10^4, 10^5, 10^6, 10^7, or 10^8 CFU/well *Salmonella enterica* serovar *Typhimurium* (ST4, ST5, ST6, ST7, or ST8, respectively) in the apical compartment for 1 h. Treatment media were removed and replaced with media containing gentamicin. Media from the apical and basolateral compartments were collected and assayed for cytokines at 5.0 h after the onset of bacterial treatment. Each bar represents the least square mean (± SEM) of four observations. Within each secretory direction, bars without common superscripts differ ($P < 0.05$). Significant accumulation of IL8 was observed in wells treated with ST at 10^6, 10^7, or 10^8 CFU/well, with greater secretion in the basolateral direction.

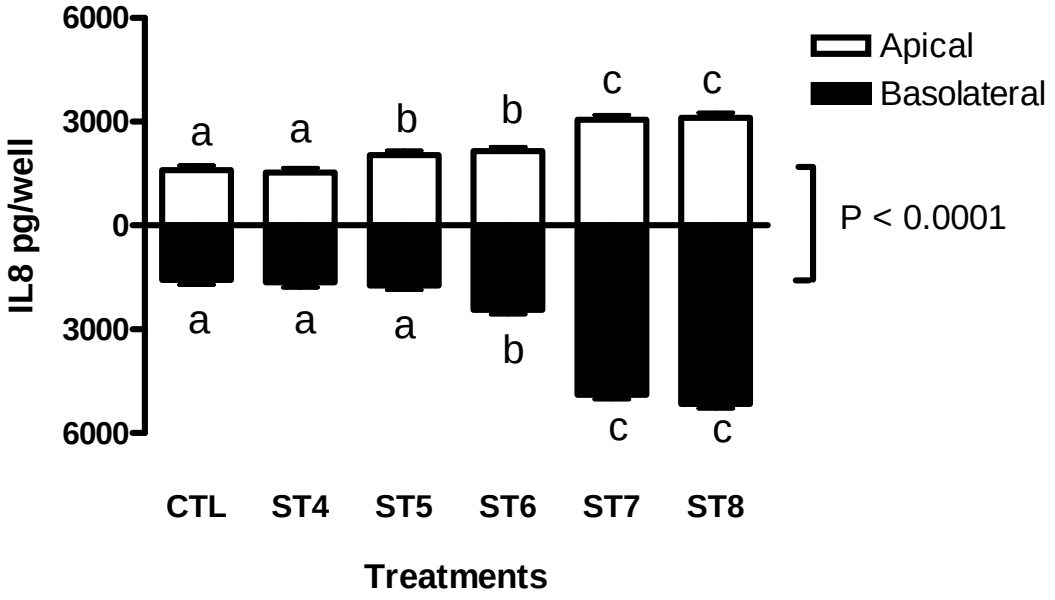


Figure 3.2 Polarized interleukin 8 (IL8) secretion by confluent porcine jejunal epithelial cell (IPEC-J2) monolayers treated with media alone (control; CTL), 10^8 CFU/well *Salmonella enterica* serovar *Typhimurium* (ST), 10^8 CFU/well Choleraesius (SC), 10^8 CFU/well *Lactobacillus reuteri* (LR), or 10^8 CFU/well *Bacillus licheniformis* (BL) in the apical compartment for 1 h. Treatment media were removed and replaced with media containing gentamicin. Media from the apical and basolateral compartments were collected and assayed for cytokines at 1.5, 3.0, and 6.0 h after the onset of bacterial treatment. Each bar represents the least square mean (± SEM) of six observations. Significant accumulation of IL8 was observed at 6 h in wells treated with ST, with greater secretion in the basolateral direction.

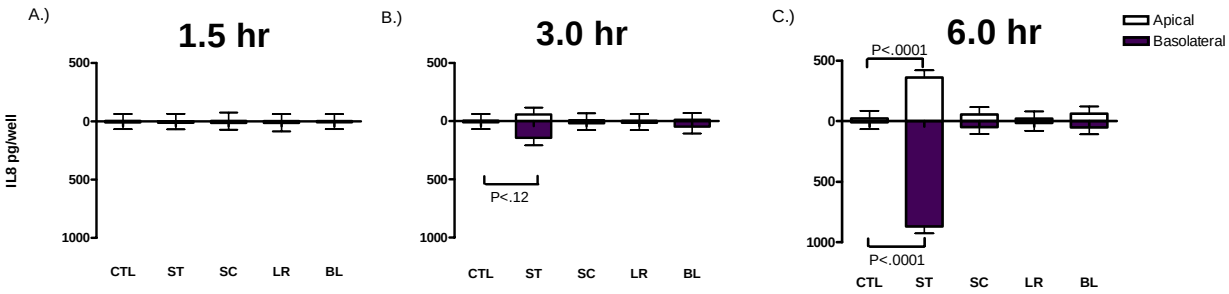


Figure 3.3 Relative abundance of interleukin 8 (IL8; panel A), macrophage inflammatory protein (MIP)3α/CC chemokine ligand-20 (CCL20; panel B) and Tumor necrosis factor-alpha (TNFα; panel C) mRNA from cultured porcine jejunal epithelial cells (IPEC-J2) treated with media alone (control; CTL), 10^8 CFU/well *Salmonella enterica* serovar *Typhimurium* (ST), 10^8 CFU/well Choleraesius (SC), 10^8 CFU/well *Lactobacillus reuteri* (LR), or 10^8 CFU/well *Bacillus licheniformis* (BL) in the apical compartment for 1 h. Total RNA extracted at 1.5, 3.0 and 6.0 h post treatment. Each bar represents the least square mean (± SEM) of three observations. Within time periods, bars without common superscripts differ ($P < 0.05$).

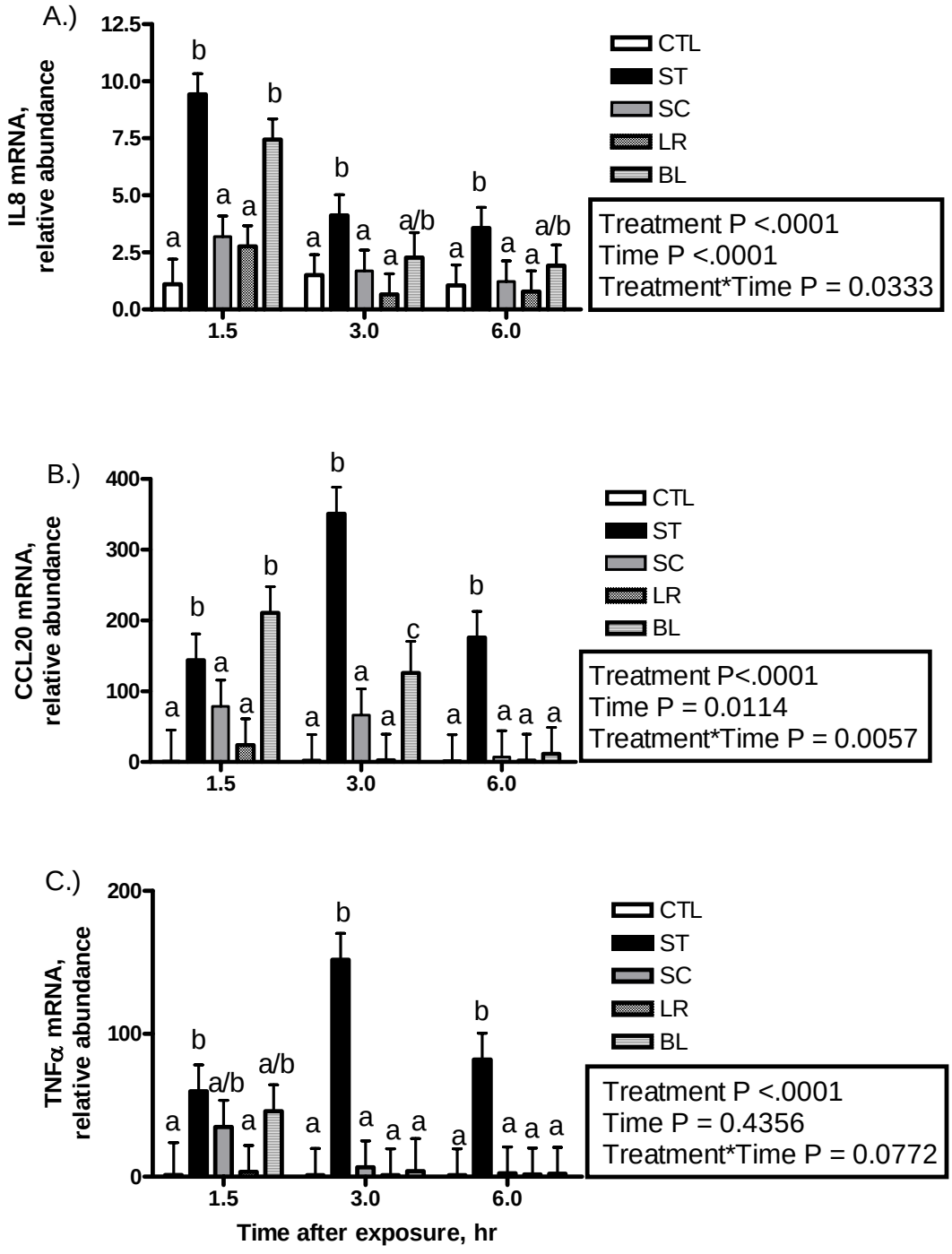
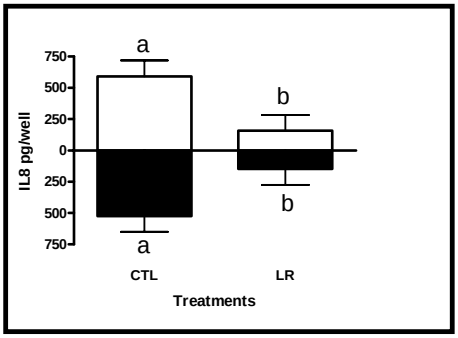

A.)
IL8 mRNA, relative abundance
12.5
10.0
7.5
5.0
2.5
0.0
CTL
ST
SC
LR
BL
Treatment P <.0001
Time P <.0001
Treatment*Time P = 0.0333
1.5
3.0
6.0
B.)
CCL20 mRNA, relative abundance
400
300
200
100
0
Treatment P<.0001
Time P = 0.0114
Treatment*Time P = 0.0057
C.)
TNFα mRNA, relative abundance
200
100
0
Treatment P <.0001
Time P = 0.4356
Treatment*Time P = 0.0772
Time after exposure, hr

Figure 3.4 Polarized interleukin 8 (IL8) secretion by confluent porcine jejunal epithelial cell (IPEC-J2) monolayers treated with media alone (control; CTL), or apical overnight exposure to 1.5 x 10^6, 1.5 x 10^7, or 1.5 x 10^8 CFU/well *Lactobacillus reuteri* followed by apical challenge with 10^8 CFU/well *Salmonella enterica* serovar *Typhimurium* (ST) for 1 h (LR6ST, LR7ST, LR8ST). Treatment with 10^8 LR alone or ST alone served as positive controls (LR and ST, respectively). Treatment media were removed and replaced with media containing gentamicin. Media from the apical and basolateral compartments were collected and assayed for cytokines at 4.0 h after the onset of ST treatment. Each bar represents the least square mean (± SEM) of four observations. Within each secretory direction, bars without common superscripts differ ($P < 0.05$). Significant accumulation of IL8 was observed in wells treated with ST regardless of the presence of LR, with greater secretion in the basolateral direction. Treatment with LR stimulated less IL8 in both the apical and basolateral compartments relative to control (insert).


a
b
b
a
750
500
250
0
250
500
750
IL8 pg/well
CTL
LR
Treatments

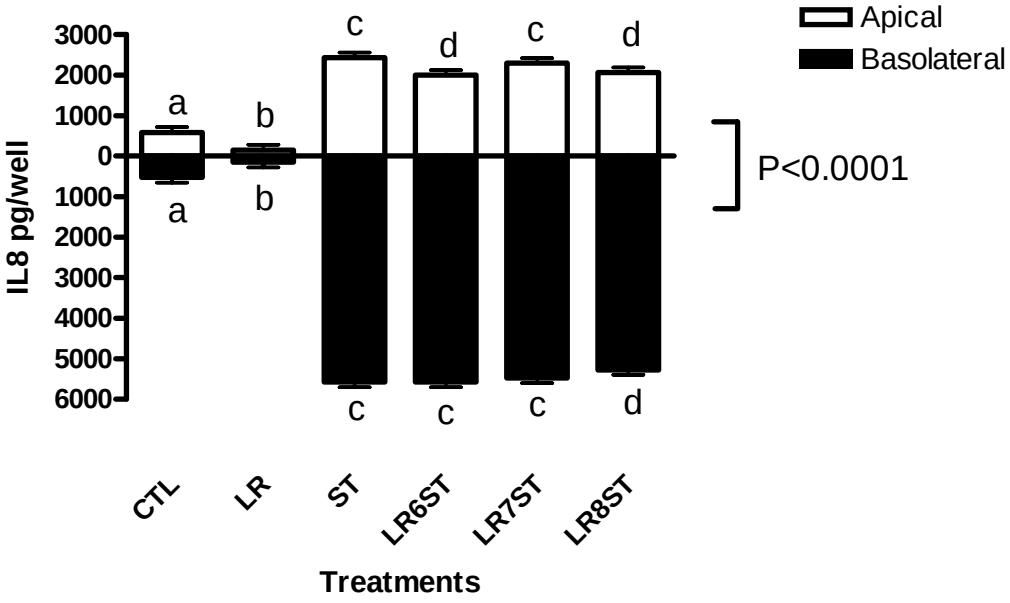
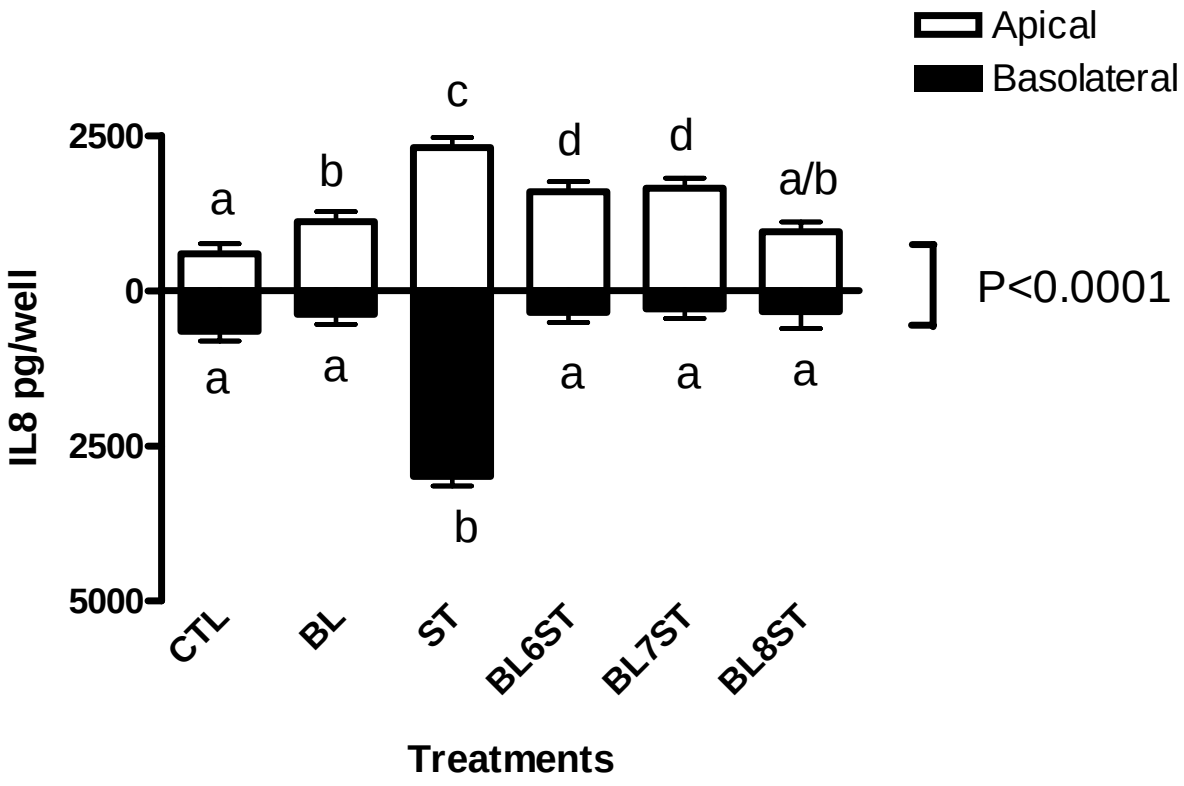

Apical
Basolateral
P<0.0001
IL8 pg/well
3000
2000
1000
0
1000
2000
3000
4000
5000
6000
a
b
c
d
c
d
a
b
c
c
c
d
CTL
LR
ST
LR6ST
LR7ST
LR8ST
Treatments

Figure 3.5 Polarized interleukin 8 (IL8) secretion by confluent porcine jejunal epithelial cell (IPEC-J2) monolayers treated with media alone (control; CTL), or apical overnight exposure to 10^6, 10^7, or 10^8 CFU/well *Bacillus licheniformis* followed by apical challenge with 10^8 CFU/well *Salmonella enterica* serovar *Typhimurium* (ST) for 1 h (BL6ST, BL7ST, BL8ST). Treatment with 10^8 BL alone or ST alone served as positive controls (BL and ST, respectively). Treatment media were removed and replaced with media containing gentamicin. Media from the apical and basolateral compartments were collected and assayed for cytokines at 5.0 h after the onset of ST treatment. Each bar represents the least square mean (± SEM) of three observations. Within each secretory direction, bars without common superscripts differ ($P < 0.05$). Significant accumulation of IL8 was observed in wells treated with ST alone with greater secretion in the basolateral direction, whereas BL treatments significantly increased accumulation only in the apical direction and inhibited ST stimulated secretion in the basolateral direction.



CHAPTER 4 - Characterization of the swine CC chemokine ligand 20 mRNA sequence and evaluation of gene expression in tissues from healthy pigs and pigs exposed to *Salmonella enterica* serovar *Typhimurium* or serovar *Choleraesuis*

ABSTRACT

The gastrointestinal epithelium represents a barrier to potentially invasive enteric pathogens, maintains a role in innate immune surveillance, and is a source of chemokine and cytokine mediators. CC chemokine ligand 20 (CCL20) is responsible for the recruitment of dendritic cells and T cells to assist in gastrointestinal immune defense. In the current study, we characterized the swine CCL20 mRNA sequence and evaluated its expression in various gastrointestinal and immune associated tissues. Cloning of CCL20 from the porcine jejunum predicted a 97 amino acid peptide. An in vivo experiment was conducted to determine tissue CCL20 gene expression comparisons among tissues from healthy pigs, and in tissues from pigs responding to the invasive swine enteropathogens *Salmonella enterica* serovar Typhimurium transformed with green fluorescent protein (STG) or serovar Choleraesuis transformed with red fluorescent protein (SCR). For this study, 24 weaned pigs were fed 10^8 CFU of STG or SCR in cookie dough, or dough without bacteria (control) on days 0, 3, 7 and 10 of the study. Pigs were sacrificed 14 d after the initial challenge and total RNA was extracted from the tonsil, jejunum, ileum, colon, mesenteric lymph node (MLN), spleen, and liver. In uninfected control pigs, substantial variation in CCL20 mRNA was observed ($P < 0.0001$), with greatest abundance in tonsil and colon (3500-fold and 5000-fold relative to spleen, respectively), about 1500-fold in jejunum, ileum, and MLN, and lowest in liver (not different from spleen). Feeding pigs STG for 14 d did not affect CCL20 mRNA abundance in tissues compared to control pigs. Feeding SCR stimulated a modest increase in CCL20 in spleen (approximately threefold increase), and about a 26-fold increase in liver ($P < 0.05$ for both, compared to control pigs), but did not affect CCL20 mRNA in other tissues. These data suggest that tissues of the porcine gastrointestinal tract and

MLN are prominent sources CCL20 in pigs, and that repeated oral exposure of pigs to host-adapted *Salmonella* serovar Choleraesuis, but not serovar Typhimurium, increased CCL20 in liver and spleen, tissues that otherwise have relatively low CCL20 expression.

1. INTRODUCTION

Healthy pigs in production settings maintain a dynamic population of immune cells in the lamina propria with close association to the gastrointestinal epithelium. This population of cells is thought to be influenced by both commensal and pathogenic microbiota resulting in chemotactic signals that recruit and maintain the appropriate immune cell types. The presence of invasive enteric pathogens can provoke increased migration of cells into the lamina propria and subepithelium, and salmonellae organisms are known swine enteric pathogens that have long been associated with neutrophil infiltration into affected mucosal sites (Rothkotter et al., 1999).

Two serovars of Salmonella enterica, serovar Typhimurium (ST) and serovar Choleraesuis (SC) account for the vast majority of salmonellosis in pigs (Schwartz, 1999; Taylor, 1999). The serovars differ in their pathogenicity to pigs with ST producing self-limiting enteritis, and SC, the so-called host adapted serovar, potentially producing systemic disease (Schwartz, 1999; Meyerholz and Stabel, 2003). There is compelling evidence that enterocytes are instrumental in generating chemotactic signals in response to enteric pathogens, including ST (Eckmann et al., 1997). Gastrointestinal enterocytes substantially outnumber microfold cells in the gut epithelium (Eckmann and Kagnoff, 2001), even in Peyer's patches (Gebert et al., 1996), which suggests that enterocytes also serve important roles in immune cell recruitment in response to salmonellae organisms. CC chemokine ligand 20 (CCL20) is one such chemokine that functions as a chemoattractant for CD34+ immature dendritic cells and CD45RO+ memory T cells, expresses major expression in the gastrointestinal tract including the colon, small intestine, and appendix (Kunkel et al., 2003; Kwon et al., 2002; Tanaka et al., 1999) and is increased after stimulation with either TNFα, IL1α, or exposure to *Salmonella* (Izadpanah et al., 2001).

Here we report on the characterization of the mRNA sequence for the putative swine CCL20 gene, also termed macrophage inflammatory protein 3α (MIP3α) (Hieshima et al., 1997). We also evaluated the constitutive distribution of CCL20 in tissues from healthy (control) pigs

obtained from an in vivo experiment designed to determine changes in expression of CCL20 mRNA in tissues of the gastrointestinal tract, mesenteric lymph node (MLN), spleen and liver following a repeated exposure to salmonellae serovars transformed with green (STG) and red (SCR) fluorescent proteins. Our working hypothesis was that the repeated low dose exposure of pigs to invasive bacterial pathogens (designed to model fecal to oral transmission of *Salmonella* in swine production settings) would trigger the upregulation of CCL20, particularly in gastrointestinal tissues.

2. METHODS AND MATERIALS

2.1 Cloning CCL20

Using human CCL20 sequences (GenBank™ accession number: BC020698) a tBlastx algorithm-based search was conducted using the sequence tag (EST) databases for the pig (TIGR Pig Gene Index, The institute for Genomic Research, http://www.tigr.org/tigr-scripts/tgi/T_index.cgi? species=pig). The EST clones (CF179066.1, CB287066.1 and CB287401.1) which contained CCL20 homologous sequences in its translated region were therefore selected for use. To identify porcine CCL20, 5' and 3' rapid amplification of cDNA ends (FirstChoice® RLM-RACE kit, Ambion® Inc., Austin, TX) in conjunction with high throughput clone selection techniques were utilized. Nested gene specific 5' and 3' RACE primers based on the EST clones were as follows: outer sense 5'-AAA ATG ATG TGC AGT AGC AAG AGT T-3', outer antisense 5'-GTC CAT ATA ATC TCA TTC CTC CAA AC-3'; inner sense 5'-CTC CGA TAT ACA GAC CAT ATT CTT CA-3', inner antisense 5'-ATC AGA TGA GCT CAG AAG TCT CAG TA-3'. Total RNA from porcine jejunal samples collected from ST challenged pigs was extracted with TRI® Reagent (Sigma-Aldrich Co., St. Louis, MO) using the manufacturer's protocol. Following total RNA isolation, the DNA-*free*™ (Ambion Inc., Austin, TX) kit was used to ensure removal of contaminating genomic DNA from all RNA samples. Samples were frozen in 50 µl of Nuclease-Free Water (Ambion Inc., Austin, TX). The 5' and 3' RACE products were obtained by PCR amplification using adapter primers provided by the kit along with the CCL20 specific primers described above. The PCR products were then ligated into pCR®4-TOPO® and transformed into One Shot® Mach1™-T1^{r} competent *E. coli* cells (TOPO TA Cloning® Kit for Sequencing, Invitrogen™, Carlsbad, CA). Plasmids were

isolated from the positive clones (QIAprep® Spin Miniprep, Valencia, CA), screened by PCR and sequenced (DNA Sequencing and Genotyping Facility, Dept. of Plant Pathology, Kansas State University, and DNA Sequencing Laboratory, Department of Microbiology and Immunology, University of Arkansas for Medical Sciences) to characterize the cDNA of porcine CCL20.

2.2 Bacterial strains and culture conditions.

ST and SC isolates from swine origin were obtained from Dr. Jerome Nietfeld, Diagnostic Medicine Pathobiology, Kansas State University, and identification of *Salmonella* serotypes was further verified by National Veterinary Services Laboratory, Ames Iowa, USA. SC was transformed with red fluorescent protein (SCR) utilizing the kanamycin resistant pDsRed-Express-1 vector (catalog no. 6994-1, BD Biosciences Clontech, Palo Alto, CA; a lac Z promoter was cloned into this vector upstream of DsRed-Expression coding sequence). ST was transformed with green fluorescent protein (STG) by first modifying the pDsRed-Express-1 vector (BD Biosciences). To modify the pDsRed-Express-1 vector, the DsRed-Express coding sequence was excised and the GFPuv coding sequence from the pGFPuv vector (BD Biosciences Clontech) was inserted into the modified pDsRed-Express-1 vector. Bacteria were transformed as described previously (Sanderson et al., 1995). Both STG and SCR were grown in Luria Bertani medium at 37°C for 24 hr, at which point bacterial populations were estimated by spectrophotometry at 600 nm optical density. Bacteria were then pelleted and resuspended in PBS at a concentration of 10^{10} CFU/ml, 10 μL of the appropriate treatment (STG, SCR, or control) was added to cookie dough balls and fed to each pig (providing 10^{8} CFU/pig). This oral dosage of bacteria was derived empirically based on the lowest range of Salmonella resulting in disease in pigs and the number of *Salmonella* potentially shed from pig feces (Fedorka-Cray et al., 2000).

2.3 Animal challenge protocol and tissue collection

This experimental protocol was approved by the Kansas State University Institutional Animal Care and Use Committee before initiation of the study. Twenty four crossbred pigs, typical of commercial pigs and approximately 5 wk of age, were used and showed no clinical signs or laboratory evidence of salmonellosis or any other enteric diseases. Pigs were penned as

groups of two in an environmentally controlled isolation facility at 25°C and under constant light with *ad libitum* access to feed and water. The diet was typical of commercial nursery diets, but was free of added antibiotics and growth promoting levels of copper and zinc. After an acclimation period of 7 d, pigs were fed 10^8 CFU STG (n=8) or STR (n=8) on days 0, 3, 7, and 10, while the control group (n = 8) received dough without added bacteria. The use of transformed bacteria was chosen to assist in the isolation of the respective pathogens after animal passage, which was utilized for the repeated challenges at d 3, 7, and 10. Samples of tonsil, jejunum, ileum, colon, MLN, spleen, and liver, were removed aseptically from weaned pigs at sacrifice 14 d after initial salmonella challenge, rapidly frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation. In addition to the evaluation of CCL20 as reported here, we also evaluated selected TLRs and proinflammatory cytokines and (or) chemokines gene expression profiles (Burkey et. al., 2006; submitted), as well as growth performance and selected immune responses in vivo (Fraser et al., 2006; submitted) from pigs in this study.

2.4 RNA extraction and reverse transcription (RT) PCR

Total RNA was extracted using TRI® Reagent (Sigma-Aldrich Co., St. Louis, MO) RNA isolation reagent as per the manufacturer's protocol. Following total RNA isolation, the DNA-*free*™ (Ambion Inc., Austin, TX) kit was used to ensure removal of contaminating genomic DNA from all RNA samples. Samples were frozen in 50 µl of nuclease-free water (Ambion Inc., Austin, TX). RNA was quantified by spectrophotometry using an optical density of 260 nm. RNA quality was verified by agarose gel electrophoresis and visualization of the 28S and 18S ribosomal RNA. Reverse transcription was carried out using TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA). Briefly, reverse transcription was carried out in a 50 µl final volume that included 25 mM $MgCl_2$, 500 µM dNTP's, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, 50 U/µL MultiScribe reverse transcriptase, and TaqMan RT buffer. The reverse transcription mixture was incubated at 25°C for 10 min, heated to 37°C for 60 min, and inactivated at 95°C for 5 min. The resultant cDNA was stored (-20°C) until used.

2.5 Real time PCR analysis for gene expression

Real-time quantitative PCR was utilized to quantify CCL20 relative to the quantity of 18S rRNA in total RNA isolated from porcine tissue samples. The PCR reactions were carried

out in 96-well plates with the appropriate forward and reverse primers (900nM), the appropriate TaqMan TAMRA probe (200 nM), PCR Mastermix (Applied Biosystems, Foster City, CA), and 3.5 μL of the cDNA sample. The porcine specific CCL20 primers and detection probes were synthesized from published GenBank sequences using Primer Express® software (Applied Biosystems, FosterCity, CA); forward primer, 5'-3': GCTCCTGGCTGCTTTGATG, reverse primer 5'-3': CAGTCAAAG TTGCTTGCTTCTGA, and probe 5'-3': CGGTGCTGCTCTACCTCTGCA. The CCL20 probe was synthesized with 6-FAM as the 5' fluorophore and TAMRA as the 3' quencher dye. Commercially available eukaryotic 18S rRNA (Applied Biosystems, Foster City, CA) primers and probe were used as an endogenous control. Assays using non-template controls and samples were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Thermal cycling parameters were utilized according to manufacturer recommendations and included 50 cycles of 15 s at 95°C and 1 min at 60°C. Relative expression of mRNA was normalized with the 18S rRNA endogenous control.

2.6 Statistical analyses

To determine basal levels of expression as well as differences in the relative abundance of CCL20 mRNA among the tissues collected from control animals, the $\Delta\Delta C_T$ method was used with the average spleen ΔC_T for CCL20 set as the reference expression (n = tissue from eight pigs). The $\Delta\Delta C_T$ values were expressed as $2^{-\Delta\Delta CT}$ to obtain relative abundance. The relative abundance values were then square root transformed to ensure homogeneity of variance and analyzed by the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC), with tissue in the model. To facilitate depiction of the data, relative abundance values were back transformed and illustrated in Figure 3.

Differences in relative abundance of CCL20 mRNA in each of the individual tissue types as a result of STG and SCR feeding were also determined using the $\Delta\Delta C_T$ method with the average control ΔC_T as the reference expression (n = tissue from eight control pigs per treatment). The $\Delta\Delta C_T$ values were expressed as $2^{-\Delta\Delta CT}$ to obtain relative abundance values, which were then square root transformed to ensure homogeneity of variance, and analyzed using the PROC MIXED procedure of SAS with treatment in the model. To facilitate depiction of the data, relative abundance values were back transformed and illustrated in Figure 4.

3. RESULTS

3.1 Characterization of cDNA encoding CCL20

The cDNA sequence obtained from RLM RACE generated a 564 nucleotide sequence (Figure 1), which translated into a 97 amino acid protein (The Sequence Manipulation Suite; http://www.ualberta.ca/~stothard/javascript/reference.html (Stothard, 2000)) confirmed to be porcine CCL20 (NCBI Blastx 2.2.12; http://www.ncbi.nlm.nih.gov/BLAST/). Results of multiple protein sequence alignment as determined by CLUSTAL W (ClustalW WWW Service at the European Bioinformatics Institute, http://www.ebi.ac.uk/clustalw, Rodrigo Lopez, Services Program; Figure 2A) with PubMed porcine (CAE11786), bovine (NP_776688), canine (BAD42361), and human (NP_004582) sequences indicating that our CCL20 had the highest sequence score (98) with the already published pig sequence (Figure 2B), followed by the bovine (82) and canine (82) sequences, and was least similar to the human sequence (76).

3.2 CCL20 mRNA distribution in healthy porcine tissues

Tissue samples, including the tonsil, jejunum, ileum, colon, MLN, spleen, and liver were collected from control animals (from the STG and SCR feeding experiment) to evaluate the relative expression of CCL20 mRNA in apparently healthy pigs (Figure 3). The lowest CCL20 expression was in the spleen and liver, and spleen was chosen as the reference tissue for computation of $\Delta\Delta C_T$. Relative abundance of CCL20 mRNA was greatest in colon and tonsil (approximately 3500-fold and 5000-fold greater respectively; $P < 0.0001$ for both, relative to spleen), followed by an approximately 1500-fold greater abundance in the jejunum, ileum, and MLN ($P < 0.0001$ for all, relative to the spleen).

3.3 CCL20 mRNA distribution in tissues from pigs STG or SCR

This experiment was designed to evaluate the effect of repeated low dose oral STG or SCR challenge on CCL20 mRNA expression in the above mentioned tissues (Figure 4). The expression of CCL20 was unaffected by either STG or SCR in the tonsil, jejunum, ileum, colon, and MLN (Figure 4: A, B, C, D, and E). However, pigs fed SCR had increased abundance of CCL20 in the spleen (approximately three-fold increase above controls; Figure 4F, $P = 0.059$) and the liver (approximately 26 fold increase above controls; Figure 4G, $P = 0.087$).

4. DISCUSSION

The chemokine CCL20, is also known as macrophage inflammatory protein 3α (MIP3α) (Hieshima et al., 1997), liver and activation regulated chemokine (LARC) (Hieshima et al., 1997), and Exudos-1 (Hromas et al., 1997), and is expressed abundantly in the gastrointestinal tract, including the colon, small intestine, and appendix. The expression of CCL20 in gastrointestinal cell lines seems to vary with the cell type and the type of stimuli. Several human intestinal cell lines express CCL20 mRNA and produce the secreted CCL20 protein; both of which are increased after TNFα, IL1α, or exposure to *Salmonella* or *Escherichia coli* (Izadpanah et al., 2001). However, differences between cell lines has also been noted, in that several have constitutive expression (e.g. HT-29, Caco-2, LS174T, and I-407), while others demonstrate regulated (HT29 and Caco-2) expression of CCL20 mRNA (Izadpanah et al., 2001). The swine intestinal epithelial line IPEC-J2 constitutively expresses CCL20 mRNA which is upregulated substantially upon exposure to both SC and ST (Skjolaas et al., 2006). In addition, IPEC-J2 cells demonstrated an upregulation of CCL20 mRNA upon exposure to the transformed bacteria utilized in this study, confirming pathogenicity of these enteropathogens after transformation (Burkey et al., 2006).

The 397 bp Sus scrofa putative CCL20 chemokine mRNA sequence as determined from the lymph node has been reported in PUBMED (http://www.ncbi.nlm.nih.gov/entrez /query.fcgi?CMD=search&DB=pubmed; accession: AJ577084, author: Ezquerra,A.). The mRNA sequence for CCL20 has also been sequenced for *Homo sapiens* NM_004591, *Bos taurus* NM_174263, *Canis familiaris* NM_001005254, *Rattus norvegicus* NM_019233, and *Mus musculus* NM_016960. We characterized and further evaluated the porcine CCL20 mRNA sequence and gene distribution in swine gastrointestinal and lymphoid tissues to confirm its sequence and to further describe its distribution and associated function in healthy young pigs as well as in pigs challenged with two common and economically important swine enteropathogens. The sequence we obtained from cloning via RLM RACE complement those from the pig reported previously (PUBMED accession AJ577084).

The in vivo expression of CCL20 expression has been described for other species, but limited information is available in the domestic pig. The normal human colon demonstrates minimal constitutive CCL20 mRNA expression, whereas inflammation induced major upregulation of its expression (Izadpanah et al., 2001). The ileum from healthy pigs also

constitutively expressed CCL20 mRNA and was increased by approximately 24 h post oral ST challenge (Skjolaas et al., 2006). In the mouse model, variable expression has been observed in the gastrointestinal tract, depending mainly on the mouse strain, tissue type, and (or) the inflammatory stimulus used. For example, BALB/c mice have constitutive CCL20 expression in the colon and appendix (Tanaka et al., 1999). Yet, the small intestine of BALB/c mice failed to demonstrate a strong constitutive expression which is in contrast to the C57BL/6 mice strain (Tanaka et al., 1999) . The expression of CCL20 is also altered upon stimulation with LPS, whereby BALB/c mice exhibited a reduced expression of CCL20 in the colon and appendix, and a strong increase in mRNA expression in the small intestine (Tanaka et al., 1999).

Swine gastrointestinal tissues and MLN collected in this study (including the jejunum, ileum, and colon) had constitutive expression of CCL20 mRNA, but we did not see an increase or decrease in CCL20 mRNA expression as a result of STG or SCR exposure in any of the gastrointestinal tissues evaluated. As noted above, pigs exposed to a single oral gavage of 10^{10} ST experienced rapid upregulation of CCL20 which occurred 24 h after challenge, with a return to basal mRNA expression by 48 h after exposure (Skjolaas et al., 2006). It is possible; therefore, that CCL20 may have been increased acutely by STG, and perhaps SCR, in gastrointestinal tissue, but waned with repeated exposure to bacteria over 14 d.

Aside from the gastrointestinal tract, the expression of CCL20 has been documented in normal human liver, lung, appendix, tonsil, lymph nodes, peripheral blood leukocytes, thymus, fetal liver, fetal lung, small intestine, colon epithelial cells, stomach, bladder, pancreas, testis, cervix epithelial cells, placenta, and skin (Schutyser et al., 2003). Our results demonstrate that aside from the aforementioned swine jejunum, ileum, colon, and MLN, the tonsil, spleen, and liver also constitutively express CCL20 mRNA, although the pig liver and spleen express the chemokine at relatively low levels.

In the current study, only SCR altered CCL20 gene expression where an increase was observed in liver and spleen. That STG failed to similarly affect CCL20 in liver and spleen may be explained by reported differences in patterns of mucosal invasion between the serovars. The so-called host adapted serovar SC is more likely to aggressively invade disparate mucosal sites and establish systemic disease than ST (Meyerholz and Stabel, 2003; Schierack et al., 2005). Moreover, of particular interest is the observation that SCR induced upregulation in tissues that otherwise demonstrated the lowest expression in healthy animals. In a separate report evaluating

growth performance of these same animals, pigs fed SCR had substantially reduced growth rate compared to pigs fed STG (Fraser et al., 2005). It is tempting to speculate that the invasive nature of SC may upregulate CCL20 and other immune mediators as activated immune cells traffic to affected tissues. Such activation may represent a substantial nutrient drain that contributes to slowed growth.

In conclusion, we have characterized the constitutive expression of swine CCL20 in various gastrointestinal and lymphoid tissues of healthy young pigs. We have also shown that repeated exposure of two *Salmonella* serovars in the pig gastrointestinal tract triggered alterations in CCL20 expression levels varying with serovar and (or) tissue type. Relative to the *Salmonella* spp. challenge, STG exposure triggered a lesser response among the tissues evaluated. These differences may partially underlie the differences between the two *Salmonella* serovars in eliciting distinct disease pathogenesis in swine.

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Figure 4.1 Putative porcine CCL20 mRNA sequence, translated by The Sequence Manipulation Suite, red indicates amino acid sequence.

```
           M  M  C  S  S  K  S  L  L  L  A  A  L  M  S  V  L
CGCCCTTAAAATGATGTGCAGTAGCAAGAGTTTGCTCCTGGCTGCTTTGATGTCGGTGCT

 L  L  Y  L  C  S  K  S  E  A  A  S  N  F  D  C  C  L  R  Y
GCTGCTCTACCTCTGCAGCAAGTCAGAAGCAGCAAGCAACTTTGACTGCTGCCTCCGATA

 T  D  H  I  L  H  P  R  F  I  M  G  F  T  Q  Q  L  A  N  E
TACAGACCATATTCTTCACCCCAGATTTATCATGGGCTTCACACAGCAGCTCGCCAATGA

 A  C  D  I  N  A  I  I  F  Y  T  K  K  K  L  A  V  C  A  D
AGCTTGTGACATCAATGCAATCATCTTTTACACAAAGAAAAAGTTAGCCGTGTGTGCAGA

 P  K  K  I  W  V  K  Q  A  V  N  I  L  S  Q  R  V  K  K  M
TCCAAAGAAGATCTGGGTGAAACAAGCCGTGAATATCCTCAGTCAAAGAGTCAAGAAGAT
  *
GTAAAAACAGATGCTCCCTGCTATGGAATTGGACACAGCCCAAGAACAAAAAGAACCTCG

CTGTGCTGGTGGTTTCACTTACACATTACTGAGACTTCTGAGCTCATCTGATCTGTGCCT

CTTTGGACTTGTCCAATTAATGAAGTTGATTCATATTGCATCATAGTTCGCTTTGTTAAG

CATCATGTTATACTCTATTTTATGTTATGTTATTTATATGTGTAGTTTTCTTGTGTGCCT

TGCTATTTAATACTAGTTTTCTACAAGGCCATT
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Figure 4.2 Putative porcine CCL20 protein sequence alignments (A) and comparisons (B) of CCL20 protein sequences for KSU_pig, to pig (pubmed: CAE11786), human (Pubmed: NP_004582), Bovine (Pubmed: NP_776688), and canine (Pubmed: BAD42361). According to ClustalW, "*" means the residues or nucleotides in that column are identical in all sequences in the alignment, ":" means that conserved substitutions have been observed, and "." means that semi-conserved substitutions are observed.

A)

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KSU_PIG         MMCSSKSLLLAALMS-VLLLYLCSKSEAASNFDCCLRYTDHILHPRFIMGFTQQLANEAC 59
pig             -MCSSKSLLLAALMS-VLLLYLCSKSEAASNFDCCLRYTDHILHPRFIMGFTQQLANEAC 58
bovine          MMCSSKNLLLAALMS-VLLLHFCSKSE-ASNFDCCLRYTERILHPSILVGFTQQLANEAC 58
canine          -MCSSKNLLLAALMMSVLLLHLCSKSEAASNFDCCLRYTEHMVPPGSIMGFTQQLANEAC 59
human           -MCCTKSLLLAALMS-VLLLHLCGESEAASNFDCCLGYTDRILHPKFIVGFTRQLANEGC 58
                 **.:*.*******  ****::*.:**:******** **:::: *  ::***:*****.*

KSU_PIG         DINAIIFYTKKKLAVCADPKKIWVKQAVNILSQRVKKM 97
pig             DINAIIFYTKKKLAVCADPQKIWVKQAVNILSQRVKKM 96
bovine          DINAVVFYTRKKLAVCADPKKKWVKQVVHMLSQRVKRM 96
canine          DINAVIFYTKKKKAVCADPKKKWVKRTVRLLSHRVKKM 97
human           DINAIIFHTKKKLSVCANPKQTWVKYIVRLLSKKVKNM 96
                ****::*:*:** :***:*:: ***  *.:**::**.*
```

(B)

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	KSU_PIG	97	2	pig	96	98
1	KSU_PIG	97	3	human	96	76
1	KSU_PIG	97	4	bovine	96	82
1	KSU_PIG	97	5	canine	97	82

Figure 4.3 Relative abundance of macrophage inflammatory protein (MIP) 3α/CC chemokine ligand-20 mRNA from healthy porcine tonsil, jejunum, ileum, colon, MLN, spleen, and liver. Each bar represents the least square mean (± SEM) of eight observations, and bars without common superscripts denote significant differences ($P < 0.05$).

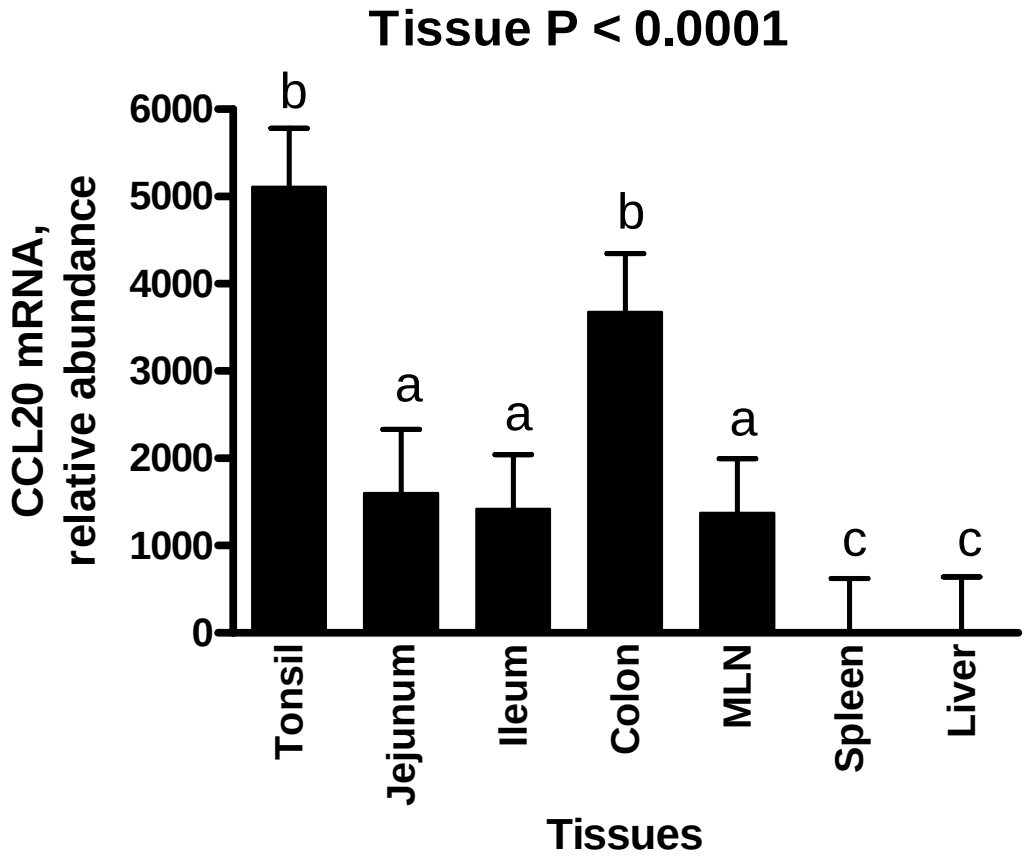
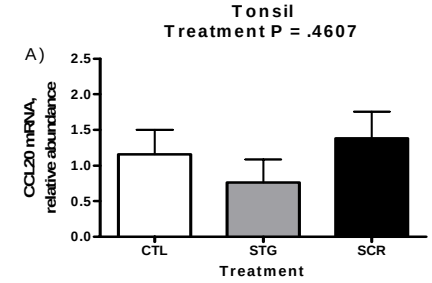
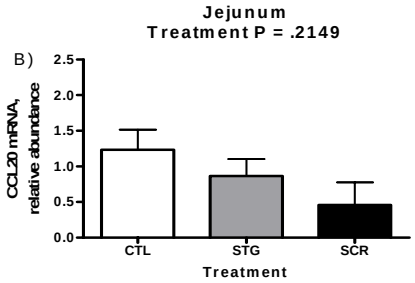
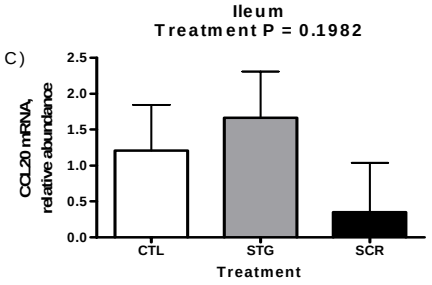
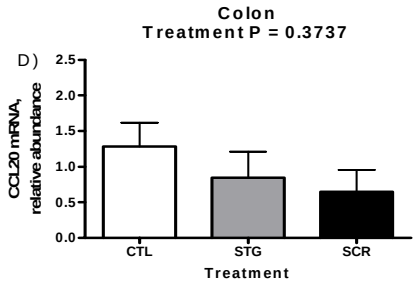


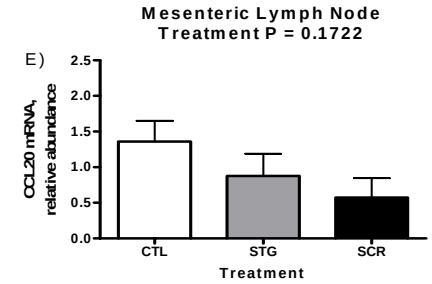
Figure 4.4 Relative abundance of macrophage inflammatory protein (MIP)3α/CC chemokine ligand-20 (CCL20) mRNA from porcine tonsil, jejunum, ileum, colon, MLN, spleen, and liver after repeated oral challenge (days 0, 3, 7, 10) with 10^8 CFU of *Salmonella enterica* serovar Choleraesuis (SCR), serovar Typhimurium (STG), or sterile media (CTL). Total RNA extracted at 14 d post initial challenge. Each treatment bar represents the least square mean (± SEM) of eight observations. Bars without common superscripts denote significant differences ($P < 0.05$).

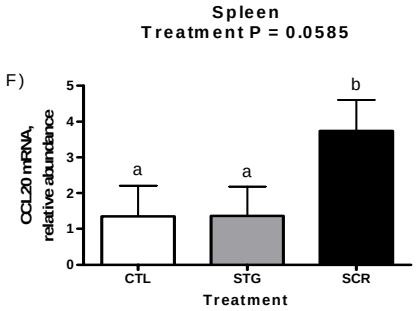

Tonsil
Treatment P = .4607
A)
CCL20 mRNA, relative abundance
2.5
2.0
1.5
1.0
0.5
0.0
CTL
STG
SCR
Treatment

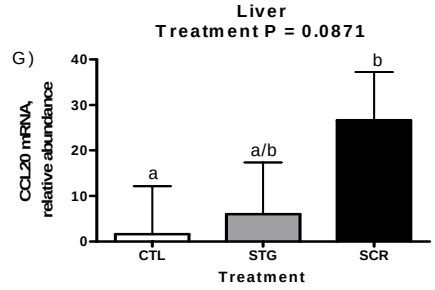

Jejunum
Treatment P = .2149
B)
CCL20 mRNA, relative abundance
2.5
2.0
1.5
1.0
0.5
0.0
CTL
STG
SCR
Treatment


Ileum
Treatment P = 0.1982
C)
CCL20 mRNA, relative abundance
2.5
2.0
1.5
1.0
0.5
0.0
CTL
STG
SCR
Treatment


Colon
Treatment P = 0.3737
D)
CCL20 mRNA, relative abundance
2.5
2.0
1.5
1.0
0.5
0.0
CTL
STG
SCR
Treatment


Mesenteric Lymph Node
Treatment P = 0.1722
E)
CCL20 mRNA, relative abundance
2.5
2.0
1.5
1.0
0.5
0.0
CTL
STG
SCR
Treatment


Spleen
Treatment P = 0.0585
F)
CCL20 mRNA, relative abundance
5
4
3
2
1
0
a
a
b
CTL
STG
SCR
Treatment


Liver
Treatment P = 0.0871
G)
CCL20 mRNA, relative abundance
40
30
20
10
0
a
a/b
b
CTL
STG
SCR
Treatment

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