Effects of Prebiotic fibre Diets on Rat Mucosal Intestinal and Systemic Immunity and *in Vitro* Mechanistic Analysis of Anti-Inflammatory Effects of *Lactobacillus* Strains on Rat and Human Intestinal Epithelial Cells

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ABSTRACT

Probiotics and prebiotics are emerging household terms, whose claimed health benefits share commonality. Their attributed health benefits include the production or induction of short chain fatty acids, maintaining bowel function, building colonization resistance (against pathogens) and treating antibiotic-associated diarrhea as well as colitis. Although both probiotic and prebiotic effects on immune system have been studied, the mechanisms of their activity are still not clearly defined and the conclusions drawn are elusive. While probiotics can act to influence the host at the cellular level, prebiotics, by definition, exert their effects indirectly through their impact on gut microbes. One purpose of this study was to investigate effects of *Lactobacillus rhamnosus* R0011 on innate immune parameters at the intestinal epithelial cell level, examining effects on both human and rat IEC. A second purpose was to define the effects of a range of prebiotic dietary fibres on the immune system at the mucosal and systemic level, using Biobreeding rats.

L. rhamnosus demonstrated the ability to decrease proinflammatory cytokine and Toll-like receptor agonist-induced IL-8 and CINC-1 production from human and rat IEC, respectively. The timing of *L. rhamnosus* R0011 addition to HT-29 IEC, relative to proinflammatory challenge, influenced its ability to decrease IL-8 production. *L. rhamnosus* was more effective at decreasing production of IL-8 from human IEC when they were pre-incubated with this bacterium and subsequently challenged with proinflammatory stimuli. Certain effects of *L. rhamnosus* R011 were also observed in the absence of proinflammatory stimuli. Viable *L. rhamnosus* induced TNF-α production from rat IEC and heat-killed *L. rhamnosus* decreased constitutive TGF-β production from rat IEC and induced IL-8 or CINC-1 production from human and rat IEC, respectively.

In Biobreeding rats, we demonstrated that oat dietary fibre significantly alters active TGF-β, CINC-1 and IL-6 levels in the colon in comparison to AIN-93G-fed rats. Wheat dietary fibre induced changes in active TGF-β, CINC-1 and IL-4 levels in the ileum in comparison to resistant starch-fed rats. Lastly, resistant starch exerted effects in the mesenteric lymph node, where changes in active TGF-β were observed in rats in comparison to AIN-93G-fed rats. Oat bran, wheat bran and resistant starch had no effects on cytokine levels in the serum or spleen of rats. Fructooligosaccharide-fed rats had a significant increase in active TGF-β levels in the colon and a significant decrease in active TGF-β levels in the spleen. Overall this suggests a FOS supplemented diet has both mucosal and systemic effects in rats, while wheat, oat and resistant starch supplemented diets had effects focused at the different locations at the mucosal level.

These results illustrate differences in the ability of different dietary fibres to target immune parameters in specific mucosal tissues along the gastrointestinal tract and differential ability to exert systemic effects. Understanding the mechanism of action of probiotics provides insight into the downstream effects of prebiotics, while investigating effects of prebiotics on the immune system provides a broader view of the outcome of changes in gut microbiota composition and activity at the host organism level.

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wheat fibre diets ($p<0.05$). There was no significant difference in IL-4 and IL-10 levels between rats fed different diets. There was a significant difference in active TGF-β levels between rats fed 5% oat fibre and rats fed AIN-93G diets ($p<0.05$). Bars with different letters are significantly different from each other (as determined by ANOVA and Bonferroni post-hoc test).

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LIST OF ABBREVIATIONS

ANOVA- Analysis of variance

APRIL- A proliferation-inducing ligand

BAFF- B cell-activating factor

BLQ- Below level of quantification

BB- Biobreeding rats

CD- Cluster of differentiation

CINC-1- Cytokine-induced neutrophil chemoattractant

Con-A- Concanavalin-A

COX- Cyclooxygenase

DC- Dendritic cell

DGGE- Degrading gradient gel electrophoresis

DMEM- Dulbecco's Modified Eagle Medium

DSS- Dextran sodium sulphate

EAE- autoimmune encephalomyelitis

ELISA- Enzyme linked immunosorbant assay

ETEC- Enterotoxigenic *E. coli*

FBS- Fetal bovine serum

FOS- Frucctooligosaccharides

GABA- Gamma-aminobutyric acid

GALTs- Gut-associated lymphoid tissue

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

GI- Gastrointestinal

GPR43- G-protein receptor 43

HLA- Human leukocyte antigen

Hsp- Heat shock protein

HT-29- Human colon adenocarcinoma cells

IBD- Inflammatory bowel disease

IEC- Intestinal epithelial cell

Ig- Immunoglobulin

IL-#- Interleukin

IL-1ra: Interleukin 1 receptor antagonist

INF- Interferon

LPS- Lipopolysaccaride

LTA- Lipoteichoic acid

MALT- Mucosa-associated lymphoid tissue

MAMPs- Microbe-associated molecular pattern

*meso*DAP - *Meso*-diaminopimelic acid

MLN- Mesenteric lymph node

MRS- de Man, Rogosa and Sharpe

MYD88- Myeloid differentiation primary response gene (88)

NF-κB- Nuclear factor kappa b

NLR- Nucleotide-binding oligomerization domain-like receptor

NOD-#- Nucleotide-binding, oligomerization domain-containing protein

PAMP- Pathogen-associated molecular patterns

PBS- Phosphate buffered saline

PP- Peyer's patch

PPAR-γ- Peroxisome proliferator-activated receptor gamma

PRR- Pattern recognition receptor

R&D- Research and Development

SAA- Serum amyloid A

Sb- *Saccharomyces boulardii*

SCFA- Short chain fatty acid

SE- Standard Error

T_H# - T helper cell

T_{Reg}- T regulatory cell

TGF- Transforming growth factor

TIRAP- Toll-interleukin 1 receptor containing adaptor protein

TLR- Toll-like receptor

TNF- Tumor necrosis factor

TRAF- TNF receptor associated factors

WHO- World Health Organization

Wt- Wild Type

INTRODUCTION

The Gut Microbiota

The gut microbiota is a complex community of thousands of organisms harbored within the human intestinal tract. There are about 500-1000 species of bacteria within the gut microbiota, contributing to a total population of about 10^{13} bacterial cells, greater than the number of cells comprising the human body. The most common species cultured from the human GI tract include *Escherichia coli*, *Klebsiella*, *Streptococcus*, *Lactobacillus, Staphylococcus*, *Bacillus, Bifidobacterium, Fusobacterium, Clostridium, Eubacterium, Peptococcus* and *Peptostreptoccus* [1]. Both anaerobes and aerobes are present within the complex gut niche, but anaerobes greatly outnumber the amount of aerobes by about 100-1000 fold [2].While there are currently varied theories about and much research into how these bacteria interact with the host, the mechanisms behind the introduction and colonization of the gut by bacteria during development are not currently fully understood.

The relationship between the host organism and the gut microbiota is a mutualistic and symbiotic one, suggesting that both the microbiota and the intestinal epithelial cells (IEC) that come in contact with these bacteria co-evolved [3]. The microbes present benefit from a constant supply of nutrients, while the host benefits from the bacteria's ability to degrade and metabolize large macromolecules into products that their body can absorb and metabolize [4] .The fermentation accomplished by bacteria produces vital nutrients that are absorbed by the intestinal epithelial cells (IEC), including vitamins and short chain fatty acids (SCFA)[5]. If these nutrients were not present then cell death would likely occur in the intestinal lining, impairing nutrient absorption and leading to malnutrition. Bacterial communities within the gastrointestinal (GI) tract of humans are reported to vary due to gender, amount of siblings, or whether a person has

been delivered via C-section [6]. This suggests that there are a large variety of influences on the human GI microbiota, including environmental and hormonal factors. The gut microbiota has large influences in maintaining the balance of health and disease within the gastrointestinal tract, as well as contributing to the development of the immune system.

The gut microbiota not only contributes to nutritional value of ingested foods, but also plays a large role in innate and adaptive immunity at mucosal surfaces, such as the intestine. The immune system has evolved in a way that allows these commensal bacteria to live in the intestine, yet the immune system can still recognize and eliminate pathogens [3].

Probiotics and Prebiotics

Probiotics are bacteria that promote host health by interacting with the intestinal mucosal barrier potentially influencing the growth and development of other intestinal microbiota as well as contributing to the homeostasis of mucosal immunity [6]. Prebiotics, however, are non-digestible food stuffs that are fermented by bacteria, producing a wide variety of products that may promote health [7]. Prior evidence indicates that probiotics and prebiotics can increase resistance to infection and improve immune responses and immunoregulation. One way in which probiotics may create a positive influence on health is through interaction with the IEC of the GI tract [6]. Probiotics also have other routes of interaction in the intestine, including contact with M cells; Peyers patches (PP) and the mesenteric lymph nodes (MLN). Through fermentation of food products, probiotics can be cultivated and consumed by humans providing an alternate route of probiotic delivery than the capsule form, in which they are often delivered.

Probiotic properties have been observed in *Lactobacillus* and *Bifidobacterium* species, which are the most common genera used in fermentation of food stuffs. Consumption of

probiotics has been observed to increase the barrier effect in the intestine as well as decrease intestinal inflammation and increase immunoregulatory activity within mucosal associated lymphoid tissues (MALT). *Lactobacillus rhamnosus* GG in particular is an excellent candidate for probiotic culture because it contains many qualities needed in order to be implemented into the mucosal microbiota; it is found naturally in humans, it has the ability to attach to human IEC and colonize within the intestine and it has resistance to acid and bile (which enables it to reach the intestine with high viability). When ingested and incorporated into the intestinal microbiota, *L. rhamnosus* GG has been shown to improve the balance of bacteria within the intestine to those that are often considered to be health promoting, and produce SCFAs [1]. Due to these properties *L. rhamnosus* GG is now one of the most studied probiotic bacterial strains.

In contrast to probiotics, prebiotics are food ingredients, such as dietary fibres, that are non-digestible and promote the growth of specific bacteria within the intestinal tract that confer health benefits in the host [7]. Prebiotics are sometimes used in medicine as a mean of providing SCFA, maintaining bowel function, building colonization resistance (against pathogens) and treating antibiotic-associated diarrhea as well as inflammatory bowel disease (colitis) [8]. The target bacteria for most prebiotic supplements are *Lactobacillus* and *Bifidobacterium*, the genera that have shown the greatest probiotic actions. The end products of fermentation ultimately provide the host with beneficial nutrients, potentially increasing overall gut health [8]. Most prebiotics are dietary fibres consisting of non-starch polysaccharides and lignin [9]. Dietary fibre has been linked to an increase in human health, although, the reasoning is not yet well understood. What is understood is that when the dietary fibre enters the intestine, it is fermented by the gut microbiota creating an array of different SCFAs [10].

SCFAs have many physiological effects when absorbed by the intestinal and colonic epithelial cells. These include, but are not limited to, increased salt and water uptake, increased mineral solubility and a decrease in luminal pH, which has been attributed with inhibiting the growth of harmful bacteria. The most common SCFAs are acetic, propionic and butyric acids. Butyric acid has been reported to prevent ulcerative colitis, a form of inflammatory bowel disease (IBD) in rat models [11] and humans [12] and compiled research suggests butyrate can prevent colonic tumors at early stages [13]. Experiments done in gnotobiotic mice (mice that have no gut microbiota) with DSS(dextran sulphate)-induced colitis models have demonstrated that supplementation with acetate lowers the symptoms of IBD by lowering levels of the colonic myeloperoxidase (MPO) and proinflammatory cytokines MIP1α and TNF-α [14]. But most importantly, SCFAs can also play a role in the modulation of the mucosal immune system. SCFA interact with the IEC by binding to G-protein coupled receptor 43 (GPR43), a critical receptor in the innate immune system [15]. There is also evidence that SCFAs interact with neutrophils, suggesting that SCFA may act through more than one route to influence actions of cells in the immune system. SCFA administration and uptake by IEC have demonstrated the ability to relieve certain symptoms of inflammatory bowel disease. Knockout mice, devoid of the gene encoding Gpr43 demonstrate increased inflammation in induced models of colitis, arthritis and asthma, providing evidence of the major role SCFA play in mucosal immunity [14]. Butyrate has also been reported to inhibit NF-κB activation and promote IκBα degradation at the IEC levels, preventing induction of proinflammatory activity [16].

SCFA production is dependent upon the dietary fibres consumed and the bacteria present within the gut microbiota. Two large groups of prebiotics are currently under investigation for their influence on gut microbiota and physiological changes in the host, including the alteration

of immune responses. These two types of prebiotics include fructans and resistant starch. Fructans are naturally occurring oligosaccharides and fructooligosaccharides found in foods such as onions, bananas, wheat, oat, artichokes and garlic. Resistant starch is found in raw potatoes and unripe foods, such as bananas [8]. Dietary fibres may also play a prebiotic role. Similarly, resistant starch, barley β-glucans, raffinose and oligofructose have demonstrated the ability to produce butyrate through their fermentation in both *in vitro* and *in vivo* (rats) [10]. When the fermentative activity of bacteria within the gut is examined in in the presence of prebiotics, bacteria from the *Bacteroidetes* phylum have been observed to produce large quantities of acetate and propionate. In comparison, bacteria from the *Firmicutes* phylum produce mainly butyrate [14].

Previous investigations with prebiotics, specifically oat bran and wheat bran, have demonstrated major trends in the alteration of the normal rat microbiota. In a study by Abnous *et al.* (2009), rats were fed either oat bran or wheat bran and their fecal microbiota was examined with 16S sequencing to observe changes in gut microbiota diversity relative to rats receiving control diet (AIN-93G) [9]. Through cultivation, researchers observed increases in CFUs of anaerobic bacteria over 28 days in both oat and wheat bran-fed rats in comparison to rats consuming the control diet. Rats fed the wheat bran diet had higher levels of *Lactobacillus acidophilus,* while oat bran fed rats had higher counts of *Bacteroides,* enterococci and *Enterobacter* species. The diversity demonstrated by cultivation did not match that observed using molecular methods however. Degrading gradient gel electrophoresis (DGGE) analysis demonstrated that by the end of the 28 day feeding trial, the composition of the microbiota differed between all diets [9]. Similarly, in a study by Bouhnik *et al.* human subject's feces were examined after the ingestion of a series of prebiotic foodtypes (short-chain

fructooligosaccharides, soybean oligosaccharides, galactooligosaccharides, and type III resistant starch) which demonstrated a greater induction of *Bifidobacterium* spp growth in comparison to controls [17]. In an experiment by Dinoto, Achmad *et al.* (2006), rats were fed both raffinose and encapsulated *Bifidobacterium breve* to observe the effects on the rat cecal microbiota [18]. Rats fed raffinose and *B. breve* as well as rats fed raffinose only had increases in the species *B. animalis* in cecal samples. In comparison, groups just fed *B. breve*, had no significant changes in the gut microbiota [18]. This study suggests that probiotics themselves may not change the microbiota to a great extent; instead, prebiotics must be consumed in order to do so. The effects of prebiotics on the intestinal microbiota has been well studied, although, much more must be done in order to fully understand the mechanisms involved, especially as many of the earlier studies relied on culture based methodology to examine the gut microbiota, thus giving an incomplete picture. It is also still unknown how prebiotic fibre-induced changes in the gut microbiota can impact the immune system of rats and humans.

Effects of Prebiotics and Probiotics on the Immune System

Prebiotics not only have the potential to influence the gut microbiota, but they also have an effect on the immune system. Certain of the bacterial species induced by prebiotic supplementation have been shown to have an effect on both mucosal immunity and systemic immunity, as well as innate and adaptive immunity, influencing proinflammatory and regulatory immune responses [3]. IEC are the primary cell type exposed to the commensal bacteria of the gut. These cells are well equipped with modes of distinguishing commensal bacteria from pathogenic bacteria. One way of recognition is through microbial-associated molecular patterns (MAMPs) using pattern recognition receptors (PRRs) [3, 6]. Activation of these receptors on IEC leads to signal transduction cascades and ultimately the production of cytokines,

chemokines and antimicrobial compounds (defensins). Two examples of PRRs are toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs). These receptors are part of the innate immune system, meaning they do not create memory and are not very specific. Instead, they are fast acting and recognize an array of molecules. The adaptive immune system is made up of cells and molecules that respond more slowly, yet are able to produce memory and are more specific. The adaptive immune system is quite finely controlled by both T and B cells. Adaptive and innate immunity cross talk by using specific cell mediators (cytokines) as well as antigen presenting cells (ex. Dendritic cells) [19].

Within the gut reside the gut-associated lymphoid tissues (GALT). These tissues are responsible for the recruitment of dendritic cells (DCs), Immunoglobulin A-producing B cells and T cells [3]. There are many tissues with immune type characteristics and responsibility within the GALT. The PP and the MLN are examples of two tissues that facilitate adaptive immune responses. These tissues house both T and B cells, which play a key role in adaptive immunity, and also provide an ideal microenvironment for activation of T and B cells by antigen. The activated B and T cells leave these tissues via the blood stream and home back to the MALT to enter the lamina propria where they interact with DCs, which sample and display antigens sampled from gut bacteria [20].

The dendritic cells present within the lamina propria are termed $CD103^+$ DCs and $CXCR1^+$DCs [21]. The $CD103^+$ dendritic cells are managed by an array of cytokines, including Thymic stromal lymphopoietin (TSLP), Transforming Growth Factor-β (TGF-β) and retinoic acid [3]. Recently, the ability of probiotic bacteria to differentiate DCs from an immature to a regulatory state has been observed. The mechanism behind this interaction begins in the intestine; IEC are stimulated by commensal bacteria within the gut, producing TGF-β and TSLP.

These cytokines interact with immature dendritic cells housed within the GALT, specifically the lamina propria, turning immature DCs into regulatory DCs, as well as controlling activity of T_{Reg} cells [22] .

Probiotics have been observed to stimulate differentiation of T helper 1 (T_H1), T helper 2 (T_H2) and T helper 17 (T_H17) cells and development of regulatory T ($T_{Reg)}$ cells ($FOXP3^+$). For example, the commensal gut bacterium *Bacteroides fragilis* has shown the ability to differentiate T Helper cells into T_{Reg} cells, which in turn produce Interleukin 10 (IL-10) TGF-β. [3]. These cytokines have regulatory roles within the immune system, often decreasing states of inflammation. Some commensal bacteria not only stimulate regulatory cells, they can also stimulate mucosal proinflammatory T cell subtypes, such as T_H17 cells. Bacteria that induce production of such compounds as serum amyloid A protein (SAA) in the ileum (produced in response to segmented filamentous bacteria) promote the differentiation of T_H17 cells [23]. These cells are located within the GALT and have been implicated in an array of inflammatory bowel diseases [6]. Induction of T_H17 cells leads to the production of such proinflammatory cytokines as IL-1β, IL-6 and IL-23[3]. Research by Zaph *et al.* (2008) demonstrated that T_H17 cell levels are increased in germ free mice [24]. This group provided evidence that gut commensal bacteria control the levels of T_H17 within the gut, which is inversely proportional to IL-25 production. IL-25 expression drives down IL-23 production, subsequently decreasing the levels of T_H17 cells within the intestine [24].

Certain probiotics have been observed to shift the balance of the immune system to a more T_H2 dominated system. T_H1 are implicated in protection against pathogens that are bacterial, viral or protozoan, whereas T_H2 have been shown to protect against helminths and ectoparasites [6]. The shift is completed via stimulation of immune cells, such as monocytes,

neutrophils and T_{Reg} cells, producing such cytokines as IL-12 and the immunoregulatory cytokine IL-10, [22]. In a study by Ezendam *et al*., rats with an autoimmune disorder termed autoimmune encephalomyelitis (EAE) were fed *Bifidobacterium animalis*, which significantly reduced the numbers of eosinophils and lymphocytes in the lungs [25]. This autoimmune syndrome, among others, is caused by inappropriate stimulation of the immune system, leading to an increased T_H1 immune response. Interestingly, *B. animalis* had no effect on immunoglobulin E (IgE), the Ig class associated with allergy, nor on cytokines associated with allergy. It was also observed that the probiotics induced a stronger T_H2 response- specifically an increase in IL-4, IL-5, IL-10 and IL-13 production [25].

Studies have shown that gnotobiotic mice develop a truncated immune system, implying that the intestinal microbiotia is imperative for a fully functioning immune system [3]. Specifically these germ free mice lacked a great number of memory T-cells ($CD4^+$) in their spleen (peripheral to the intestine) and contained less and smaller germinal centers (locations in lymph nodes that contain actively proliferating T and B cells). These mice also expressed a T_H2-type profile.

The effects of the gut microbiota on the systemic or peripheral immune system are not yet well documented, although recent investigations by Clarke *et al.* (2010) suggest that the microbiota play a large role in systemic immunity [26]. This study discovered a major role of peptidoglycan in innate immunity, showing that this compound primes neutrophils, enhancing their ability to destroy certain pathogens, specifically *Streptococcus pneumonia* and *Staphylococcus aureus*. The mechanism involves the PPR called nucleotide-binding, oligomerization domain-containing protein-1(Nod1). NOD1 recognizes *meso*-diaminopimelic acid (*meso*DAP). Peptidoglycan is translocated from the gut and is recognized by neutrophils in

the bone marrow. This study demonstrated that neutrophil activity was directly correlated with peptidoglycan concentrations within the blood [26]. Nod1$^{-/-}$ mice showed much higher signs of susceptibility to pneumococcal sepsis, demonstrating Nod1 and the gut microbiota's critical role in the systemic innate immune system [26].

Another example of the influence of the gut microbiota on the immune system are the changes associated with segmented filamentous bacteria (SFB). These bacteria are present within the rodent gut, and induce strong IgA responses compared to rodents that lack these bacteria. Rodents without SFB also lack mucosal T_H17 cells and are unable to control the growth of pathogens within their intestine, as shown in models using *Citrobacter rodentium* infection. This suggests that SFB play a critical role in priming or developing mucosal intestinal immunity. SFB have also been shown to increase the production of T_H1 cells in the mucosal intestinal lymphoid tissues by increasing production of IL-12 by DCs. Both T-cell subsets T_H1 and T_H17 recruit and activate phagocyte cell types such as macrophage and neutrophils, which ultimately remove pathogenic bacteria [6]. Recently, SFB have also been identified within humans, which will have a large impact on the research avenue of the role of SFB within the gut [3].

Current research has shown that different sections of the intestine respond differently to prebiotics or probiotics. A study by Gaboriau-Routhiau *et al.* (2009) has shown that in germ-free mice colonized with either mouse fecal microbiota or cultivated microbiota, the ileum responds most with respect to cytokine production (mRNA expression) compared to the duodenum, cecum and colon. Mice that were fed conventional mouse feces experienced significant increases in expression of IL-1β, IL-6, TNF-α, INF-γ, IL-17 and IL-10, as well as CD3ε and Foxp3, compared to germ free mice. The colon, in contrast, only had significant increases in IL-17 and Foxp3 levels in comparison to germ free mice [27]. This research avenue is currently unexplored

and further experiments could help create a model of intestinal specific responses influenced by probiotics and prebiotics.

Effect of Probiotics on Toll-like-Receptors

TLRs are a very important component of the innate immune system. As mentioned previously, they contribute to the homeostasic relationship of commensal intestinal bacteria with the host and to recognition of pathogens. The TLRs within the gut are a means through which the host can observe the presence of the bacteria colonizing the intestine and monitor the population of bacteria. They recognize conserved molecules that bacteria express or release, an example being lipopolysaccaride (LPS). Once recognized they aid in the release of cytokines that regulate the immune response of the GALT [28]. TLRs also have many other important physiological roles such as effects on IEC growth, secretion of defensins and downstream IgA production. Although they are very important receptors present in the gut, they are also linked to disease. Faulty TLR signaling has been linked to colon cancer and inflammatory bowel disease, such as Crohn's and colitis (lack of TLR5) [29].

The signaling cascade behind TLRs is often controlled by the myeloid differentiation primary-response protein 88 (MYD88) pathway. Once TLRs are activated, MYD88 is recruited leading to the phosphorylation (by kinases IKKα and IKKβ), ubiquitylation and therefore degradation of IκB by 26S proteasomes [30]. IκB acts as an inhibitor of NF-κB, keeping it from entering the nucleus. Once IκB is degraded NF-κB is freed, entering the nucleus as a promoter for the transcription of proinflammatory cytokines and adhesion molecules [6]. Activation of TLRs on both IEC and DCs ($CD103^{+}$) also has greater downstream effects on adaptive immune responses, leading to the production of B cell-activating factor (BAFF), a proliferation-inducing ligand (APRIL) and IL-4, which promote both T cell dependent and T cell-independent IgA

class-switching [3]. Zaph *et al*. (2007) have demonstrated that knockout mice with deletions in the gene encoding IKK-β have great susceptibility to the parasite *Trichuris* [31]. Normally, an infection with *Trichuris* would induce a T_H2 cytokine profile (IL-4, IL-5 and IL-13). But under knockout conditions, a T_H1 profile is induced, leading to the production of proinflammatory cytokines INF-γ, TNF-α and IL-17. This demonstrates the importance of the NF-κB pathway for mucosal immune homeostasis [31]. Below is a chart outlining the different types of TLRs and the pathogen-associated molecular patterns (PAMPs) they recognize.

Table 1. Overview of relevant immune receptors involved in innate recognition.

Receptor	**Motif Recognized**	**Comments**	**Reference**
TLR2	Lipoteichoic acid (LTA), yeast zymosan	MYD88 and TRAF6, NF-κB activation	[32], [29]
TLR3	Double-stranded RNA	MYD88 and TRAF6, NF-κB activation	[29]
TLR4	Lipopolysaccaride (LPS)	MYD88, TIRAP and TRAF6, NF-κB activation	[32], [29]
TLR5	Flagellin		[32], [29]
TLR6	Mycoplasmal macrophage-activating lipopeptide (MALP-2), Soluble tuberculosis factor (STF), Phenol-soluble modulin (PSM), *B. burgdorferi* outer surface protein A lipoprotein (OspA-L)	MYD88 and TRAF6, NF-κB activation	[29]
TLR9	CpG oligodeoxynucleotides	MYD88 and TRAF6, NF-κB activation	[32], [29]
		Expressed intracellularly	
NOD2	Muramyl dipeptide	NF-κB activation	[6]
		Expressed intracellularly	

Probiotic bacteria, when in contact with IEC, often decrease proinflammatory cytokine production (IL-8, IL-1β, TNF-α) due to the blocking of NF-κB action, and an increase in barrier function. But, certain probiotics have also been shown to decrease apoptosis and to increase β-defensin release, heat shock protein (Hsp) production, mucin expression and sIgA [33]. Though the actions and outcomes of certain probiotics are known, different species and strains have been observed to act through different mechanisms. This includes a decrease in NF-κB activation or increase in mediators of MAPK transduction pathways, such as p38 and ERK1/2 leading to a decrease in proinflammatory cytokine production and an increase in production of such anti-inflammatory cytokines as IL-10 [32, 33]. Another theory suggests that common gut bacteria, like *B. thetaiotaomicron* antagonize the activation of NF-κB by enhancing nuclear transport of its p65 (RelA) subunit by forming a complex with PPAR-γ. PPAR-γ is responsible for the export of this subunit from the nucleus, inhibiting the ability of NF-κB to enter the nucleus to induce proinflammatory cytokine expression [30]. It has also been observed that non-pathogenic *Salmonella* inhibit NF-κB by blocking its degradation by protein IκBα [4], limiting NF-κB translocation into the nucleus. Many bacteria are able to alter their surface MAMPS to avoid recognition by PRRs. They may also alter the transcriptional and posttranscriptional levels of PRRs, varying their expression. It is also hypothesized that certain intestinal alkaline phosphatases alter the MAMPs of bacteria, so they are not as recognizable by PRRs, limiting the extent of the immune response [4].

Not only have probiotics been observed to modulate the immune response via effects on cytokine production, but they have also been observed to influence the degree of expression of TLRs. In a study by Vizoso Pinto and Rodriguez Gomez (2009) it was discovered that the intestinal cell line HT-29 becomes sensitized to bacteria after pre-exposure to TNF-α [32]. The

proinflammatory chemokine IL-8 levels were higher in those cells treated with the TNF-α and Lactobacilli than the cells treated with TNF-α alone. The same results were observed when IEC were pre-treated with *S. typhimurium* in order to activate TLRs, and then treated with Lactobacilli. IEC pretreated with Lactobacilli then exposed to *S. typhimurium* had higher IL-8 expression than cells treated with *S. typhimurium* alone. In the same study, it was determined that exposure to probiotic Lactobacilli (*L. rhamnosus* GG and *L. plantarum*), increased mRNA expression levels of both TLR2 and TLR9. In comparison, TLR4 mRNA levels in HT-29 cells were consistent with baseline controls. Interestingly, this group also showed that Lactobacilli increased TLR5 expression, which recognizes flagellin. Lactobacilli species do not contain flagellin, which led researchers to believe that these bacteria are influencing TLR transduction [32].

Different cells within the body express different TLRs based on their location and external environment. In addition, some cells types express different TLR levels on their basal and apical surface. The reasons pertaining to the presence and absence on certain cell types is not understood. However, it is known that TLR expression is highly controlled by the presence and absence of certain cytokines. IEC are among a group of cells that have been investigated to determine the types of TLRs present and how their expression is controlled. It has been noted that INF-γ and TNF induce TLR4, but the observed results are contradictory [32]. Studies have shown that TLR expression depends on the location within the intestinal tract. In the colon, TLR2 and TLR4 are expressed at low levels, whereas TLR5 is expressed highly. In the small intestine TLR3 is highly expressed, although almost all the TLRs are expressed throughout the intestine (TLR1, TLR2, TLR3, TLR5 and TLR9) [29].

Secreted Immune Altering Bioative Factors from Probiotic Bacterial Strains

Lactobacillus GG (LGG) has been observed to be effective in treating rotavirus diarrhea in infants and children as well as antibiotic-associated diarrhea. This particular strain has also demonstrated the ability to protect against such virulent pathogens as *Cryptosporidium parvum*, *Helicobacter pylori* and *Candida in vivo* [34]. It has also been associated with anti-carcinogenic properties, due to a decrease in colon cancer in treated murine models. In experiments conducted by Tao *et al.* (2006) it was observed that peptides produced by *Lactobacillus* GG induced the production of cytoprotective heat shock proteins in murine IEC [34]. This was determined by measuring the activation of the MAPK family, which is involved in signal transduction of many immunostimulatory compounds. Tao *et al.* also attempted to characterize the secreted factor. This study demonstrated that the factor is very heat stable, pH sensitive and less than 10kDa. However, it was sensitive to proteases (pepsin, trypsin and protease K) suggesting the bioactive factor is a protein/peptide [34].

In contrast, prior research has indicated that anti-inflammatory molecules released from bacteria like *Bifidobacterium* and lactic acid bacteria are polyamines [35]. It has been documented that polyamines have a major role in humans, such as their roles in synthesis and stabilization of DNA, RNA and proteins, cell proliferation and differentiation, and the regulation of enzyme activity. It is believed that they also have a key role in development and maturation of intestinal mucosal tissue. Previously, it has been determined that after consumption of the yogurt LKM512 by human volunteers, polyamine concentrations increased in the intestine, indicating that perhaps they play a part in the health benefits associated with probiotics, including control of intestinal inflammatory responses. In other experiments, it has been observed that polyamines

suppress inflammation. For example, it has been documented that the polyamine spermine down regulates macrophage activation and therefore inflammation [35].

In contrast, Silva *et al.* (1987) concluded that an antimicrobial substance from *Lactobacillus* GG may be of a fatty acid nature. This antimicrobial substance has been observed to inhibit growth of other bacteria in the gut microbiota. Cultures of *Lactobacillus* GG were demonstrated to be heat stable, small molecular weight, resistant to proteases and pH sensitive indicating that this substance is most likely a short-chain fatty acid [36].

The probiotic activity of *Lactobacillus acidophilus* LB has also been investigated in some detail and this strain has been shown to be highly antagonistic against *Salmonella in vivo* and *in vitro*. This bacterium secretes a heat-stable antimicrobial compound into spent culture supernatant of LB (LB-SCS). It has been observed that treatment with LB-SCS kills intracellular *Salmonella*. It has been previously noted that when a pathogen adheres to or attempts to adhere to epithelial cells that there is a release of many cytokines. These cytokines include IL-6, -7, -9, -10 and TNF-α. When infection occurs, there is often the activation of three protein kinases ERK, JNK, p38. These signal transduction pathways lead to the activation of NF-κB and AP-1 leading to the release of IL-8. As mentioned previously, IL-8 is a chemokine that attracts lymphocytes to areas of infection. LB-SCS has been shown to down regulate *S. enterica* serovar Typhimurium SL134 induced IL-8 production from Caco-2 IEC, demonstrating that this unknown bioactive factor controls cytokine expression induced by pathogen adhesion to IEC [37]. It was also observed that the LB-SCS decreased trans-cellular passage and intracellular growth of *S. enterica* serovar Typhimurium. The mechanism through which the factor in LB-CFCS acts has been investigated and it is believed to damage bacterial membranes, preventing infection.

Coconnier-Polter *et al.* (2005) observed that *Salmonella* exposed to LB-CFCS lost intracellular ATP, resulting in decreased viability [38].

Lactobacillus species are not the only microorganism that produces probiotic factors that influence the host immune response. *Saccharomyces boulardii* (Sb) is a species of non-pathogenic yeast. It has been used in the past to treat human gastrointestinal disorders, such as antibiotic associated diarrhea and recurrent C*lostridium difficile* infection [39]. The probiotic factor released from Sb and the mechanism through which it interacts with its host is currently unknown. Some theories suggest that it competes with pathogens for nutrients; inhibits pathogen adhesion, strengthens enterocyte tight junctions, neutralizes bacterial virulence factors and enhances the mucosal immune response. Sougioultzis *et al.* (2006) hypothesized that Sb modulates the immune response by the host through effects on NF-kB signaling [39]. Testing the effect of Sb culture supernatant (SbS) on IL-8 production by human monocytes and enterocytes indicated that SbS inhibited Il-1β and TNF-α induced IL-8 production from HT-29 IEC. SbS was also observed to prevent IκBα degradation, reducing NF-κB- DNA binding in IL-1β and LPS-induced THP-1 IEC. The bioactive factor in SbS was characterized by boiling, lipid extraction, density gradient ultra-centrifugation and gel filtration. The results indicated that the factor was heat stable, not a lipid, small (<1 kDa) and water soluble [39].

Objectives

Chapter 1: Determining the effects of *Lactobacillus rhamnosus* R0011 on Inflammatory Responses

Aim of Study: To observe effects and mechanistic action of *L. rhamnosus* R0011 in the presence of proinflammatory compounds on HT-29 IEC. This was primarily done by measuring production of IL-8, a proinflammatory cytokine.

(i) Comparison of Inflammatory Responses Induced by Tumor Necrosis Factor Alpha (TNF-α) and Interleukin-1 Beta (IL-1β) and down regulation with Viable and Heat-killed *L. rhamnosus* R0011

Previous investigations into the use of *L. rhamnosus* R0011 have demonstrated a decrease in constitutive inflammatory cytokine production by HT-29s, and this decrease was observed with heat-killed R0011[40]. The same has been demonstrated with LPS-induced IL-8 production [41]. Therefore, the efficiency of heat-killed and viable R0011 at altering IEC IL-8 production was compared, to determine whether *L. rhamnosus* must be alive in order to have the same efficiency at decreasing proinflammatory parameters.

(ii) Determining effects of probiotic strains on TLR-induced proinflammatory cytokine production

Decreases in TLR agonist-induced IL-8 production would be anticipated if these strains of probiotic bacteria were able to down regulate TLR activation and block NF-κB transduction. To examine this, the ability of specific *Lactobacillus* strains to down regulate IL-8 production induced by different TLR agonists was compared. As for objective (i), viable *L. rhamnosus* was compared to heat-killed strains in order to understand mechanisms of immune alteration.

(iii) Further characterization of immunomodulatory effects of *L. rhamnosus* using the primary rat IEC-6 cell line

This objective was similar to objective (i), in that the goal was to determine the effects of *L. rhamnosus* R0011 on proinflammatory cytokine production, but used a nontransformed rat IEC cell line (IEC-6). IEC-6 IEC were exposed to similar conditions as the HT-29 IEC, in order to observe *L. rhamnosus*' ability to down regulate CINC-1 production, and to modulate TGF-β and TNF-α production.

Chapter 2: Determining the Impact of Dietary fibre on the immune system of rats

Aim of Study: To compare mucosal and systemic effects of different dietary fibres on the immune system of rats at the level of the intestinal tract by measuring serum antibody levels and cytokines in rat tissue. Another aim was to compare effects of dietary fibres on immunological biomarker concentration based on location within the intestinal tract.

(iv) Trial 2: Oat or wheat bran diet effects on antibody levels in rats

Rats fed both oat and wheat bran developed different microbiota community profiles in their gut, as observed by Abnous *et al.* (2009) [9]. Antibody levels (IgG1 and IGg2a) in the serum were measured for comparison of effects of these dietary fibres on systemic antibody production.

(v) Trial 3: Fructooligosaccharides (FOS) wheat bran, oat bran and resistant starch diet effects on antibody levels and cytokine profiles in rats

Antibody and cytokine levels in rats fed wheat bran, oat bran or resistant starch, were quantified. Specifically, IgG1 and IgG2a were measured in the serum. Based on the previous investigations it was hypothesize that varied types of dietary fibres would induce a diverse set of

bacteria species within different portions of the gastrointestinal tract and therefore have varying impacts on the immune system. Therefore, both colon and ileum tissues were compared for TGF-β (active and total), IL-4, IL-6, IL-10 levels. In the MLN both active and total TGF-β levels were measured. For investigations into the systemic effect of the dietary fibres, cytokine profiles were measured in the serum and spleen, including serum CINC-1 and splenic TGF-β, CINC-1, IL-4, IL-6, IL-10 and IL-2. Several other serum cytokines were assayed however many were below the level of quantification.

MATERIALS AND METHODS

Cell Culture

The HT-29 human colorectal adenocarcinoma adherent cell line (ATCC # HTB-38) was maintained with RPMI 1640 (Sigma, Canada) containing 10% fetal bovine serum (FBS, Sigma, Canada), and 0.5 mg/mL gentamicin sulphate (Sigma, Canada), which constituted HT-29 culture media. The cells were grown and maintained in 250 mL, 75 cm^2 tissue culture flasks (Cellstar, Greiner Bio-One, Mississagua, Ontario). The HT-29 cells were sub-cultured by first rinsing with Phosphate Buffered Saline (PBS), followed by the addition of 2-3 mL of 20% trypsin EDTA (Sigma, Canada), and subsequently diluted in HT-29 culture media. The cells were then centrifuged for 10 minutes at 300 x *g* at room temperature. The supernatant was removed and the pellet resuspended in 2 mL of HT-29 culture media for passaging. For preparation of HT-29 IEC for challenge with cytokines or TLR agonists, the pellet was resuspended in RPMI 1640 after centrifugation. Cultures were grown and maintained in a humidified incubator (Thermo Corporation, Toronto) at 37°C and 5% CO_2.

The IEC-6 primary rat cell line (ATCC CRL-1592) was maintained Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Canada) containing 10% FBS (Canada), 0.5 mg/ml gentamicin sulphate (Sigma, Canada), and 0.1 units/mL bovine insulin (Sigma, Canada). They were passaged and incubated in the same manner as the HT-29 IEC.

Bacterial Strains

Lactobacillus rhamnosus (R0011) and *Lactobacillus helveticus* R0052 were obtained from Institut Rosell Inc. (Montreal, Quebec) in the industrially packaged and lyophilized form (CFU/g). Bacteria were weighed aseptically, washed with PBS and centrifuged at 3000xg for 20 minutes. This was repeated 3x and then the bacteria were diluted to a concentration of $1x10^9$

CFU/mL in RPMI 1640, DMEM or PBS. This stock was used for addition to cell culture assays. For some assays, bacteria were heat-killed in a 70°C water bath for 60 minutes and a sample was streaked on de Man, Rogosa and Sharpe (MRS) (Difco, BD, Canada) plates and incubated at 37C to confirm death. For production of the broth, 400 µL of the bacterial suspension at $1x10^9$ cfu/mL was transferred into 19.6 mL of and incubated in a shaking incubator at 37°C, at a speed of 200-220 RPM for 17 hours. From this suspension, 1 mL was transferred to 9 mL of RPMI-1640 or DMEM and incubated at 37°C, at a speed of 200-220 RPM for 23 hours. The pH of the broth was then measured and the corresponding control broth was adjusted to the same pH. The broths were then centrifuged at 3000g for 20 minutes filtered through a 0.22 um filtered (PROgene, Ultident Scientific, Canada) to remove bacteria. After growth, bacterial strains or conditioned broth were stored in a refrigerator at 4°C.

Challenge of HT-29s and IEC-6s with proinflammatory stimuli

Challenge of the HT-29 and IEC-6 IEC was carried out in 96 well plates at a concentration of $5x\ 10^5$ cells/mL diluted in the appropriate cell culture media. The plates were cultured and incubated overnight to allow a confluent IEC monolayer to form and cells were then treated with proinflammatory cytokines and bacteria or their products. In the case of LPS challenges, IEC were also incubated with 10% FBS, to allow optimal responses to this TLR4 agonist. Titration of proinflammatory agonists and determination of the time needed to induce production of cytokines from HT-29 and IEC-6 IEC were performed in order to determine optimal concentrations and time points for supernatant collection (Table 2). Cells were incubated for 6 or 24 hours, depending on the time needed to induce specific cytokines, in a humidified incubator at 37°C and 5% CO_2. Negative controls represent IEC and media only, and therefore constitutive IL-8 production, in the absence of proinflammatory stimuli. After incubation the

supernatants were collected and stored at -80°C until ELISAs were performed. All samples and challenges were carried out with technical triplicates.

Table 2. Concentrations of proinflammatory cytokines and TLR agonists used to induce responses from IEC.

Cytokine/ TLR Agonist	Concentration	Product
Human TNF-α	50 ng/mL	Peprotech
Human IL-1β	30 ng/mL	Sigma
Polyinosinic-polycytidylic acid (poly(I:C)	40 μg/mL	Sigma
Flagellin	100 μg/mL	Enzo Life Sciences
LPS	20 ng/mL	Sigma
CpG oligodeoxynucleotide	0.1-10 μg/mL	Imgenex
Zymosan A(Yeast cell was extract)	100μg/mL	Sigma
Murine TNF	100 ng/mL	Biovision

Animal Care and Diets

Animal care protocols were approved by the Heath Canada Ottawa Animal Care Committee and by the UOIT Animal Care Committee. Treatment and housing was performed according to Canadian Council on Animal Care (CCAC) guidelines. The rats used in this study were Biobreeding (BB) weanling rats (aged 28-42 days).These rats were obtained and raised at Heath Canada's Animal Resource Division facility. Feeding trials lasted 34 days, however, a week prior to the beginning of the trials, rats were fed control diets. In Trial 2, rats were fed 5% oat fibre, 5% wheat fibre or AIN-93G control diets (American Association of Cereal Chemists, USA). In Trial 3 rats were fed 5% oat fibre, 5% wheat fibre, 5% resistant starch or AIN-93G. The baseline diet for both trials was AIN-93G. However, one additional diet was added, 5% fructooligosaccharides (FOS) (Encore Technologies, USA), for which the control and baseline diet was Purina Chow 5001 (Purina Mills, Pembroke, ON). At the end of the trial rats were

euthanized using O_2/CO_2. Rats were then dissected and various tissues were flash frozen in RNA later (Invitrogen) and shipped to UOIT where they were stored at -80°C.

Tissue Homogenization

Tissues (colon, ileum, spleen, MLN) were removed from -80°C storage and quickly suspended in PBS containing 1% protease inhibitor cocktail (Sigma-Aldrich, USA) at 5mL/1g. They were then homogenized using the Tissuemiser (Fisher Scientific, USA) until homogeneous. The homogenate was then centrifuged (Eppendorf, 5415D) for 30 minutes at 16,100xg. The pellet was discarded and the supernatant was centrifuged twice more. The homogenized tissues were stored at -80°C until ELISAs were performed.

ELISA for Cytokine and Antibody Quantification

DuoSet ELISA kits for IL-4 (DY504), IL-6 (DY506), IL-10 (DY522), TGF-β1 (DY240), CINC-1 (DY515), and IL-1β (DY501) were obtained from Research and Development (R&D) (USA). The ELISA kit for IL-8 was obtained from Invitrogen (San Diego, California), IL-2 (CRC0023) and TNF-α (KRC301) were obtained from Invitrogen . Antibody ELISA kits, for measurement of IgG1 and IgG2, were obtained from Bethyl labs. All steps were completed following manufacturer's instructions. ELISAs were completed using Greiner full well or half well plates. Tetramethylbenzidine (TMB) (Sigma, Canada) was used as the substrate. A wavelength of 450 nm was used to read the plates on a Synergy HTTR microplate reader using KC4 version 3.4 software (Bio-tek Instrumentation, Nepean, ON).

Viability Assays for *in vitro* Studies

Viability of IEC (HT-29 and IEC-6), were monitored by both visual inspection and neutral red staining (Sigma) after incubation periods with bacteria and bacterial products. The protocol for

neutral red staining was carried out as described by Repetto *et al.* (2008) [42]. Briefly, after incubation of IEC (at a concentration of $5x10^5$ cells/mL) with proinflammatory inducers, bacteria or their products, supernatants were removed and a 1% (v/v) neutral red solution diluted in cell culture media was added to each well. The plate was then placed at 37°C for 2 hours. Thereafter supernatants were removed, the wells washed with cell culture media and the destain solution (50% ethanol, 49% water, 1% acetic acid) added. After addition of the destain solution the plate was placed on a shaker for approximately 20 minutes, then the destain solution was removed and placed in a 96 well ELISA plate. The absorbance (540 nm) was read using the Synergy HTTR microplate reader using KC4 version 3.4 software (Bio-tek Instrumentation, Nepean, ON).

Statistical Analysis

The data were analyzed using a one way ANOVA (with Bonferrroni post-hoc test), a Kruskal-Wallis test (with Dunn post-hoc test) or Student's t-test using the Instat program. All error bars indicate standard error of the mean (SEM).

RESULTS

Effects of *Lactobacillus rhamnosus* R0011 on Inflammatory Responses of Human Epithelial cells (IEC)

Decreased production of IL-8 by HT-29s IEC when co-incubated with TNF-α or TLR agonists and *L. rhamnosus* R0011

Original experiments were performed using both *L. rhamnosus* R0011 and *L. helveticus* R0052. However, *L. rhamnosus* R0011 was more efficient at decreasing proinflammatory cytokine production from TNF-α treated IEC. Therefore it was selected for further characterization. HT-29 IEC were co-incubated with either TNF-α, IL-1β, poly (I:C), flagellin or LPS and *L. rhamnosus* R0011, either viable or heat-killed, for 6 hours. There was a significant decrease in TNF-α induced IL-8 production by HT-29 IEC incubated with viable R0011 (Figure 1). However, this was not observed when IL-1β was used as the proinflammatory stimulus (Figure 2), where *L. rhamnosus* R0011 did not induce a decrease in IL-8 production. A slight decrease in TLR agonist-induced IL-8 production was also observed when HT-29 IEC were incubated with viable R0011 and poly (I:C) (Figure 3), flagellin (Figure 4) or LPS (Figure 5), however this decrease was not significant.

In contrast, heat-killed *L. rhamnosus* R0011 did not significantly down regulate IL-8 production induced by any of the stimuli. However, a common pattern was observed between the effects of viable and heat-killed *L. rhamnosus* R0011 in that heat-killed *L. rhamnosus* R0011 demonstrated the ability to decrease IL-8 production from HT-29 IEC, although their efficacy was low. Additional TLR agonists, zymosan A (TLR2) and CPG (TLR9), were incubated with HT-29 IEC in an attempt to induce IL-8 production, however, the HT-29 IEC were unresponsive

to both agonists. IL-1β production was also examined from HT-29 IEC using various inducers; although there was no detection of IL-1β production.

Decreased IL-8 production by HT-29 IEC pre-incubated with *L. rhamnosus* R0011and challenged with TNF-α, IL-1-β or TLR agonists

HT-29 IEC were pre-incubated with *L. rhamnosus* R0011 for 18 hours before a 6 hour challenge with the various proinflammatory inducers. As depicted in Figures 6 to 9 there was a greater degree of decrease in IL-8 production observed in comparison to concurrent addition of *L. rhamnosus* R0011 and proinflammatory stimuli with IEC. However, a decrease in HT-29 IEC cell viability was observed when treated with viable *L. rhamnosus* R0011 over the 24 hour incubation period (Table 3). Pre-incubation of HT-29 IEC with *L. rhamnosus* R0011 efficiently reduced IL-1β induced IL-8 production by HT-29 IEC (Table 4). Interestingly, the heat-killed *L. rhamnosus* R0011 appeared to increase IL-8 production, as well as CINC-1 production by from IEC-6 IEC relative to constitutive levels, suggesting that there is a proinflammatory effect of R0011 over long incubation periods.

Inability of *L. rhamnosus* R0011 to decrease IL-8 production when HT-29 IEC are pre-incubated with TNF-α or TLR agonists

In an attempt to characterize *L. rhamnosus* R0011's interaction with IEC, HT-29 IEC were incubated with TNF-α or TLR agonists for an hour before the addition of *L. rhamnosus* R0011, and then incubated for 5 hours before cytokine measurement (Table 5). *L. rhahmnosus* R0011 showed a diminished ability to reduce IL-8 production by HT-29 IEC when added after proinflammatory agonists.

Decreased IP-10 production from HT-29 IEC when co-incubated with *L. rhamnosus* and TNF-α

HT-29 IEC were coincubated with TNF-α and *L. rhamnosus* R0011 for 24 hours in order to determine effects on IP-10 production. Figure 10 illustrates that a decrease in IP-10 levels when HT-29 IEC were concurrently treated with *L. rhamnosus* R0011 and TNF-α.However this decrease was not significant. Unfortunately, production of IP-10 was low after 24 hours, which may have contributed to the lack of significance.

Decreased CINC-1 production by IEC-6 IEC when co-incubated with *L. rhamnosus* and TNF-α or a TLR agonist

IEC-6 IEC were co-incubated with TNF-α and/or *L. rhamnosus* R0011 (viable or heat-killed) for 6 hours. As observed in Figure 11, there was a significant decrease in TNF-α induced CINC-1 production when IEC-6 IEC were incubated with viable *L. rhamnosus* R0011 in comparison to the positive control. IEC-6s were also co-incubated with the TLR agonist; flagellin (Figure 12) and *L. rhamnosus* R0011 for 24 hours. Treatment of IEC-6s with viable *L. rhamnosus* R0011 alone also significantly increased TNF-α production after 6 hours (Figure 13). Similarly, treatment with the *L. rhamnosus* R0011 alone for 24 hours increased TGF-β production (Figure 14). Contrary to the effects observed on HT-29 IEC, IEC-6 IEC produced CINC-1 when incubated with the TLR agonist zymosan A (Table 6), and *L. rhamnosus* demonstrated to ability to decrease this TLR2 induced CINC-1 production (Table 7). However, similar to the effects observed on HT-29 IEC, IEC-6 IEC were unable to produce IL-1β or IL-6 when treated with any of the proinflammatory inducers.

Impact of Dietary fibre on the Immune system of Rats

Effect of prebiotic fibre diets on serum IgG1 and IgG2a levels in BB rats

Total levels of antibody isotypes IgG1 and IgG2a were quantified in the serum of BB rats from trials 2 and 3. In trial 2, wheat bran and oat bran fed rats had lower amounts of serum IgG2 (Figure 15). However, there was no difference in serum IgG1 (Figure 16) levels between rats receiving different fibre diets. In trial 3 FOS-fed mice had increases in both serum IgG1 (Figure 17) and IgG2a (Figure 18) levels, however, this increase was not statistically significant. Wheat bran, oat bran and resistant starch diets had no statistically significant effect on serum IgG1 (Figure 19) or IgG2a (Figure 20) levels. However, an increase in serum IgG1 was observed in oat bran or wheat bran-fed rats.

Effect of fibre diets on TGF-β production within the MLN of BB rats

Active and total TGF-β were quantified in the MLN. As depicted in Figure 21, there was a significant increase in active TGF-β in resistant starch fed rats, in comparison to rats receiving the AIN-93G control diets. There was no significant difference in total MLN TGF-β (Figure 22) concentrations between rats fed any of the diets.

Effect of prebiotic fibre diets on cytokine production of selected systemic tissues of BB rats

Cytokine levels were analyzed in two systemic locations, the spleen and serum. In the serum, CINC-1 was quantified (Figure 23) and there were no significant differences between FOS-fed and Purina chow control-fed rats, or between any rats fed of the other dietary fibres and AIN-93G (Figure 24).

In the spleen, for rats fed the FOS supplemented diets, active TGF-β (Figure 25), IL-6 (Figure 25), CINC-1 (Figure 25), IL-4 (Figure 25), IL-10 (Figure 25) and total TGF-β (Figure 26) were quantified. The only significant difference between FOS diet-fed and Purina chow-fed control rats was observed with active TGF-β (Figure 25). Splenic active TGF-β was lower in FOS fed rats in comparison to rats fed the control, Purina chow. In rats fed oat bran, wheat bran and resistant starch, splenic active TGF-β (Figure 27), IL-4 (Figure 27), CINC-1 (Figure 27), IL-6 (Figure 27), IL-10 (Figure 27), total TGF-β (Figure 28) and IL-2 (Figure 29) were quantified. There was no significance observed in the spleen concentrations of any of the tested cytokines. However increases in splenic total TGF-β (Figure 28) and IL-4 (Figure 27) in oat bran-fed rats were observed, which corresponded with a decrease in splenic CINC-1 levels (Figure 27). It is important to note that although significance cannot be determined for splenic IL-2 (Figure 29), due to most rats having IL-2 concentrations below the level of detection, there was a difference observed between AIN-93G or wheat and resistant starch diets. In the spleen, IL-10 was below the level of detection for rats fed any of the diets.

Effect of fibre diets within the ileum of BB rats

Within the ileum, another mucosal tissue, cytokines quantified included active TGF-β (Figure 30), CINC-1 (Figure 30), IL-4 (Figure 30), total TGF-β (Figure 31) and IL-10 (Figure 32) for rats fed FOS or Purina chow control diets. There were increases in ileal CINC-1, IL-10, active TGF-β and total TGF-β in FOS-fed rats in comparison to the rats fed Purina chow diet; however, they were not significant. No difference was observed in ileal IL-4 levels and IL-6 was below the level of detection.

In rats fed wheat bran, oat bran or resistant starch the cytokines quantified were active TGF-β (Figure 33), IL-4 (Figure 33), CINC-1 (Figure 33), total TGF-β (Figure 34) IL-10 (Figure 35) and IL-6 (Figure 36). Rats fed wheat bran had significantly higher ileal concentrations of active TGF-β, CINC-, IL-4 and IL-10 in comparison to resistant starch fed-rats. These cytokines were also up-regulated in oat bran-fed rats, in comparison to AIN-93G-fed rats, although no significance was observed between rats fed the supplemented diets and control.

Effect of fibre diets within the colonic tissue of BB rats

Cytokine levels were quantified in the colon, a mucosal tissue. The cytokines quantified for rats fed FOS and Purina chow diets were active TGF-β (Figure 37), IL-4 (Figure 37), CINC-1 (Figure 37), IL-10 (Figure 37), IL-6 (Figure 37) and total TGF-β (Figure 38). In FOS-fed rats, active TGF-β was significantly increased in comparison to rats fed the Purina chow diet. This corresponds with the decrease in colonic CINC-1 observed in rats fed FOS diets, although not significant.

The cytokines quantified for rats fed AIN-93G, wheat bran, oat bran and resistant starch were active TGF-β (Figure 39), IL-4 (Figure 39), CINC-1 (Figure 39), IL-10 (Figure 39), IL-6 (Figure 39) and total TGF-β (Figure 40). In rats fed AIN-93G, wheat bran, oat bran or resistant starch supplemented diets, significant differences were observed in cytokine levels for oat bran-fed rats. Oat bran-fed rats had significantly higher levels of colonic CINC-1 in comparison to resistant starch fed rats. Oat bran-fed rats also demonstrated significantly higher levels of colonic IL-6 and active TGF-β in comparison to AIN-93G fed rats. There is a definite effect in colon of rats fed oat bran diets, with higher colonic tissue concentrations of cytokines compared to rats

fed other diets. This pattern was further supported when considering the effects of the oat bran diet on colonic IL-10 and IL-4 levels, although the differences were not significant.

FIGURES

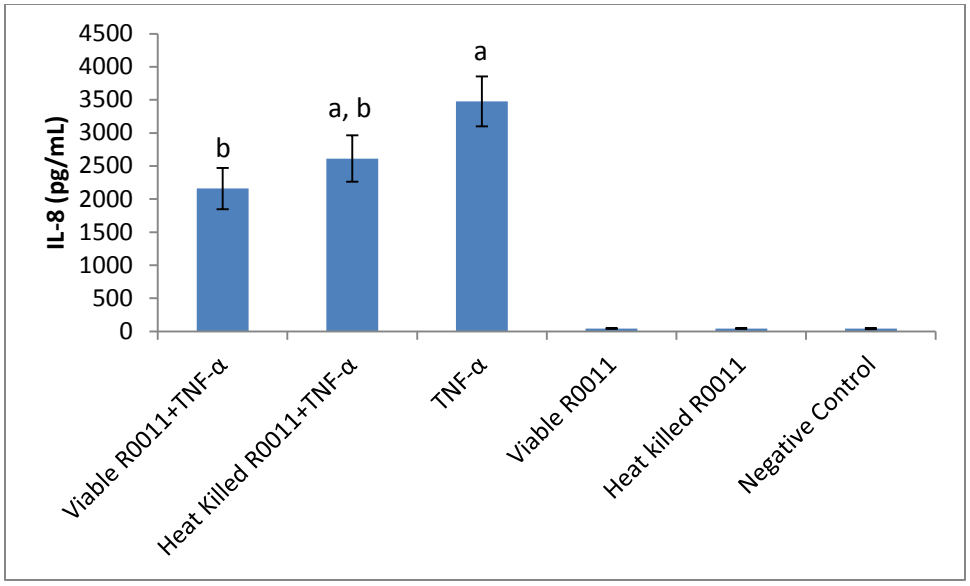

Figure 1. IL-8 production by HT-29 IEC after co-incubation with *L. rhamnosus* R0011and TNF-α for 6 hours, shown as mean concentration in pg/mL ± SEM (n=7). There was a significant difference in IL-8 production from HT-29 IEC treated with TNF-α alone and TNF-α with viable *L. rhamnosus* R0011(p-value <0.01). Statistical analysis compared only TNF-α treated IECBars with different letters are significantly different from each other (as determined by ANOVA and Bonferroni post-hoc test).

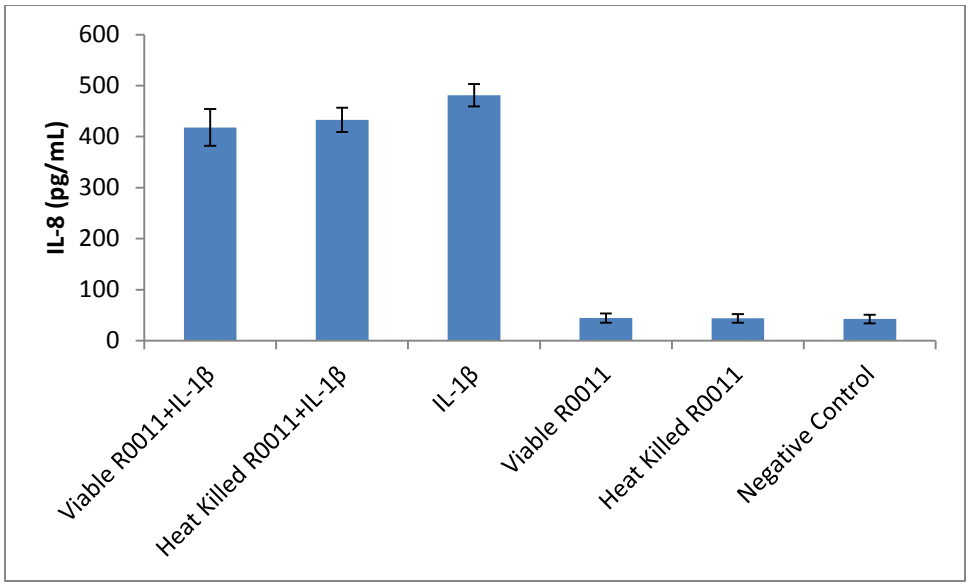

Figure 2. IL-8 production by HT-29 IEC after co-incubation with *L. rhamnosus* R0011and IL-1β for 6 hours, shown as mean concentration in pg/mL ± SEM (n=7)**.**

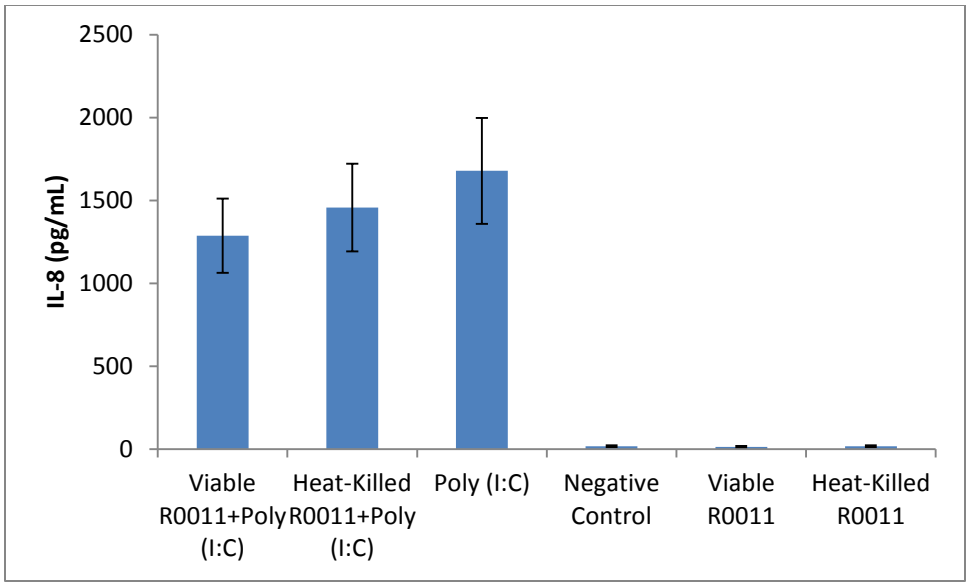

Figure 3. IL-8 production by HT-29 IEC after co-incubation with *L. rhamnosus* R0011and poly (I:C) for 6 hours, shown as mean concentration in pg/mL ± SEM (n=3-4)**.**

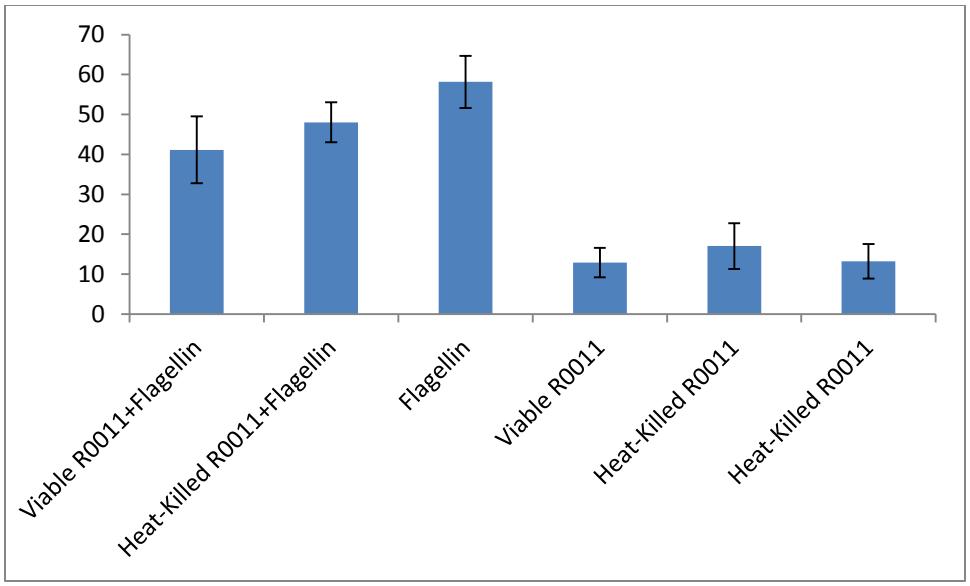

Figure 4. IL-8 production by HT-29 IEC after co-incubation with *L. rhamnosus* R0011and flagellin for 6 hours, shown as mean concentration in pg/mL ± SEM (n=3-4)**.**

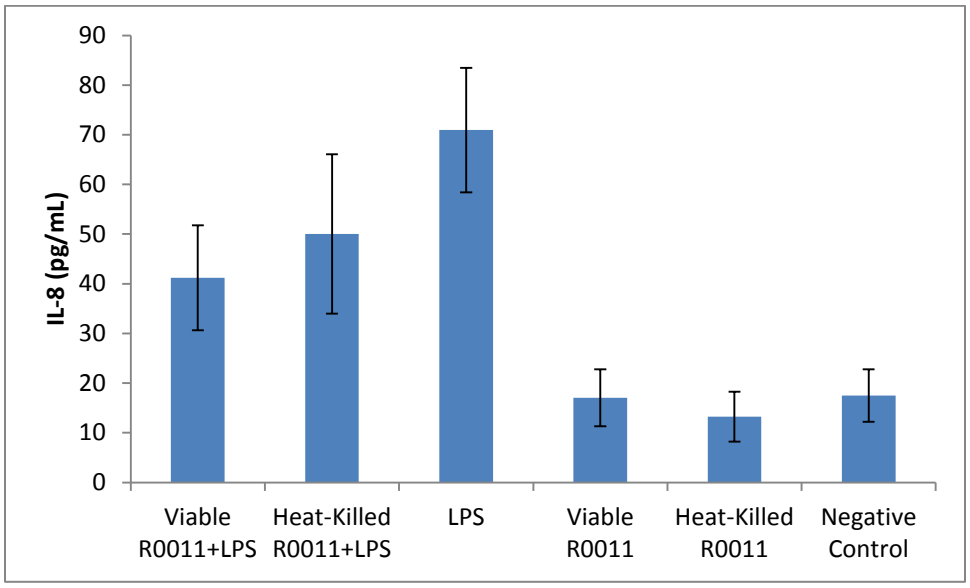

Figure 5. IL-8 production by HT-29 IEC after co-incubation with *L. rhamnosus* R0011and LPS for 6 hours, shown as mean concentration in pg/mL ± SEM (n=3-4)**.**

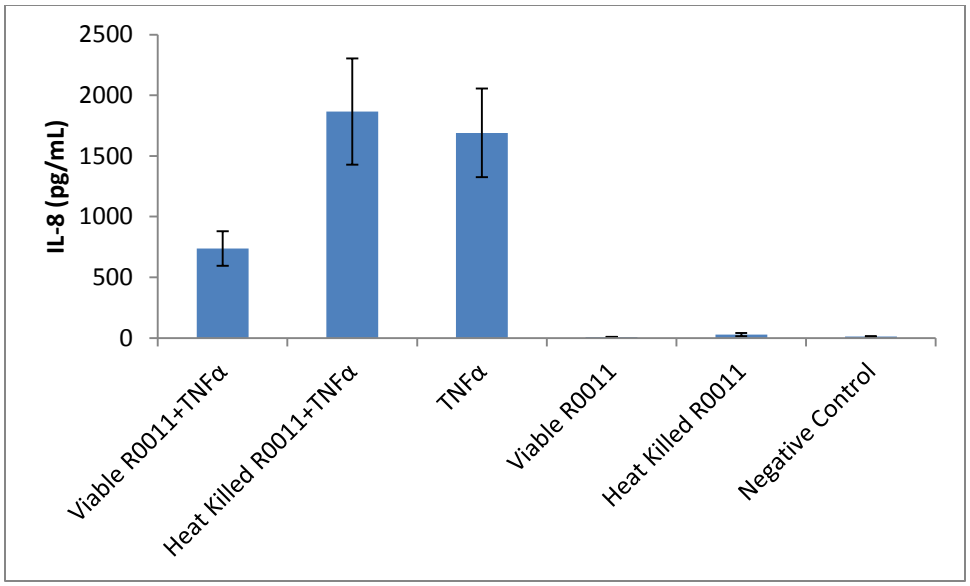

Figure 6. IL-8 production by HT-29 IEC after pre-incubation with *L. rhamnosus* R0011 for 18 hours and subsequent addition of TNF-α for 6 hours, shown as mean concentration in pg/mL ± SEM (n=4). There was a statistical trend (p=0.1) observed in IL-8 production from HT-29 IEC between TNF-α alone and HT-29 IEC treated with viable or heat-killed *L. rhamnosus* R0011 and TNF-α (as determined by an ANOVA and Bonferroni post-hoc test). Statistical analysis compared only TNF-α treated IEC.

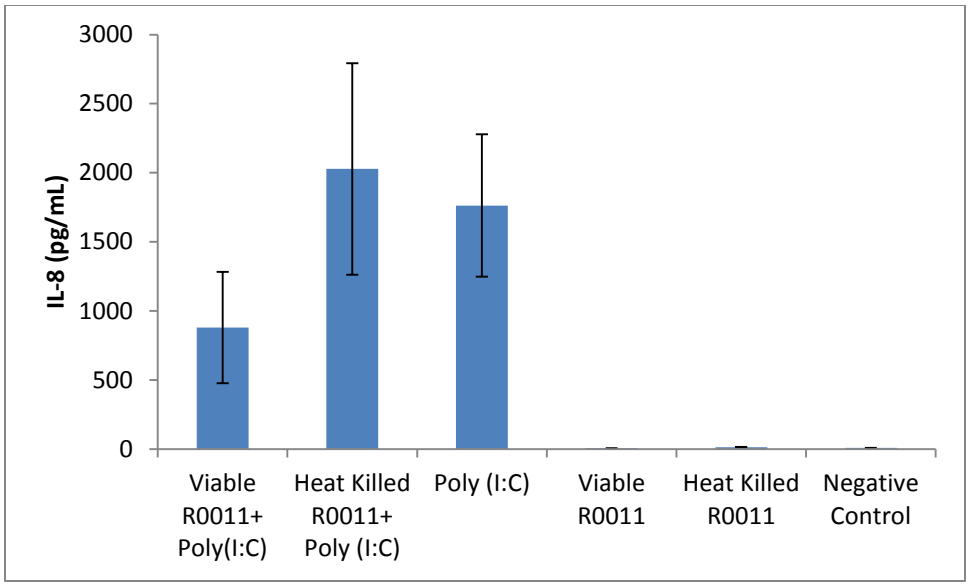

Figure 7. IL-8 production by HT-29 IEC after co-incubation with *L. rhamnosus* R0011 and poly (I:C) for 24 hours, shown as mean concentration in pg/mL ± SEM (n=4)**.**

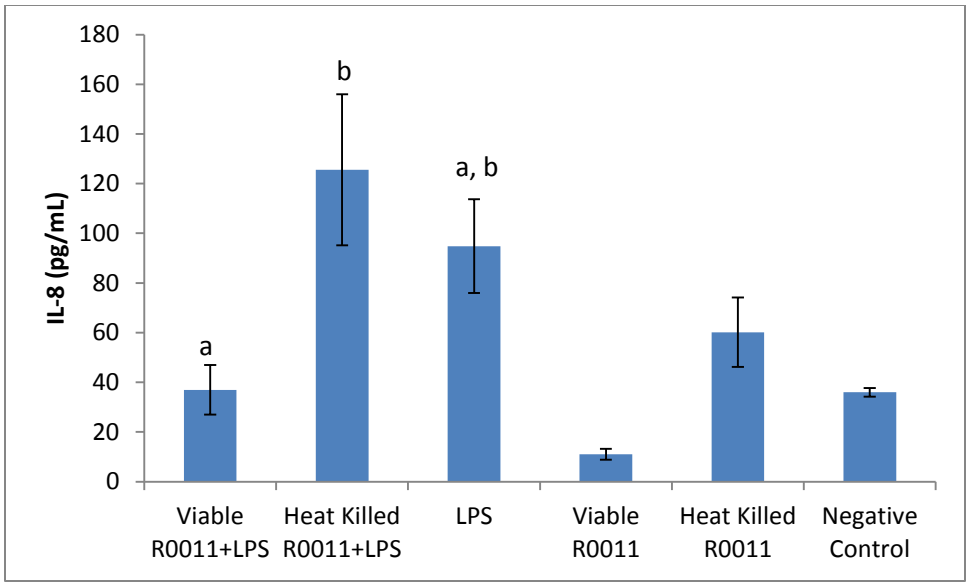

Figure 8. IL-8 production by HT-29 IEC after pre-incubation with *L. rhamnosus* R0011for 18 hours and subsequent addition of LPS for 6 hours, shown as mean concentration in pg/mL ± SEM (n=3). There was a statistical trend (p=0.066) observed in IL-8 production from HT-29 IEC between TNF-α alone and HT-29 IEC treated with viable or heat-killed *L. rhamnosus* R0011 and TNF-α. There was a significant difference in IL-8 production from HT-29 IEC treated with viable *L. rhamnosus* R0011 compared to those treated with heat-killed *L. rhamnosus* R0011, as measured by an ANOVA ($p<0.05$). Statistical analysis compared IEC treated with LPS separately from controls. Bars with different letters are significantly different from each other (as determined by ANOVA and Bonferroni post-hoc test).

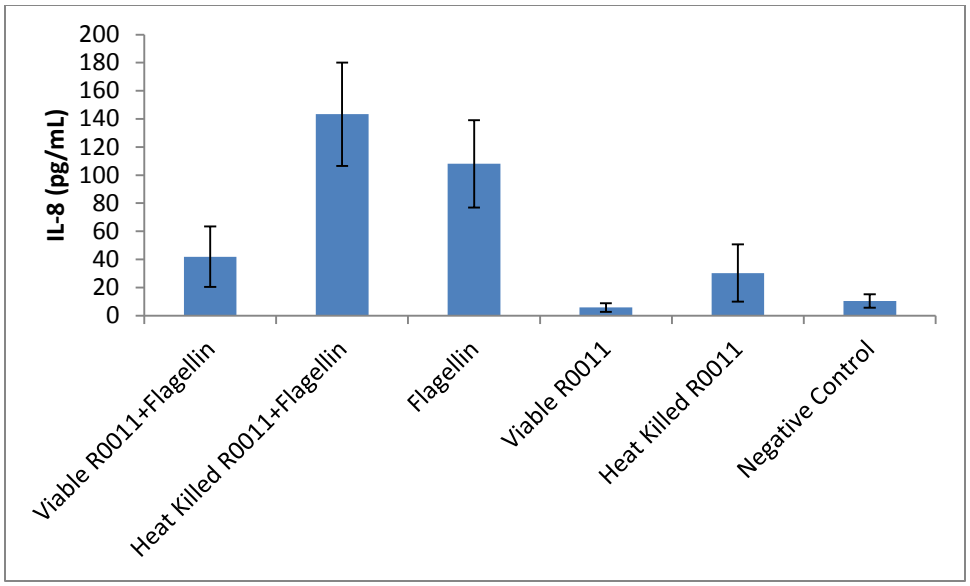

Figure 9. IL-8 production by HT-29 IEC upon pre-incubation with *L. rhamnosus* R0011 for 18 hours and subsequent addition of flagellin for 6 hours, shown as mean concentration in pg/mL ± SEM (n=3)**.**

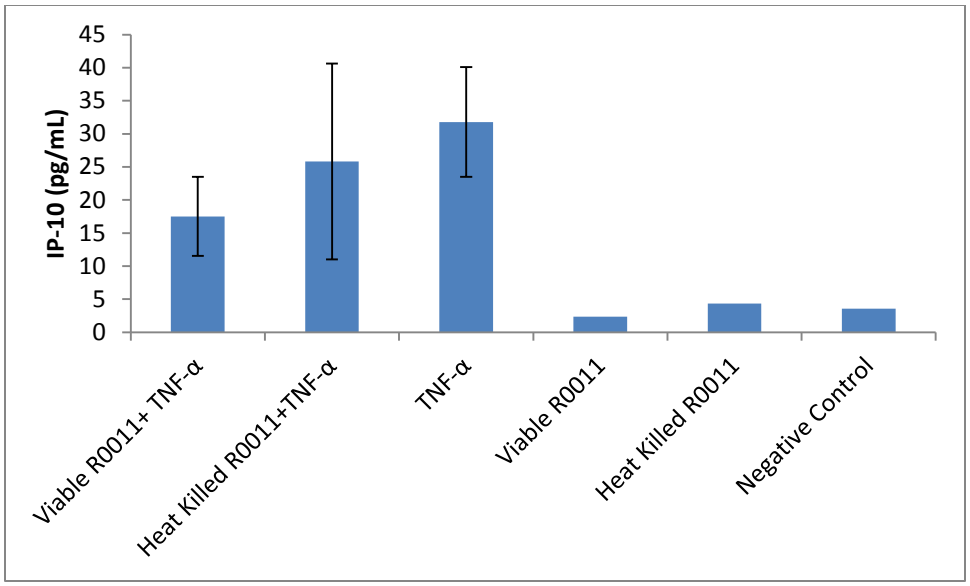

Figure 10. IP-10 production by HT-29 IEC after co-incubation with *L. rhamnosus* R0011 and TNF-α for 24 hours, shown as mean concentration in pg/mL ± SEM (n=3)**.**

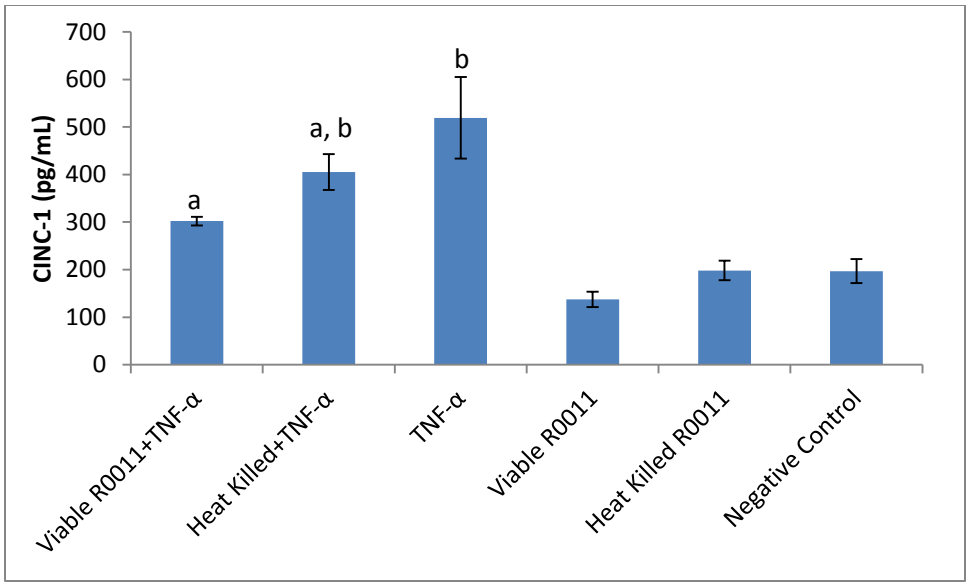

Figure 11. CINC-1 production by IEC-6 IEC after co-incubation with *L. rhamnosus* R0011 and TNF-α for 6 hours, shown as mean concentration in pg/mL ± SEM (n=4). There was a significant difference in IL-8 production from IEC-6 IEC treated with TNF-α alone and IEC-6 IEC treated with viable *L. rhamnosus* R0011 and TNF-α ($p<0.05$). Statistical analysis compared only TNF-α treated IEC. Bars with different letters are significantly different from each other (as determined by ANOVA and Bonferroni post-hoc test).

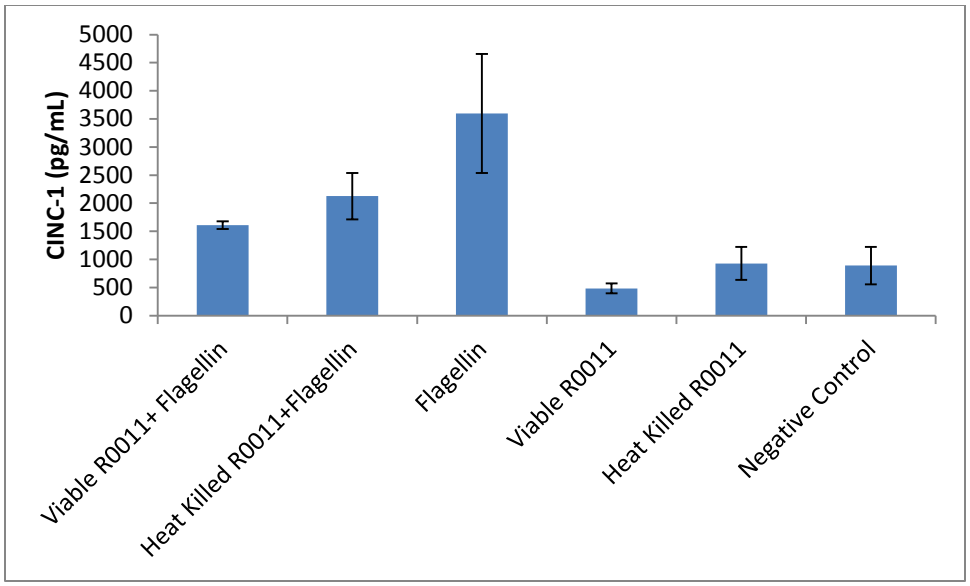

Figure 12. CINC-1 production by IEC-6 IEC after co-incubation with *L. rhamnosus* R0011 and flagellin for 24 hours, shown as mean concentration in pg/mL ± SEM.

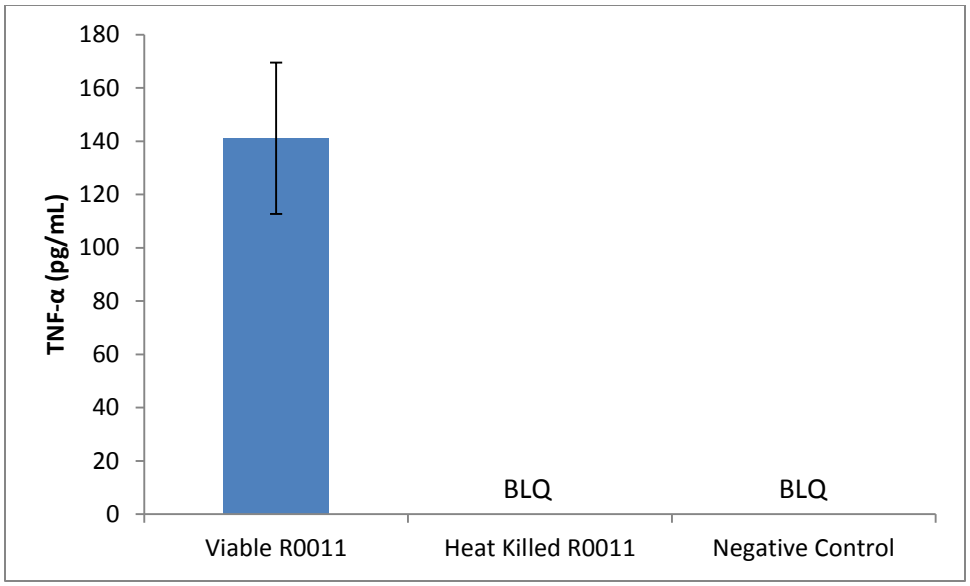

Figure 13. TNF-α production by IEC-6 IEC after incubation with viable or heat killed *L. rhamnosus* R0011 for 6 hours, shown as mean concentration in pg/mL ± SEM (n=3). TNF-α levels for both heat-killed *L. rhamnosus* and negative control were below the level of detection (BLQ).

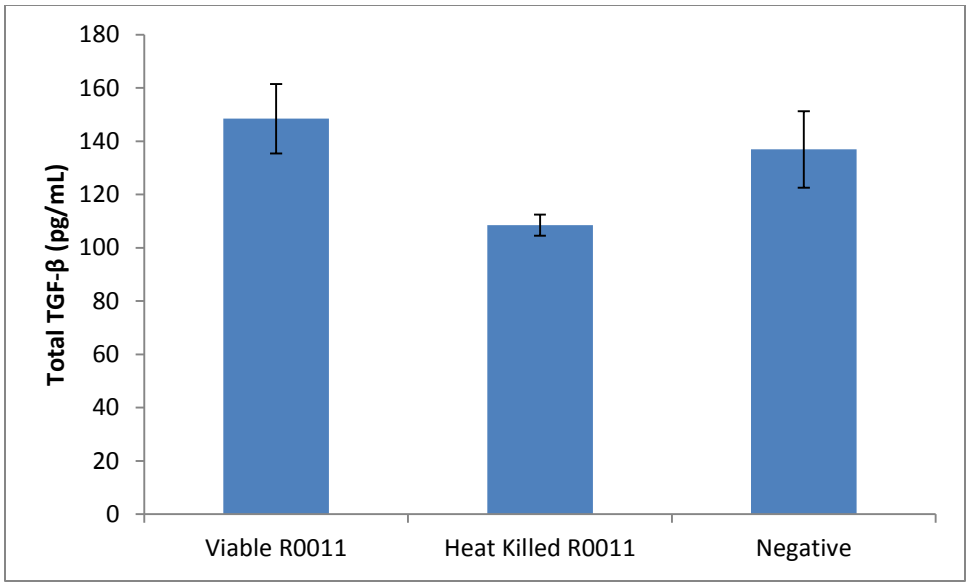

Figure 14. Total TGF-β production by IEC-6 IEC after incubation (in the absence of proinflammatory stimuli) with *L. rhamnosus* R0011 and for 24 hours, shown as mean concentration in pg/mL ± SEM (n=5).

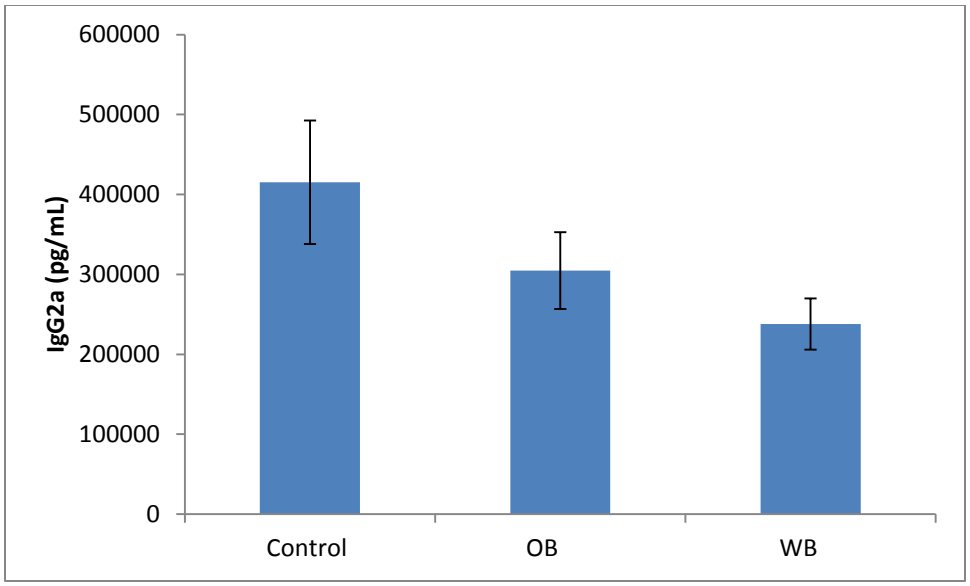

Figure 15. Quantification of IgG2a (pg/mL) within the serum of trial 2 rats. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre or 5% wheat fibre. The bars represent the mean ± SEM (n=6). There was no significant effect of diet, as determined by an ANOVA.

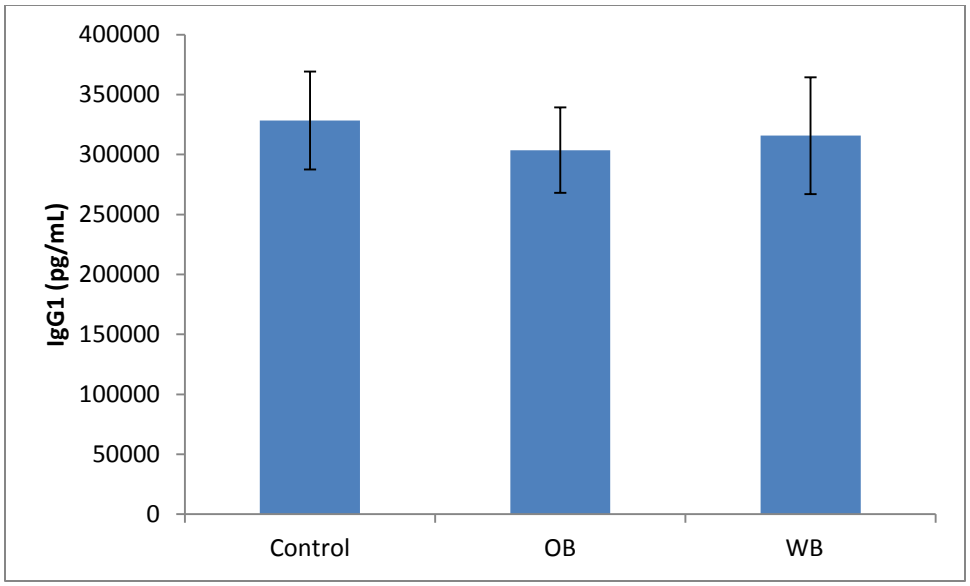

Figure 16. Quantification of IgG1 (pg/mL) within the serum of trial 2 rats. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre or 5% wheat fibre. The bars represent the mean ± SEM (n=6). There was no significant effect of diet, as determined by an ANOVA.

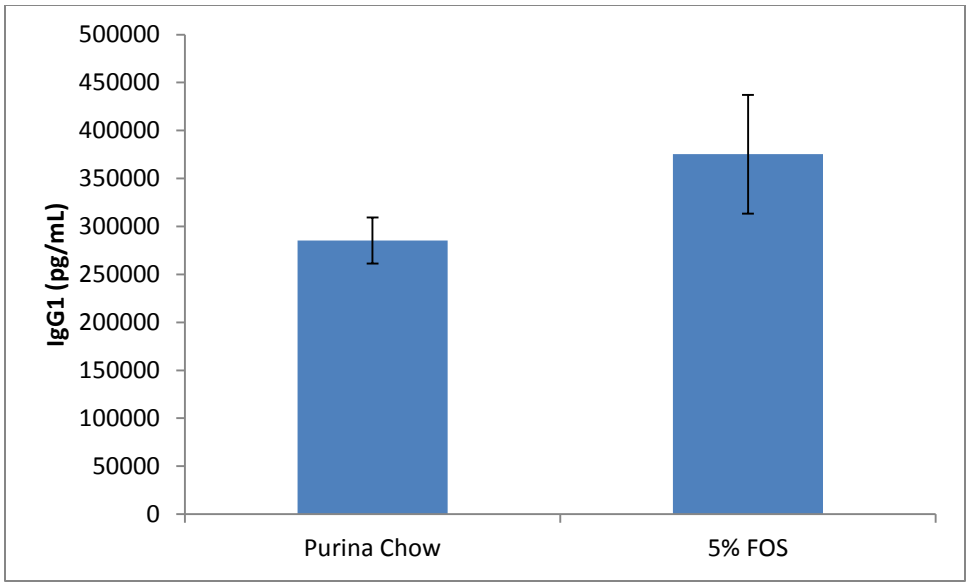

Figure 17. Quantification of IgG1 (pg/g) within the serum of trial 3 rats. Rats were fed either Purina chow alone or Purina chow supplemented with 5% FOS. The bars represent the mean ± SEM (n=6-7). There was no significant effect of diets, as determined by a Student's t-test.

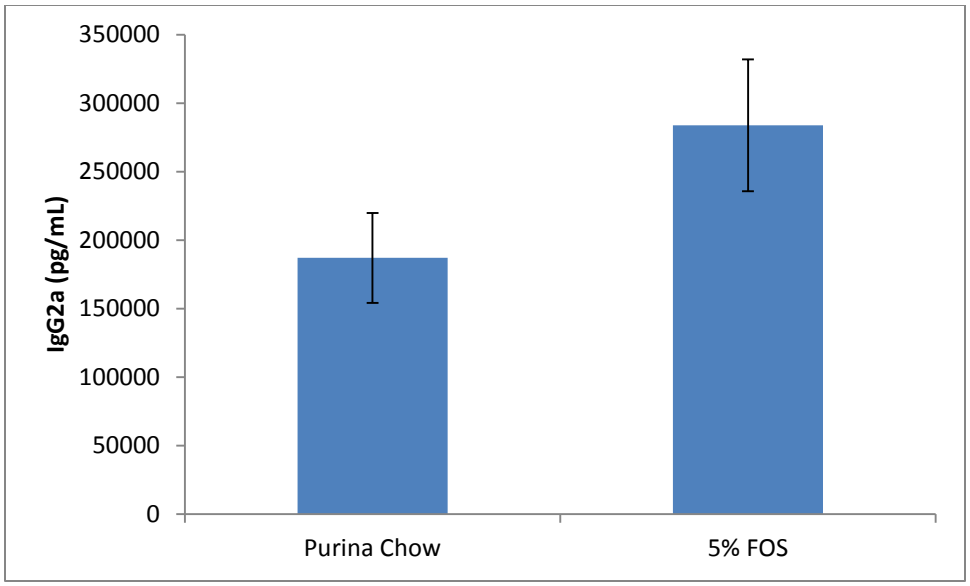

Figure 18. Quantification of IgG2a (pg/mL) within the serum of trial 3 rats. Rats were fed either Purina chow alone or Purina chow supplemented with 5% FOS. The bars represent the mean ± SEM (n=6-7). There was no significant effect of diets, as determined by a Student's t-test.

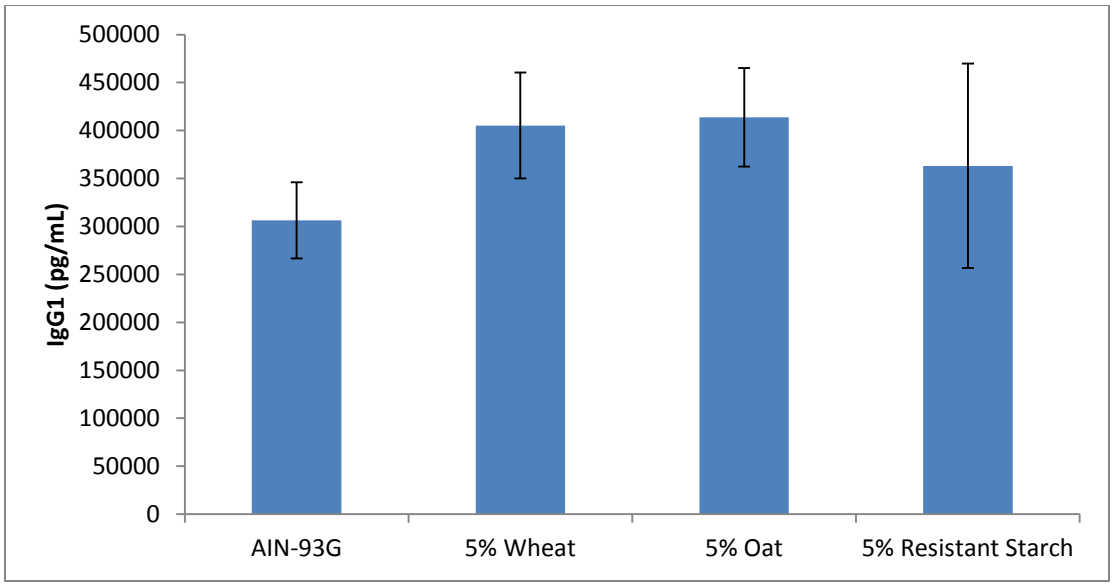

Figure 19. Quantification of IgG1 (pg/mL) within the serum of trial 3 rats. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SEM (n=10-12). There was no significant effect of diet, as determined by an ANOVA.

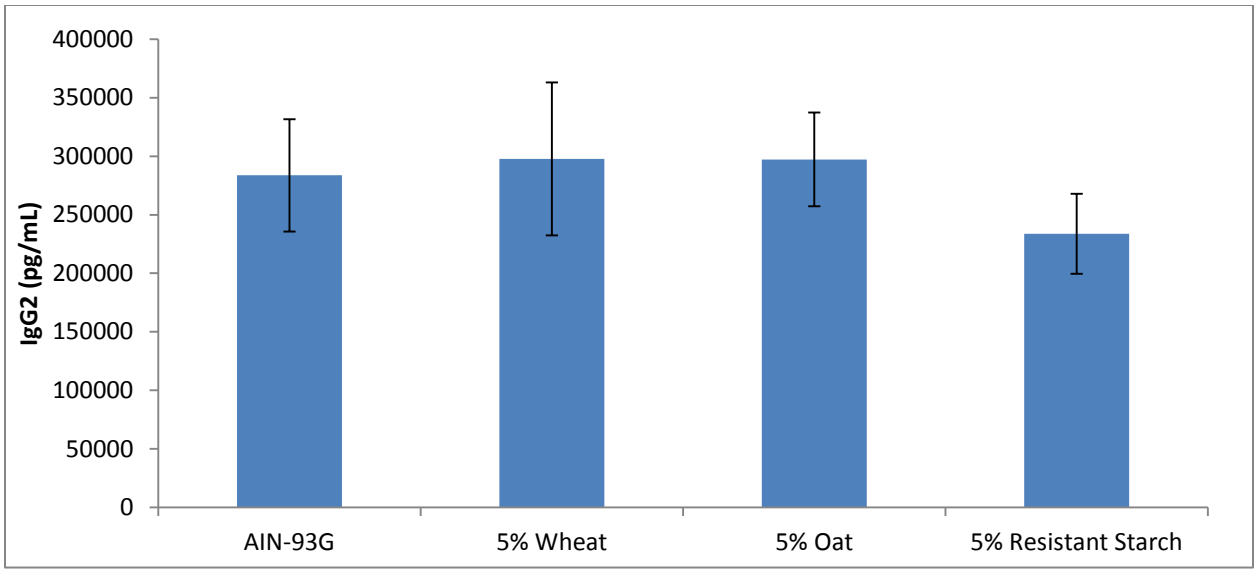

Figure 20. Quantification of IgG2a (pg/mL) within the serum of trial 3 rats. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SEM (n=10-12). There was no significant effect of diet, as determined by an ANOVA.

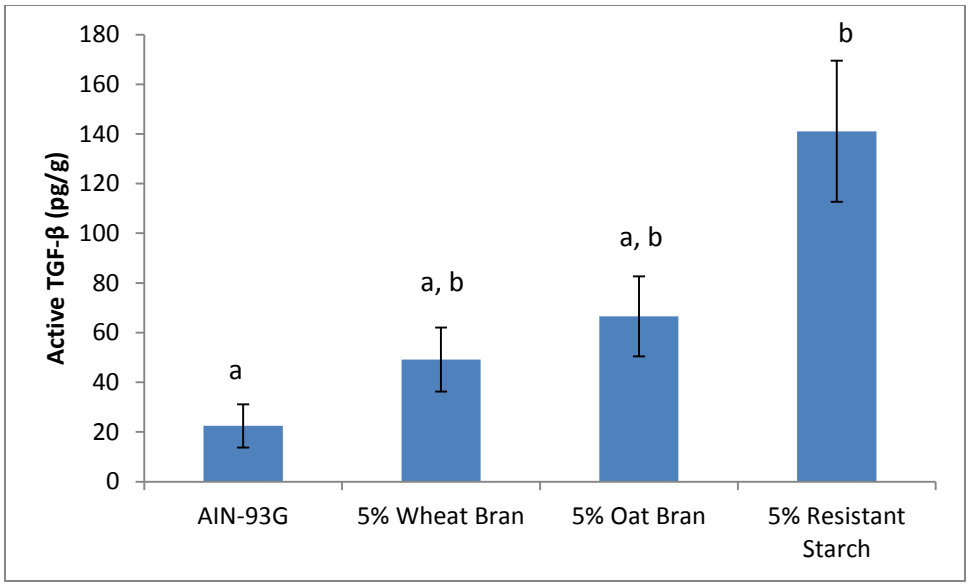

Figure 21. Quantification of active TGF-β (pg/g) within the MLN. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=4-6). TGF-β was BLQ in the MLN with one AIN-93G and one wheat bran-fed rat. These rats were assigned a value of 0 pg/g. There was a significant difference observed between rats fed-resistant starch and AIN-93G control diets ($p<0.01$). Bars with different letters are significantly different from each other (as determined by a Kruskal-Wallis test and Dunn post-hoc test).

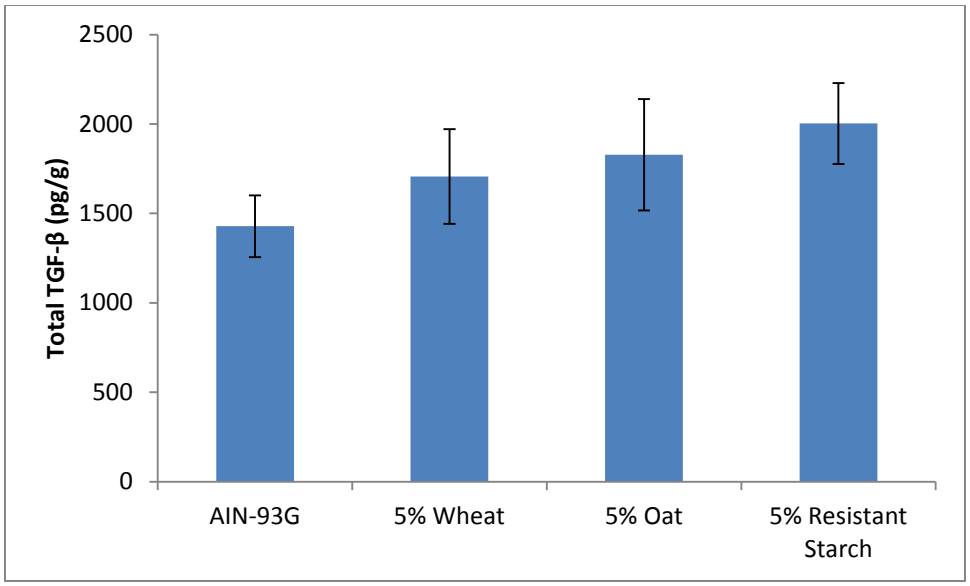

Figure 22. Quantification of total (active+latent) TGF-β (pg/g) within the MLN. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=4-6). There was no significant effect of diet, as determined by an ANOVA.

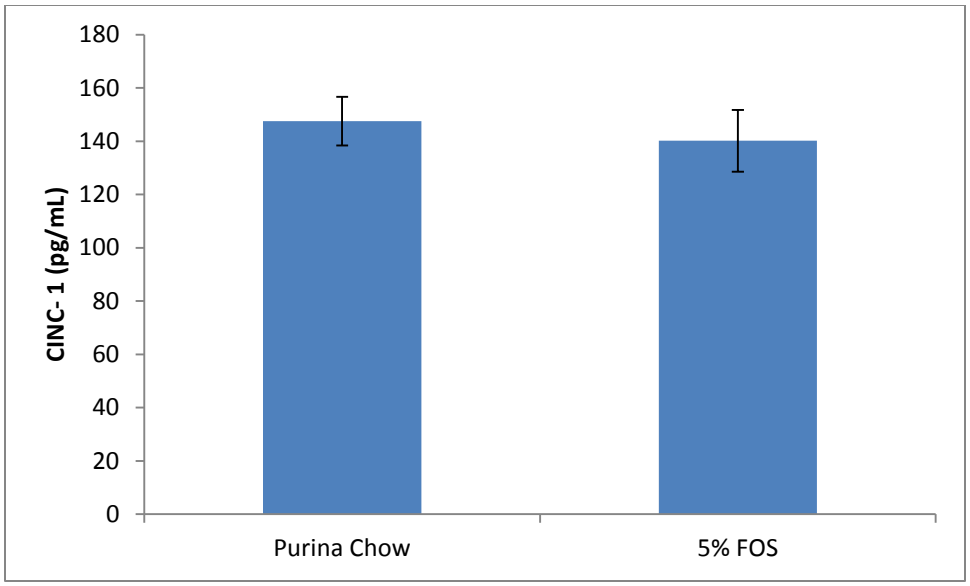

Figure 23. Quantification of CINC-1 (pg/mL) within the serum. Rats were fed either Purina chow alone or Purina chow supplemented with 5% FOS. The bars represent the mean ± SE (n=6). There was no significant effect of diets, as determined by a Student's t-test.

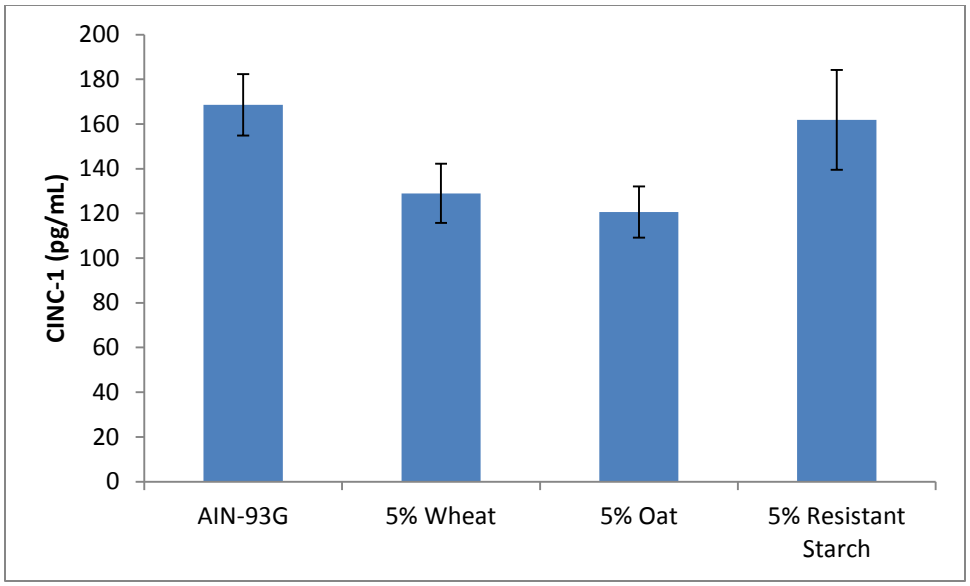

Figure 24. Quantification of serum CINC-1 (pg/mL). Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=5-9). There was no significant effect of diet, as determined by an ANOVA.

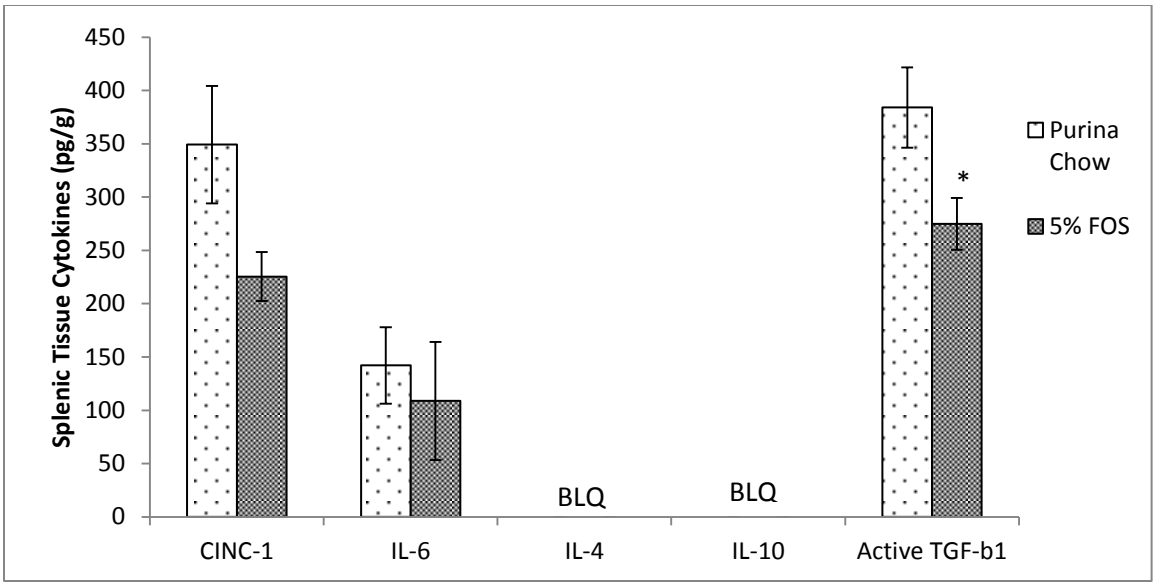

Figure 25. Quantification of CINC-1, IL-6, IL-4, IL-10 and active TGF-β (pg/g) within splenic tissue. Rats were fed either Purina chow alone or Purina chow supplemented with 5% FOS. The bars represent the mean ± SE (n=5-6). Both IL-4 and IL-10 cytokine levels were BLQ. There was no significance effect on CINC-1 and IL-6 levels between rats fed the different diets, as measured by a t-test. There was a significant difference in between rats fed Purina chow and rats fed the 5% FOS diet ($p<0.05$). Bars with asterisks denote significance (as determined by a Student's t-test).

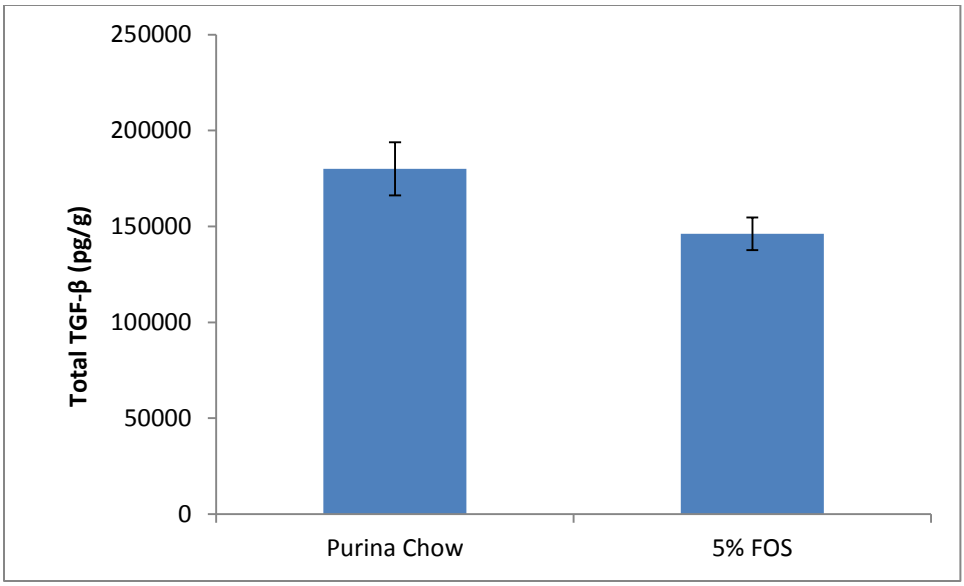

Figure 26. Quantification of total TGF-β (pg/g) within the spleic tissue. Rats were fed either Purina chow or Purina chow supplemented with 5% FOS. The bars represent the mean ± SE (n=5-6). There was no significance effect of diet, as determined by a student's t-test.

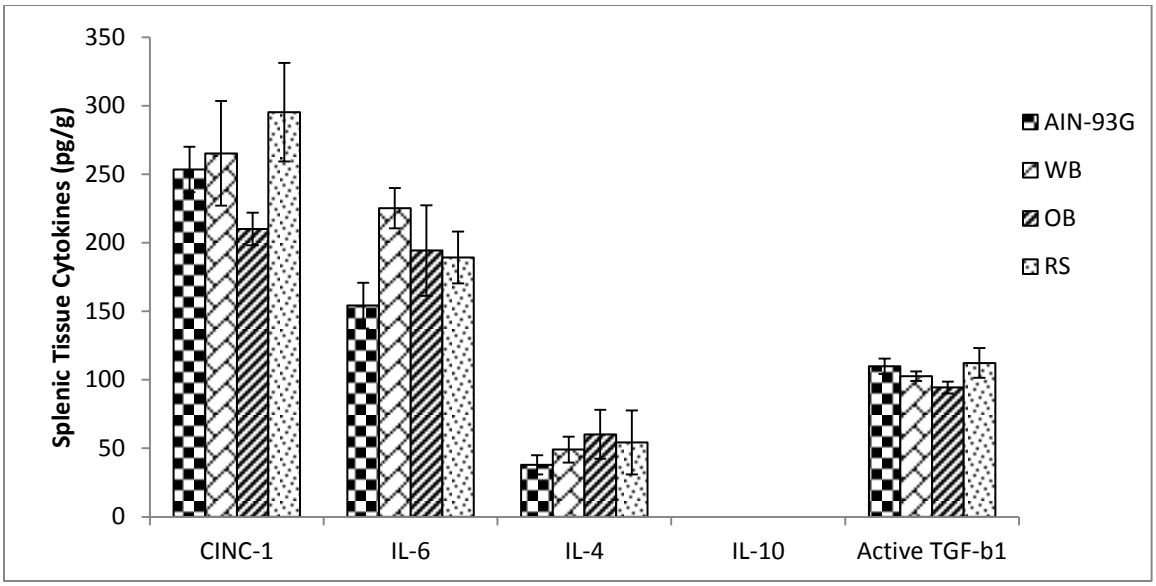

Figure 27. Quantification of CINC-1, IL-6, IL-4, IL-10 and active TGF-β (pg/g) within splenic tissue. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=5-6). IL-10 cytokine levels were BLQ. IL-4 was BLQ in the splenic tissue of one oat bran-fed rat. This rat was assigned a value of 0 pg/g. There was no significant effect of diet on any cytokine levels, as determined by an ANOVA and a Kruskal-Wallis test (for IL-4 values).

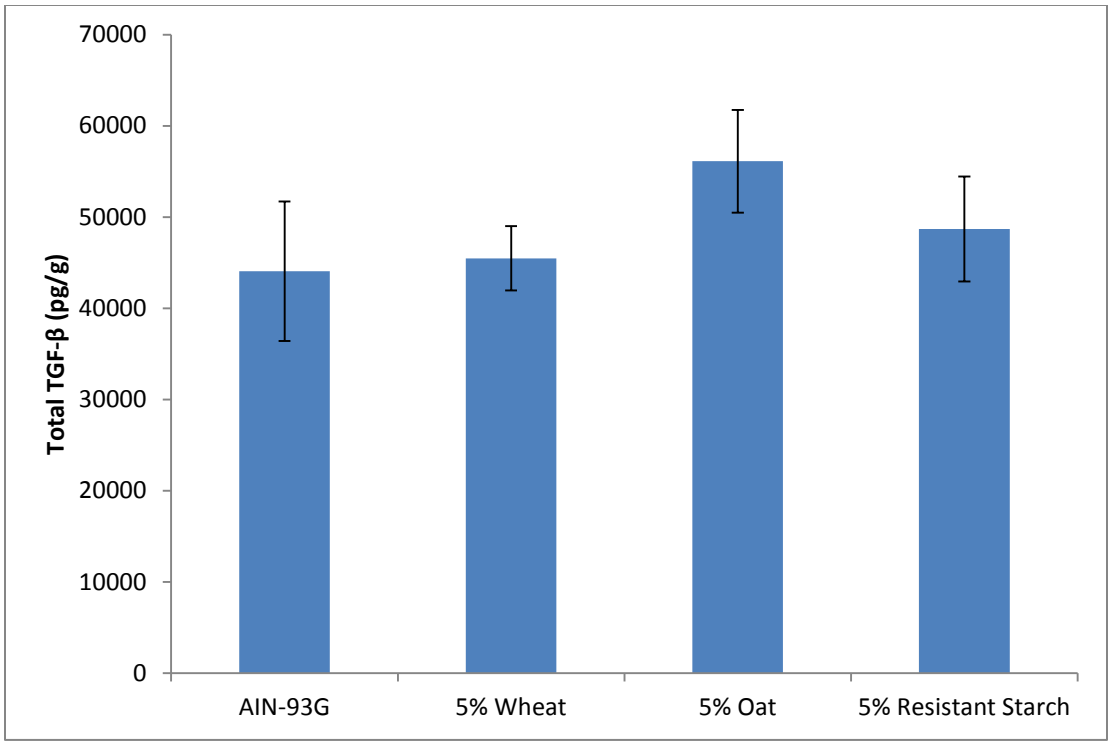

Figure 28. Quantification of total (active+latent) TGF-β (pg/g) within splenic tissue. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=5-6). There was no significant effect of diet, as determined by an ANOVA.

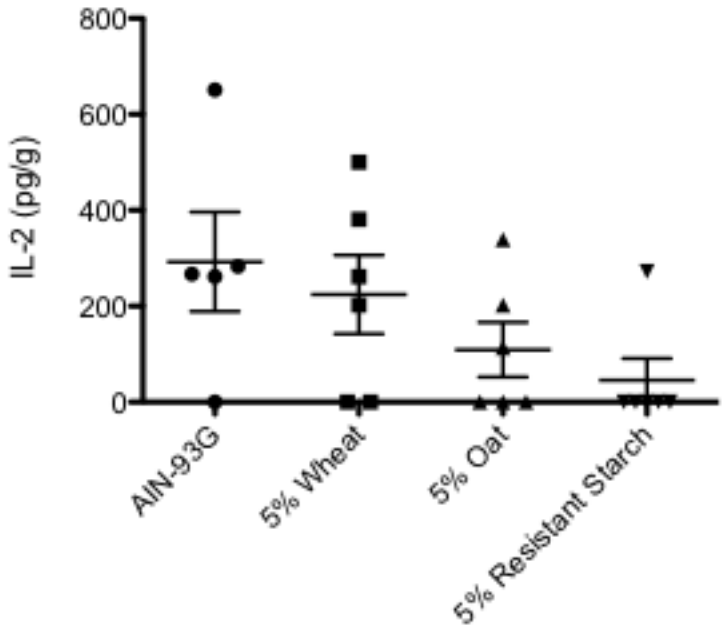

Figure 29. Quantification of IL-2 (pg/g) within splenic tissue. Rats were fed either AIN-93G alone or supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean and ± SE (n=5-6). Those rats that are 0 pg/g represent IL-2 in rats that were BLQ. There was no significant effect of diet, as determined by a Kruskal-Wallis test and Dunn post-hoc test.

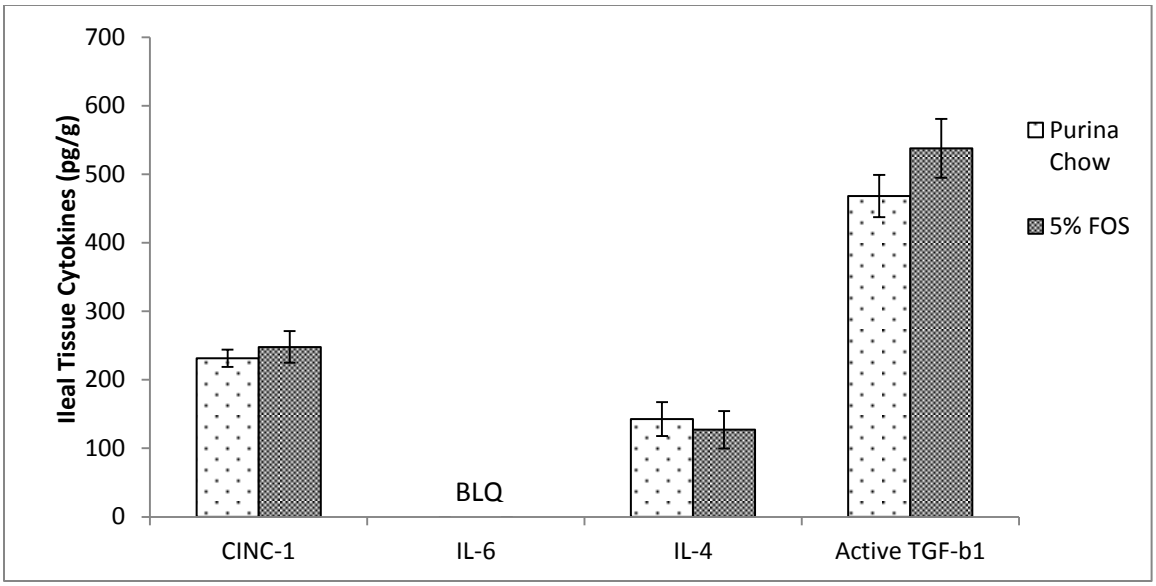

Figure 30. Quantification of CINC-1, IL-6, IL-4 and active TGF-β (pg/g) within the ileal tissue. Rats were fed either Purina chow or Purina chow supplemented with 5% FOS. The bars represent the mean ± SE (n=5-6). IL-6 cytokine levels were BLQ. There was no significance effect of the diets on any cytokine level.

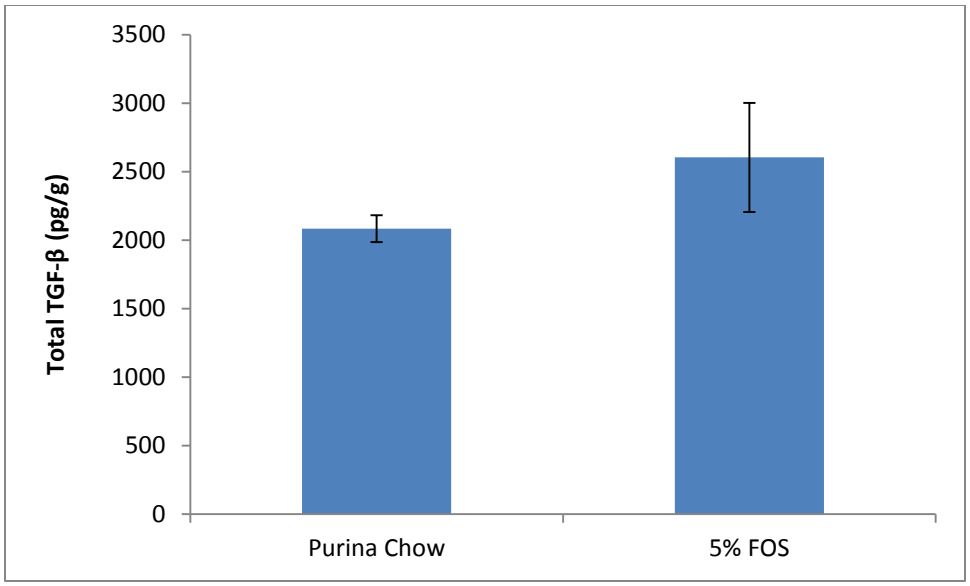

Figure 31. Quantification of total (active+latent) TGF-β (pg/g) within the ileal tissue. Rats were fed either Purina chow or Purina chow supplemented with 5% FOS. The bars represent the mean ± SE (n=6). There was no significance observed between the diets, as determined by a Student's t-test.

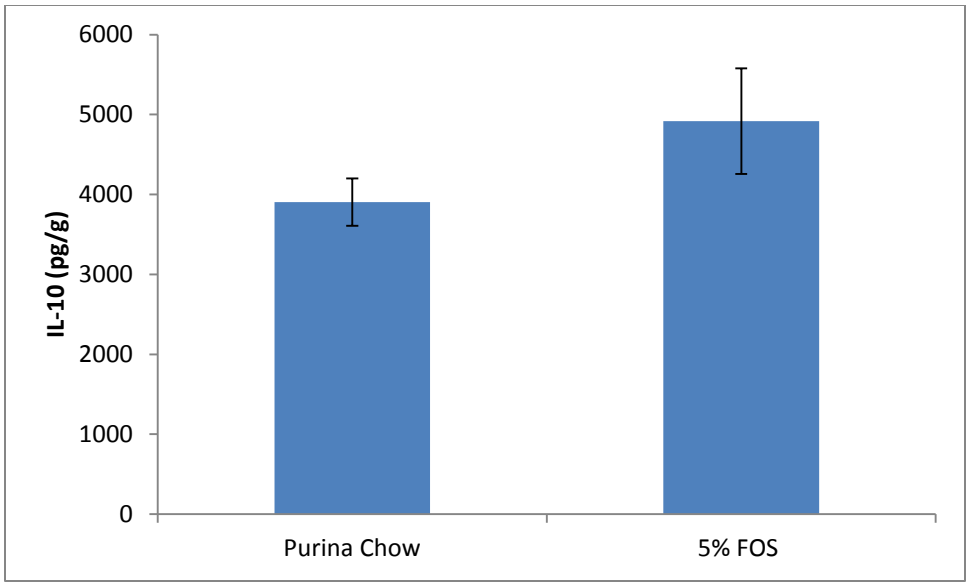

Figure 32. Quantification of IL-10 (pg/g) within the ileal tissue. Rats were fed either Purina chow or Purina chow supplemented with 5% FOS. The bars represent the mean ± SE (n=6). There was no significance effect of diet, as determined by a Student's t-test.

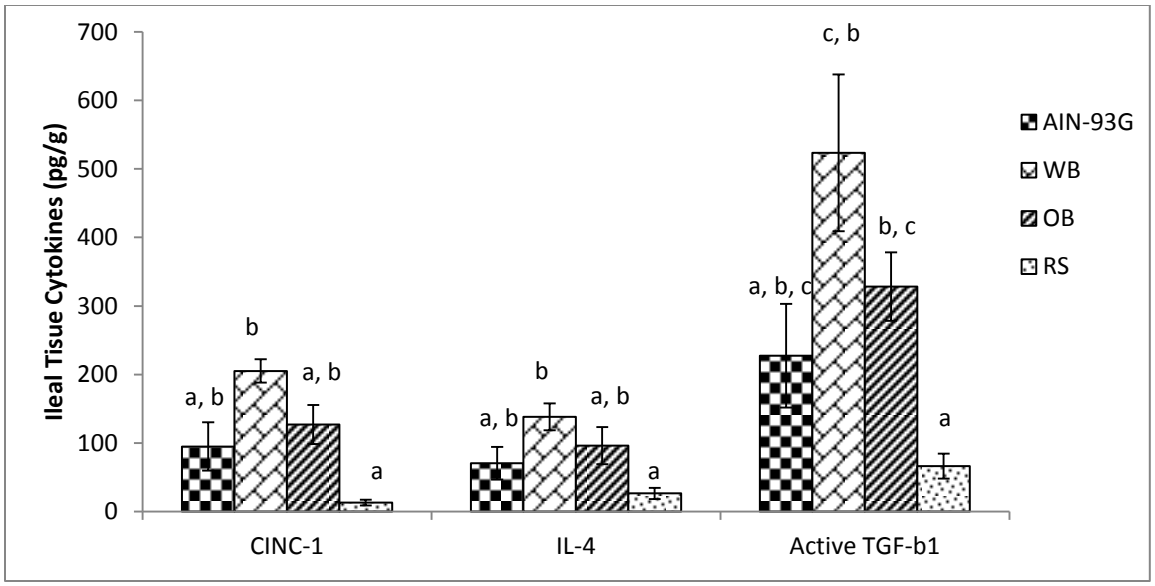

Figure 33. Quantification of CINC-1, IL-4 and active TGF-β (pg/g) within ileal tissue. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=4-8). There was a significant difference in CINC-1 levels between rats fed 5% wheat fibre and 5% resistant starch diets ($p<0.01$). IL-4 was BLQ in the ileal tissue for one AIN-93G and one resistant starch-fed rat. These rats were assigned a value of 0 pg/g. There was a significant difference in IL-4 levels observed between rats fed 5% wheat fibre and rats fed 5% resistant starch diets ($p<0.01$). Active TGF-β was BLQ in the ileal tissue of one AIN-93G and one resistant starch-fed rat. These rats were assigned a value of 0 pg/g. There were significant differences observed between rats fed 5% wheat fibre and 5% resistant starch diets ($p<0.01$), 5% oat fibre and 5% resistant starch diets ($p<0.05$). Bars with different letters are significantly different from each other (as determined by a Kruskal-Wallis test and Dunn post-hoc test).

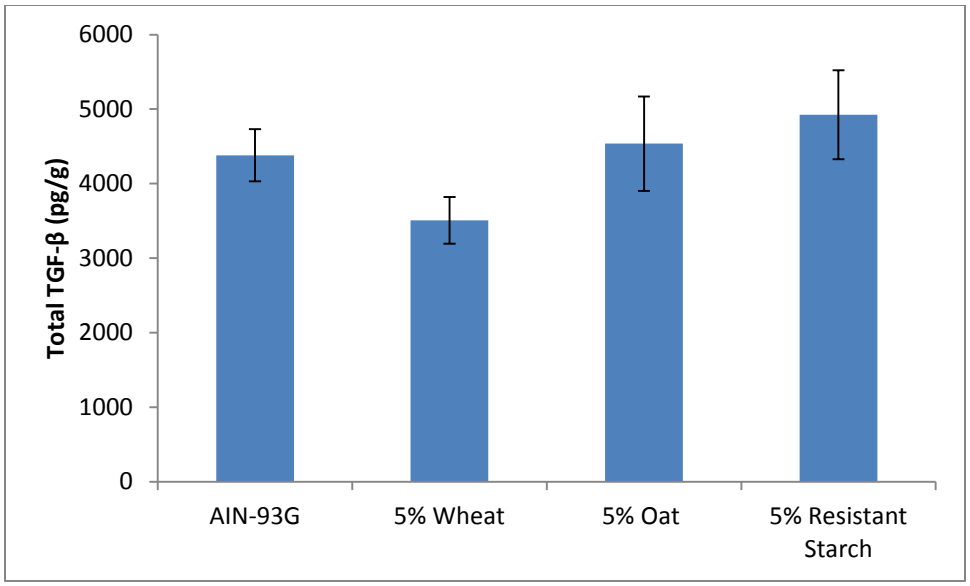

Figure 34. Quantification of total (active+latent) TGF-β (pg/g) within ileal tissue. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=6-8). There was no significant effect of diet, as determined by an ANOVA.

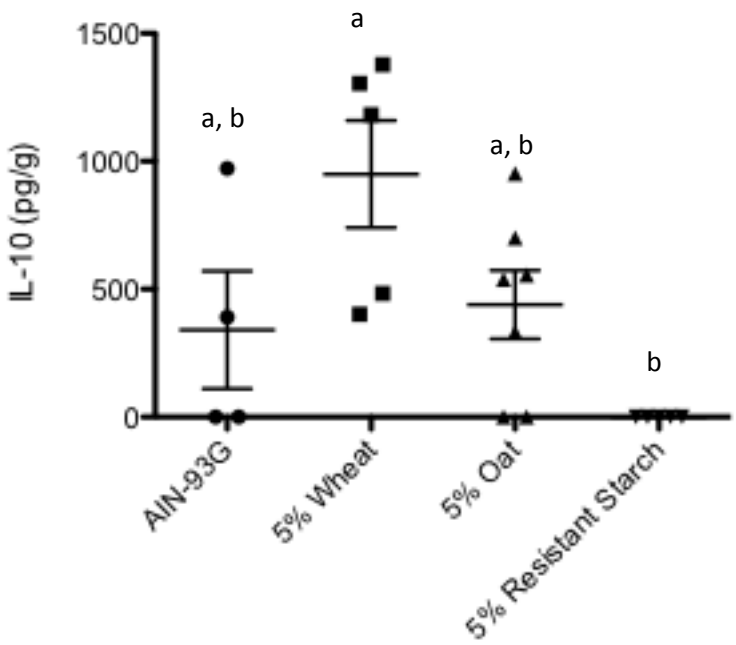

Figure 35. Quantification of IL-10 (pg/g) within ileal tissue. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=4-6). Those data points that were BLQ were assigned a value of 0pg/g. There was significance observed between 5% wheat-fed rats and 5% resistant starch-fed rats ($p<0.01$). Bars with different letters are significantly different from each other (as determined by a Kruskal-Wallis test and Dunn post-hoc test).

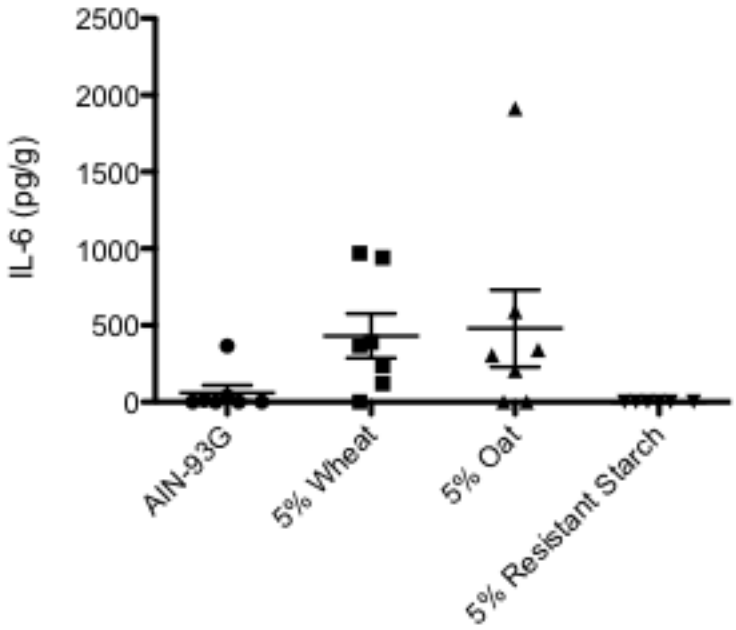

Figure 36. Quantification of IL-6 (pg/g) within ileal tissue. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean and ± SE (n=4-6). Data points that were BLQ were assigned a value of 0 pg/g. There was no significance observed between rats fed different diets (as determined by a Kruskal-Wallis test and Dunn post-hoc test).

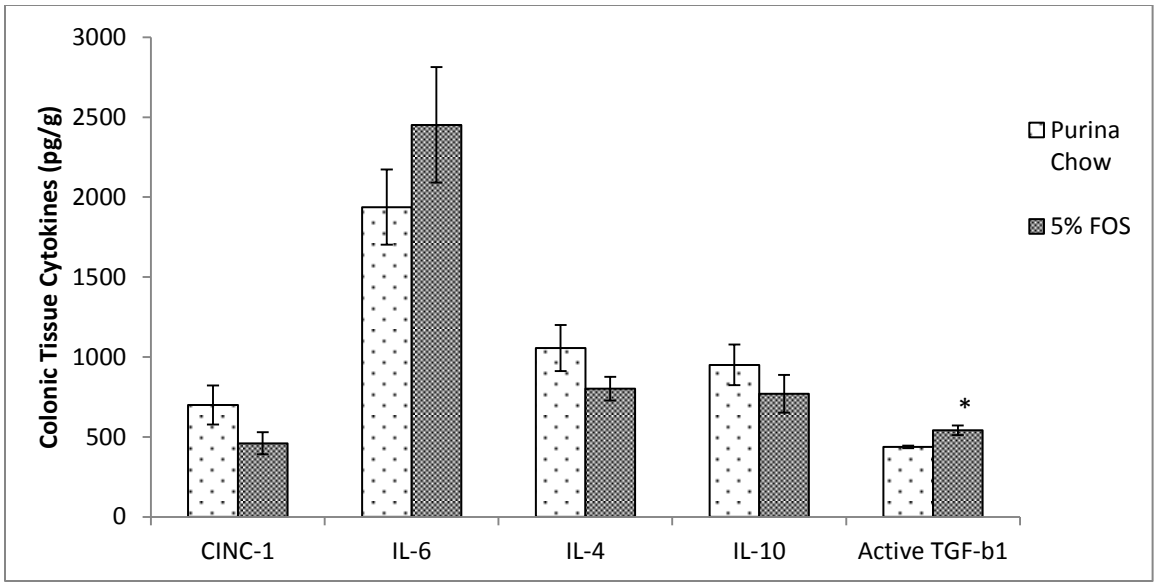

Figure 37. Quantification of CINC-1, IL-6, IL-4, IL-10 and active TGF-β (pg/g) in colonic tissue. Rats were fed either Purina chow or Purina chow supplemented with 5% FOS. The bars represent the mean ± SE (n=3-7). There was no significant difference in CINC-1, IL-6, IL-4 and IL-10 levels between rats fed different diets. There was a significant difference in active TGF-β between rats fed Purina chow and rats fed the 5% FOS diet ($p<0.05$). Bars with asterisks denote significance (as determined by a Student's t-test).

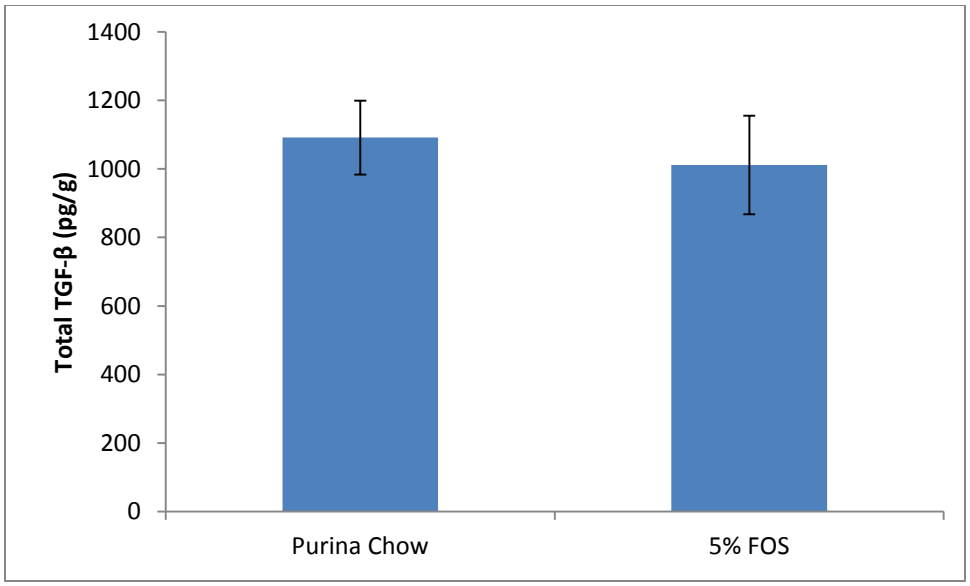

Figure 38. Quantification of total (active + latent) TGF-β (pg/g) within colonic tissue. Rats were fed either Purina chow or Purina chow supplemented with 5% FOS. The bars represent the mean ± SE (n=5-6). There was no significant effect of diet, as determined by a Student's t-test.

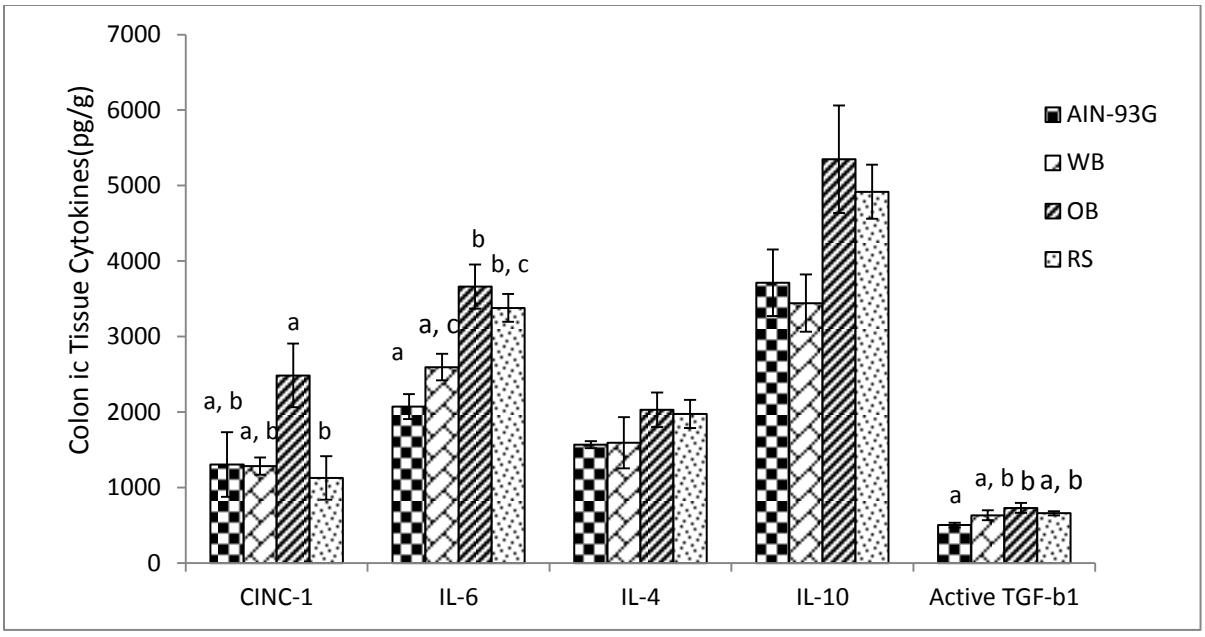

Figure 39. Quantification of CINC-1, IL-6, IL-4, IL-10 active TGF-β, and IL-4 (pg/g) within colonic tissue. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=3-8). There was a significant difference in CINC-1 levels between rats fed 5% oat fibre and 5% resistant starch diets ($p<0.05$). There was a significant difference observed between rats fed 5% oat fibre and rats fed AIN-93G diets ($p<0.01$), 5% resistant starch and AIN-93G ($p<0.01$) and 5% oat fibre and 5% wheat fibre diets ($p<0.05$). There was no significant difference in IL-4 and IL-10 levels between rats fed different diets. There was a significant difference in active TGF-β levels between rats fed 5% oat fibre and rats fed AIN-93G diets ($p<0.05$). Bars with different letters are significantly different from each other (as determined by ANOVA and Bonferroni post-hoc test).

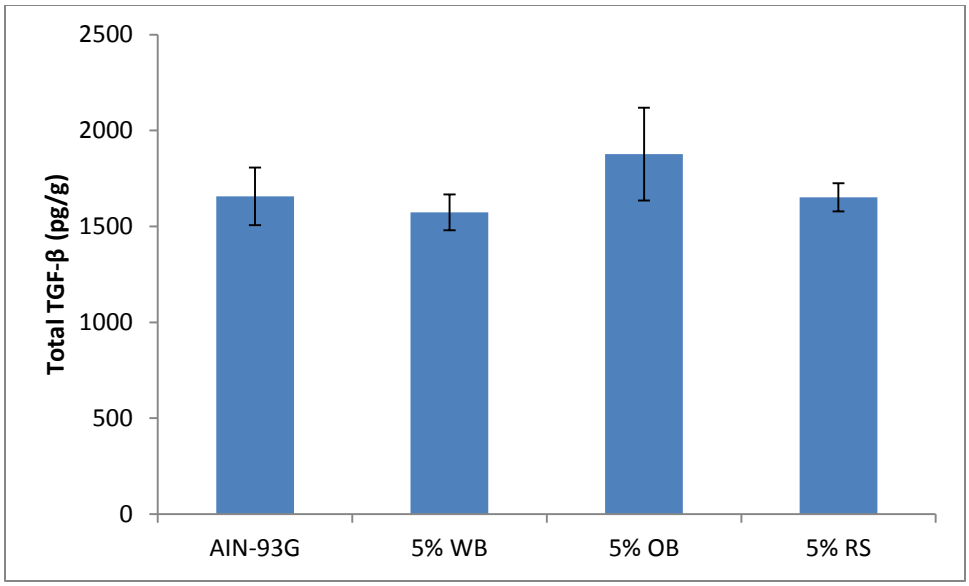

Figure 40. Quantification of total (active+latent) TGF-β (pg/g) within colonic tissue. Rats were fed either AIN-93G alone or AIN-93G supplemented with 5% oat fibre, 5% wheat fibre or 5% resistant starch. The bars represent the mean ± SE (n=3-6). There was no significant effect of diet, as determined by an ANOVA.

TABLES

Table 3. Viability of HT-29 IEC and IEC-6 IEC after incubation with *L. rhamnosus* R0011.

Incubated Components	6 hours	24 hours
	HT-29 IEC	
Viable R0011	0.20[1] (107%[2])	0.20 (81%)
Heat-killed R0011	0.19(102%)	0.23 (92%)
Negative control	0.19	0.25
	IEC-6 IEC	
Viable R0011	0.035 (116%)	0.07 (116%)
Heat-killed R0011	-	-
Negative control	0.03	0.06

[1]OD at 540nm

[2]Percent viable relative to control

Table 4. IL-1β induced IL-8 production by HT-29 IEC pre-incubated with *L. rhamnosus* R0011 for 18 hours.

Incubated Components	IL-8 (pg/mL)[1]
IL-1β	804.89
Viable R0011+ IL-1β	565.88
Heat-killed R0011+IL-1β	889.59
Viable R0011	10.81
Heat-killed R0011	37.367
Negative Control	9.96

[1]Average of 2 replicates

Table 5. IL-8 induction from HT-29 IEC pre-incubated with TLR agonists.

Cytokine/Proinflammatory Inducer	Viable R0011+Inducer	Heat-killed+Inducer	Positive Control	Negative Control	Viable R0011 alone	Heat-killed R0011 alone
TNF-α[1]	570.87[3]	774.76	739.60	4.43	5.27	5.47
IL-1β	382.89	445.10	303.93	4.43	5.27	5.27
Poly::IC	433.34	467.48	417.87	3.94	3.39	5.04
LPS[2]	20.78	23.74	21.57	11.62	7.13	9.58
Flagellin	41.56	60.56	38.45	4.43	5.27	5.47

[1]N of 1

[2]N of 2

[3]pg/mL

Table 6. IL-8 and CINC-1 induction from HT-29 IEC and IEC-6 IEC, respectively, in response to zymosan A after 24 hours of incubation.

Zymosan A Concentration[1]	**IL-8 from HT-29s**[2]	**CINC-1 from IEC-6s**[3]
0	BLQ[4]	612.61
5	BLQ	979.27
10	BLQ	1220.51
20	BLQ	1326.67
50	BLQ	1512.62
100	BLQ	1632.14
200	BLQ	1650.38
500	BLQ	1627.94

[1]ng/mL

[2]pg/mL

[3]pg/mL

[4]Below level of quantification

Table 7. CINC-1 induction from IEC-6 IEC when co-incubated with *L. rhamnosus* R0011 and zymosan A after 24 hours of incubation.

Incubated Components	CINC-1[2]
Zymosan A[1] positive control	2827.32
Viable R0011+zymosan A	1335.19
Heat-killed R0011+zymosan A	3227.19
Viable R0011 alone	176.04
Heat-killed R0011 alone	884.00
Negative control	521.07

[1]Incubated at 100 ug/mL

[2]ng/mL

DISCUSSION

Analysis of Anti-Inflammatory Effects of Lactobacillus Strains on Rat and Human IEC

The term ‘probiotic’ in recent years has become a household term. However, many people do not truly know what a probiotic is, or the ways in which they confer health benefits. The current definition used by Health Canada and the World Health Organization (WHO) to define a probiotic is “live microorganisms which when administered in adequate amounts confer a health benefit to the host." The health benefits attributed to probiotics include colonization resistance, enhancement of mucin production, preventing the binding of pathogens and, treatment of diarrhea [43]. The WHO definition stresses that a probiotic must be living in order to confer its health benefits within the host organism. However, probiotic strains that have been killed and administered to the host also have been observed to exhibit physiological effects [44]. In a study by Li *et al.* (2009), both heat-killed and viable *L. rhamnosus* GG demonstrated the ability to decrease CINC-1 in the liver and plasma of LPS-challenged rats [44]. Similarity, the results here demonstrate a decrease in proinflammatory cytokines produced by human and rat IEC when incubated with viable *L. rhamnosus* R0011 and proinflammatory inducers. In comparison, there was no significant decrease of proinflammatory cytokine production by human and rat IEC when incubated with proinflammatory inducers and heat-killed R001. However, the overall effect of heat-killed *L. rhamnosus* R0011 produced a similar pattern to that seen with viable *L. rhamnosus* R0011, as heat-killed R0011 decreased CINC-1 and IL-8 production by rat and human IEC, respectively, though the effect was not as marked as with the viable *L. rhamnosus* R0011. This pattern demonstrates some efficacy of heat-killed *L. rhamnosus* R0011 to alter proinflammatory cytokine induction from IEC. However, this pattern was not apparent when IEC were incubated with heat-killed *L. rhamnosus* R0011 for longer periods of time

(24hr), in the absence of a standard pro-inflammatory inducers. In fact, an increase in constitutive CINC-1 and IL-8 production by IEC was observed when incubated with heat-killed *L. rhamnosus* R0011 for 24 hours. This could suggest that the heat-killed bacteria are unable to produce an immune altering bioactive factor produced by viable *L. rhamnosus* R0011. Therefore, exposure to PAMPS on heat-killed *L. rhamnosus* R0011 bacteria, over the incubation time, increases IEC proinflammatory output, which is not down-regulated, due to the absence of the bioactive factor. This, however, does not explain the effects of heat-killed *L. rhamnosus* on IEC during co-incubation studies. Perhaps, the decrease in IL-8 and CINC-1 observed during co-incubation with proinflammatory inducers and heat-killed *L. rhamnosus* is due to a surface compound interacting with the IEC. This surface compound could be loosely bound to the cell membrane, so a decrease in CINC-1 and IL-8 is observed when HT-29 or IEC-6 IEC is treated with heat-killed *L. rhamnosus* R0011. Perhaps viable *L. rhamnosus* is able to produce more of this membrane factor, and so viable *L. rhamnosus* R0011 are more effective at decreasing proinflammatory cytokine production. In addition a loosely associated membrane factor could also be released into culture medium, which would account for effects observed with the cultured media of this strain. Pre-incubation of HT-29 and IEC-6 IEC with *L. rhamnosus* R0011down regulated IL-8 and CINC-1 production, respectively, more so than did co-incubation with *L. rhamnosus* R0011. Extended incubation times of viable *L. rhamnosus* R0011 with HT-29s seemed to have detrimental effects. Viability itself did not seem affected; however there was reduced IEC growth after 24 hours in comparison to control wells not containing viable *L rhamnosus* R0011. The lack of growth by HT-29 IEC could be due to the drop in pH of wells containing viable bacteria, due to the increase in lactic acid and SCFAs produced by viable *L. rhamnosus* R0011. Interestingly, Mariadason *et al*. (2001) demonstrated that butyrate caused

differentiation and apoptosis in Caco-2 IEC [45]. Butyrate has also been demonstrated to cause apoptosis in HT-29s [46]. However, butyrate has differential effects on colon cancer cells lines versus IEC *in vivo*. While the HT-29 IEC cell line is a well-established model for study of human IEC, it is a colon cancer cell line, while the rat IEC-6 is primary cell line. This difference in response to butyrate might explain why the HT-29 IEC were affected by the presence of *L. rhamnosus* and the IEC-6 cell line was not.

Based on the numerous modes of action reported for probiotics, current evidence suggests their activity can differ greatly between genus, species and even strain of bacteria. This could explain why multistrain probiotics are often more effective at altering immunity, as more immune-related pathways would be affected [47]. While many probiotics have been demonstrated to alter the pathways involved with NF-κB-mediated signaling, some more novel pathways are being exposed. For example, Hörmannsperger *et al.* (2009) demonstrated that a strain of *Lactobacillus casei* induced posttranslational degradation of the chemokine IP-10 (CXCL-10) by TNF-α-stimulated Mode-K cells (HEK293), but the expression and synthesis of IP-10 was not affected [48]. However, the results of the pre-incubation with proinflammatory stimuli suggest that this is not the way in which *L. rhamnosus* R0011 decreases IL-8 production, as no decrease in IL-8 was observed when HT-29s were first treated with TNF-α, followed by addition of viable *L. rhamnosus* R0011.

The bacteria *Lactobacillus rhamnosus* GG has been studied with great intensity due to the associated claims of improved host health. It is, arguably, the most intensely studied probiotic strain to date. *L. rhamnosus* GG has demonstrated the ability to decrease chemokine expression, prevent the detrimental effects of *E. coli* O157:H7 on IEC, as well as prevent apoptosis in IEC. This probiotic confers its effects, in part, by blocking NF-κB activation [49]. Though *L.*

rhamnosus GG has been demonstrated to secrete bioactive immune altering peptides [50], the mechanisms behind its probiotic effects are still unclear. Sánchez et al (2009) has developed a method for identifying proteins secreted from probiotic strains. Their approach involves growth of the Lactobacillus strain in MRS broth, then precipitating the proteins followed by analysis using tandem MS/MS [51]. From this approach, his group has identified novel proteins secreted by several Lactobacillus strains. Specifically, this study determined that *Lactobacillus rhamnosus* GG secreted a cell wall hydrolase and also glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The cell wall hydrolase was linked with the ability of the strain to prevent damage to the epithelial cell layer, while the authors suggest GAPDH is involved in adhesion to intestinal receptors [52]. Using an alternative method, Yan *et al.* (2007) identified two proteins secreted by *L. rhamnosus* GG, which they termed p75 and p40. These proteins reduced damage in IEC caused by TNF- α and decreased TNF-α induced IEC apoptosis [53].

There has been speculation that some probiotics also alter immune responses through a TLR2-mediated pathway. This has been demonstrated for a variety of different probiotic strains. Shimazu *et al.* (2012) used a strain of *Lactobacillus jensenii*, which down regulated proinflammatory cytokine production and NF-κB activation from porcine IEC stimulated with enterotoxigenic *E. coli* (ETEC) or LPS. These investigators later showed that the protective ability of *Lactobacillus jensenii* was partially TLR2 mediated [54]. Similarly, Ciorba et al. (2012) investigated the effects of the probiotic *L. rhamnosus* GG and its conditioned media on radiation damage in the gut. These investigators found that both the bacteria and its conditioned media protected from epithelial apoptosis, and also lead to improved crypt cell survival in wild type mice. However, the protective effects of *L. rhamnosus* GG and its conditioned media were lost in MyD88$^{-/-}$, TLR2$^{-/-}$ and COX-2$^{-/-}$ (cyclooxygenase-2 $^{-/-}$) mice [55]. This demonstrates that

the bacterium is conferring its effects through a TLR2 mediated pathway. Similarly, secreted factors from probiotic strains have also shown the ability to interact with DCs. Hoarau *et al.* (2006) demonstrated that supernatants from *Bifidobacterium breve* caused DCs to mature and in doing so increased expression of CD83, CD86 and HLA-DR (human leukocyte antigen-DR) through a TLR2-dependent mechanism [56].

A key TLR2 agonist, located on the cell wall of gram positive bacteria, is lipoteichoic acid. Thus, if probiotic bacteria are exerting effects through the TLR2 pathway, it could be due to the presence of this TLR2 agonist in their cell wall. However, other evidence casts doubt on the role of TLR2 in anti-inflammatory effects of probiotics. A study by Claes *et al*. (2010) examined effects of knocking out a gene involved in the synthesis of lipoteichoic acid (resulting in loss of cell wall D-alanine esters) in *L. rhamnosus* GG. Wild-type (wt) *L. rhamnosus* GG had no anti-inflammatory effect in mice with dextran sodium sulphate (DSS) induced colitis. In fact, wt *L. rhamnosus* GG increased the disease severity. However, the knockout strain of *L. rhamnosus* GG demonstrated the ability to decrease disease severity by increasing body weight (in 3% DSS treated mice) and decrease inflammation (in 1% DSS treated mice). This demonstrates that the anti-inflammatory effects of *L. rhamnosus* GG were not due to lipoteichoic acid [57].

Zymosan A, a TLR2 agonist, demonstrated the ability to decrease CINC-1 production from IEC-6s. In comparison, HT-29s were unresponsive to zymosan A, producing no IL-8 upon stimulation. The discrepancy in the response to zymosan between HT-29s and IEC-6s could be due to differential expression of the TLR2 receptor on the apical surface between cell lines and/or species. Interestingly, *L. rhamnosus* R0011 decreased production of CINC-1 from zymosan A treated IEC-6s. Although TLR stimulation typically leads to NF-κB activation, this effect of zymosan is interesting, due to the fact that some probiotics have demonstrated the

ability to decrease proinflammatory responses through the TLR2 receptor. This further suggests that the mechanism used by *L. rhamnosus* R0011 to decrease CINC-1 production by IEC-6 and is not TLR2 mediated.

Neither IEC-6s nor the HT-29 IEC responded to CpG DNA, a TLR 9 agonist. This lack of response to CpG DNA has been documented previously in bronchial epithelial cells, where IL-8 was only induced when CpG acted synergistically with IL-1β [58]. Logically, the abundance of CpG within the gut would be astronomical; therefore perhaps IEC are desensitized to this agonist or TLR9 expression is low, to prevent constant inflammation in the gut.

An increase in TNF-α production was observed from IEC-6s after 24 hours of incubation with viable *L. rhamnosus* R0011. This may suggest that this bacterium has proinflammatory effects on IEC in the absence of other stimuli. Production of TNF-α from mammalian cells has been observed with several probiotic strains. For example, strains of the probiotic *Bifidobacteri*a have demonstrated the ability to significantly increase TNF-α and IL-6 production by macrophages [59]. This method of immune alteration, in an *in vivo* model, would be effective in increasing host responses to pathogens, by recruiting other immune cells, and would be most effective if the bacterium or its by products were to cross into the PP. Interestingly, a decrease in TGF-β production was observed in IEC-6s treated with heat-killed IEC-6s, which corresponds with the increases in CINC-1 production observed after 24 hours of incubation with *L. rhamnosus* R0011.

The effects of *L. rhamnosus* R0011 on cytokine production from HT-29 and IEC-6 IEC could be due to adherence to IEC, as demonstrated by Wallace *et al.* [40]. The anti-inflammatory effects may not only due to a secreted factor, but, also the direct interaction between bacteria and

IEC. However, a study has demonstrated that there is no link between probiotic efficacy and adhesion properties [60], giving further weight to evidence suggesting that at least certain strains of probiotics exert their effects through secreted factors.

Effects of Prebiotics on Mucosal and Systemic Immunity

The use of the term prebiotic has become more popular in recent history. Prebiotics are claimed to reduce the incidence of strokes, diabetes, obesity, etc. [61]. One of these claimed effects is the alteration of the immune system, in a beneficial sense. However, the current definition of a prebiotic does not include effects on the immune system. This is partially due to the unknown mechanisms through which prebiotics confer their effects on the host. The effects of prebiotics must be defined in order to better understand the mechanisms in which they act, to validate claims of their health benefits, and to administer them more effectively. Previously, dietary fibres have demonstrated the ability to increase $CD4^+$ T cell numbers in the MLN, increase IgA secretion in cecum, and increase the number of PPs, to provide as a few examples [59]. The results here however, demonstrate a novel aspect of dietary fibre, not demonstrated previously.

Dietary FOS alters systemic immune parameters

The spleen was investigated for effects of dietary fibre on systemic immunity. This tissue has a major role in the mammalian immune system. It is a secondary lymphoid tissue that harbours vast amounts of both mature and immature T and B cells. It is a site a where antigens meet immature T and B cells and activation and maturation of these cells occurs. Thus, a great amount of immunoglobulin and cytokine production occurs in the spleen [62]. These functions make the spleen a tissue of interest for investigating diet-based changes in immune activity.

Interestingly, FOS-fed rats did have a change in active TGF-β levels in the spleen. TGF-β concentrations decreased in comparison to rats fed the corresponding control diet, Purina chow. The decrease in TGF-β levels could be indicating that FOS has a proinflammatory systemic effect, reflected in lower levels of this regulatory cytokine. Previous studies have demonstrated that FOS can have proinflammatory qualities in the spleen. In a series of experiments by Benyacoub *et al.* (2008) it was demonstrated that a FOS-supplemented diet increased proinflammatory cytokine output (IL-12, INF-γ and TNF-α) from mouse splenocytes challenged with LPS. This study also demonstrated the efficacy of using FOS as an adjuvant for a salmonella vaccine, where these proinflammatory properties could be an advantage [63]. Currently, there are patents for using FOS as an adjuvant.

There were no significant effects of wheat bran, oat bran and resistant starch supplemented diets on the spleen with respect to cytokine concentrations in this study. There is very little research to date which delves into the effect of these specific fibres on systemic immunity. Most prebiotic fibre research focuses on the effects of inulin and FOS. However, a study by Yamazak *et al.* (2008) did examine the effects of wheat bran extracts on peripheral blood mononuclear cells from human blood. This study demonstrated that that the wheat fibre by its self (no fermentation) increased production of IL-10 and inhibited Concanavalin A (Con-A)-induced production of IL-5, IL-13 and INF-γ. The induction of IL-10 was TLR4 dependent, indicating that wheat contains some type of LPS-mimicking compound [64]. Therefore SCFA independent and gut microbiota independent effects of dietary fibre on immune parameters are possible.

Wheat bran, oat bran, resistant starch and FOS alter mucosal immune parameters

The dietary fibres used in the present study demonstrated the ability to alter immune parameters at a mucosal level, with fibre-dependent differences observed with respect to control diets or between different fibre-supplemented diets. Mucosal tissues are those that provide a barrier for the microbiota, and so were a key location examined for effects on the immune system. The mucosal tissues investigated were the ileum, colon and MLN. The ileum is the lower portion of the small intestine, the colon is the large intestine and the MLN is a lymph node, responsible for draining the MALT [65]. Appendix 1 depicts the layout of the GI tract.

The MLN is a T cell and B cell rich node allowing sampling of antigen from the gut. This is a key location for T and B cells to undergo activation and proliferation once they have encountered antigen within the gut [66]. The MLN also serves as a key location to "firewall" gut bacteria, using sIgA to prevent further translocation of commensals from the MLN into systemic locations [65]. Changes in T cell or APC activity as reflected by the MLN cytokine profile can thus serve as a useful indicator of potential effects of dietary fibre on the mucosal immune system. Within the MLN, the resistant starch diet significantly increased production of active TGF-β in comparison to all other diets and control. This suggests that resistant starch is inducing an anti-inflammatory effect in this tissue, an action that could possibly translate into increased T_{reg} cell maturation [67]. Effects of a resistant starch-supplemented diet on mucosal immunity and specifically on the MLN have been previously explored. Resistant starch has demonstrated the ability to significantly increase the $CD4^+/CD8^+$ ratio in the MLN [68]. This effect could correspond with an increase in TGF-β, similar to the observations outlined here with resistant starch-fed rats. In the same study, the authors documented increased IL-4 production from MLN lymphocytes when stimulated with Con-A. In a study by Bassaganya-Riera *et al.* (2011) it was

demonstrated that resistant starch could alleviate the symptoms associated with inflammatory bowel disease in IL-10 deficient mice. A resistant starch-supplemented diet decreased INF-γ production by $CD4^+$ T cells, increased IL-10 expression in the spleen, and also increased PPAR-γ expression in the colon [69]. The results here demonstrate the effects of resistant starch diet in rats at baseline levels, and have demonstrated the ability to significantly alter cytokine levels in the MLN. It would suggest that in a proinflammatory state, resistant starch might have the potential to counter inflammatory effects by increasing production of regulatory cytokines such as IL-10 and TGF-β.

The ileum is often associated with changes in gut immunity or homeostatic mechanisms. This is mostly due to the fact that surrounding the ileum are the PPs [66]. The PPs contain many germinal centres, up to 250, in which T and B cells are located, waiting for antigen presentation from antigen presenting cells or direct contact with bacteria and/or their secreted products [70] [71]. The PPs have similar functions as the MLN, except on a smaller scale. Rats fed the wheat bran supplemented diet had significant alterations in ileal cytokine profiles in comparison to rats fed the AIN-93G control diet and the other fibre diets. Significant increases in ileal concentrations of active TGF-β, CINC-1 and IL-4 were observed between rats fed wheat bran in comparison to those fed the resistant starch supplemented diets.

Collaborators have demonstrated that resistant starch diets in rats decrease bacterial load and diversity within the gut in comparison to wheat and oat diets, as well as in comparison to the AIN-93G control diet (personal communication, M. Kalmokoff). This could lead to less fermentation and decreased production of SCFAs. On the other hand, wheat bran significantly increases bacterial load and diversity [9] as well as increasing production of SCFA relative to control non-prebiotic diets [72]. The decrease in microbiota diversity and bacterial load

associated with resistant starch could explain the decrease in cytokines within the ileum observed in resistant starch-fed rats, while the increase with wheat bran can be attributed to the greater microbial load and microbiota diversity induced by this fibre. Although microbial load and diversity were measured in the colon, the effects still might have been great in the ileum. It does not, however, explain the effects of the resistant starch diet on the MLN of rats. Alternatively, wheat bran contains many food antigens, which could lead to an immune inducing effect and potentially a proinflammatory response. Related to this observation is the study by Yamazaki *et al.* (2008), which demonstrated the presence of a LPS-like molecule in wheat, which interacts with TLR4. Activation of TLR4 in the gut could lead to a proinflammatory response, as observed by an increase in CINC-1 [64].

The increase in IL-10 and active TGF-β levels in the ileum of rats fed wheat bran diets indicates that there is possibly an anti-inflammatory effect or response induced by the dietary fibre. The increase in ileal CINC-1 levels in rats fed wheat bran-supplemented diet could be due to homeostatic mechanisms and may not represent a proinflammatory response. The increase could also be linked to the increase in bacterial load observed previously with wheat bran fed mice [9]. If this were the case, more bacterial antigen and proinflammatory agonists would be present in the ileum, potentially leading to a proinflammatory response, and an increase in CINC-1.

In rats, between the ileum and colon, lies the cecum. The most fermentation occurs in this tissue, hence rats are known as ‘cecal fermenters’ [73]. Therefore it is possible that SCFA are produced from cecal fermentation can enter the colon and have immune altering effects at this location. The colon contains the most bacteria of all locations along the GI tract, suggesting that there would be considerable interaction between this tissue and the microbiota [74]. Rats fed the

oat-supplemented diet had higher colonic tissue levels of active TGF-β, IL-6, IL-10 and CINC-1 in comparison to the control, AIN-93G diet. Similarly, Metzler-Zebeli *et al.* (2012) found that a β-glucan diet increased the production of colonic IL-6 in pigs. The investigators attributed this increase in colonic IL-6 to the production of butyrate within the colon by the microbiota [75]. Interestingly, the study by Abnous *et al.* (2009) observed an increase in diversity and quantity of the microbiota in rats fed not only a wheat diet, but also an oat bran diet. As with the increase in diversity in wheat bran-fed rats, the difference in cytokine profiles in oat bran-fed rats could be attributed to this alteration in microbiota and greater production of SCFAs. The increase in colonic tissue levels of TGF-β and IL-10 would suggest that IL-6 has an anti-inflammatory role in this context, potentially leading to B and T cell maturation and antibody class switching, activities supported by IL-6 [76].

Similar to oat bran-fed rats, resistant starch-fed rats also had alterations in colonic cytokine levels in comparison to the AIN-93G control diet-fed rats. Increases were observed in colonic levels of IL-4, IL-10, IL-6 and TGF-β for resistant starch-fed rats in comparison to those receiving the control diet. Interestingly, the resistant starch diet increased these cytokine parameters without increasing CINC-1 production in comparison to control. In contrast oat bran-fed rats an increase in CINC-1. This suggests that perhaps wheat bran acts through a separate pathway than resistant starch. However, the proinflammatory effect may be needed in order to induce downstream effects in immunity.

The only statistically significant difference in cytokine levels observed between the FOS-fed rats and the Purina chow control-fed rats was in active TGF-β in the colon. This increase in colonic active TGF-β levels corresponded with a decrease in CINC-1 levels; however, this difference was not statistically significant. This suggests that within the colon, FOS may have an

anti-inflammatory effect. This indication has been demonstrated previously. Inulin, another type of fructan, has demonstrated the ability to lower disease severity and inflammation in chemically-induced colitis in rats. This anti-inflammatory effect corresponds with an increase in LAB numbers in the cecum as well as in production of colonic lactate and butyrate [77]. A similar study by Hoentjen *et al.* (2005) demonstrated that the combination of inulin and FOS (Synergy 1) decreased the colitis disease severity score in genetically predisposed (HLA-B27) 'colitis' mice. Similarly they observed increases in *Lactobacillus* and *Bifidobacerium* numbers in the cecum. Most importantly, they also found that a Synergy 1-supplemented diet decreased cecal IL-1β and increased cecal TGF-β [78]. These findings show a similar pattern to the decrease in colonic CINC-1 and increase in colonic TGF-β observed in our study.

There was a general pattern for cytokine production profiles in FOS-fed rats within the ileum. Greater production of most cytokines in ileal tissue was observed in FOS-fed rats compared to rats receiving the Purina chow control diet; however these differences were not statistically significant. The reason these cytokines measurements did not attain statistical significance could reflect certain properties of the background diet, Purina chow. Purina chow is a commonly used food source for experimental rodents. However, the composition is somewhat variable (changes between batches). In addition, Purina chow contains soy products, including flavonoids and estradiols. These compounds have been document to modify immune responses [79]. Therefore, Purina chow could potentially be masking immune alterations of FOS, which might otherwise be more evident, as observed with the other dietary fibres. In comparison the AIN-93G control has a known and precisely defined amount of carbohydrates, proteins, fat, vitamins, minerals [80]. AIN-93G also has a smaller amount of flavonoids and estradiols, which makes it more applicable to nutritional immunology studies. Further study of the effects of FOS

using the AIN-93G control diet would therefore be potentially valuable in delineating the true extent of its effects on mucosal and systemic immune parameters.

Overall, based on the results shown here, FOS seems to have some anti-inflammatory effects at the mucosal level, while however showing a proinflammatory effect at the systemic level. Previous research has demonstrated that this dichotomy in fact could be accurate. Wheat, oat and resistant starch, in comparison to control, have no effects systemically, but have very significant effects at the mucosal level. The results here seem to suggest that specific dietary fibres can target different gastrointestinal locations, depending on their fermentability and capacity to influence microbiota changes and diversity in the gastrointestinal tract.

However, the effects observed with the prebiotic diets may not all be due to bacterial fermentation and the resulting SCFA production. In a study by Zenhom *et al.* (2011) the effects of prebiotic fibres themselves, without the fermentation of bacteria was investigated. Prebiotics alone (oligosaccharides, α3-sialyllactrose and FOS) showed potential anti-inflammatory activity by reducing IL-12, IL-8, TNF-α and NF-κB expression as well as increased PPAR-γ expression in the Caco-2 human IEC cell line [81]. When prebiotics are ingested in combination with certain probiotic strains immune alterations have also been reported to occur. Roller *et al.* (2004) have demonstrated in rats that when prebiotics (inulin and FOS) and probiotics (*L. rhamnosus and B. lactis*) are combined they increased production of IgA in the ileum and decreased oxidative burst activity in blood neutrophil. However, prebiotics by themselves did not have these effects; instead they enhanced production of IL-10 in the PP and IgA in the cecum. This type of evidence suggests that combined delivery of probiotics and prebiotics has greater potential to influence the host immune system than delivery of either alone [82]. However, much remains to be learned about the effects of longer-term incorporation of prebiotics and dietary fibre into the diet.

Future Research

Future studies could examine the effect of *L. rhamnosus* R0011 on NF-κB specific cell pathways. The use of a cell line expressing specific TLR receptors and NF-κB activation reporter systems would be of use in this context. This approach would demonstrate which TLRs are essential for the effects observed with *L. rhamnosus* R0011, as well as confirm that NF-κB is indeed being down regulated in *L. rhamnosus* treated IEC. It would also be of interest to investigate whether the increase in TNF-α observed with viable *L. rhamnosus* R0011-treated IEC-6s is NF-κB dependent, by using NF-κB specific inhibitors. Certain strains of bacteria can influence the expression of TLRs by IEC and thus one could investigate whether *L. rhamnosus* R0011 influences TLR expression on HT-29s, IEC-6s, and on other cell types where TLR expression is important, such as macrophages and dendritic cells.

In addition, recent preliminary data suggests that *L. rhamnosus* R0011may release the neurotransmitter gamma-aminobutyric acid (GABA). Investigating the possible communication between bacteria and parasites within the gut could be of value as this is an understudied area of research, and may provide insight into interactions between the gut microbiota and parasites, and potentially into novel anti-parasite strategies. Lastly, investigating the effects of dietary fibre in challenged models (ex. immunized rats) could provide more information about dietary effects on immunity. Specifically, immunization would activate the typical events in an immune response, and so provide a useful approach to more fully delineate the effects of dietary fibre on immunity and potentially reveal the downstream effects of cytokine changes in mucosal tissues.

Conclusion

The *L. rhamnosus* R0011 strain has demonstrated the ability to decrease proinflammatory indicators to a large variety of selected proinflammatory inducers that would typically be encountered by the immune system. The current data suggests that this decrease in proinflammatory cytokine production is not due to post-translational degradation, and may reflect blocking of a cellular pathway upstream of translation.

In addition, the results shown in this thesis demonstrate that the effect of different dietary fibres is tissue specific. The data suggests that dietary resistant starch affects cytokine profiles in the MLN, wheat fibre affects cytokine profiles in the ileum and oat affects cytokine profiles in the colon. However, the potential overall health benefits of this alteration in cytokine levels remains to be determined.

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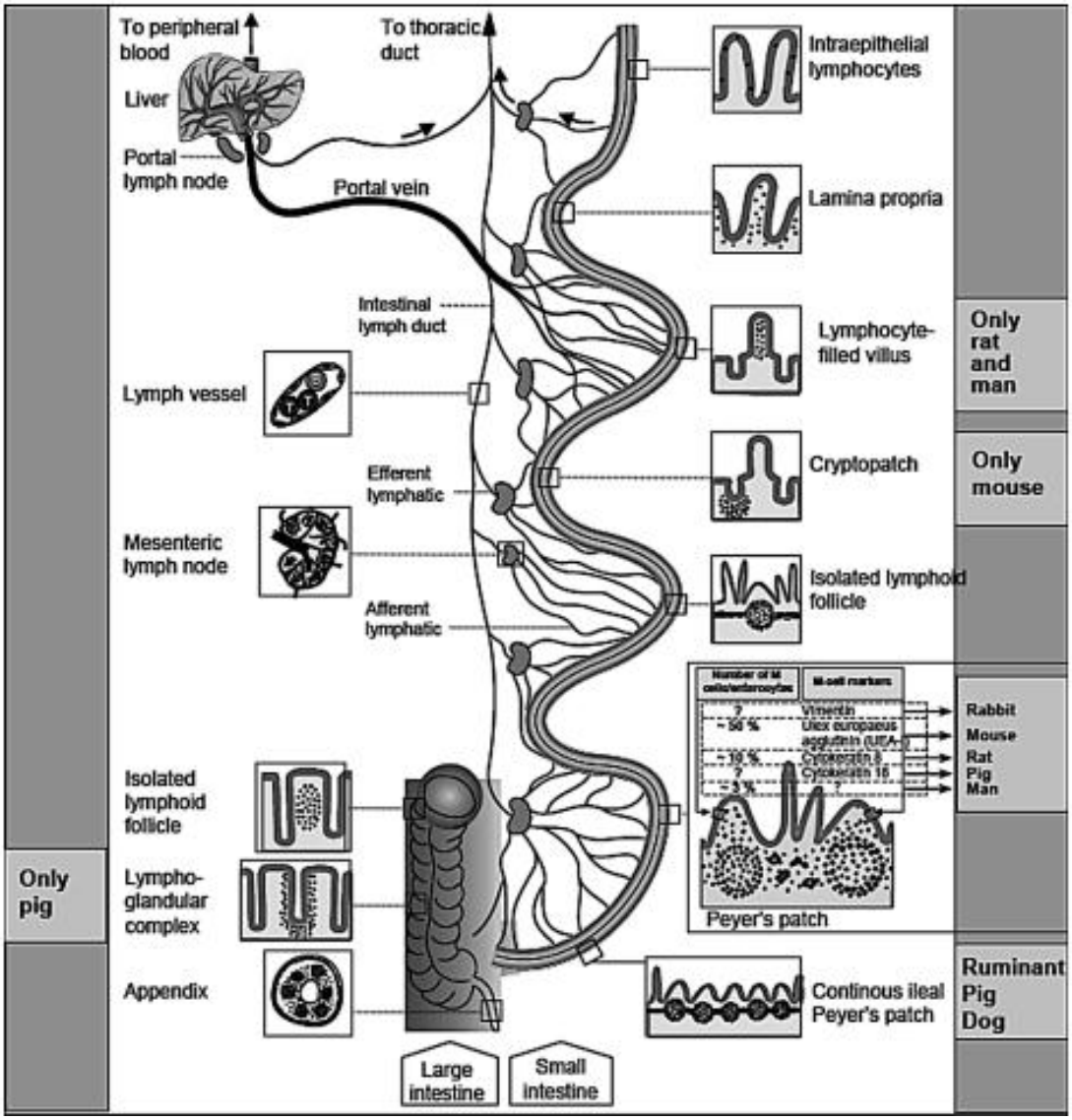
APPENDIX



From Brandtzaeg (2010) [70]