

**Immunomodulatory Effects of Dietary Fibre Supplementation: Effects on Cytokine  
and Antibody Production and Lymphocyte Population Profiles**

By

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## ABSTRACT

Gastrointestinal microflora has been shown to have a bi-directional relationship with the host immune system. A variety of fermentable carbohydrate polymers largely pass through the small intestine, providing fermentable substrates for gut microflora. Dietary fibre supplementation may provide a strategy for manipulating the intestinal bacterial profile, changing the interaction with the mucosal immune system, thereby modulating the host immune system. We used a BBc rat animal model to evaluate the effects of oat bran and wheat bran dietary fibre on the immune system. Previous collaborative efforts have shown that these dietary fibres can change the intestinal microflora, with wheat bran fibre showing a greater ability to influence colonic microbial community diversity. We have shown that dietary wheat bran fibre led to reduced IL-4 levels in the liver and T lymphocyte numbers in the Mesenteric Lymph Node and may be involved in reduced IgA levels in the cecal contents. In addition, IgA in the cecal contents was decreased while MLN B cell numbers increased in response to dietary wheat bran fibre. It was observed that neither wheat bran or oat bran treatments exerted any pro-inflammatory effects, with oat bran actually improving antioxidant status. These results suggest that both oat and wheat bran fibre treatments induce changes in the intestinal microflora, and that the microflora changes due to wheat fibre are associated with immunomodulatory effects on the host. This type of dietary fibre supplementation could ultimately provide a potential strategy for promoting health through microflora-associated effects on the immune system.

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## LIST OF ABBREVIATIONS

- APRIL – A proliferation inducing ligand
- ANOVA – Analysis of variance
- APC – Antigen presenting cell
- BAFF – B cell activation factor
- BBc – Biobreeding control rats
- BCMA - B cell maturation antigen
- BLyS – B lymphocyte stimulator protein
- BSA – Bovine serum albumin
- CCAC – Canadian council on animal care
- DC – Dendritic cell
- ELISA – Enzyme linked immunosorbant assay
- FBBG - Filamentous brush border glycocalyx
- FBS – Fetal bovine serum
- FOS – Fructooligosaccharides
- GALT – Gut associated lymphoid tissue
- GRAS – Generally regarded as safe
- HBSS – Hank’s buffered salt solution
- HRP – Horseradish peroxidase
- ICAM1 – Intracellular adhesion molecule 1
- IEC – Intestinal epithelial cells
- Ig - Immunoglobulin

IL-#- Interleukin

IFN $\gamma$  – Interferon gamma

KRPG – Krebs ringer phosphate buffer with glucose

LAB – Lactic acid bacteria

LP – Lamina propria

MEM – Minimal essential media

MLN – Mesenteric lymph node

NK – Natural killer cell

PBS – Phosphate buffered saline

PP – Peyer's patch

SE – Standard Error

SEM – Standard error of the mean

TACI - Transmembrane activator and calcium modulator and cyclophilin ligand activator

TC – Cytotoxic T cell

TGF – Transforming growth factor

TNF – Tumour necrosis factor

## INTRODUCTION

In recent years, there has been an increase in the focus on interactions between intestinal bacteria and the intestinal mucosal immune system and the impact on host health. The targeted interactions are often referred to as immunomodulatory effects. There are various measures for determining immunomodulatory effects, including cytokine analysis (Galdeano and Perdigon, 2004), antibody production (Roller *et al.*, 2004; Fung *et al.*, 2000), and lymphocyte proliferation (Mohamadzadeh *et al.*, 2005), as well as CD4 and CD8 T-cell lymphocyte ratios (Watzl *et al.*, 1999). Much of the research in this field has arisen from studies of the effects of probiotics, with additional studies addressing effects of prebiotics. Prebiotics have the potential for similar impact as probiotics, since they are based on utilizing the natural intestinal bacteria to achieve positive benefits. Probiotics can be defined as viable microorganisms, when administered in sufficient amounts, alter the intestinal microflora profile and confer a health benefit on the host organism (FAO and WHO, 2002; Galdeano and Perdigon, 2004; Dieleman *et al.*, 2003). Prebiotics are often described as non-digestible oligosaccharides (Morelli *et al.*, 2003) which have a positive impact on the host by stimulating particular bacteria in the intestinal tract (Roberfroid, 2000). A further adaptation of this research is the development of synbiotics, which are a combination of both probiotics and prebiotics (Morelli *et al.*, 2003; Le Leu *et al.*, 2005).

### *Bacterial presence in the gut*

There are over 400 species of bacteria living within the human gastrointestinal tract (Fang *et al.*, 2000; Simon and Gorbach, 1986). These intestinal bacteria have been shown to be critical to numerous aspects of host health, including effects on the immune system (Roberfroid, 2000) (which is the focus of my research), inflammatory bowel disease (Dieleman *et al.*, 2003), and even protection against toxins present in contaminated food (Watzl *et al.*, 1999). Many genera of Lactic Acid Bacteria (LAB) such as *Lactobacillus*, as well as *Bifidobacterium* species are present in the intestine (Isolauri *et al.*, 2001; Wallace *et al.*, 2003), and it is these genera on which most prebiotic and probiotic research has been performed. However, *Lactobacilli* and *Bifidobacteria* do not represent the majority of the colonic population, which includes several other genera, such as *Clostridia* and *Bacteroides*. Much of the colonic microflora is as yet unculturable (Eckberg *et al.*, 2005), and can be effectively identified and mapped only by current molecular methods.

It has been found that supplementation or modification of the intestinal microflora using probiotics can have beneficial effects on the host. In cases where the normal bacterial profile has been disrupted, replenishment of the natural intestinal microflora can help to limit negative effects, such as oral antibiotic therapy (Dieleman *et al.*, 2003). Not all cases of positive probiotic and prebiotic effects are the result of simply restoring microbial populations of the gut to normal levels however. Immunomodulatory benefits have also been observed from the supplementation of the existing microflora with additional bacteria. Supplementation with *Lactobacillus casei* and *Lactobacillus bulgaricus* has been reported to increase the activity of macrophages (Isolauri *et al.*,

2001), while *L. bulgaricus* and *Lactobacillus acidophilus* derived from yogurt have been shown to increase antibody production, specifically IgG and IgM (Mohamadzadeh, 2005). It has also been shown that supplementation with *Lactobacillus rhamnosus* GG can potentially modify levels of mucosal cytokine production (Dieleman *et al.*, 2003). However, in many cases, probiotic effects are strain specific. There has been some evidence that probiotic bacteria must be viable in order to stimulate cytokine and antibody production (Galdeano and Perdigon, 2004), although some debate surrounds this. Another issue is providing biologically relevant levels of probiotic bacteria, such as those found in yogurt, which currently provide the most common intake of probiotics, and which in some cases do not have sufficient quantities of cells to provide the expected beneficial effects (Akalin *et al.*, 2007). This means that other alternatives to providing probiotic bacteria as a supplement to enhance the immune system should be considered.

### *Prebiotic fibres*

Prebiotics have the potential to have a significant impact as an alternative to consumption of probiotics. The premise behind prebiotics is that they pass through the human digestive system undigested, and therefore can be used as substrates by intestinal bacteria. Examples of prebiotics include  $\beta$ -glucans from oats (Su *et al.*, 2007), inulin from chicory root (Roller *et al.*, 2004; Su *et al.*, 2007; Roberfroid, 2001), fructooligosaccharides (FOS) from beans (Su *et al.*, 2007), and even indigestible resistant starches (Sajilita *et al.*, 2006). These prebiotics are then hydrolyzed and fermented by bacteria with the appropriate metabolic activity, allowing changes in growth and proliferation of certain bacteria, including *L. acidophilus* (Su *et al.*, 2007; Aryana *et al.*,

2007), *Bifidobacterium animalis lactis* (Su *et al.*, 2007), *L. casei* (Su *et al.*, 2007), *Streptococcus thermophilus* (Akalin *et al.*, 2007), and *L. bulgaricus* (Akalin *et al.*, 2007), and ultimately influencing gut microbial community profiles

$\beta$ -glucans are a major constituent of oat bran (Tungland and Meyer, 2002) and barley (Angelov *et al.*, 2006), and are a well fermented dietary fibre (Tungland and Meyer, 2002).  $\beta$ -glucans are the primary source for monomeric glucose within the soluble portion of oat fibre (Hansen *et al.*, 1992). They are also considered to be the major functional material within cereal fibres (Angelov *et al.*, 2006). In fact, oat and barley have the highest  $\beta$ -glucan percentage of the cereals (Wood and Beer, 1998; Manthey *et al.*, 1999).  $\beta$ -glucan has been used for a variety of functional purposes, including food processing, where it is used for thickening, stabilizing, and the gelling of food (Burkus and Temelli, 1999). It has been used for this purpose within low fat cheeses (Brennen and Cleary, 2005). In addition, it has been shown to increase the viability of probiotic *Bifidobacterium animalis ssp. lactis* (Vasilijevic *et al.*, 2007), indicating that  $\beta$ -glucans can be utilized as a fermentable substrate by certain Bifidobacteria (Wood and Beer, 1998; Jaskari *et al.*, 1998) and other lactic acid bacteria (LAB) (Johansson *et al.*, 1998).

Inulin is the major fibre constituent of wheat bran, and consists of short chained  $\beta$  1-2 linked fructose units, with terminal glucose units (Le Blay *et al.*, 1999). It is considered a fructo-polysaccharide due to the relatively large chain length (Le Blay *et al.*, 1999). It is almost wholly resistant to degradation by mammalian digestive enzymes, but is readily fermented by various bacteria in the large intestine (Roberfroid and Delzenne, 1998), including bifidobacteria (Gibson *et al.*, 1995). It has been shown in rats that inulin

prebiotic fibre supplementation can increase colonic IL-10 and interferon-gamma (IFN $\gamma$ ) (Roller *et al.*, 2004). Additional health benefits have been reported with inulin intake such as increased iron absorption, critical due to the common deficiency of dietary iron (Yeung *et al.*, 2005). Inulin is generally regarded as safe (GRAS) at high intake levels and therefore provides an optimal dietary fibre source without safety concerns for over-ingestion (Coussement, 1999). The average inulin intake of Americans is around 5g/ day (Moshfegh *et al.*, 1999), while safe intakes can range upward of 30g/day without any observed adverse effects (Coussement, 1999).

Frucooligosaccharides also consist of short chain  $\beta$ 1-2 linked fructose units, with terminal glucose units (Le Blay *et al.*, 1999). The difference between inulin and FOS is chain length, with FOS having a degree of polymerization of 3-10, and is considered to be an oligosaccharide (Englyst *et al.*, 1995). FOS is estimated to be fermented up to 83% (Scholz-Ahrens *et al.*, 2001), and can stimulate growth of both lactobacilli and bifidobacteria (Le Blay *et al.*, 1999). FOS has been reported to have effects on the immune system, including upregulated IgA secretion in the Peyer's Patches (PP), as well as increased secretion of both Th1 cytokines (IFN $\gamma$ ) and Th2 cytokines (IL-10) (Hosono *et al.*, 2003). FOS has also been shown to modify the cultivatable fecal bacterial phylotypes, increasing numbers of inulin fermenting bacteria, particularly clostridia strains (Gourgue-Jeannot *et al.*, 2006). This re-emphasizes the impact that dietary fibre has on the diversity of the intestinal bacterial community profile.

Resistant starches are considered to be those starch products which are either fully or partially resistant to enzymatic degradation within the human small intestine (Englyst *et al.*, 1990). Resistant starches, while only fermented at 46%, are able to stimulate the

growth of bifidobacteria (Scholz-Ahrens *et al.*, 2001). Sources of resistant starch include potatoes, maize, peas, and bananas (Tunland and Meyer, 2002). Unlike other dietary fibres discussed, resistant starches do not fall under one defined description, but rather different types of resistant starch have been classified (Englyst *et al.*, 1992). There appears to be a disagreement in the number of classifications necessary, as certain reports define three types of RS, while others classify RS into four different categories. Utilizing the four category classification, type 1 RS includes starches physically inaccessible to enzymes, such as those in seeds, while type 2 RS is enzymatically resistant to  $\alpha$ -amylase until gelatinized (Tunland and Meyer, 2002). Conversely, type 3 RS consists of previously digestible gelatinized starch which, due to the heating and cooling of food processing, has become retrograded, and therefore resistant (Tunland and Meyer, 2002). Type four RS consists of those starches produced primarily by commercial processes (Tunland and Meyer, 2002). Despite these specific differences, resistant starch is chemically similar to other starches, containing glucose subunits linked by either  $\alpha$ -D-(1-4) or  $\alpha$ -D-(1-6) bonds (Sajilata *et al.*, 2006). Amylose, which is the main linear polymer of RS, consists of long chains of glucose linked by  $\alpha$ -D-(1-4) bonds (Sajilata *et al.*, 2006). Amylopectin, which is more highly branched, also consists of  $\alpha$ -D-(1-6) bonds, and is usually a larger molecule than amylose (Sajilata *et al.*, 2006). Resistant starches, while relatively new in the field of dietary fibre research, have been shown to have a variety of positive benefits. Within the large intestine, resistant starch can increase the amount of mucin produced, which provides protection against infection of the gut by foreign microbes (Toden *et al.*, 2007). It is reportedly implicated in reduced risk of colon cancer (Le Leu *et al.*, 2005; O'Keefe, 2008), although this could be disputed, since increased

dietary intake of resistant starch was concurrent with reductions in red meat and animal fat in the diet (O'Keefe, 2008).

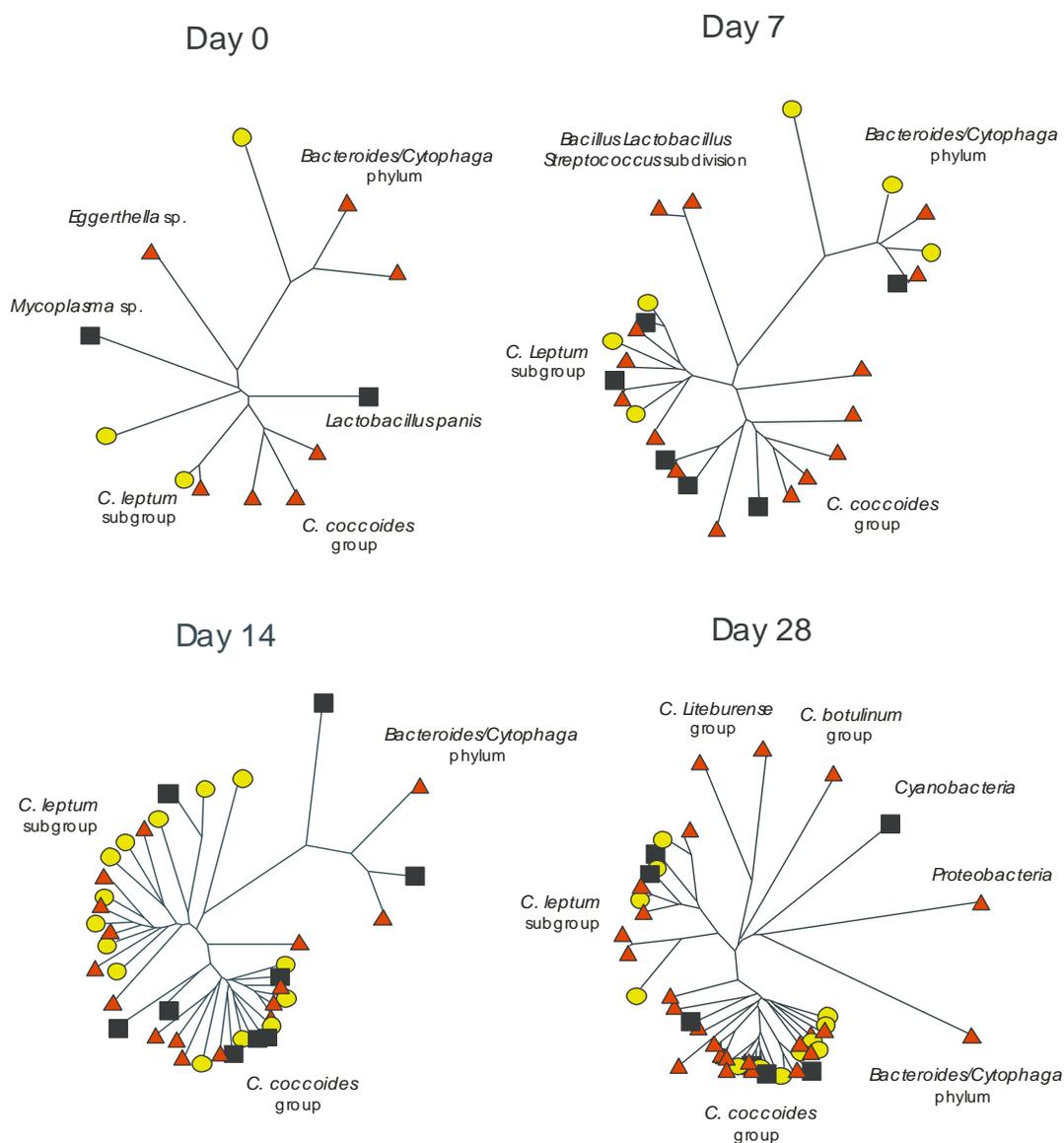
One major problem with many studies is that they are performed using *in vitro* based techniques, which do not consider the possible interactions between the intestinal bacteria and the impact on the host. Examples include Su *et al.*, (2007), Aryana *et al.*, (2007), and Akalin *et al.*, (2007). Su *et al.*, (2007) looked at several different prebiotics and their effects on probiotic growth *in vitro*. However, they did not explore whether these prebiotics would then have any effect on the mucosal immune system or Gut-Associated Lymphoid Tissue (GALT). A limited number of studies have used *in vivo* techniques and as a whole are still insufficient to provide comprehensive or conclusive data on the effect of the many different prebiotics available. This leaves many gaps in the current knowledge about prebiotic fibres and their immunomodulatory effect *in vivo*. In addition, much of the research available looks at inulin type fibres, meaning many of the other prebiotic fibres are left relatively unstudied. This leaves many gaps in current knowledge regarding the potential application of dietary fibre supplementation for modification of the immune system.

*Colonic bacteria community profiles can be modified through intake of dietary fibre*

Many studies have shown that the intestinal bacterial profile can be modified by dietary supplementation with different types of fibre. A review by Tunland and Meyer (2002), lists numerous dietary fibres with effects on intestinal microflora, such as: gluco-oligosaccharides, isomaltose, inulin, and FOS. Many studies have reported increased growth of *Bifidobacterium* strains due to dietary fibre supplementation with fibres such as

inulin (Gibson, 1998; Roberfroid *et al.*, 1998). Even less commonly used prebiotics such as lactulose have been shown to increase growth of intestinal lactobacilli species (Madsen *et al.*, 1999). In another study, FOS fed to rats was reported to increase the total concentrations of intestinal LAB (Le Blay *et al.*, 1999). Inulin has been shown to increase *Bifidobacteria* in humans (Kruse *et al.*, 1999). The research described in this thesis is a continuation of collaboration between several labs exploring effects of dietary fibre on colonic microbial communities. Previous work carried out through this collaboration has included identification of novel bacterial phylotypes (determined by 16S sequencing) within the BBc rat colon as a result of feeding oat bran or wheat bran as dietary fibre. It was found that while both oat bran and wheat bran induced changes in the gut microflora profile, at the end of the feeding trial, wheat bran had produced substantially more measurable novel phylotypes than oat bran (Abnous *et al.*, 2009) (Fig. 1). This indicates that wheat bran fermentation in the intestine leads to increased colonic community diversity. Many studies investigate only a limited range of types of bacteria when examining changes in bacterial diversity resulting from dietary fibre administration or *in vitro* systems assessing effects of fibre on bacterial growth. This approach may be insufficient for determining the true extent of changes occurring in colonic bacterial populations, considering the diversity of the population and the fact that many of these bacteria are unculturable. It has been shown that inulin can be fermented by species of *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Enterococcus*, and *Lactobacillus*. However, each showed different fermentation efficiency, and yielded different growth potentials as a result (Roberfroid, 2001). Use of an animal model is thus

well suited for determining the changes that dietary fibre supplementation can have on the intestinal microflora profile, and any resultant immunomodulatory effects.



**Figure 1.** Unrooted phylogenetic tree representing phylogenetic relationships within colonic contents of BBc rats. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Samples were collected on Day 0, 7, 14, and 28 after start of feeding trial. Data represents unique phylotypes for control (■), oat bran (●) or wheat bran (▲) for one trial ( $n = 8$ ). Particular phylotypes, groups, or phyla identified include: *Bacteroides/Cytophaga* phylum, *Lactobacillus panis*, *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Mycoplasma* species, *Eggerthella* species, *Bacillus-Lactobacillus-Streptococcus* subdivision, *Clostridium botulinum*, *Cyanobacteria*, *Proteobacteria*, and *Clostridium lituse-burense*. Figure adapted with permission from Martin Kalmokoff (Abnous *et al.*, 2009).

*Bacterial interaction with the intestinal mucosal immune system*

The bacteria present in the lower intestinal tract have a number of different host cell types with which they can interact and potentially induce immunological effects. Some cell types within the immune system are only capable of interacting directly with a foreign antigen, while others, such as dendritic cells and intestinal epithelial cells (IEC) possess the ability to transport the antigen across the intestinal epithelial layers to the interior of the body and interact with lymphoid tissues present.

One type of cell, making up most of the surface area of the intestinal tract, is the intestinal epithelial cell (IEC). IEC in fact have a dual role, in that they not only allow nutrients across the membrane, but simultaneously provide a physical barrier to protect the host from infection by foreign pathogens (Nagler-Anderson, 2001). IEC contain intercellular tight junctions between cells, which restrict passage of most small molecules through the epithelial layer (Madara, 1998). One specialized type of IEC are enterocytes, which are covered with microvilli on the luminal side (Kraehenbul and Neutra, 2000). These microvilli are covered with glycoproteins which create the Filamentous Brush Border Glycocalyx (FBBG), which can absorb nutrients without allowing transport of foreign pathogens (Kraehenbul and Neutra, 2000). Mucous is produced by specialized IEC, and serves as another physical barrier in host defence against pathogens. IEC are able to respond directly to foreign pathogens by producing cytokines, which are capable of stimulating an immune response in the GALT (Pitman and Blumberg, 2000).

Another type of cell present within the epithelial layer are microfold cells (M cells) (Nagler-Anderson, 2001). M cells are similar to IEC with the exception that the membrane bound glycoproteins on the luminal side are absent (Nagler-Anderson, 2001).

M cells are responsible for “sampling” the contents of the intestinal lumen and possess the capacity to transport bacteria and antigens across the epithelium through vesicles (Nagler-Anderson, 2001). They can then pass the sampled material into the GALT to awaiting APCs, which can then present antigens to T cells in the GALT (Nagler-Anderson, 2001).

The GALT can be divided into two distinct regions: inductive and effector (Nagler-Anderson, 2001). In the inductive regions called Peyer’s Patches (PP), antigen transported into the PP by cells is taken up and processed by APC such as macrophages and DCs (Nagler-Anderson, 2001). A recent discovery is that DCs are also capable of extending through the epithelium to sample external antigens directly, and are capable of forming tight junctions with IEC to maintain the integrity of the intestinal epithelium (Nagler-Anderson, 2001), thus providing an additional route for entry of bacteria and antigens into the GALT. After antigens are brought into the PP, they can then be presented to T lymphocytes, and can also stimulate B lymphocytes. Activated B and T lymphocytes then travel through the blood or lymph, via the MLN, recirculating and ultimately homing back to GALT effector sites such as the lamina propria (LP) (Brandtzaeg *et al.*, 1999), where a high density of IgA+ memory B cells are located. Memory cells in these “effector” locations are therefore capable of rapidly initiating an immune response through production of antibodies and cytokines.

There are different isotypes or classes of antibodies which B cells can produce, including IgA, IgM, and IgG. IgA can be produced systemically as a monomer, or secreted as a dimer (Nagler-Anderson, 2001). Secretory IgA plays a critical role in the defense against pathogens at mucosal surfaces because it can bind to pathogens prior to

entry into the body, eliminating them by immune exclusion (Macpherson and Uhr, 2004). Secretory IgA may also limit over-stimulation of the immune response, since it only weakly activates the complement system, which is involved in pro-inflammatory responses (Kaetzel *et al.*, 1991). The importance of secretory IgA in limiting translocation of commensal bacteria into the body has been recognized only relatively recently (Macpherson *et al.*, 2000). Secretory IgA in the MLN serves to compartmentalize intestinal commensals away from the systemic immune system, acting as a “firewall” to prevent unnecessary and potentially harmful activation of systemic responses to commensals (Macpherson and Smith, 2006). Another class or isotype of antibody is IgM, which is the first isotype produced in response to infection (Stavnezer, 1996). IgG is produced later as a result of class-switching, as is IgA, and the majority of antibodies produced during a secondary or memory response are either IgG (at systemic locations) or IgA (at mucosal locations) (Stavnezer, 1996). Class-switching to IgE can also occur, although IgE is normally present at low levels, and its production is involved in type 1 hypersensitivity reactions (allergies) (Stavnezer, 1996). While B cells only produce one type of antibody at any one time, different antibody isotypes can be made through a process called class switching. This is a process where genes encoding the antibody heavy chain of IgM are switched for genes encoding heavy chains from other antibody classes (Litinskiy *et al.*, 2002).

There are many different cytokines, each with a different role to play in the immune system. IL-2 is produced primarily by Th1 cells, and is involved in pro-inflammatory type responses such as activating macrophages, NK, and TC cells (Nielsen and Pedersen, 1997). IL-4 is produced primarily by Th2 cells and is involved in

regulating antibody production by B cells as well as in the differentiation of Th0 cells into Th2 cells (Rincon *et al.*, 1997). IL-10 is also a Th2-derived cytokine (Rimoldi *et al.*, 2005) and along with TGF $\beta$ , is able to assist in class switching to IgA (Fagarasan and Honjo, 2003). TGF $\beta$  is produced mostly by B cells and APC, and is the primary activator of class switching to IgA (Stavnezer, 1996; Fayette *et al.*, 1997). TNF $\alpha$  is primarily a monocyte-derived cytokine (Flach *et al.*, 1998) which is involved in pro-inflammatory Th1 responses (Coffman *et al.*, 1988).

### *Research Objectives*

The broad objective of this research project was to investigate the potential for different dietary fibres to influence an *in vivo* intestinal microbial community, and to examine associated effects on the immune system. The current study will analyze effects on cytokine and lymphocyte profiles in different tissues, at both systemic and mucosal locations. This will provide evidence as to the extent of immune system modification as a result of intake of dietary fibre present in different fibre sources, including oat, wheat bran, resistant starch and FOS. Analysis of liver and spleen tissues allows determination of whether specific cytokines are increased systemically and not just locally within the mucosal tissues. Analysis of the following cytokines was performed:

- IL2 – Mainly pro-inflammatory with anti-inflammatory implications
- IL4 – Anti-inflammatory and involved in class switching
- IL6 – Both pro-inflammatory and anti-inflammatory roles
- IL10 – Anti-inflammatory cytokine
- TNF $\alpha$  – Pro-inflammatory cytokine

Analysis of the spleen, blood, and mesenteric lymph node lymphoid populations allow for determination of the immunomodulatory impact on both systemic and mucosal immunity, including changes in T helper cell populations (CD4 and CD8). Analysis of mesenteric lymph node and cecal tissue provides information regarding effects on the intestinal mucosal immune system. And finally, cecal and fecal IgA analysis provides information regarding changes in antibody secretion within the gastrointestinal tract. Related changes in antibody isotype profiles at the systemic level can be made based on serum antibody measurements.

Ultimately, the information gained is expected to provide insight into the capacity for prebiotics to be used in the modification of the immune system. It is expected that dietary oat bran and wheat bran will significantly impact the immune system of the rats used in this study. This may help to develop more specific studies targeting particular fibres present within supplements such as oat and wheat bran ultimately providing support for nutritional supplementation with prebiotics in order to enhance or modify the immune response.

## MATERIALS AND METHODS

### *Animal Care and Diets*

All animal care protocols were approved by the Health Canada Ottawa Animal Care Committee prior to commencement of respective studies. Treatment and housing was performed according to Canadian Council on Animal Care (CCAC) guidelines.

Biobreeding (BBc) weanling rats (aged 28-42 days old) were obtained from the rat colony at Health Canada's Animal Resource Division facility (Ottawa, Canada).

Three separate trials were performed, with rats being fed *ad libitum*. For trials 1 and 2, rats were divided into control, oat bran, and wheat bran groups. The control diet was an AIN-93G formulation (Test Diet, USA), while the oat and wheat bran groups were supplemented with 5% oat or wheat bran dietary fibre (American Association of Cereal Chemists, USA), respectively. Diets were maintained at 20% protein, 58-63% carbohydrate, 7% fat, 5% dietary fibre, 0-2% water, and 0-1% ash. Dietary fibre was maintained at 5% utilizing wood cellulose within control group, and energy density of the diets were comparable within 1% of each other. For trial 3, additional diets were added. A 5% resistant starch group was included which utilized the prior control group. An additional group and control were added separately. This diet consisted of Purina Chow 5001 (Purina Mills, Pembroke, Ont.) for control, or 5% fructooligosaccharides (FOS) (Encore Technologies, USA) supplemented in the Purina chow. A separate control group was required for this experimental diet based on previous work performed at the Health Canada facility. Feeding trials were 34 days in length, with initial weaning onto control diet for one week prior to commencement of time course studies performed in

collaborating laboratories. Euthanasia was performed using O<sub>2</sub>/CO<sub>2</sub> asphyxiation at percentages and times determined by the CCAC.

#### *Tissue Homogenization procedure*

Spleen and liver tissues were initially placed in 5mL/1g Phosphate Buffered Saline (137mM NaCl, 2.68mM KCl, 1.01mM Na<sub>2</sub>HPO<sub>4</sub>, 1.38mM KH<sub>2</sub>PO<sub>4</sub>) containing 1% protease inhibitor cocktail (Sigma-Aldrich, USA). Tissues were homogenized using Tissuemiser (Fisher Scientific, USA) for 1 minute, followed by centrifugation (Eppendorf 5415D) for 30 minutes at 16,100 g. The supernatant was collected, then centrifugation and collection was repeated twice more. The samples were then stored at -80°C until use.

#### *Fecal and cecal sample preparation*

Samples were weighed and diluted 1g/2mL with 10g/L BSA in PBS. Samples were vortexed, incubated for 10 minutes at room temperature, then centrifuged at 4000g for 30 minutes. Supernatants were collected and frozen at -80°C until use.

#### *Cytokine analysis from tissue homogenates*

Quantitative analysis of cytokines within tissue homogenates was performed using cytokine specific commercial Enzyme Linked Immunosorbant Assay (ELISA) kits (Biosource, USA) using modified manufacturer's protocols and analyzed on a Synergy HTTR microplate reader using KC4 version 3.4 software (Bio-Tek, USA).

All manufacturer volumes added to ELISA plates were halved due to usage of half-volume ELISA plates to conserve available samples. Initial coating of wells with primary antibody for IL-2, IL-10, and TNF $\alpha$  was at concentrations of 1.25 $\mu$ g/mL using Coating Buffer B (4.3g NaHCO<sub>3</sub> and 5.3g Na<sub>2</sub>CO<sub>3</sub> in 1L H<sub>2</sub>O at pH 9.4). For coating with IL-4 antibody, concentration was 1.25 $\mu$ g/mL in Coating Buffer A (8.0g NaCl, 1.13g Na<sub>2</sub>HPO<sub>4</sub>, 0.2g KH<sub>2</sub>PO<sub>4</sub>, and 0.2g KCl in 1L H<sub>2</sub>O at pH 7.4). Plates were coated with 50 $\mu$ L per well and incubated in fridge at 4°C overnight for 12-18 hours.

Washing was performed by tapping plate onto absorbent paper, filling well with wash buffer (9.0g NaCl and 1mL Tween 20 in 1L H<sub>2</sub>O at pH 7.4), and tapping wells clear onto fresh absorbent paper.

Unbound well surfaces were blocked with 150 $\mu$ L per well of assay buffer (8.0g NaCl, 1.13g Na<sub>2</sub>HPO<sub>4</sub>, 0.2g KH<sub>2</sub>PO<sub>4</sub>, and 0.2g KCl in 1L H<sub>2</sub>O at pH 7.4) for 1 hour at room temperature.

Standards were prepared using serial dilutions to provide concentration ranges of 1000pg/mL to 15.625pg/mL, with the blank containing only assay buffer. Samples for the following cytokines were diluted using assay buffer as previously determined from titration assays: Liver IL-2 – 1 in 10, Liver IL-4 -1 in 5, Liver IL-10 – 1 in 2, Liver TNF $\alpha$  – 1 in 2, and all spleen samples were undiluted. The biotinylated detection antibodies were prepared using the following dilutions: IL-2 – 0.125 $\mu$ g/mL using assay buffer with 5% Fetal Bovine Serum (FBS), IL-4 – 0.050 $\mu$ g/mL using assay buffer with 5% FBS, IL-10 – 0.125 $\mu$ g/mL using 5% FBS, and TNF $\alpha$  – 0.37 $\mu$ g/mL using only assay buffer. Liquid was removed from wells by inverted tapping of plate onto absorbent paper. After wells were cleared, 50 $\mu$ L of either standard or sample were added to appropriate wells in

triplicate. Then 25µl of detection antibody was also added to all wells, and the plate was gently tapped on the sides to aid in mixing.

The plates were then incubated for 2 hours at room temperature, and those assays using undiluted samples were incubated on a shaker at 600 rpm as determined in previous titration assays, to assist in increased diffusion of reactions due to the viscous nature of the undiluted samples. The wells were then washed 5 times.

The streptavidin conjugated horseradish peroxidase (HRP) enzyme for each cytokine kit was prepared by dilution in assay buffer to these concentrations: IL-2 – 0.15µg/mL, IL-4 – 0.2µg/mL, IL-10 – 0.2µg/mL and TNFα – 0.3µg/mL. The HRP solution was added at 50µl/well and incubated for 30 minutes at room temperature. The plate was then washed 5 times.

To each well, 50 µl of Tetramethylbenzidine (TMB) (Sigma Aldrich, Canada) was added and incubated for various times (15-25 minutes) to achieve by visual observation, colour development corresponding to final optical density ranges of 1.0-2.0, optimal for the microplate reader. After sufficient incubation time, 50µl of 1.8N H<sub>2</sub>SO<sub>4</sub> reaction stop solution was added to each well. The absorbance was then measured at 450nm wavelength, and results calculating using the software 4-parameter curve fit for standard.

#### *Immunoglobulin analysis from fecal and cecal sample*

Analysis of immunoglobulins from fecal and cecal samples was performed using immunoglobulin specific commercial ELISA kits (Bethyl Laboratories, USA) using

manufacturer's protocols and analyzed on a Synergy HTTR microplate reader using KC4 version 3.4 software (Bio-Tek, USA).

All volumes recommended by the manufacturer for ELISAs were halved due to usage of half-volume ELISA plates to conserve available samples. Wells were initially coated with 50  $\mu$ l of primary antibody for either IgA, IgG, or IgM, diluted to concentrations of 10 $\mu$ g/mL using coating buffer (0.05M carbonate-bicarbonate, at pH 9.6). Plates were incubated for 60 minutes at room temperature.

Wells were washed three times using wash solution (50mM Tris, 0.14M NaCl, 0.05% Tween 20, at pH 8.0). To all wells, 100 $\mu$ l of blocking solution (50mM Tris, 0.14M NaCl, 1% bovine serum albumin (BSA), at pH 8.0) was added and incubated for 30 minutes at room temperature. The wells were then washed three times.

Standards were diluted using sample diluents (50mM Tris, 0.14mM NaCl, 1% BSA, 0.05% Tween 20, at pH 8.0) to create standard curves with the following ranges: IgA – 1000 to 15.625ng/mL, IgG – 2000 to 31.25 ng/mL, IgM – 500 – 7.8ng/mL. Samples were diluted using sample diluent as follows: IgA - 1 in 30, IgG – undiluted, and IgM – undiluted. The standards and samples were added at 50 $\mu$ l/well in triplicate and incubated for 60 minutes at room temperature. Wells were then washed 5 times.

The HRP antibody conjugates were diluted in sample diluent to prepare the following concentrations as determined in previous titration assays: IgA - 10ng/mL, IgG – 5ng/mL, IgM – 1 ng/mL. To each well, 50 $\mu$ l of working HRP solution was added and incubated for 60 minutes at room temperature. Wells were again washed 5 times.

To each well was added 50µl TMB substrate (Sigma Aldrich, Canada), and incubated for 13 minutes, at which time 50µl/well of 2M H<sub>2</sub>SO<sub>4</sub> was added. The absorbance was then measured at 450nm wavelength, and results calculating using the software 4 parameter curve fit for standard.

### *Primary Cell Culture Preparation*

Spleen and mesenteric lymph nodes (MLN) were removed from animals sacrificed at the Health Canada ARD in Ottawa, Canada. The animals were sacrificed and dissected for tissues over a period of three days, referred to as Tissue sets A, B, and C respectively.

The spleens were chopped into 3-4 pieces and immediately immersed in MEM media (Sigma Aldrich, Canada) containing 2.2g/L sodium bicarbonate, 100mg/L kanamycin (Sigma-Aldrich, Canada), 100U/L of penicillin (Sigma-Aldrich, Canada), and 50µg/L streptomycin (Sigma-Aldrich, Canada) at pH 7.1 and 10% fetal bovine serum (FBS).

The MLN were screened through 70um cell dissociation strainers (BD Biosciences, Canada) using rubber plunger tips and washed with MEM. All cells were shipped on ice and processed at UOIT within 24 hours.

Cell preparation for primary culture is outlined in Appendix A. Spleen pieces were screened through cells strainers, and washed with MEM. The cells were centrifuged for 10 minutes at 300g and supernatant removed by vacuum aspiration. The cells were then re-suspended in 5mL Tris buffered ammonium chloride lysis buffer (7.47g/L NH<sub>4</sub>Cl, 2.06g/L Tris base) in order to remove erythrocytes. Cells were

incubated for 2 minutes before 9mL MEM with 10% FBS was added. Cells were centrifuged for 10 minutes at 300g, and supernatant was removed by vacuum aspiration. Cells were re-suspended in 1mL MEM with 10% FBS and counted using a hemocytometer. Cells were adjusted to  $10^6$  cells/mL. For preparation of MLN cells, cells were centrifuged at 300g for 10 minutes, then re-suspended in 1mL MEM with 10% FBS, counted, and adjusted to  $10^6$  cells/mL.

After adjustments of all cell concentrations, cells were divided into two groups: whole cell populations for use in immunoglobulin and cytokine production, and macrophage isolation for nitric oxide production, phagocytic analysis, and hydrogen peroxide production analysis.

Due to limitations imposed by poor cell yields, tissue sets B and C were treated differently than tissue set A. Tissue set A had adherence performed for all three expected assays, while for tissue sets B and C, whole cell populations were utilized for the phagocytosis and respiratory burst assays. The adherence step for isolation of macrophages was performed on tissue culture treated cell culture dishes (Corning brand, Sigma-Aldrich, USA). Adherence step was for 90 minutes at 37°C and 5% CO<sub>2</sub>. After adherence, cells were removed from plate using 8mL trypsin and incubated for 5 minutes at 37°C and 5% CO<sub>2</sub>. Cells were washed and collected from plate using MEM with 10% FBS, and separated into two tubes. The cells were then centrifuged for 10 minutes at 300 g. One tube for each cell set was re-suspended in MEM with 10% FBS, while the other was re-suspended in KRPG for the respiratory burst assay. Cells were counted and adjusted to  $10^6$  cells/mL for use in experimental assays.

### *Nitric Oxide*

After preparation of macrophages for primary culture, cells were plated at  $10^6$  macrophages per mL, and challenged with  $10\mu\text{g/mL}$  or either *Escherichia coli* lipopolysaccharide (LPS) O55:B5 (Sigma-Aldrich, Canada) or Pansorbin cells (*Staphylococcus aureus*, Calbiochem, USA). Cells were incubated for 3 days at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in 96 well plates. After incubation, cells were centrifuged at 300g for 10 mins prior to collecting supernatant for analysis.

Analysis of macrophage nitric oxide production from cell cultures was quantified using a commercially available nitrate colorimetric assay kit (Cayman Chemical, USA) analyzed on a Synergy HTTR microplate reader using KC4 version 3.4 software (Bio-Tek, USA). A linear standard curve was prepared using the included  $\text{NaNO}_3$  standard, diluted with assay buffer to give final concentrations of 0-35  $\mu\text{M}$  and standard volumes of  $40\mu\text{l}$ . The samples were diluted in half with assay buffer and  $40\mu\text{l}$  added to wells in triplicate. Blank wells were plated in duplicate by using  $200\mu\text{l}$  of assay buffer only. After adding samples and standards,  $10\mu\text{l}$  each of nitrate reductase cofactor preparation and nitrate reductase enzyme preparation was added to all wells except blank wells. Plate was incubated 1 hour at room temperature and covered. Griess reagents R1 and R2 were added to all wells except blank and incubated for ten minutes. Absorbance was then measured at  $540\text{nm}$ , and results calculated using the software linear curve fit for standard.

### *Respiratory Burst*

Analysis of the reactive oxygen species hydrogen peroxide was quantified using a commercially available fluorometric assay kit (Molecular Probes, Invitrogen, USA) and analyzed on a Synergy HTTR microplate reader using KC4 version 3.4 software (Bio-Tek, USA). Stock reaction buffer was prepared by diluting 5X reaction buffer with reverse osmosis (RO) water. The horseradish peroxidase stock solution was prepared by adding one vial of HRP to 1mL of stock reaction buffer. Amplex red reagent was prepared by adding 60µl of dimethyl sulfoxide (DMSO) to one vial of amplex red. Reaction mixture was prepared by combining 25µl amplex red, 50µl HRP, and 4.925mL of reaction buffer. To appropriate wells of a black sided 96 well plate, 90µl of the reaction mixture was added, and warmed for 10 minutes at 37°C. For tissue set A, 20 µl of 10<sup>4</sup> macrophages per mL suspended in KRPG were added to the all wells except negative control, which received 20µl KRPG only. For tissue sets B and C, 20 µl of 10<sup>6</sup> cells/mL suspended in KRPG were added per well. Challenge wells also received 10µg/mL phorbol 12-myristate 13 acetate (PMA) (Sigma Aldrich, Canada), which can induce respiratory burst effects. Preliminary testing was performed using LPS 055:B5 at a 10µg/mL concentration, similar to previous studies (Bitzinger *et al.*, 2008), however insufficient responses were observed. Fluorescence was immediately measured from plate bottom at 530nm excitation, and 590nm emission. The plate was then incubated at 37°C and 5% CO<sub>2</sub> and read again after 30 and 60 minutes.

### *Phagocytosis*

Analysis of macrophage phagocytic activity from cell cultures was quantified using the commercially available Vybrant fluorometric kit (Molecular Probes, Invitrogen, USA) and analyzed on a Synergy HTTR microplate reader using KC4 version 3.4 software (Bio-Tek, USA). After preparation of cells for primary culture, cells were plated at either  $10^4$  macrophages (Tissue set A) or  $10^6$  cells (Tissue sets B and C). All samples and controls were plated black sided 96 well plates in triplicate. Negative control wells had 100 $\mu$ l of MEM, while positive control baseline wells had 100 $\mu$ l of cells. Challenged wells also received 20 $\mu$ g/mL of LPS 055:B5. The plate was quickly spun at 300-400g, and incubated for 1 hour at 37°C and 5% CO<sub>2</sub>. The fluorescent *E. coli* suspension was prepared by adding one vial of concentrated Hank's Balanced Salt Solution (HBSS) to one vial of fluorescein labeled *E. coli* K12 particles. This solution was diluted 1:10 with Milli-Q water and solution was sonicated and vortexed to eliminate precipitates. Supernatant was removed from wells by vacuum aspiration and 90 $\mu$ l of fluorescent suspension added to all wells. Plates were incubated for 2 hours at 37°C and 5% CO<sub>2</sub> to allow for phagocytosis of fluorescent particles. Trypan blue was used to quench any extracellular fluorescence from remaining particles and was prepared by diluted 1:5 with Milli-Q water and sonicated to eliminate precipitates. After incubation, fluorescent suspension was removed by vacuum aspiration, and 90 $\mu$ l trypan blue suspension added to each well. After incubating for 1 minute at room temperature, trypan blue was removed by vacuum aspiration and fluorescence was measured from the bottom of the plate at 485nm excitation and 520nm emission wavelengths.

### *Flow Cytometric analyses*

Flow cytometric analysis was performed at Health Canada in Ottawa, Ontario using a Becton Dickinson fluorescent activated cell sorter (FACS) and standard operating procedures. Gating techniques were standard and similar to those use in Shingadia *et al.* (2001). Fluorescent antibodies were initially titrated to correct dilutions for optimal detection. Antibodies used included, CD3, CD4, CD8, CD161a, CD45RA, CD172, CD68, CD25, Foxp3, IgA, IgG, and IgM. All antibodies were obtained from Biolegend, USA.

### *Statistics*

Analysis of variance (ANOVA) was performed using either Graphpad Instat for one-way ANOVA with Tukey's *post-hoc* test or Graphpad Prism for two-way ANOVA with Bonferroni's *post-hoc* test where  $p < 0.05$ . Graphs were created using Sigmaplot and error bars represent Standard Error of the Mean (SEM). Data for separate trials was combined if no significant difference between trials was detected using a two-way ANOVA ( $p > 0.05$ ).

## RESULTS

### *Dietary Fibre supplementation with wheat or oat fibre does not induce pro-inflammatory responses*

In order to assess the potential for increased inflammatory response due to dietary fibre supplementation, several different markers and tissues were assessed. No significant changes in liver TNF $\alpha$  levels were observed due to either oat bran or wheat bran fibre supplementation relative to controls (Fig. 2). Splenic TNF $\alpha$  and IL-6 were also tested but were below the level of detection by ELISA. Additionally, IL-2 levels were not significantly increased over basal levels in either the liver (Fig. 3) or the spleen (Fig. 4) in response to either fibre treatment relative to controls.

### *Dietary fibre does not change systemic tissue IL-10 Levels*

In order to determine the ability of dietary fibre to induce changes in regulatory cytokines, IL-10 levels were quantified in both liver and spleen tissues. Oat bran and wheat bran did not induce significant changes in liver IL-10 levels in either Trial 1 (Fig. 5) or Trial 2 (Fig. 6). Levels of splenic IL-10 also remained unchanged in response to either dietary treatment for both Trial 1 (Fig. 7) and Trial 2 (Fig. 8) when compared to controls.

### *Effects of dietary fibre treatment on Immunoglobulin levels*

Several different measures were taken in order to determine local effects of wheat or oat fibre treatment within the intestinal immune system, including measurement of intestinal IgA. It was observed that IgA levels within the cecal contents decreased

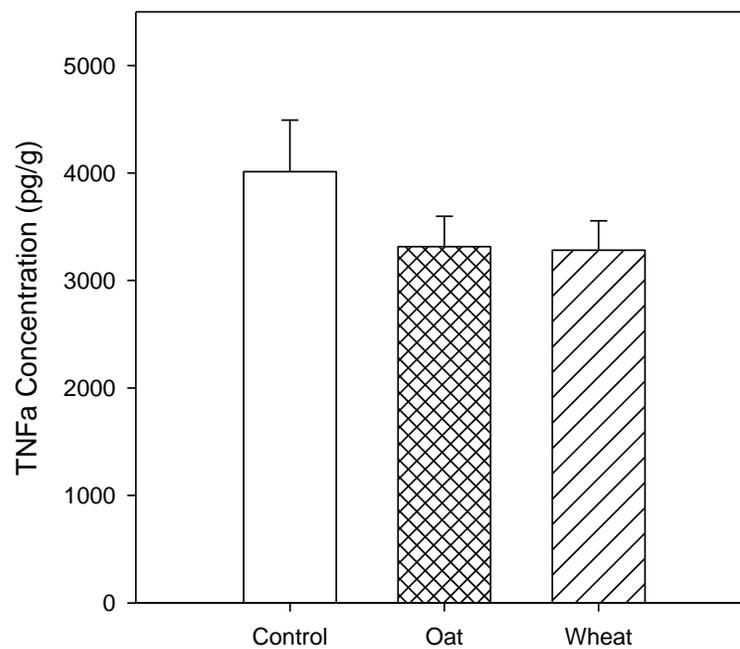
significantly due to wheat bran fibre supplementation (Fig. 9). However, no significant differences were observed for IgA measured in fecal samples (Fig. 10). Cecal IgM and IgG levels were below the limit of detection by ELISA, a finding which is typical for intestinal immunoglobulin isotypes.

#### *Effects of fibre on lymphocyte and macrophage numbers*

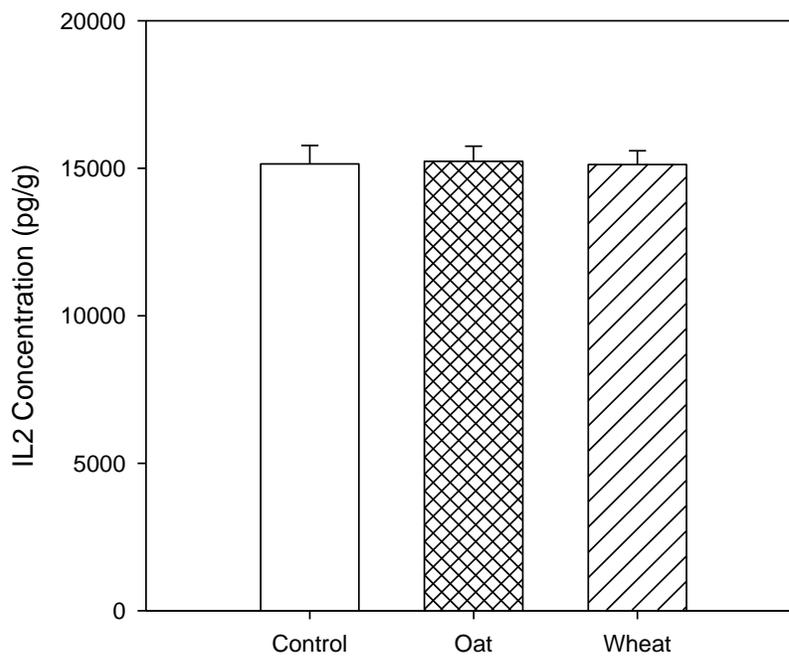
Effects of wheat and oat bran supplementation on B and T cell numbers, macrophage/neutrophil numbers and on NK numbers were compared in spleen, MLN and in blood by flow cytometry. Numbers of helper and cytotoxic T cells were also determined by enumerating CD4<sup>+</sup> and CD8<sup>+</sup> T cells respectively. No changes were observed in cell population profiles in blood (Table 1). However, double positive (CD4<sup>+</sup>CD8<sup>+</sup>) T cells in the spleen showed a significant increase for wheat bran treatment in comparison to both the control and oat bran treatments (Table 2). The major effects were observed in MLN lymphocyte population profiles (Table 3). Dietary wheat bran supplementation produced a significant decrease in total T cell numbers within the MLN as determined by the number of CD3<sup>+</sup> cells (Fig. 11). This change in total T cell number appeared to reflect a decrease in numbers of CD4<sup>+</sup> T cells, and not CD8<sup>+</sup> T cells, with numbers of CD4<sup>+</sup> T cells in the MLN being significantly lower in wheat bran than in oat bran treated rats, although not significantly different from controls (Table 3). This decrease in MLN T cell numbers was also concurrent with an increase in B cell numbers in rats receiving wheat bran treatment (Fig. 12).

*Effects of dietary fibre on systemic tissue IL-4 levels*

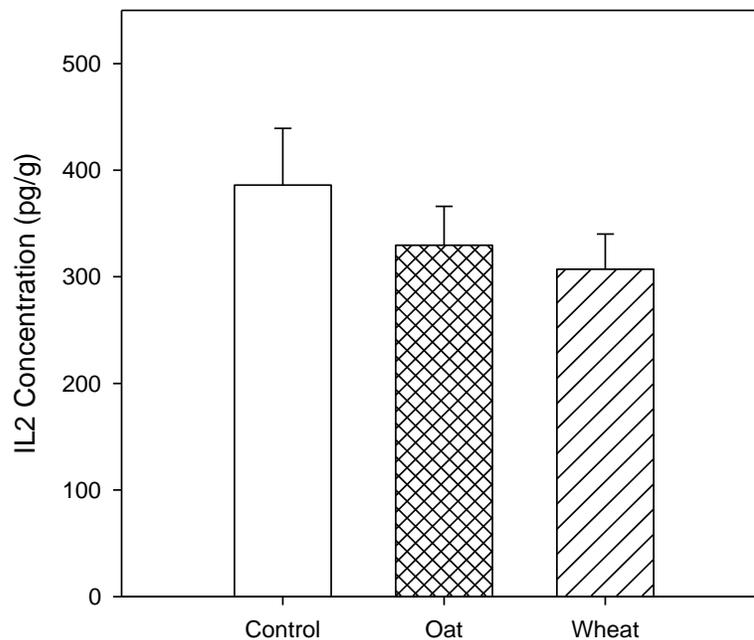
A trend ( $p < 0.1$ ) towards decreased liver IL-4 levels was observed, and the treatments were subsequently compared to control using T-tests to identify which treatment was producing the effect. It was determined that wheat bran significantly decreased liver IL-4 levels, indicating systemic effects of wheat bran fibre treatment (Fig. 13). However, these effects were not seen in the spleen where no significant differences in IL-4 levels were observed for either Trial 1 (Fig. 14) or Trial 2 (Fig. 15).



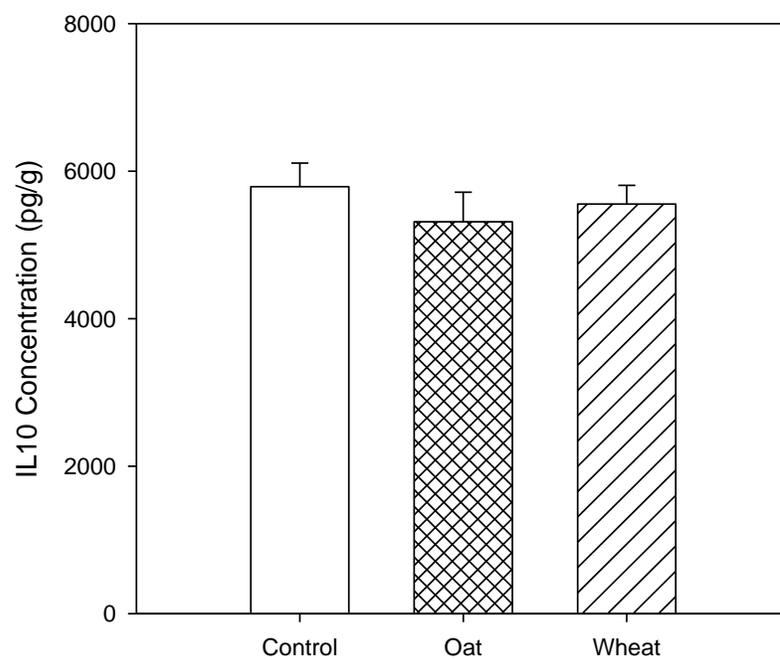
**Figure 2.** TNF $\alpha$  levels in BBc rat liver. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of two trials ( $n = 16$ ). Effects of dietary fibre were not significant ( $p = 0.26$ ) as determined by two-way ANOVA.



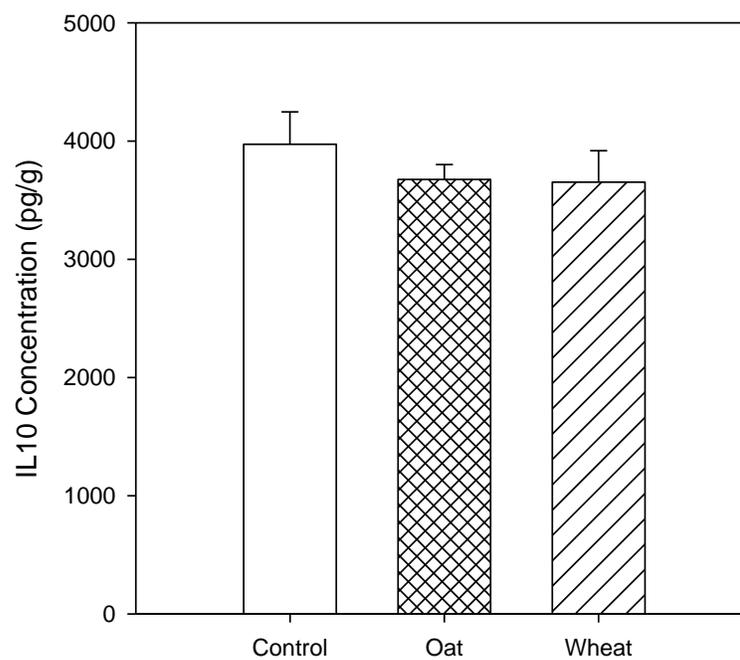
**Figure 3.** IL-2 levels in BBc rat liver. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of two trials ( $n = 14$ ). Effects of dietary fibre were not significant ( $p = 0.93$ ) as determined by two-way ANOVA.



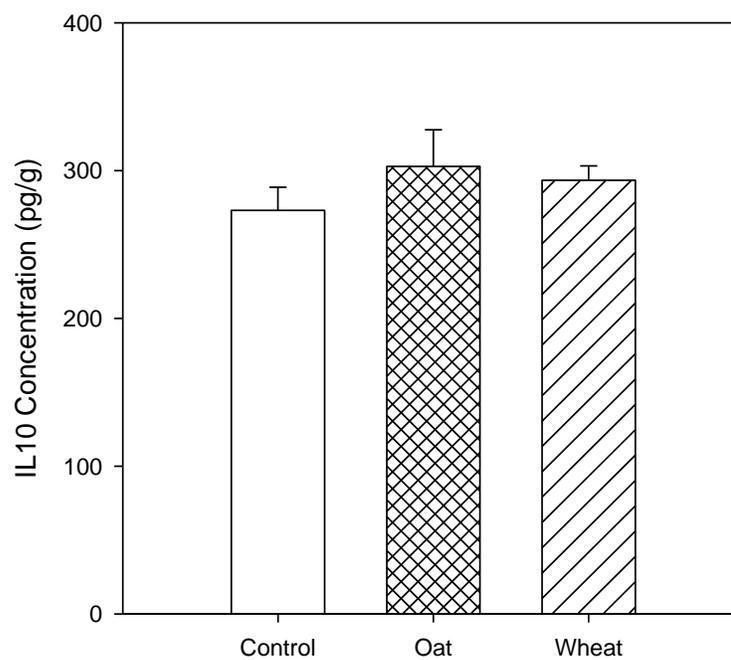
**Figure 4.** IL-2 levels in BBC rat spleen for Trial 2. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 7$ ). Effects of dietary fibre were not significant ( $p = 0.41$ ) as determined by one-way ANOVA.



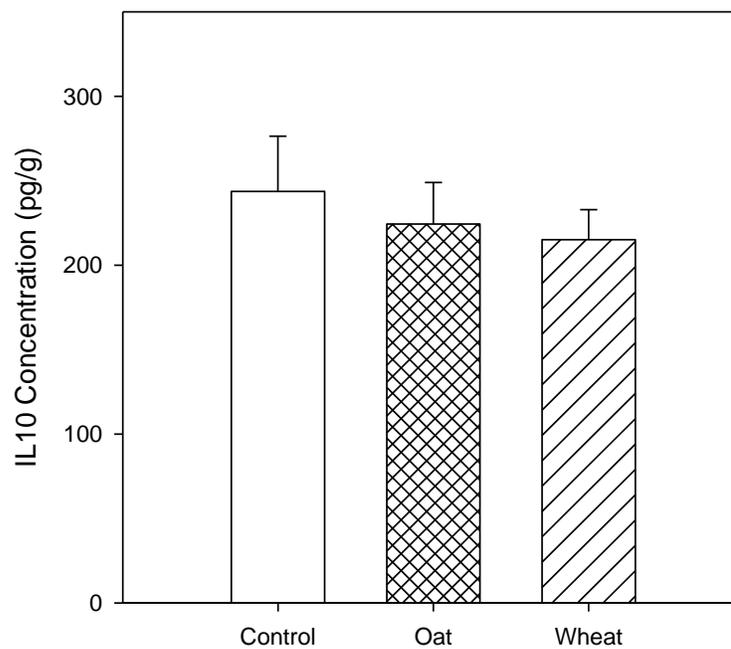
**Figure 5.** IL-10 levels in BBc rat liver for Trial 1. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 8$ ). Effects of dietary fibre were not significant ( $p = 0.60$ ) as determined by one-way ANOVA.



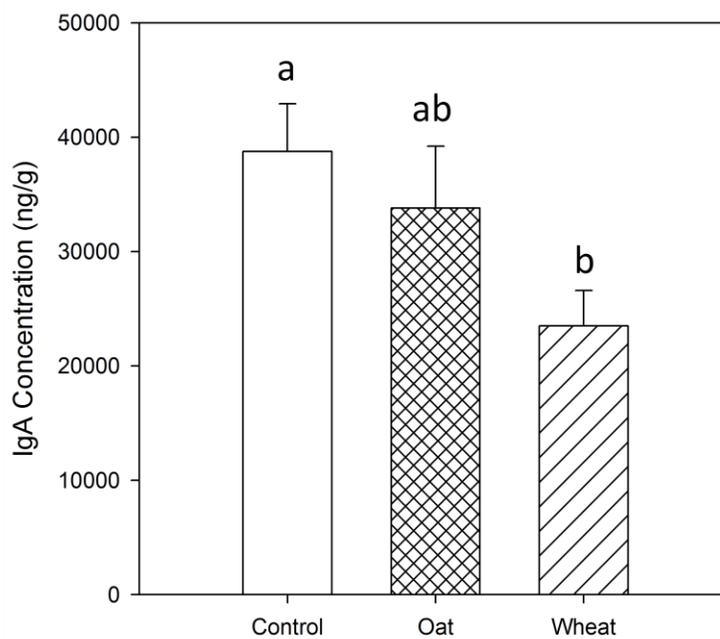
**Figure 6.** IL-10 levels in BBc rat liver for Trial 2. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 8$ ). Effects of dietary fibre were not significant ( $p = 0.56$ ) as determined by one-way ANOVA.



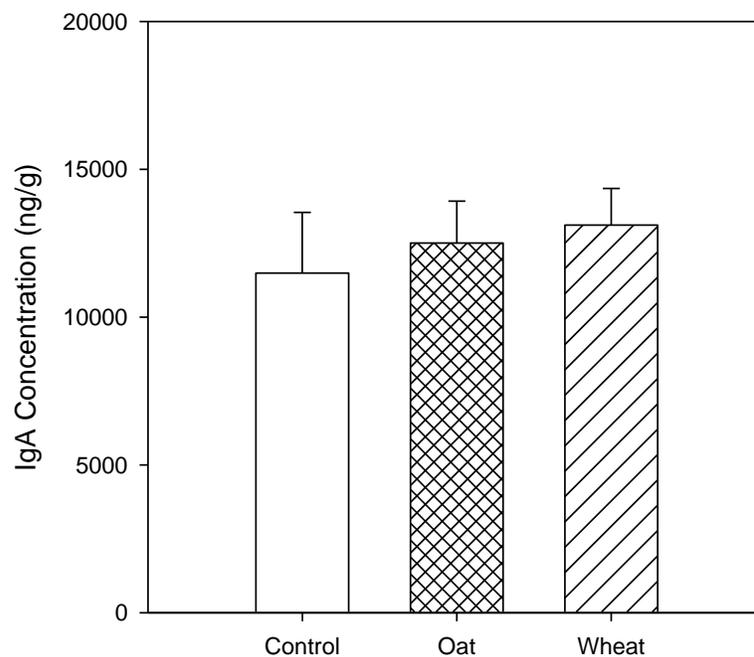
**Figure 7.** IL-10 levels in BBc rat spleen for Trial 1. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 5$ ). Effects of dietary fibre were not significant ( $p = 0.50$ ) as determined by one-way ANOVA.



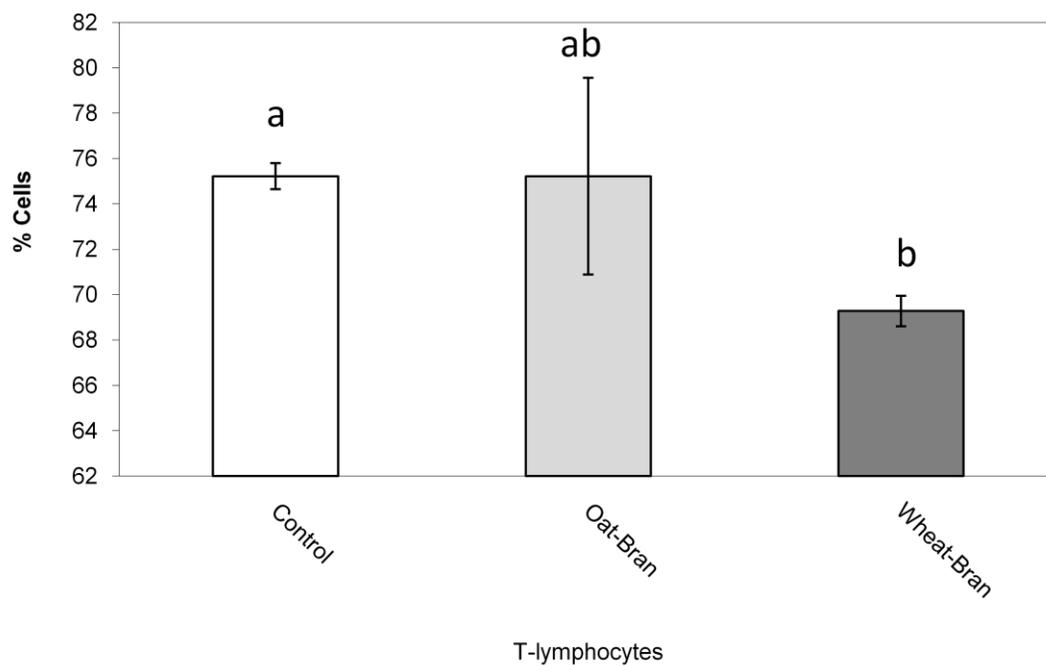
**Figure 8.** IL-10 levels in BBc rat spleen for Trial 2. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 7$ ). Effects of dietary fibre were not significant ( $p = 0.73$ ) as determined by one-way ANOVA.



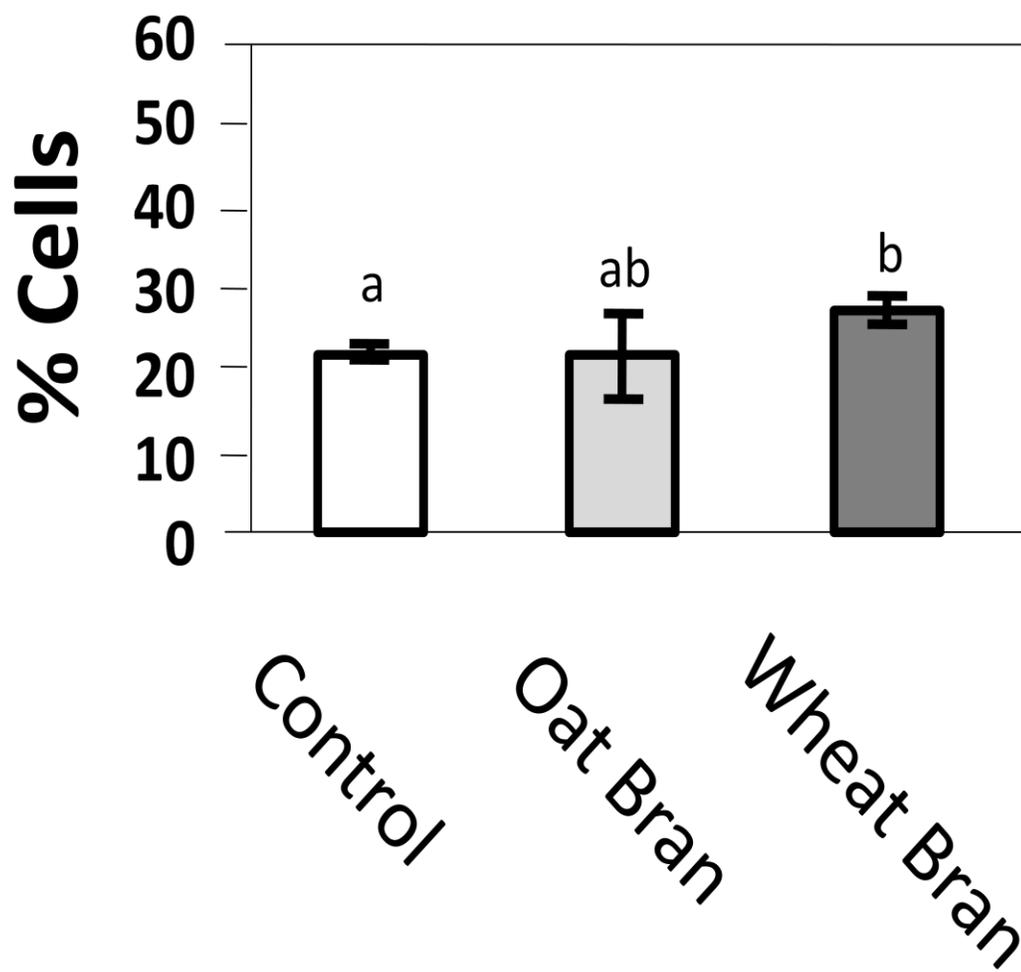
**Figure 9.** IgA levels in BBc rat cecal contents. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of two trials ( $n = 16$ ). Values with different letters (a,b) differ significantly ( $p < 0.05$ ) as determined by two-way ANOVA using Bonferroni's test.



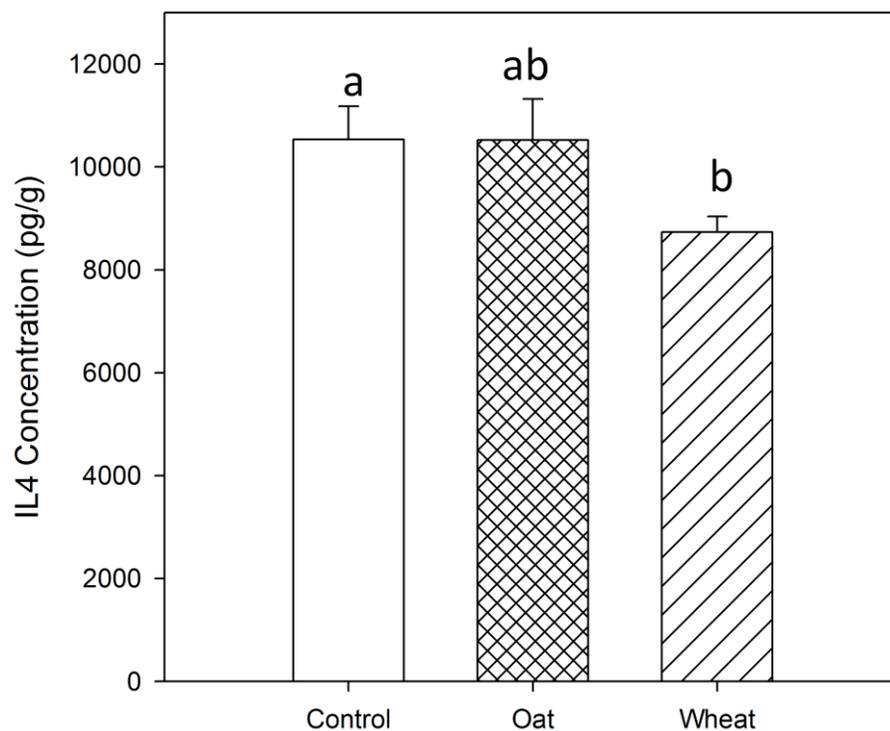
**Figure 10.** IgA levels in BBc rat fecal contents for Trial 2. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial (Control:  $n = 6$ , Oat:  $n = 5$ , Wheat:  $n = 7$ ). Effects of dietary fibre were not significant ( $p = 0.76$ ) as determined by one-way ANOVA.



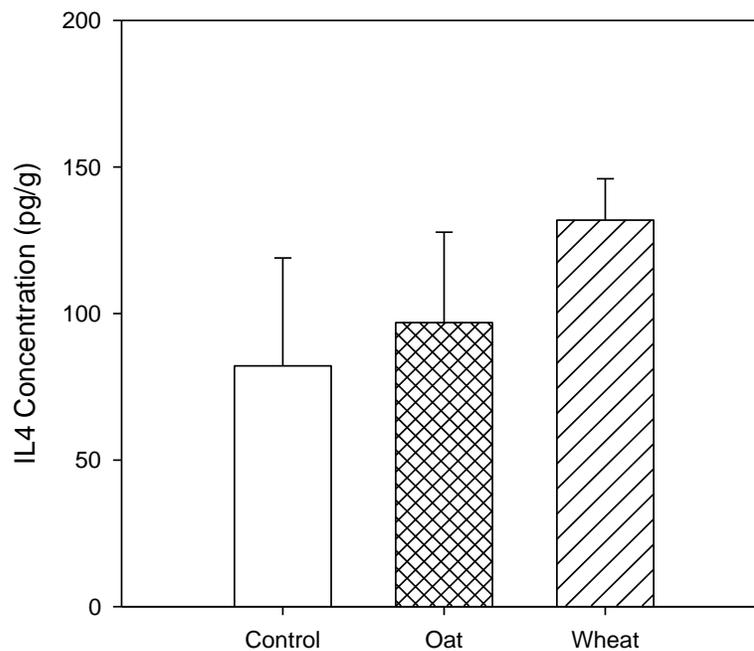
**Figure 11.** T lymphocyte levels (CD3+) in BBc rat MLN. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 5$ ). Values with different letters (a,b) differ significantly ( $p < 0.05$ ) as determined by one-way ANOVA performed using Tukey's test.



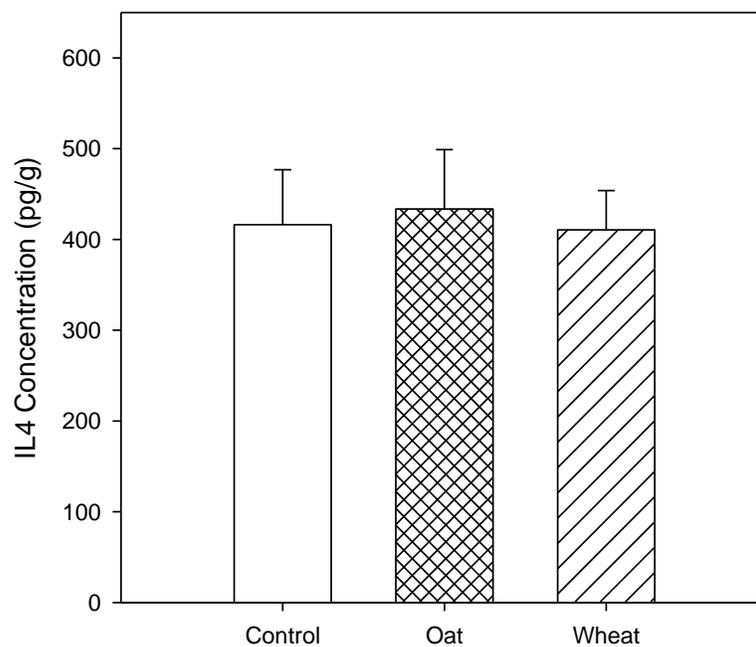
**Figure 12.** B lymphocyte levels in BBc rat MLN. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 5$ ). Values with different letters (a,b) differ significantly ( $p < 0.05$ ) as determined by one-way ANOVA performed using Tukey's test.



**Figure 13.** IL-4 levels in BBc rat liver. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of two trials ( $n = 16$ ). Values with different letters differ significantly ( $p < 0.05$ ) as determined by t-test. A trend towards significance was observed using two-way ANOVA ( $p < 0.1$ ).



**Figure 14.** IL-4 levels in BBc rat spleen for Trial 1. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 5$ ). Effects of dietary fibre were not significant ( $p = 0.48$ ) as determined by one-way ANOVA.



**Figure 15.** IL-4 levels in BBc rat spleen for Trial 2. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 7$ ). Effects of dietary fibre were not significant ( $p = 0.96$ ) as determined by one-way ANOVA.

**Table 1.** Mononuclear cell population profiles in Blood Samples<sup>1</sup>

Treatment	Total T Cells (CD3+)	T Helper cells (CD3+CD4+)	Cytotoxic T cells (CD3+8+)	Double Positive T Cells (CD4+8+)	Monocyte Macrophage (CD3-CD172+)	NK Cells (CD3-CD161a+)
Control	64.3±1.1	47.1±0.7	16.8±0.3	2.9±0.1	60.5±1.9	1.7±0.1
Oat	63.2±1.2	46.8±0.9	16.2±0.4	2.9±0.1	62.1±1.7	1.7±0.1
Wheat	62.3±0.6	45.5±0.7	16.2±0.4	3.0±0.1	62.2±2.4	1.3±0.2

<sup>1</sup>Data are shown as a mean percentage of total cell population ± SE, *n* = 5

**Table 2.** Mononuclear cell population profiles in Spleens<sup>1</sup>

Treatment	Total T Cells (CD3+)	T Helper cells (CD3+CD4+)	Cytotoxic T cells (CD3+8+)	Double Positive T Cells (CD4+8+)	NK Cells (CD3-CD161a+)
Control	49.4+/-2.3	32.7+/-2.2	13.3+/-0.5	1.58+/-0.2	2.02+/-0.3
Oat	48.2+/-0.6	31.5+/-0.4	13.1+/-0.5	0.74+/-0.1	1.05+/-0.1
Wheat	50.8+/-1.8	35.5+/-1.7	14.8+/-0.5	2.78+/-0.5 <sup>2</sup>	1.53+/-0.4

<sup>1</sup> Data are shown as a mean percentage of total cell population +/- SE,  $n = 5$

<sup>2</sup> Differs significantly from Control and Oat treatments ( $p < 0.05$ )

**Table 3.** Mononuclear cell population profiles in Mesenteric Lymph Nodes<sup>1</sup>

Treatment	T Helper cells (CD3+CD4+)	Cytotoxic T cells (CD3+8+)	Double Positive T Cells (CD4+8+)	Monocyte/Macrophage (CD3-CD172+)
Control	55.6+/-0.4	16.8+/-0.5	1.0+/- 0.0	26.2+/-1.5
Oat	56.9+/-1.4	16.6+/-0.5	1.0+/-0.0	28.1+/-1.7
Wheat	52.5+/-0.5 <sup>2</sup>	15.1+/-0.3	1.0+/-0.1	27.6+/-2.1

<sup>1</sup> Data are shown as a mean percentage of total cell population +/- SE,  $n = 5$

<sup>2</sup> Differs significantly from Oat treatment ( $p < 0.05$ )

## DISCUSSION

There has been an increase in the study of dietary fibre effects on health parameters in recent years, including effects on heart health, anti-cancer properties, and blood lipid levels. One study found that resistant starch in combination with *B. lactis* would assist in pro-apoptotic responses to carcinogen exposure (Le Leu *et al.*, 2005). In recent years there has been increased research into the immunomodulatory effects of dietary fibre, but there are still gaps and problems with some of this research, including contradictions in study results which may reflect the different designs of the experiments used (Scholz-Ahrens *et al.*, 2001). As one review stated, evidence is only preliminary that modification of colonic microflora is able to provide health benefits (Roberfroid, 2000). For example, one study has found that FOS fermentation does not increase *Bifidobacteria* growth, which opposes findings in other studies and common conceptions about the impact of FOS on gut microbial communities (Gourgue-Jennot *et al.*, 2006).

A clinical study found that synbiotic supplementation with *Bifidobacteria lactis*, *Lactobacilli acidphophilus*, and inulin was able to increase levels of the probiotics (Casiraghi *et al.*, 2007), but they did not investigate whether any immune effects resulted. One interesting study tested the effects of combining oats and LAB to create a “health drink” with properties of both prebiotics and probiotics (Angelov *et al.*, 2006). Their justification of this as a health drink appears to be that whole grain oats are associated with beneficial health effects, as are LAB, with the prebiotic fibres in the oats acting as fermentation substrate for the LAB. However, they provided no evidence that the combination would have a positive effect on the immune system. Instead, they simply tested whether the oats were a suitable substrate for the LAB, and did not examine any

potential bioactivity and immunomodulatory effects they might have. As well, many studies investigating dietary fibre have been performed *in vitro*, an approach that does not allow for effective analysis of effects on gastrointestinal community diversity or subsequent effects on the immune system.

Thus the aim of this research was to determine whether dietary supplementation with different types of fibres can modify the immune response in a BBc rat model, to determine the range of these effects, and through collaborative work, to consider these effects in concert with the impact on colonic microbial community diversity.

*Dietary oat bran and wheat bran do not stimulate pro-inflammatory effects*

Within the immune system, there are two major patterns of cytokine production, those produced by Th1 cells and primarily responsible for pro-inflammatory responses, and those produced by Th2 cells which generally involve regulation and suppression of the inflammatory response, as well as regulating antibody production.

Within the pro-inflammatory Th1 category, both IL-2 and TNF $\alpha$  play critical roles (Dang *et al.*, 2008). Reduced serum TNF $\alpha$  levels have been associated with increased fecal *Bifidobacterium* levels in elderly humans (Roller *et al.*, 2004). The cascade of factors resulting from pro-inflammatory markers such as IL1 $\beta$ , IL6, and TNF $\alpha$  is enormous, with significant negative repercussions (Jeschke *et al.*, 2004). In excess, these cytokines can cause host damage by modifying production of other cytokines, chemokines, adhesion molecules (such as intracellular adhesion molecule 1 (ICAM-1) (Wang *et al.*, 2006) and angiogenesis (O'Keefe, 2008).

The release of TNF $\alpha$  within the intestinal mucosa can lead to a significant increase in localized inflammatory cells such as macrophages, which can subsequently result in impairment of mucosal function such as mucin production (Neu *et al.*, 2002). The positive role of TNF $\alpha$  in the immune system should not be overlooked however. TNF $\alpha$  is one of the initial cytokines produced in response to bacterial infection, and is therefore critical in priming and activating the immune system to respond to foreign pathogens (Karlsson *et al.*, 2002). In fact, despite the previous mention of tumorigenesis resulting from TNF $\alpha$  overproduction, in moderation it is also capable of protection against tumour development (Gomez-Flores *et al.*, 1999), in part through induction of apoptosis (Sellers and Fisher, 1999). This shows that proper immunomodulation of this cytokine is critical to health. In this study, no changes in TNF $\alpha$  were observed due to either oat bran or wheat bran supplementation, suggesting that neither treatment increases pro-inflammatory responses.

Another cytokine measured was IL-2. While typically considered a Th1 cytokine, it also plays a role in controlling B cell activity by co-stimulating CD40 activated B cells to produce antibodies (Litinskiy *et al.*, 2002). In addition IL-2 has been associated with optimal induction of Foxp3<sup>+</sup> regulatory T cells (Davidson *et al.*, 2007; Zheng *et al.*, 2007), a function which is typically anti-inflammatory. However, IL-2 can also be associated with a pro-inflammatory Th1 profile, and Th1 cells are the primary producers of IL2 (Fiorentino *et al.*, 1989). In fact the pro-inflammatory response resulting from elevated IL-2 levels is sufficient to cause measurable damage to intestinal tissue (Dang *et al.*, 2008). IL-2 is involved in increased activity of natural killer cells (Nielsen and Pedersen, 2007), as well as increased nitric oxide (NO) production of macrophages

activated by T cells producing IL-2 (Tao and Stout, 1993). Several studies have shown that changes in the intestinal microflora (Coffman *et al.*, 1988) can impact the levels of IL-2 produced. It has previously been shown that certain *E. coli* strains are capable of inducing murine dendritic cells to produce IL-2 (Granucci *et al.*, 2001). In contrast, *Bifidobacterium infantis* 35624 has been shown to decrease the levels of IL-2 produced in the spleen (Sheil *et al.*, 2006). However, since neither oat or wheat fibre treatments produced any significant changes in IL-2 production relative to controls in either the liver or spleen tissues, IL-2 does not appear to be a factor in the potential immunomodulatory effect of dietary oat bran or wheat bran supplementation.

Oxidative stress markers were also examined in this study. Reduced paraoxonase activity in plasma (Appendix A) would reflect an increase in oxidative damage associated with inflammation (Camps *et al.*, 2009). Since paraoxonase activity is responsible for reducing oxidized LDL levels (Aviram and Rosenblat, 2004), LDL oxidation (Appendix B) is another measure that can be used to determine the level of pro-inflammatory responses. However, these two biomarkers were unaffected by either wheat or oat bran treatment, suggesting that these dietary regimens do not cause oxidative stress. In addition, in the oat bran group, serum antioxidant activity increased significantly, indicating improved antioxidant status. In turn, this shows that oat bran may have anti-inflammatory effects, but as this is the only significant result obtained for the oat bran treatment group, this remains uncertain.

*Regulatory IL-10 is not increased due to oat bran or wheat bran supplementation*

It has previously been reported that dietary fibre in the form of inulin can significantly increase IL-10 production in the intestinal immune system (Roller *et al.*, 2004). The cytokine IL-10 is an important regulator of pro-inflammatory responses, and is critical in inhibiting feedback mechanisms found within the T cell system (Li and Flavell, 2008). IL-10 is capable of effecting this inhibition in multiple ways. First, it can directly inhibit the maturation of antigen presenting cells (APC) which are responsible for presenting antigen to T cells and their subsequent differentiation (Li and Flavell, 2008). As well, the actual process of naïve T cell differentiation can be inhibited by IL-10 (Li and Flavell, 2008). In addition, these T cells may subsequently produce IL-10, introducing a negative feedback mechanism capable of limiting a pro-inflammatory cascade induced by activation of Th1 and cytotoxic T cells (Li and Flavell, 2008). As no significant differences in systemic IL-10 were found in response to either oat bran or wheat bran treatments, there is no evidence that IL-10 contributes to immunomodulatory effects of these dietary fibre supplements. It is possible that differences in IL-10 levels are present at the mucosal level, which the future trial will address through cytokine analysis of cecal tissue.

*Wheat Bran Fibre significantly decreases IgA levels in Cecal contents*

The role for IgA within the body is extremely varied. The increased bacterial diversity observed within the wheat bran group indicates the ability of dietary fibre supplementation to change the intestinal bacterial profile. This may be influenced by

changes in IgA levels, since changes in bacterial communities are followed by IgA secretion, providing a feedback mechanism for controlling novel phylotypes (Suzuki, 2007). Therefore over time, as bacterial diversity changes, IgA secretion responds by increasing to control overgrowth, possibly suppressing microflora changes and maintaining homeostasis. This would explain the changes observed during the time course study on novel phylotypes shown in Figure 1. This possibility was not addressed in previous trials, as only one time point for fecal samples was taken. The future trial 3 analyses will include measurement of multiple time points, to determine if there is chronological effect on IgA secretion.

Secretory IgA is the form which is secreted across mucosal membranes and is commonly released as a dimer (Corthesy, 2007). This dimer is formed by connecting two monomers together with a J chain, a polypeptide produced by those cells which secrete antibodies (Cerutti, 2008). Without the production of J chains, only monomeric IgA is produced, which cannot be transported as the secreted form, and a significant decrease in foreign pathogen elimination is observed (Mora and Von Andrian, 2008). J chains are required for secretion because they interact with the polymeric immunoglobulin receptor, which is responsible for transporting antibodies across the cell surface of mucosal epithelial cells (Mostov and Deitcher, 1986). It is therefore possible that the observed decreased cecal IgA levels reflect changes in the transcriptional regulation of J chain polypeptides (Brandtzaeg, and Johansen, 2005). This is also likely, since IgA<sup>+</sup> B cells in lymphoid tissues, in particular the MLN, show decreased basal levels of J chain expression when compared to other regions of the gut mucosa (Brandtzaeg *et al.*, 2005; Brandtzaeg *et al.*, 1999; Bjerke and Brandtzaeg, 1990). Thus, even though B cell

numbers were increased, a subsequent decrease in J chain regulation may lead to an overall decrease in secreted IgA. J chain transcriptional regulation is mediated by different cytokines, including IL-2, IL-4, IL-5, and IL-6 (Brandtzaeg and Johansen, 2005), which may subsequently modify levels of secreted IgA by indirectly affecting levels of J chain expression.

#### *Wheat Bran Fibre decreases IL-4 levels systemically*

The distal nature of the liver in relation to the intestinal immune system makes it suitable for studying systemic effects on the immune system of the host. It has previously been shown that a variety of physiological effects can be linked to microbial fermentation and interaction with the intestinal mucosa. Short chain fatty acids resulting from carbohydrate fermentation, especially acetate, propionate and butyrate are capable of producing noticeable effects on the liver, including decreased fatty acid synthesis and esterification (Delzenne *et al.*, 2000). The liver also has the greatest population of macrophages in the body, which are capable of inducing significant systemic effects through cytokine release (Ayala *et al.*, 1991; Ayala *et al.*, 1991). In addition, Kupffer cells, specialized macrophages within the liver are also capable of exerting local effects in the liver (Cerutti, 2008). This may have particular implications in instances where bacteria are carried by dendritic cells across intestinal mucosa and to systemic tissues such as the liver, a situation where liver macrophages would play a role in pathogen clearance (Rescigno *et al.*, 2000). The link between mucosal and systemic immunity is further reinforced by a study illustrating that in the absence of a large portion of the large intestine, B cells were completely absent from the liver (Zarabi and Rupani, 1986). Thus,

the mucosa-associated lymphoid tissue (MALT) and systemic tissues do not operate in complete isolation – interactions between these compartments are ongoing. Therefore, the significant changes observed in liver IL-4 in this study could reflect events in the MALT and lend support to the hypothesis that dietary fibre, and the resulting changes in microflora profiles can exert systemic effects as well as local effects at the mucosal level.

IL-4, along with other cytokines including TGF $\beta$ , IL-10, IL-5 and IL-6, is involved in regulating IgA class switching by B cells (Brandtzaeg and Johansen, 2005). IL-4 is also involved in IgG class switching (Litinskiy *et al.*, 2002), although in this study, levels of cecal and fecal IgG were below limits of detection, so potential effects on IgG could not be determined. In addition, as previously discussed, IL-4 is involved in J chain transcriptional regulation, and decreased levels of IL-4 may lead to decreased levels of J chain polypeptide production, subsequently reducing the levels of secreted IgA as seen in the cecal contents.

The reduced levels of hepatic IL-4 may reflect a more widespread effect of dietary fibre on IL-4 that would also be evident in mucosal tissues. Although these were not available for the present study, future work aims to assess the effects of dietary wheat bran fibre on IL-4 levels in MLN, cecal and intestinal tissues. It is also possible that the decreased hepatic IL-4 levels are directly responsible for the decreased cecal IgA levels, due to the potential for direct interactions between the intestinal mucosa and the liver through the hepatic portal vein. Antigens have been shown to pass directly from the intestinal mucosa to the liver through the hepatic portal vein, bypassing the primary bloodstream altogether (Macpherson and Smith, 2006). There are limited serum samples still available from this trial that can be assessed for Ig isotype profiles, as well as

cytokines IL-4 and TGF $\beta$  if volume permits, allowing determination of whether hepatic IL-4 levels reflect in changes in the circulating IL-4.

*Effects of dietary fibre on lymphocyte populations in the Mesenteric Lymph Nodes*

There are several different sites in the intestinal mucosa where bacteria and antigens can be sampled, but they are sampled primarily in the Peyer's patches (Kunkal *et al.*, 2003). The MLN is a key location for IgA+ B cells (McWilliams *et al.*, 1977), and has also been shown to serve a "firewall" role, preventing bacterial translocation, including translocation of commensals, through IgA production (Macpherson *et al.*, 2000). We observed a significant decrease in total T lymphocyte populations in the MLN in response to wheat fibre treatment, which appears to reflect a decrease in CD4+ Th cell numbers.

It has been shown in other studies that certain lactobacilli are able to modify T-lymphocyte activity (Easo *et al.*, 2002), and their subsequent production of cytokines responsible for antibody production. Therefore, it is possible that the changes in microflora associated with wheat bran fibre supplementation lead to changes in T cell activity and cytokine production, leading to reduced numbers of T lymphocytes in the MLN.

Given the importance of Th cells in supporting B cells, our finding that dietary wheat bran led to increased levels of B cells in the MLN may appear contradictory in light of the decreased levels of T cells also found in the MLN. Th cells are commonly involved in B cell activation, and a decrease in Th cells could potentially cause a decrease in B cell numbers, even though this was not the case. However, there are two main

mechanisms by which B cells are activated, those which are T cell-dependent those which are independent of T cells (Brandtzaeg and Johansen, 2005).

If the activation of B cells were solely T cell dependent, than a decrease in T cell numbers could lead to a reduction in B cell numbers. However, B cells can be activated through a T cell-independent mechanism involving binding of B cell receptors for B cell activation factor (BAFF), B cell maturation antigen (BCMA), along with Transmembrane Activator and Calcium modulator and Cyclophilin ligand activator (TACI) (Litinskiy *et al.*, 2002). Several different types of APC express B lymphocyte stimulator protein (BLyS), which binds to these receptors on B cells (Litinskiy *et al.*, 2002). In addition, APC produce A Proliferation-Inducing Ligand (APRIL), which binds to BCMA, and can induce a pathway for anti-apoptosis and proliferation that is independent of T cells (Litinskiy *et al.*, 2002). In fact, APRIL is also involved in T cell independent class switching and upregulation of IgA secretion (Litinskiy *et al.*, 2002). Thus, the decrease in MLN T lymphocyte numbers concurrent with an increase in B lymphocyte numbers may be explained through activation of T cell-independent mechanisms, potentially due to the observed changes in colonic microflora

In addition, our finding that wheat bran fibre led to a decrease in cecal IgA concurrent with an increase in MLN B cell numbers may also appear contradictory. However, an increase in B cell numbers does not necessarily imply an increase in IgA+ B cells. In the first trial, only total B cell numbers were enumerated. The third trial in progress is attempting to address this limitation by quantifying the percentage of IgA+ B cells relative to IgG+ and IgM+ B cells, in order to better clarify the effects of dietary fibre on B cell populations.

It is also necessary to consider to the effect of T helper cells in the effects observed. The observed decrease in CD4+ T helper cells could play a role in the apparent discrepancy between increased MLN B cell numbers accompanied by decreased cecal IgA. Despite the increased number of B cells, they may produce less IgA due to a lack of T helper cell support, including decreased production of IL-4. Reduced IL-4 production would be expected to result in less effective class switching. Taken together, these results suggest that T helper cell-dependent processes were also influenced by wheat bran intake, and were manifested in reduced B cell production of IgA. This is further support for the hypothesis that dietary fibre supplementation, specifically wheat bran, is capable of immunomodulation and that this effect is potentially linked to changes in the intestinal microflora profile.

#### *Future directions*

Based on the research so far, several questions remain to be addressed. First, is IL-4 directly involved in the observed effects on cecal IgA? In order to answer this question, two follow-up measures will be analyzed. Cytokine analysis has not been performed thus far on serum, so serum samples will be tested for both IL-4 and TGF $\beta$ , as both of these cytokine are involved in class switching and so can influence IgA production. Serum sample volume permitting, immunoglobulin isotype profiles of the serum samples will also be analyzed, to determine whether changes in IgM, IgG, or IgA are evident at the systemic level. Another question relates to the increase in B cell numbers in the MLN concurrent with the decrease in IgA in response to wheat bran treatment. It is possible that the elevated B cell numbers do not reflect an increase in

IgA+ B cells, a hypothesis which is being explored in the third trial by determining numbers of IgM+, IgG+ and IgA+ B cells by immunofluorescence and flow cytometry. It is expected that the results of this future work will allow for a clearer picture of the immunomodulatory effects of dietary fibre supplementation.

One additional direction which should be explored is utilizing a diseased model, such as exhibiting inflammatory bowel disorder. This would allow for the observed effects to be evaluated in a challenged animal, where the benefits of immunomodulatory effects from dietary fibre supplementation can be directly observed.

#### *Work in progress*

Additional experiments are currently in progress for continuation of this research. A third feeding trial has been performed at the Animal Research Division facility at Health Canada in Ottawa, Ont. This feeding trial is similar in approach to the previous two trials, with the inclusion of two additional experimental groups (resistant starch and inulin) and two corresponding appropriate control diets.

Experiments have been performed to determine the level of lymphocyte and macrophage activity within both spleen and MLN. Primary culture of cells from both these tissues has been performed for whole cell populations and for isolated macrophages. Whole cell populations were utilized for determination of macrophage phagocytic activity, respiratory burst measured through hydrogen peroxide production, as well as both antibody and cytokine production. Macrophages were isolated for quantifying nitric oxide production.

In addition, data on the effects of dietary fibre on lymphocyte and macrophage populations was collected by flow cytometry for spleen, MLN and blood populations, for the markers listed in Appendix E.

Both mucosal (MLN, colon, jejunum, ileum, cecum) and systemic (spleen) tissues were collected for cytokine analysis, and serum and plasma were collected for analysis of cytokine profiles and immunoglobulin isotypes.

Cecal contents were collected at the end of the feeding trial, and fecal pellets were collected at intervals throughout the trial to allow for analysis of IgA throughout the trial, at time points corresponding to sampling for colonic microbial population diversity profiles.

Serum was also analyzed for the following markers which are indicative of liver damage: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, and blood urea nitrogen levels.

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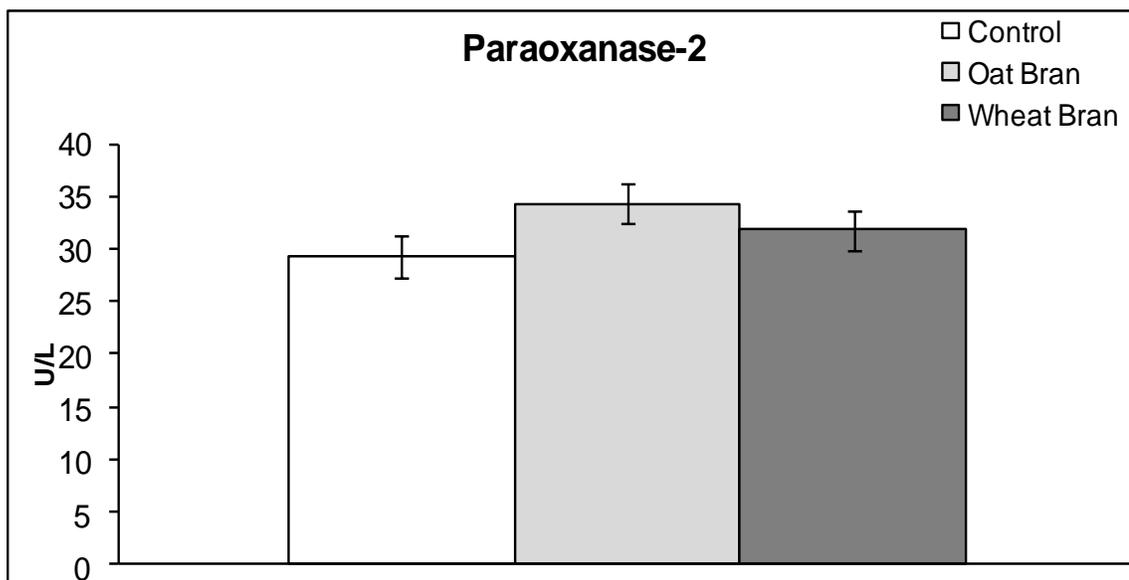
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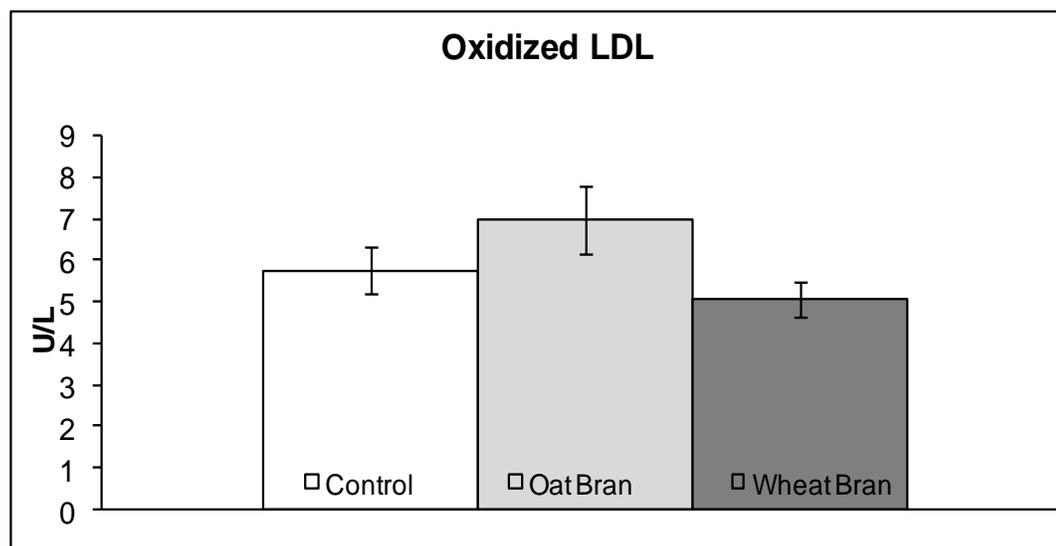
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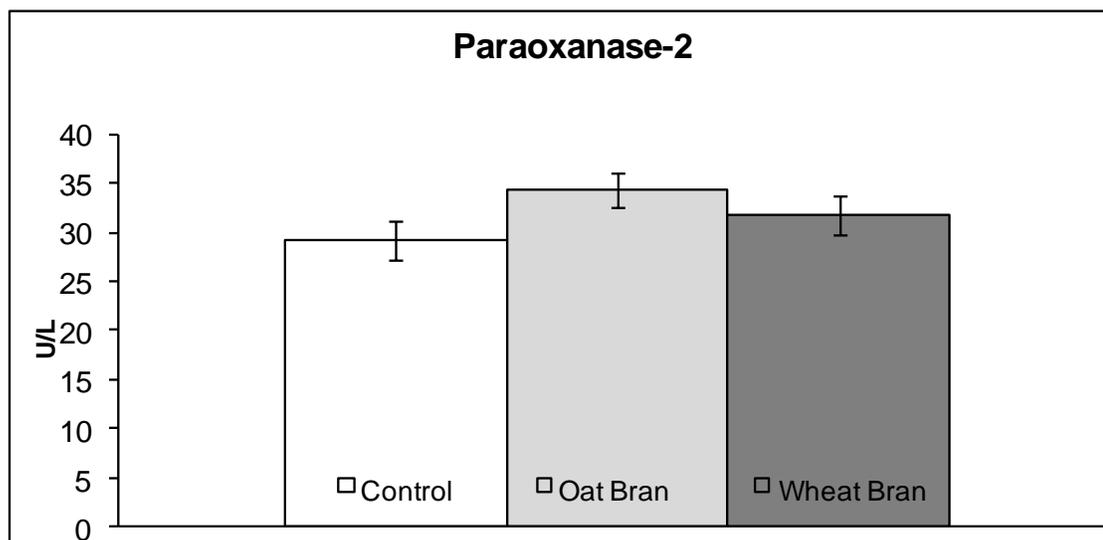
APPENDICES*Appendix A – Additional measures from feeding trial 1 – Paraoxanase Levels*

Levels of paraoxonase activity of BBc rat plasma. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 10$ ). Effects of dietary fibre were not significant ( $p > 0.05$ ) as determined by one-way ANOVA.

*Appendix B – Additional measures from feeding trial 1 – Oxidized LDL Levels*

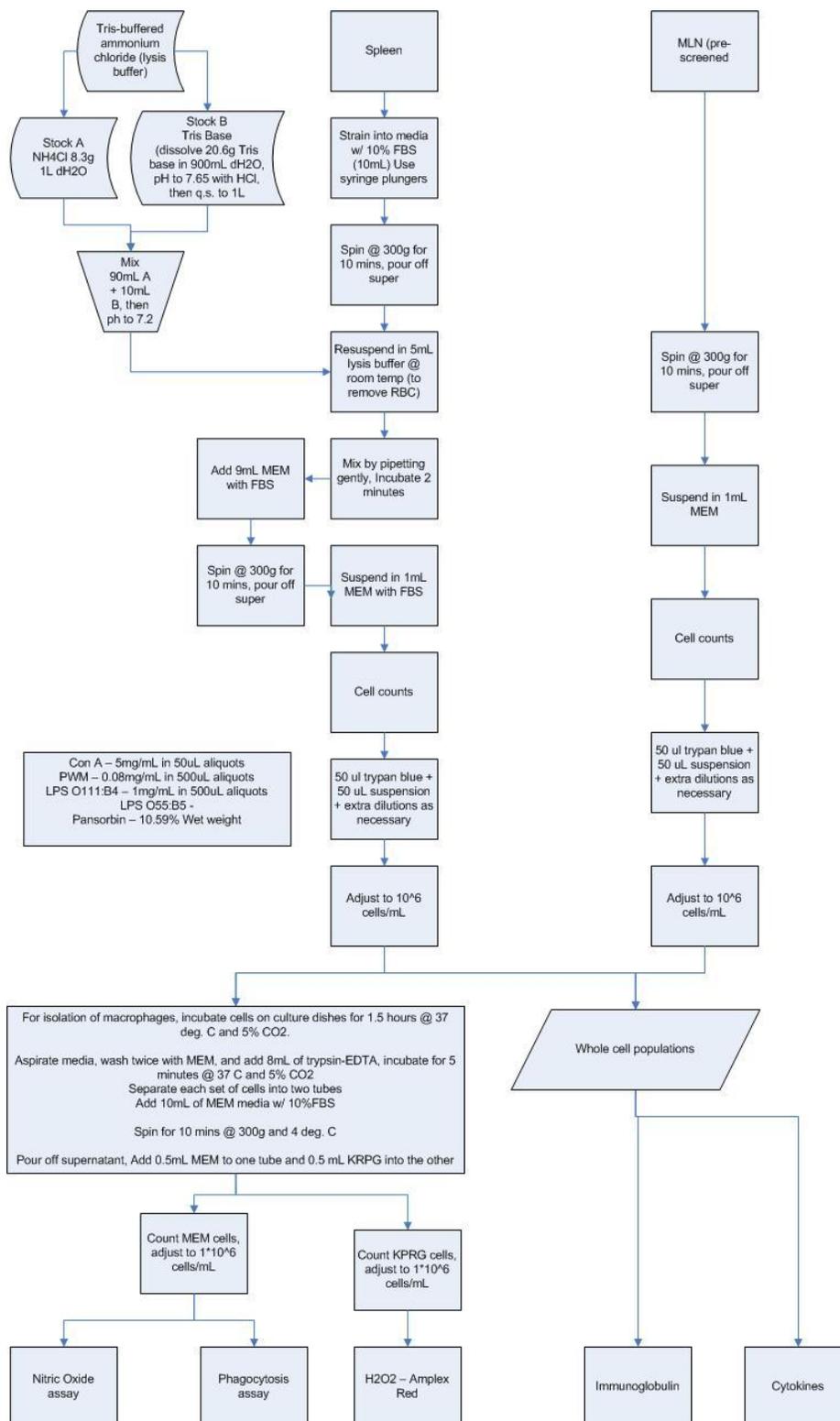


Levels of oxidized LDL in BBc rat plasma. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 10$ ). Effects of dietary fibre were not significant ( $p > 0.05$ ) as determined by one-way ANOVA.

*Appendix C – Additional measures from feeding trial 1 – Total Antioxidant Status*

Concentration of total antioxidants in BBc rat plasma. Rats were fed control diet alone, or control diet supplemented with either oat or wheat fibre. Data are means ( $\pm$ SEM) of one trial ( $n = 10$ ). Value with different letters (a,b) differ significantly ( $p < 0.05$ ) as determined by one-way ANOVA performed using Tukey's test.

Appendix D – Flowchart for primary cell culture preparation



*Appendix E – Flow Cytometry Targets and Markers*

<b>Cells targeted</b>	<b>Cell markers</b>
T Cells	CD3, CD4, CD8
T Cells, B Cells, NK Cells	CD3, CD45, CD45RA, CD161a
Macrophage	CD3, CD45, CD68, CD172
Immunoglobulins	CD45RA, IgA, IgG, IgM
Foxp3	CD3, CD4, CD25, Foxp3
Foxp3 Isotype Control	CD3, CD4, CD25, Isotype AF647