Influence of dietary interventions on the immune system: An investigation of the impact of omega-3 fatty acids, dietary fermentable materials and high energy diets

by

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Abstract

Dietary interventions vary in ability to influence immune activity. Separate investigations were conducted to determine the immune impact of omega-3 fatty acids, dietary fermentable materials (DFM) and high energy (HE) diets. Omega-3 fatty acid impact on bovine immunity was examined by comparing effects of fish oil and microalgae dietary supplements in the context of different feed management systems (pasture versus confinement). Cattle consuming microalgae had higher antibody production and cattle in the pasture system had higher anti-keyhole limpet hemocyanin (KLH) and total antibody responses on all diets. Overall, effects of omega-3 supplements on bovine immunity were influenced by feed management system with heightened impact on antibody production observed with a pasture system.

A double-blind placebo-controlled, randomized crossover clinical trial involving 30 healthy adults was conducted to examine impacts of β2-1 fructans on immunity. Subjects consumed an oligofructose-enriched inulin or maltodextrin supplement for a 28-day period (5 g, 3× daily with meals). β2-1 fructan supplementation significantly increased serum endotoxin and IL-4 concentrations, circulating percentages of $CD282^{+}/TLR2^{+}$ myeloid Dendritic cells (mDC) and *ex vivo* responsiveness to a Toll-like receptor 2 (TLR2) agonist, while serum IL-10 concentrations decreased.

A rat trial was designed to examine effects of a HE diet supplemented with varied DFM (7% wheat bran, oat bran, resistant starch type II, or fructooligosaccharide (FOS)) on obesity-associated biomarkers in male Sprague-Dawley rats. After 2 weeks of HE diet consumption, rats were segregated into obese-prone and obese-resistant phenotypes and shifted to DFM-supplemented HE diets for an additional 11-12 weeks. Obese-prone rats

had higher serum insulin and leptin concentrations than obese-resistant rats. FOS-fed rats had decreased splenic percentages of B cells, natural killer cells, T lymphocytes, memory T cells and cytotoxic T cells compared to rats on the control diet. High-energy diets had differential impacts on systemic and mucosal cytokines in both phenotypes; however, DFM had relatively less influence on cytokine and adipokine profiles associated with diet-induced obesity. Taken together, dietary interventions are capable of subtly influencing varied immune outcomes in animal models and in human subjects, however the interpretation of these dietary influences may be context-dependent.

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List of Abbreviations

AAFC	Agriculture and Agri-Food Canada
AIF	Atlantic Innovation Fund (Atlantic Canada Opportunities Agency)
AIN-93G	American Institute of Nutrition Growth Purified Diet
ANOVA	Analysis of Variance
BFA	Brefeldin A
BLQ	Below Level of Quantification
BSA	Bovine Serum Albumin
CBA	Cytometric Bead Array
CINC	Cytokine-Induced Neutrophil Chemoattractant
CRP	C-Reactive Protein
DC	Dendritic cell
DFM	Dietary Fermentable Material
DHA	Docosahexaenoic Acid
DIO	Diet-Induced Obesity
DR	Diet-Induced Obesity Resistant
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Eicosapentaenoic Acid
FOS	Fructooligosaccharide
G-CSF	Granulocyte Colony-Stimulating Factor
GALT	Gut-Associated Lymphoid Tissue
GHQ	General Health Questionnaire
GI	Gastrointestinal

GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HDL	High-Density Lipoprotein
HEPES	4-(2-hydroxyethyl)-1piperazineethanesulfonic acid
HSD	Honest Significant Difference
Ig	Immunoglobulin
IEC	Intestinal Epithelial cell
IEL	Intraepithelial Lymphocyte
IFN-α, IFN-γ	Interferon-alpha, Interferon-gamma
IL	Interleukin
IL-1Ra	Interleukin-1 Receptor antagonist
IP-10/CXCL10	Interferon gamma-Induced Protein 10/ C-X-C Motif Chemokine Ligand 10
ITF	Intestinal Trefoil Factor
KLH	Keyhole Limpet Hemocyanin
LAL	Limulus Amebocyte Lysate
LAP	Latency-Associated Protein
LBP	Lipopolysaccharide Binding Protein
LDL	Low-Density lipoprotein
LPS	Lipopolysaccharide
LoQ	Limit of Quantification
LSD	Least Significant Difference
MALT	Mucosa-Associated Lymphoid Tissue
mDC	Myeloid Dendritic cells

MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Node
MyD88	Myeloid Differentiation Factor 88
NF-κB	Nuclear Factor kappa-Light-Chain-Enhancer of Activated B cells
NK	Natural Killer cell
NOD	Nucleotide-binding oligomerization domain receptor
NSAC	Nova Scotia Agricultural College (collaborators on AIF Trial #7)
P3C	Pam3Cys
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear cells
pDC	Plasmacytoid Dendritic cells
pIgR	Polymeric Immunoglobulin Receptor
PPAR	Peroxisome Proliferator-Activated Receptor
PRR	Pattern Recognition Receptor
PUFA	Polyunsaturated Fatty Acids
qPCR	Real-Time Polymerase Chain Reaction
rANOVA	Repeated-Measures ANOVA
rbc	red blood cell
REML	Restricted Maximum Likelihood
RPMI	Roswell Park Memorial Institute
RS	Resistant Starch Type II
sCD40L/TNFSF5	Soluble Cluster Differentiation 40 Ligand/Tumour Necrosis Factor Superfamily Member 5

sICAM-1/sCD54	Soluble Intercellular Adhesion Molecule-1/Soluble Cluster Differentiation 54
SCFA	Short Chain Fatty Acids
sIgA	Secretory Immunoglobulin A
SST	Serum Separation Tubes
TGF-β1	Transforming Growth Factor beta-1
T_H	T-helper cell
TLR	Toll-Like Receptor
TMB	3,3'5,5' tetramethylbenzidine
TMR	Total mixed rations
TNF-α	Tumor Necrosis Factor-alpha
TRAM	TRIF-Related Adaptor Molecule
T_{reg}	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
wbc	white blood cell

Chapter 1: Evaluating Effects of Dietary Supplemented Fatty Acids on Bovine Immunity

Introduction

Dietary Polyunsaturated Fatty Acids and the Immune System

Polyunsaturated fatty acids (PUFA) are present in varying amounts in the human diet. Common sources of PUFA in the diet are fish oils, which are rich supplies of eicosapentaenoic acid (EPA) 20:5(n-3) and docosahexaenoic acid (DHA) 22:6(n-3, **Appendix A, Figure A1**). Unsaturated fatty acids are essential for the maintenance and functioning of cell membranes since fatty acids incorporate into the membranes to provide structure and fluidity (Calder, 1998). Increasing evidence suggests that dietary intake of PUFA can alter cell membrane composition, leading to changes in cell signaling and mediator production which would thereby influence immune responses. Elements of the immune system that are potentially modified by PUFA intake include suppression of lymphocyte, natural killer (NK) cell and macrophage activities (Caroprese *et al*., 2009; Yaqoob and Calder, 1995) as well as decreased production of pro-inflammatory cytokines (Mullen *et al.*, 2010). However, the effect of PUFA supplementation on responses to immunization and antibody production are not well understood.

Dietary fatty acids may be supplied to cattle in an effort to increase energy concentration in feeds (Fouladi-Nashta *et al.*, 2009), improve calf weight gain *(Garcia et al.,* 2014) improve milk quality (Franklin *et al.*, 1999) and also to modulate immune responses (Caroprese *et al.,* 2009; Farran *et al*., 2008). Feeding cattle sources of omega-3 fatty acids has been shown to elevate fatty acid profiles within plasma and milk (Fouladi-Nashta *et al.*, 2009). Evidence suggests the omega-3 PUFA source and the DHA/EPA ratio

may modulate the immune response distinctively (Caroprese *et al.*, 2009; Farran *et al.*, 2008). For example, in a previous study by Caroprese *et al*. (2009) it was demonstrated that cattle fed flaxseed had higher anti-ovalbumin immunoglobulin (Ig) G in response to immunization than cattle fed fish oil, which differed in its fatty acid composition. It has also been proposed that cattle fed flaxseed may have altered eicosanoid production by inflammatory cells in comparison to cattle fed soy or tallow (Farran *et al*. 2008). Analysis of milk from cattle fed fish oil displays EPA concentrations twice that of DHA (Kitessa *et al.*, 2004), which is not surprising given that fish oil itself has a higher EPA to DHA ratio. Dietary microalgae is an excellent source of omega-3 fatty acids and is a more concentrated source of DHA than fish oil, while containing less EPA (Abughazaleh *et al.*, 2009). A previous study has shown that dietary marine microalgae feeding altered the fatty acid profile of bovine milk by increasing the concentration of DHA (Franklin *et al.*, 1999), possibly enhancing the nutritive quality of the milk.

Few studies have examined the fatty acid profiles of plasma after a dietary algae feeding regime, and there have not been any published studies to date which have investigated the bovine immunization response after consumption of dietary microalgae. There is a need to improve immunization strategies in cattle as they become immunosuppressed during transport or after entering new feedlots (Estrada *et al.*, 1999), making the cattle more susceptible to infection. Increasing interest into the effects of PUFA in immunization strategies prompted this investigation into the immune responses of cattle fed dietary omega-3 fatty acids. Research into the enhancement of immune responses in cattle will not only benefit the beef and dairy industries, but also further our understanding of the potential role of PUFA in immunization strategies.

PUFA & Bovine Immunization Responses

KLH is a standard T-cell dependent antigen that is frequently used in investigating responses to immunization in cattle (Estrada *et al.*, 1999; Fligger *et al.*, 1997). KLH is considered a powerful immunogen and it is isolated from the hemolymph of the Californian Pacific coast sea mollusc *Megthura crenulata* (Harris and Markl, 1999). It is unlikely that dairy cattle will have highly specific natural antibodies for KLH, however they may produce a measurable amount of lower affinity natural antibodies capable of recognizing the KLH antigen (Harris and Markl, 1999; van Altena *et al*., 2016; van Knegsel *et al.*, 2007). Upon immunization with KLH, KLH-specific T cells will recognize the processed and presented antigen and facilitate the production of antibodies from KLH-specific B cells.

In order to investigate bovine responsiveness to immunization, antibody production profiles must be measured. The main antibody isotypes of interest in this research project were IgG, IgM and IgA. IgG is the predominant circulating Ig and cattle have two subclasses of this immunoglobulin, IgG_1 and IgG_2 (Butler *et al.*, 1971). IgM is the antibody mainly responsible for the primary immune response and it is also the first antibody produced following immunization (Mix *et al.*, 2006). IgG and IgM are quantifiable in serum, and are generally measured after systemic immunization. IgA is present in measurable but relatively low concentrations in serum since it is a mucosally secreted antibody, and it is the predominant isotype capable of neutralizing antigen at mucosal surfaces and in secretions (Pabst, 2012). For this reason, IgA is usually quantified in milk or colostrum.

The characteristic response to primary immunogenic stimulus occurs 5-10 days following immunization, with a small peak in antibody production (Liu *et al*., 1991). The primary production of antibody isotypes would follow the order of IgM > IgG > IgA. The secondary response occurs more rapidly (after a booster dose of immunogenic stimulus), usually within 1-3 days with a much larger peak in antibody production (Liu *et al*., 1991). The production of antibody isotypes would follow the order of IgG > IgA > IgM in the secondary response. Antibody production generally returns to protective baseline levels after a period of thirty days.

Previously, dietary consumption of omega-3 PUFA has been shown to increase titres of IgM and IgG in Sprague Dawley rats, and this is more pronounced with DHA supplementation than with EPA supplementation (Sugano *et al*., 2000). The immune response to antigens during omega-3 dietary enrichment has been well studied in chickens, and the results of these experiments assist in the current understanding of immune responses during PUFA supplementation. A previous study investigated the immune response of broiler chicks while supplementing the diet with different sources of omega-3 PUFA, either from tuna oil or sunflower oil (Maroufyan *et al.*, 2012). The main findings of the study were that the omega-3 PUFA profiles were significantly increased in the plasma of chicks receiving fish oil supplements, and the antibody titres and interleukin-2 (IL-2) concentrations increased with dietary PUFA enrichment. Increased IL-2 production could explain the increased antibody titres, since IL-2 is a key cytokine involved in developing adaptive responses. IL-2 is also a key cytokine for T cell activation (Popmihajlov *et al*., 2012), and so it indirectly facilitates immunoglobulin production. In another dietary PUFA study involving chickens, it was discovered that birds fed diets

enriched in α-linolenic acid, an omega-3 fatty acid, had increased titres of specific antibodies after KLH immunization (Sijben *et al*., 2001). However, when the animals were fed both linoleic acid (an omega-6 fatty acid) in combination with α-linolenic acid, lower anti-KLH antibody titres were found. These results suggest that α-linolenic acid may have involvement in antigen presentation, while linoleic acid may hinder the antigen presentation process (Sijben *et al.*, 2001). These studies in chickens illustrate the capability of PUFA to modulate the immune system and provide a platform to further study immune functions of fatty acids in cattle.

Flaxseed is also a source of omega-3 fatty acids that may potentially modify bovine immune responses. Farran *et al*. (2008) investigated the immune responses of cattle fed diets containing flaxseed (rich source of α-linolenic acid, an omega-3 fatty acid) or soy (rich source of linoleic acid, an omega-6 fatty acid). Cattle fed flaxseed had significantly higher omega-3 fatty acid concentrations in the plasma (Farran *et al*., 2008), confirming that dietary interventions may alter plasma lipid profiles. In addition, cattle fed flaxseed had lower concentrations of tumour necrosis factor-alpha (TNF-α) in response to injection of lipopolysaccharide (LPS) and lower body temperatures than cattle receiving the soy supplement (Farran *et al*., 2008). Another study investigated the immune response of cattle fed either flaxseed or fish oil as omega-3 supplements (Caroprese *et al.*, 2009). Cattle fed flaxseed produced higher titres of IgG antibodies specific for the immunizing antigen, and also displayed significantly lower levels of IL-10 than cattle fed fish oil. IL-10 is a regulatory cytokine which inhibits T_H1 cells as well as macrophage activity (Borish, 1998). A lower concentration of this cytokine was interpreted to indicate suppression of T_H2 cytokine production, although it could have also indicated decreased T_{reg} activity. In

another study which solely studied the bovine immunization response after omega-3 fish oil supplementation found increased secondary IgG responses to ovalbumin (Ballou and DePeters, 2008). The findings from these studies provide evidence that the fatty acid source as well as differing amounts of EPA and DHA concentrations in omega-3 supplements modulate distinctive aspects of the immune response. Further studies are required to determine appropriate EPA and DHA ratios for optimal immunization responses.

Chapter 1 Study Objectives

To evaluate effects of dietary supplemented fatty acids on the bovine immune system, an experiment was designed to examine impacts of feeding dairy cattle omega-3 PUFA supplements in two separate feed management systems on responsiveness to immunization. This trial was named AIF Trial #7, after the Atlantic Innovation Fund, and it was carried out in 2008. In a previous experiment (AIF Trial #6), influences of dietary algae on bovine immune responses to KLH immunization were evident, and this provided the rationale for continuing with KLH immunization investigation in Trial #7. The omega-3 fatty acid sources for this trial were dietary microalgae or fish oil. Pasture (grazing cattle in open pasture) and confinement (cattle kept in barn tie stalls) were the feed management systems utilized in the trial. It was hypothesized that cattle receiving the microalgae supplement would have increased responsiveness to KLH immunization compared to cattle on the fish oil supplement or control diet, since the microalgae had a distinctively high DHA to EPA ratio. Cytokines and immunoglobulins measured while investigating the impact of supplementing the diet with fatty acids on response to immunization with KLH are shown in **Table 1-1**.

Objective 1: to directly examine the effects of supplementing fatty acids in the feeding regime on the bovine immune system, and specifically on the T-cell dependent antibody response to KLH immunization and cytokine production.

Objective 2: to investigate differences in adaptive immunological responses to KLH immunization between pasture or confinement feed management systems.

Methods

AIF Trial #7 Experimental Design

AIF Trial #7 took place in 2008 as a collaborative project with the researchers at the Nova Scotia Agricultural College (NSAC). The other researchers involved in the project were interested evaluating molecular effects of the dietary fatty acid supplements, lipid metabolic pathways, milk quality, health parameters of the calves and placenta quality. My primary interests involved measuring the adaptive immune response to immunization and potential alterations in immune activity. The trial was constructed as a 2×3 factorial design involving 48 female Holstein dairy cows over a period of 120 days. Cattle were blocked according to parity so that experimental analysis could take place over a typical lactation period.

The dietary supplements used in the trial were a control (no lipid supplement provided), a rumen protected fish oil or a rumen protected microalgae. Dietary supplements were provided to cattle in a 300 g dose per day. The fish oil supplement was Epax® 5500 (Pronova Biocare A.S., Lysaker, Norway), a highly refined and enriched fish oil containing a minimum DHA and EPA content of 55%. Fatty acid analysis of the fish oil supplement conducted by Dr. Payam Vahmani (PhD candidate at NSAC during the time of the trial) revealed nearly equal concentrations of DHA and EPA, with the DHA content as 12.33 g/100 g of total fatty acids and EPA content as 15.85 g/100 g of total fatty acids (Vahmani *et al*., 2013). The microalgae supplement was the same algae species used previously in AIF Trial #6 (proprietary), and characteristically has a high DHA content and low EPA content. Microalgae DHA content was 24.23 g/100 g of total fatty acids and the EPA content was 1.61 g/100 g of total fatty acids (Vahmani *et al.*, 2013). The main difference

between supplements were that the fish oil had half of the DHA content of the microalgae supplement, while the microalgae had one tenth of the amount of EPA content of the fish oil supplement, and the control lacked DHA and EPA. Both omega-3 fatty acid supplements were protected against ruminal biohydrogenation using a proprietary process by project collaborators at the NSAC. A ruminant nutritionist was involved in the trial and balanced the diets to ensure the feeds met cattle nutritional requirements. Details on the fatty acid composition as well as the ingredient and nutritive composition of the feeds have been published previously (Vahmani *et al.*, 2013; Vahmani *et al.*, 2014). Cattle began feeding on the dietary supplements 30 days before calving and continued for 90 days following parturition, making the trial a total of 120 days.

There were two feed management systems utilized in this trial; pasture or confinement. In the pasture feed management system, cattle were allowed to graze through a rotation of two pastures, and were brought into the barn twice daily to receive concentrated dietary supplements. Cattle designated to be in the confinement feed management system were fed total mixed rations (TMR) twice daily at *ad libitum* intake while housed in the barn tie stalls, and received the concentrated dietary supplements prior to feedings. The cattle in the confinement management system have been referred to as the TMR group. All cattle had continuous access to water and were milked twice daily following parturition. The pasture management system represented traditional farming practices, whereas the confinement/TMR management system represented industrial farming practices. Cattle were maintained in the feed management systems for the same duration as dietary supplement feeding period. The sample numbers were unbalanced between pasture (n = 23) and TMR (n = 25) management systems due to the sudden death

of a cow (twisted stomach) in the pasture system and an extra cow in the TMR system. The segregation among diets was as follows: on pasture control n = 8, microalgae n = 8, fish oil n = 7; and on TMR control n = 9, microalgae n = 8 and fish oil n = 8.

To measure adaptive immune responses, cattle were immunized with KLH, a standard T cell dependent antigen commonly used to determine humoral responses to immunization (Estrada *et al.*, 1999). KLH (Sigma Aldrich, St. Louis, MO, USA) was reconstituted and diluted in phosphate-buffered saline under aseptic conditions. Each cow received a total KLH dosage of 1 mg in 50 mL of solution per immunization, which was separated into two 25 mL intramuscular injections. Cattle were injected with a 25 mL dosage under the skin of each shoulder, which was gently rubbed following injection for distribution and leakage prevention. Cattle received dietary supplements in their feed management systems for a total of 39 days before they were administered their primary immunization with KLH. Cattle received a booster dose of KLH 14 days after primary immunization, which represented 53 days on dietary supplements in the feed management systems. Blood was drawn from the tail vein into vacutainer tubes without preservative, which was necessary for the measurement of fatty acid composition and hormone assays by the other researchers involved on the project. Blood and milk were drawn from cattle at baseline (prior to initial KLH injection), 1, 7 and 14 days following primary immunization, 7 days following secondary immunization, and on the final day of the trial. Serum and milk samples were stored at -80°C until analysis. Cattle immunizations, blood draws and milking were carried out by animal research technicians at NSAC.

Measurement of Antibody Production During KLH Immunization

Antibody levels of all 48 cows were measured in order to evaluate whether fatty acid supplementation had an effect on the response to immunization, and also to determine if there were differences in immune responses of cattle consuming different omega-3 fatty acid supplements or on distinct feed management systems. Antibody concentrations were measured at four specific days during the trial, which represent the immunization baseline (day 0), primary response (7 days after initial KLH immunization), secondary response (21 days after initial KLH immunization and 7 days following KLH booster) and long-term response (51 days after initial KLH immunization and final day of the trial). These KLH time points with corresponding days on supplement/feed management system and days since calving are shown in **Table 1-2**. Concentrations of IgM, IgG and its subclasses were measured in serum. IgA concentrations were measured in milk since IgA is the predominant mucosal isotype, and there was interest in determining if any dietary effects were detectable at the mucosal level. Antibody profiles were analyzed using Bovine Ig enzyme-linked immunosorbent assay (ELISA) Quantitation Sets (Bethyl Laboratories Inc., Montgomery, TX, USA) for IgA, IgM, IgG, IgG_1, and IgG_2 isotypes. Total Ig concentrations were measured as directed by the manufacturer and were carried out using 96-well high-binding full area microplates (Greiner Bio-One, Frickenhausen, Germany). In order to measure KLH-specific antibody concentrations including anti-KLH IgM, anti-KLH IgG and its subclasses anti-KLH IgG_1 and anti-KLH IgG_2, and anti-KLH IgA, ELISA plates were coated with 10 µg/mL of KLH antigen in 0.05 M carbonate-bicarbonate buffer pH 9.6 overnight and blocked with 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20 pH 8.0. In separate sequential steps, samples were diluted and incubated on the ELISA plate, and then

HRP-conjugated bovine Ig detection antibodies were added to the assay. The chemical substrate, 3,3'5,5' tetramethylbenzidine (TMB; Sigma Aldrich, St. Louis, MO, USA) was added and the colorimetric reaction was stopped with the addition of 1.8 N H_2SO_4. The optical density of the reactions in the microplates were read at a wavelength of 450 nm on a Synergy HT multi-detection microplate reader using KC4 v3.4 analysis software (BioTek Instruments Inc, Winooski VT, USA). The Ig and anti-KLH Ig concentrations were calculated in unknown samples from a standard curve.

Measurement of Cytokine Activity During KLH Immunization

Cytokines IL-4, IFN-γ, active and total TGF-β1 were chosen for analysis in order to determine possible mechanisms of immunomodulation as well as shifts in the balance of T_H1 and T_H2 cell responses that may have been induced by the omega-3 supplements or feed management systems, as these cytokines are involved in Ig class switching. Cytokine concentrations were measured in serum using the bovine IL-4 ELISA reagent kit (Thermo Fisher Scientific, Waltham, MA, USA), IFN-γ and TGF-β1 DuoSet ELISA Development kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's procedures. Active and total TGF-β1 concentrations were quantified separately due to the requirement of a sample activation procedure. "Active" TGF-β1 was measured in serum as any other cytokine, since this form of TGF-β1 was already in its immunoreactive form. However, in order to measure the "total" amount of TGF-β1, activation of latent TGF-β1 was necessary since this cytokine cannot be measured by ELISA with the attachment of its latency-associated protein (LAP), which inhibits TGF-β1 bioactivity (Annes *et al*., 2003; Kropf *et al.*, 1997). Once the LAP has been cleaved, the total amount of TGF-β1 can be quantified,

including both biologically active and previously latent forms, since antibodies used in the ELISA bind only to the active form of the cytokine only. The sample activation procedure was carried out in microcentrifuge tubes treated with Sigmacote® (Sigma Aldrich, St. Louis, MO, USA), which is a solution of chlorinated organopolysiloxane in heptane that prevents TGF-β1 protein from binding to the surface of the tubes. In each tube, 20 µL of 1N HCl was added to 40 µL of serum and incubated for 10 minutes at room temperature. Acidification was neutralized with the addition of 20 µL of 1.2 NaOH/0.05M 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES), and samples were subsequently diluted in reagent diluent for the analysis of "total" concentrations of TGF-β1. The time points chosen for cytokine analysis in serum included KLH d0, d1, d7, d14, d21 and d51.

Organ tissues were collected from 24 cows involved in the trial, while the remaining 24 cows were led to pasture retirement. Spleen and caecum tissues were analyzed for 12 cows in the TMR group and 12 cows in the pasture group (n = 4 on fish oil, n = 4 on microalgae and n = 4 on control for each feed management system). The spleen was chosen to examine immune activity at the systemic level, and the caecum was chosen to examine activity at the level of the mucosa-associated lymphoid tissue (MALT). These tissues were homogenized in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) with the addition of 1% Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA). Tissues were suspended in the buffer at a ratio of 1:5 (wt/vol) and then homogenized using a VWR®200 homogenizer (VWR International, Radnor PA, USA) and centrifuged (17 200 × g for 30 min at 4°C). Supernatants were collected and stored at -80°C until cytokine analysis by the same ELISAs used in the analysis of serum samples (IL-4, IFN-γ and active/total TGF-β1).

These tissue measures were representative of immune activity from the final day of the trial, 51 days after immunization with KLH and 120 days after receiving dietary supplements in the feed management systems. All cytokine ELISAs were carried out using the same microplates, plate reader and analysis software as the Ig ELISAs.

Statistical Analyses

For the analyses of the Ig data, assistance was received from a statistician, Sherry Fillmore, Statistical Project Manager with Agriculture and Agri-Food Canada (AAFC, Kentville, Nova Scotia), since she had history working with previous AIF trials. This statistician's expertise was required due to blinding by dietary supplement identity throughout experimentation and to the complexity of the trial design with varying lactation cycles. The statistician has access to documents detailing the life history of the cattle; which included situations of multiparity and general health information that could have led to the exclusion of certain cows from the analysis. Ig data analysis was carried out using Genstat v14.0 software (VSN International, Hemel Hepstead, UK). The data was first transformed using a log10 transformation, and then analyzed by a restricted maximum likelihood model (REML) since the dataset was unbalanced, followed by Fisher's least significant difference (LSD) multiple range test for comparisons within feed management system and supplement groups. In the evaluation of Ig changes across days, linear regression was used to collect linear and quadratic coefficients across time. The Ig plots demonstrating immunization responses to KLH were created using SigmaPlot v13.0 (Systat Software Inc., San Jose, CA, USA).

The statistical analysis program SigmaPlot v13.0 (Systat Software Inc., San Jose, CA, USA) was used to analyze cytokine data collected during this trial. Data was analyzed

by two-way analysis of variance (ANOVA) to examine differences between omega-3 fatty acid supplements and control, pasture and TMR feed management systems as well as feed management system and supplement interactions (feed system × suppl.). ANOVA analysis was followed by Tukey's pairwise multiple comparison tests. Data was transformed if it did not pass normality (Shapiro-Wilk) or equal variance (Brown-Forsythe) tests, using common transformations available in the software program. A P value of < 0.05 was considered statistically significant. All results in tables are presented as the mean ± SEM.

Results

Assessment of Antibody Production in Response to KLH Immunization

Immunoglobulin concentrations were measured in order to quantify antibody production in response to the bovine KLH immunization. Total antibody concentrations were measured for each immunoglobulin isotype in addition to antibody concentrations specific for the KLH antigen.

Total IgM and anti-KLH IgM production in response to KLH immunization was measured in serum and is displayed in **Figure 1-1**. There was an overall effect of feed management system on total IgM concentrations ($P < 0.001$), cattle grazing in pasture had higher IgM concentrations than those fed TMR while housed in barn tie stalls. On KLH d21 (secondary response), cattle in the pasture feed management system displayed higher total IgM concentrations than those in the TMR system ($P < 0.001$). Supplement did not have a significant effect on total IgM concentrations, and no interactions of feed system × supplement were found. An overall effect of feed management system was also evident on anti-KLH IgM concentrations ($P < 0.001$). Similar to total IgM concentrations, cattle on TMR had decreased anti-KLH IgM production in comparison to cattle grazing in pasture. On KLH d7 (primary response, $P < 0.001$) and KLH d21 (secondary response, $P = 0.001$), cattle in the pasture feed management system had higher anti-KLH IgM concentrations than those in the TMR system. There was an effect of supplement on anti-KLH IgM concentrations ($P = 0.043$); cattle on microalgae displayed higher concentrations than those on control, however cattle on fish oil did not differ from those on control or microalgae. There was an overall interaction of feed system × supplement on anti-KLH IgM concentrations ($P = 0.049$).

Total IgG and anti-KLH IgG production in response to KLH immunization was measured in serum and is displayed in **Figure 1-2**. There was an overall effect of feed management system on total IgG concentrations ($P < 0.001$), cattle grazing in pasture had higher IgG concentrations than those fed TMR while housed in barn tie stalls. On KLH d21 (secondary response), there was a significant difference between total IgG concentrations based on feed management system ($P < 0.001$), with cattle in the pasture group having higher concentrations than those in the TMR group. Supplement had an effect on total IgG concentrations ($P = 0.003$); cattle on the microalgae supplement had the highest total IgG concentrations overall. There was a significant interaction of feed system × supplement overall on total IgG concentrations ($P = 0.008$). An overall effect of feed management system was also evident in concentrations of anti-KLH IgG ($P < 0.001$). Similar to total IgG concentrations, cattle on TMR had decreased anti-KLH IgG production in comparison to cattle on pasture. On KLH d21 (secondary response), cattle in the pasture feed management system displayed higher anti-KLH IgG concentrations than those in the TMR system ($P < 0.001$). There was an effect of supplement on anti-KLH IgG concentrations ($P = 0.041$); cattle on fish oil or algae displayed higher concentrations than cattle on control in pasture, while the opposite pattern occurred in cattle on TMR. There was an overall interaction of feed management system × supplement on anti-KLH IgG concentrations ($P = 0.044$), and this interaction was also evident at KLH d21 ($P = 0.014$).

Anti-KLH IgG_1 and anti-KLH IgG_2 production in response to KLH immunization was measured in serum and is displayed in **Figure 1-3**. There was an overall effect of feed management system on anti-KLH IgG_1 concentrations ($P < 0.001$); cattle grazing in pasture had higher anti-KLH IgG_1 concentrations than those fed TMR while housed in barn tie

stalls. This difference between anti-KLH IgG_1 concentrations due to feed management system was apparent on KLH d7 (primary response, $P = 0.005$), KLH d21 (secondary response, $P < 0.001$) and KLH d51 (long-term response, $P = 0.001$). Supplement had overall trend ($P = 0.065$) on anti-KLH IgG_1 concentrations; cattle on fish oil or microalgae supplements tended to have increased concentrations compared to those on control. There was a significant interaction of feed management system × supplement overall on anti-KLH IgG_1 concentrations ($P = 0.023$). An overall effect of feed management system was also evident on anti-KLH IgG_2 concentrations ($P = 0.047$); cattle on pasture displayed higher concentrations than those on TMR. On KLH d21 (secondary response), cows in the pasture feed management system had significantly higher anti-KLH IgG_2 concentrations than those in the TMR system ($P = 0.002$). In common with anti-KLH IgG_1 concentrations, supplement showed a trend toward significance ($P = 0.059$) on anti-KLH IgG_2 concentrations; cattle on the microalgae supplement tended to have increased anti-KLH IgG_2 concentrations compared to control, however cattle on fish oil did not differ from those on control or microalgae. On KLH d7 (primary response), there was an effect of supplement ($P = 0.031$), and cattle on fish oil and microalgae had higher anti-KLH IgG_2 concentrations than those on control. There was a significant interaction of feed management system × supplement overall on anti-KLH IgG_2 concentrations ($P = 0.013$), and this was also evident on KLH d7 (primary response, $P = 0.026$).

Total IgA and anti-KLH IgA production in response to KLH immunization was measured in milk and is displayed in **Figure 1-4**. There was an overall effect of feed management system on total IgA concentrations ($P = 0.003$); cattle grazing in pasture had higher IgA concentrations than those fed TMR while housed in barn tie stalls. Supplement

had an effect on total IgA concentrations ($P = 0.003$); cattle on the fish oil supplement had significantly lower concentrations than those on control or microalgae. On KLH d51 (long-term response), cattle on fish oil had the lowest IgA concentrations ($P = 0.038$). There was a significant interaction of feed system × supplement ($P = 0.049$) on IgA concentrations at KLH d7 (primary response). There was also an overall effect of feed management system on anti-KLH IgA concentrations ($P < 0.001$). Similar to total IgA concentrations, cattle on TMR had lower anti-KLH IgA production, while cattle on pasture had higher concentrations overall, but the supplement effect was not statistically significant. Anti-KLH IgA concentrations were significantly different between feed management systems on KLH d7 (primary response, $P = 0.045$), d21 (secondary response, $P < 0.001$) and d51 (long-term response, $P < 0.001$). There was no significant effect of supplement on anti-KLH IgA production.

Assessment of Cytokine Activity in Response to KLH Immunization

Cytokine concentrations were measured in order to determine the level of immune activity in response to bovine KLH immunization. Examination of changes in patterns of cytokine concentrations over the course of the immunization schedule allowed for determinations of shifts in the balance of T_H1 and T_H2 cell responses. Analysis of cytokine data also provided a means to assess how the omega-3 supplements or feed management systems influenced immune activity.

IL-4 was chosen for analysis due to its active role in adaptive immunity, in the differentiation of naïve $CD4^+$ T cells into helper T_H2 cells and in antibody isotype switching to IgG_1. IL-4 is considered to be a signature T_H2 cytokine. Concentrations of IL-

4 were measured in serum over five different time points throughout the trial (**Table 1-3**); however, concentrations of this cytokine were very low and even below the level of quantification (BLQ) in some circumstances. This made statistical analysis difficult since there only two cows on control and a single cow on microalgae in the pasture group that displayed IL-4 production on KLH d0, a single cow on control and a single cow on fish oil in the pasture group that displayed IL-4 production on KLH d1, while no cattle displayed measurable serum IL-4 concentrations on KLH d7, and a single cow on microalgae in the TMR group had a measurable IL-4 concentration on KLH d21. Increased serum IL-4 concentrations were observed on KLH d51, however there were no differences in concentrations attributable to feed management system or supplement. IL-4 concentrations were also quantified in splenic and caecal tissue; however, no differences were found between tissue concentrations in cattle from separate feed management systems or supplements.

IFN-γ was chosen for analysis due to its role in cell-mediated immunity and inflammatory responses and is considered to be a T_H1 signature cytokine. Concentrations of IFN-γ were measured in serum over six different time points throughout the trial (**Table 1-4**); however, concentrations of this cytokine were below the level of detection on KLH d0, 1, 7, 21 and 51. The only time point with measurable serum IFN-γ concentrations was KLH d14 (the day of the KLH booster dose), although no differences in concentrations were found due to feed management systems or supplements. In common with the IL-4 concentrations quantified in splenic and caecal tissue, no differences were found between IFN-γ tissue concentrations in cattle from separate feed management systems or supplements.

TGF-β1 was chosen for analysis since is an important regulatory cytokine, which influences antibody class switching in B cells to IgA and acts as a T_{reg} cytokine through monocyte activation inhibition. Concentrations of active TGF-β1 (**Table 1-5**) were measured in serum over six different time points throughout the trial, as well as splenic and caecal measurements at the trial endpoint. On KLH d0, serum active TGF-β1 concentrations were higher overall in cattle in the pasture feed management system than TMR (P = 0.015), and concentrations of active TGF-β1 were different between feed management systems in cattle on the fish oil supplement (P = 0.009). No differences in active TGF-β1 concentrations between feed management systems or supplements were found on KLH days 1, 7 or 14. On KLH d21, there was an overall effect of feed management system (P = 0.006) on active TGF-β1 concentrations, with cattle grazing in pasture having higher concentrations than those fed TMR in barn tie stalls, and this difference was most apparent in cattle on the control diet (P = 0.024). The overall effect of feed management system (P < 0.001) on active TGF-β1 concentrations was present again on KLH d21, with cattle in pasture having higher concentrations than those fed TMR. These feed management system-associated differences in active TGF-β1 concentrations were apparent in cattle on the control (P = 0.044) and microalgae supplement (P < 0.001). In the spleen, cattle in the TMR feed management system had higher active TGF-β1 concentrations than cattle in the pasture system. An effect due to feed management system on active TGF-β1 concentrations was present within caecal tissue, with cattle in the pasture system having higher concentrations than those in the TMR system (P < 0.001). This difference between active TGF-β1 concentrations by feed management system was present

within each diet; pasture grazing cattle displayed higher concentrations than TMR-fed cattle on control ($P = 0.020$), fish oil ($P < 0.001$) and microalgae ($P = 0.010$) supplements.

Concentrations of total TGF-β1 (**Table 1-6**) were also measured in serum over six different time points throughout the trial, as well as in splenic and caecal tissue. On KLH d0, total TGF-β1 concentrations were higher overall in cattle in the pasture feed management system than those in the TMR system ($P = 0.046$). Within cattle on the fish oil supplement, those in the pasture group had higher total TGF-β1 concentrations than those in the TMR group ($P = 0.039$). There was a supplement effect on concentrations of total TGF-β1 ($P = 0.021$), with cattle on fish oil having higher concentrations than those on microalgae ($P = 0.037$), and those on microalgae having lower concentrations than those on control ($P = 0.047$). Within the TMR feed management system, microalgae-fed cattle had lower total TGF-β1 concentrations than those on control ($P = 0.010$). On KLH d1, total TGF-β1 concentrations were higher overall in cattle in the pasture feed management system than TMR ($P = 0.042$). No differences in total TGF-β1 concentrations between feed management systems or supplements were found on KLH days 7 or 14. On KLH d21, total TGF-β1 concentrations were higher overall in cattle in the pasture feed management system than those in the TMR system ($P = 0.025$), and on the microalgae supplement, concentrations of total TGF-β1 were higher in cattle in the pasture system than those in the TMR system ($P = 0.026$). A similar pattern was found again on KLH d51, where total serum TGF-β1 concentrations were higher overall in cattle in the pasture feed management system than in the TMR system ($P = 0.009$), and on the microalgae supplement, concentrations of total TGF-β1 were higher in cattle grazing in pasture that those which were TMR-fed ($P = 0.029$). In the spleen, an overall supplement effect was present in total

TGF-β1 concentrations (P = 0.007), with cattle on the microalgae supplement having higher concentrations than those on fish oil (P = 0.019) or control (P = 0.012). Within the pasture feed management system, splenic total TGF-β1 concentrations were highest in the cattle fed the microalgae supplement compared to those on the fish oil supplement (P = 0.021) or control (P = 0.047). An effect due to feed management system on total TGF-β1 concentrations was present within caecal tissue, with cattle in the pasture system having higher concentrations than those in the TMR system (P < 0.001). This difference between total TGF-β1 concentrations by feed management system was present within each diet; pasture grazing cattle displayed higher concentrations than TMR-fed cattle on control (P = 0.070) and microalgae (P = 0.004), and lower concentrations than TMR-fed cattle on the fish oil supplement (P = 0.023).

Discussion

The rationale for continuing experimentation with supplementing the bovine diet with omega-3 fatty acids in the form of algae stemmed from previous AIF trials. Results obtained by our laboratory during AIF Trial #6 demonstrated that macroalgae supplementation with Tasco® (Acadian Seaplants Ltd., Dartmouth, Nova Scotia, Canada), an animal feed containing the brown alga *Ascophyllum nodosum*, increased KLH specific antibody production in dairy cattle (unpublished data). Tasco® has been previously shown to influence the bovine immune system, as cattle displayed increased macrophage activity when they entered new feed lots after feeding on Tasco® (Allen *et al*., 2001). The division of cattle by separate feed management systems (grazing in pasture or confined while fed TMR) was introduced in AIF trial #7 to reflect realistic cattle housing methods utilized in Canada.

Cattle in both feed management systems and on all dietary supplements responded to KLH immunization, however differences in the magnitude of response were observed. IgM are the first class of antibodies produced in the primary response to immunologic stimulus (Baumgarth *et al*., 2005; Liu *et al.*, 1991) and are the first line of defence against viral and bacterial antigens (Ehrenstein and Notley, 2010). The IgM antibody class is capable of strong complement activation and is highly polyvalent due to its pentameric structure, further enabling its ability to neutralize antigen (Ehrenstein and Notley, 2010). Total IgM concentrations measured in serum at all timepoints were distinctively higher in the cattle which grazed pasture in comparison to those which were confined in barn tie stalls and fed TMR, and this difference was most pronounced at the secondary response (21 day following immunization). Total IgM concentrations in the pasture group displayed

a classic immunization response. However, cattle in the TMR group did not display the typical immunization response pattern, and their total IgM response profile resembled that of their total IgG response profile which was flattened and did not follow a characteristic curve pattern. Cattle fed the microalgae supplement displayed higher total IgM responses than cattle on fish oil or control, though there was no significant effect of supplement on total IgM concentrations. A similar pattern was observed again in examining anti-KLH IgM concentrations, where cattle on pasture responded more robustly than cattle on TMR at primary and secondary response measurements, and followed the typical immunization response pattern. The anti-KLH IgM response was highest in cattle in the microalgae group. Production of IgM and anti-KLH IgM by cattle in the trial were far greater than other isotypes measured, and this would have increased the immune capacity in the event of viral or bacterial antigen exposure, particularly at the primary response. The cattle in the pasture feed management system or cattle receiving microalgae supplements had the highest serum total IgM and anti-KLH IgM response to immunization.

IgG antibodies are found predominantly in blood and have involvement in effective viral antigen neutralization (Butler *et al.*, 1971). IgG also facilitate opsonisation and are produced more slowly than IgM after a primary response to immunogenic stimulus (Liu *et al*., 1991; Mix *et al*., 2006). In general, all subclasses of IgG are capable of activating complement, although they may do so using classical, alternative or both forms of activation (Mix *et al*., 2006). Cattle which grazed pasture had higher total IgG responses to immunization overall in comparison to those which were confined in barn tie stalls and fed TMR, and this effect was specifically noted at the secondary response measure. Cattle in pasture displayed a classic immunization response with increasing titers at primary and

secondary responses, while returning to lower concentrations (maintained above baseline) over time. Although cattle in the TMR group did respond to immunization, they did not display the expected immunization response pattern for total IgG concentrations, and their pattern appeared to be more flattened in comparison. Microalgae-fed cattle produced stronger total IgG responses than those fed fish oil supplements or control. Cattle in pasture displayed higher anti-KLH IgG responses than those on TMR overall, and this effect was most pronounced at the secondary response. The production of anti-KLH IgG antibodies appeared to resemble the characteristic immunization response curve more than the total IgG antibodies, although this was not surprising given the fact that total IgG concentration increased due to anti-KLH IgG production. Cattle receiving both omega-3 supplements had higher anti-KLH IgG responses than those on control.

IgG_1 antibodies are produced by B2 cells in response to antigen when T_H2 cells release IL-4, and this isotype is capable of strong classical and alternative complement activation (Butler *et al*., 1971; Mix *et al*., 2006). Cattle in the pasture management system had significantly higher anti-KLH IgG_1 responses overall than those on the TMR system. Since cattle on the omega-3 supplements only tended to have higher anti-KLH IgG_1 responses than those on control, this implies that antibody production was more strongly influenced by feed management system. The anti-KLH IgG_1 response to immunization followed a typical pattern. IgG_2 antibody production by B2 cells is enhanced by IFN-γ production by T_H1 cells, a process which actually inhibits IgG_1 production (Mix *et al*., 2006). IgG_2 antibodies are capable of classical and alternative complement activation. Cattle in the pasture management system had significantly higher anti-KLH IgG_2 responses than those in the TMR system, and cattle on the microalgae supplements had higher

responses than those on control. Production of anti-KLH IgG_2 did not follow the characteristic immunization response pattern. The concentrations of anti-KLH IgG_2 were much lower in quantity than anti-KLH IgG_1, which was surprising since IgG_2 is usually the predominant IgG isotype in bovine serum (Butler *et al*., 1971).

A previous study conducted by van Altena *et al.* (2016) investigated the ability of naturally produced antibodies specific for KLH in dairy cattle to recognize common microbial structures. These cattle had not been previously immunized with KLH; however, they did have non-specific natural antibodies that bound KLH. It was discovered that cattle with higher titers of natural anti-KLH IgM displayed higher IgM binding capacity to *Escherichia coli* and *Salmonella typhimurium* overall (van Altena *et al*., 2016). Cattle which produced high or low titers of natural anti-KLH IgG had equal IgG binding capacity to *E. coli* (van Altena *et al*., 2016). This study concluded that cattle producing higher natural anti-KLH IgM and anti-KLH IgG concentrations had enhanced ability to bind pathogen-associated molecular patterns (PAMPs), and also a reduced chance in developing mastitis (van Altena *et al*., 2016). The majority of naturally produced antibodies are of the IgM isotype (Baumgarth *et al*., 2005), and the cattle in AIF Trial #7 displayed almost 63 fold higher IgM production than IgG. The KLH-specific antibodies were also produced in higher quantities belonging to the IgM isotype, which was almost 10 fold higher than the IgG isotype at the primary response, and this was typical of a primary response to immunization. Since cattle with higher natural anti-KLH IgM titers have been shown to have reduced incidence of mastitis (van Altena *et al*., 2016), cattle in the pasture feed management system while consuming the microalgae supplement likely had increased antibody binding capacity to PAMPS and decreased risk of developing mastitis.

IgA is predominantly a mucosal isotype produced by B1 cells, and contributes to mucosal immunity while neutralizing antigen (Liu *et al*., 1991; Pabst, 2012). Since IgA is the major Ig in mucosal secretions (Butler *et al*., 1971; Guidry *et al*., 1980), it was quantified in bovine milk. Total IgA responses were highest overall in cattle that grazed pasture, and microalgae-fed cattle had higher concentrations than fish-oil fed cattle. This effect remained at the long-term response measure. Total IgA concentrations did increase from the baseline to KLH d7, however total IgA concentrations did not follow the typical pattern of an antibody response to immunization with a rapid increase during the secondary response followed by a gradual decline in antibody concentrations specific for the immunizing antigen. In fact, the responses were completely inconsistent between supplements, and the TMR-fed cattle seemed to display diminishing total IgA concentrations over the KLH immunization schedule while the cattle in the pasture group appeared to increase total IgA concentrations. Anti-KLH IgA responses were higher in the pasture group of cattle, and this pattern was consistently observed over primary, secondary and long-term responses.

The presence of anti-KLH IgA production in milk suggests that a mucosal immune response occurred during systemic KLH immunization. In order to further explore impact of these dietary supplements and feed managements on the mucosal immune response, other mucosal samples besides milk should be collected for measurement and analysis of Ig concentrations, such as nasal secretions (Corbeil *et al*. 1984), salivary secretions (Sakaquchi *et al*. 2013), or faecal samples (Tolleson *et al*. 2013). The total Ig concentrations within milk collections may have fluctuated naturally over the weeks following parturition, with the possibility of steadily decreasing antibody transfer to the

calf influencing the total Ig concentrations in milk more strongly than the KLH immunization did. It is also important to consider that the responses over primary, secondary and long-term responses may have differed if the KLH was administered mucosally instead of the intramuscular (systemic) injection. However, it is possible that mucosal KLH immunization may not have induced a stronger Ig production response since the delivery of antigen to a mucosal surface may also induce tolerance, and this route of antigen tolerization has been shown in cattle (Weiss *et al*. 2006).

Specific cytokine concentrations were analyzed an effort to examine mechanisms of immunomodulation and shifts in the balance of T_H1 and T_H2 cell responses. IL-4 was chosen as a T_H2 marker in the analysis of KLH immunization parameters because its signaling regulates the differentiation of naïve $CD4^+$ T cells into helper T_H2 cells, (Paul, 1991), it is involved in B cell isotype class switching to IgG_1 (Pinchuk *et al*., 2003) and is considered a T_H2 signature cytokine. It was difficult to measure serum IL-4 as it was undetectable at the primary and secondary responses. At the long-term response, IL-4 was detectable in serum, spleen and caecum, but no differences between feed management system or omega-3 supplements were found. IFN-γ was chosen as a T_H1 marker in the analysis of KLH immunization parameters since it has involvement in cell-mediated immunity, and is considered a T_H1 signature cytokine (Schroder *et al.*, 2004). Similar to IL-4, it was difficult to measure IFN-γ in serum since it was undetectable at most points throughout the trial. Splenic and caecal IFN-γ concentrations measured at the end of the trial did not differ between feed management systems or dietary PUFA supplements. The fact that the IFN-γ tissue concentrations did not differ at the end of the trial may not however be representative of tissue IFN-γ concentration fluctuations throughout the trial.

Unfortunately, it was not possible to take these tissue measurements during the trial. It was interesting that IFN-γ concentrations were highest in serum at KLH d14, which was the day cattle received their booster KLH dose, perhaps reflecting a heightened T_H memory response, as the blood was drawn within hours of the cattle receiving the KLH injections. If IL-4 and IFN-γ concentrations had shown change, IL-4/IFN-γ production ratios would have been calculated to determine if there was a shift towards either T_H1 cell mediated activity or T_H2 humoral activity (Becker, 2006; Paul, 1991; Roman *et al.*, 1997). However, this was not possible because serum concentrations of these T_H1 and T_H2 cytokines were not detectable at several time points during the trial. Serum cytokine quantification is often not the optimal measure to monitor the activity of IL-4 and IFN-γ (Albers *et al.*, 2005). While tissue measures are preferable, they are not always possible.

TGF-β1 was chosen in the analysis of KLH immunization parameters because it is an important T_{reg} cytokine (Tran, 2012), it inhibits monocyte activation, influences antibody class switching to IgA in B cells and it can be also be considered a T_H2 cytokine (Li *et al.*, 2006; Rubtsov and Rudensky, 2007). At baseline, secondary and long-term response measurement points, cattle in the pasture feed management system produced higher serum concentrations of active TGF-β1 than those in the TMR system. In splenic tissue (representing a systemic measure), cattle in the TMR system displayed higher active TGF-β1 concentrations than those in the pasture system. In caecal tissue (representing a mucosal measure), the opposite pattern emerged and cattle in the pasture system on each diet produced higher active TGF-β1 concentrations than those in the TMR system. It is acknowledged that TGF-β1 may have also been produced not only by immune cells but also by the intestinal epithelial cells of the caecal tissue, and this may represent direct

effects of the diet in the pasture management system. At baseline, one day following initial immunization with KLH, secondary and long-term measurement points, cattle in the pasture feed management system produced higher serum concentrations of total TGF-β1 than those in the TMR system. Higher total TGF-β1 concentrations were also evident in the caecal tissue of cattle in the pasture group. At the baseline measure, cattle consuming fish oil had the highest serum total TGF-β1 concentrations, while cattle which consumed microalgae had the highest splenic total TGF-β1 concentrations suggesting a long-term on impact of this supplement on systemic TGF-β1 production. These patterns of serum active and total TGF-β1 production do follow the total IgA and anti-KLH IgA responses measured in milk, which were most strongly influenced by the pasture feed management system.

A previous study conducted by Garcia *et al.* (2014) measured immune responses in newborn Holstein calves which received unsaturated fatty acid supplements in the form of either high or low linoleic acid (an omega-6 PUFA). The *ex-vivo* IFN-γ production by isolated peripheral blood mononuclear cells (PBMCs) after stimulation with concanavalin A was measured, and it was found that PBMCs isolated from calves on high linoleic acid had increased production of IFN-γ. The study conclusions were that calves receiving the high linoleic acid supplement had earlier development of T_H1 activity due to increased capacity to produce IFN-γ (Garcia *et al.*, 2014).

An objective of AIF Trial #7 was to measure whether or not there were shifts in the balance of T_H1 and T_H2 activity based on systemic or mucosal concentrations of IL-4, IFN-γ, active and total TGF-β1. Since there were no apparent differences due to feed system or supplement on concentrations of IL-4 and IFN-γ, it was difficult to determine a direct T_H1/T_H2 shift based on these cytokines. However, active and total TGF-β1 concentrations

in serum and tissue were highest in cattle in the pasture feed management system, and TGF-β1 is an important regulatory cytokine with an ability to influence T_H1 and T_H2 activity. Cattle in the pasture system also produced higher concentrations of total and anti-KLH specific antibodies, which suggests heighted T_H2 activity. Additionally, there was a shift in the balance of anti-KLH IgG_1 and anti-KLH IgG_2 concentrations. IgG_2 production is associated with T_H1 activity and IgG_1 production is indicative of T_H2 activity (Mix *et al.*, 2006). IgG_2 antibodies are typically more predominant in bovine serum than IgG_1 (Butler *et al.*, 1971); however, cattle produced higher serum concentrations of anti-KLH IgG_1 than anti-KLH IgG_2 throughout the response to KLH immunization. The total increase in Ig production and anti-KLH IgG_1/IgG_2 antibody prevalence further indicates a shift towards T_H2 activity. Specifically, cattle in the pasture management system and those supplemented with microalgae (higher DHA and lower EPA content than fish oil) demonstrated a response indicative of shift towards T_H2 activity greater than those in the TMR system or on the control.

Studies have previously investigated differences between pasture and TMR management systems in dairy cattle, but their focus has mainly been on milk production and overall milk quality (Bargo *et al.*, 2002; Kolver and Muller, 1998). It has been reported that cattle in pasture have lower milk production (Kolver and Muller, 1998), and cattle in confinement fed TMR produce milk with higher fat and protein content (Bargo *et al.*, 2002). The differences between these feed systems on cattle immunity and various health parameters are under-studied. Diverse variables may contribute to the impact of feed system. For example, it is possible that differences in exercise, social interaction or isolation and barn-associated stressors may influence immune activity. AIF trial #7 results

demonstrate distinct differences between these two feed management systems with respect to bovine immunization responses, suggesting that feed management systems do have an impact on immune parameters, although the mechanism behind these differences remains to be determined.

Microalgae and fish oil have been previously supplemented in the bovine diet, however the extent to which they may influence bovine immunity has not been thoroughly investigated. A study which investigated microalgae feeding found that cattle produced milk with higher DHA content while on the microalgae supplement, and this milk was considered to have higher nutritional quality (Franklin *et al*., 1999). In a separate study investigating the effects of supplementing the bovine diet with fish oil, it was found that fish oil consumption did not influence the expression of genes involved in lipid metabolism in skeletal muscle or in the liver (Gessner *et al*., 2016). There have not been any negative impacts of omega-3 fatty acid supplementation on bovine health or metabolism parameters reported in the literature. After analyzing data collected during AIF Trial #7, cattle on the omega-3 supplements produced higher concentrations of total IgA, total IgG, anti-KLH IgG, anti-KLH IgG_1/IgG_2, and anti-KLH IgM than cattle on control, and production was most often higher in cattle which consumed microalgae. These findings demonstrate that the source of omega-3 and the DHA/EPA ratio may differentially modulate immune activity, as suggested by Caroprese *et al*. (2009) and Franklin *et al*. (1999). Given that the KLH formulations did not contain any adjuvant, supplementing the bovine diet with omega-3 fatty acids, specifically microalgae, could be an effective strategy in optimizing responses to routine vaccinations. Since polysaccharides from algae have been proposed to activate TLR4 (Zhang *et al.* 2016), this could be a possible adjuvant mechanism involved

in the enhancement of the response to immunization induced by dietary microalgae supplements.

It was acknowledged that the group of cattle which grazed in pasture may have had more exercise than the cattle fed TMR while housed in barn tie stalls. In a human meta-analysis study investigating the efficacy of exercise prior to influenza vaccination, it was determined that the level of exercise did not influence the efficacy of the vaccination (Grande *et al*., 2016). The influence of exercise on cattle immunization parameters has not been well documented, and the amount and frequency of cattle movement or exercise was not recorded during the AIF Trial #7, making it difficult to equate the immunological findings with exercise. Experimentation on the metabolic properties of these cattle was conducted by Dr. Payam Vahmani (PhD candidate at NSAC during the time of the trial), and he calculated the net energy balances between feed management systems and supplements. There were no significant differences in net energy balance between cattle grazing pasture and cattle fed TMR in barn tie stalls, and no significant differences between cattle on fish oil or microalgae supplements (Vahmani *et al*., 2014). However, cattle grazing pasture did have lower blood glucose concentrations and lower energy intake overall ($P \geq 0.13$, Vahmani *et al*., 2014).

In future trials investigating immunization responses in cattle on various supplements or feed management systems, it will be important to consider the timing of sample collections, the types of samples that should be collected, and the immunizing agent. In the current trial, serum and milk were collected for Ig concentration measurements at baseline, 7, 14, 21 and 51 day following KLH immunization. After Ig analysis, the timing of these sample collections did represent strong differences between

the two feed management systems, however it did not allow for an investigation of the potential impact on kinetics of the anti-KLH response. In order to examine this aspect of the response to immunization, blood should be collected every one to two days so that potential differences in the time to peak antibody responses may be compared between cattle on different dietary supplements and feed management systems. For a full investigation of a mucosal response to immunization, other samples in addition to milk during a typical lactation cycle should be collected, such as salivary or nasal secretions and faecal samples. It would also be interesting in future feed management studies to vaccinate cattle with an infectious agent that would be relevant to cattle, such as J-VAC® (Merial Canada Inc., Baie d'Urfé, Québec, Canada). This bovine vaccine is protective against *E. coli* mastitis, and also endotoxemia caused by *E. coli* and *S. typhimurium*. This vaccination strategy may be useful in studying whether pasture grazing cattle have higher concentrations of antibodies that will protect against frequently encountered infectious agents.

In conclusion, the pasture feed management system affected the magnitude of the bovine response to KLH immunization to a greater extent than the TMR system. The cattle which grazed pasture produced higher total and anti-KLH antibody concentrations over primary, secondary and long-term responses. Additionally, dietary omega-3 supplementation improved the bovine response to KLH immunization compared to control, and microalgae supplementation (higher DHA/lower EPA ratio than fish oil) was more frequently associated with higher total and anti-KLH antibody production than fish oil. The pasture feed management system and microalgae supplement supported a shift toward T_H2 activity during KLH immunization, and cattle in these groups displayed higher

concentrations of serum and tissue TGF-β, an important cytokine involved in immunoregulation. The pasture management system and supplementation with dietary microalgae may lead to improvement of current bovine immunization strategies. This information may be useful for the design of feed supplements to optimize immune responses to routine immunization programs in dairy cattle and to improve the overall quality of bovine health.

Table 1-1. Cytokines and immunoglobulin isotypes measured for determination of the impact of dietary fatty acids on bovine immunity.

Cytokine	Cellular Source	Cellular Target & Role	References
IFN-γ	Interferon-γ is produced by activated NK cells, $CD4^+$ and $CD8^+$ T lymphocytes	Impact on macrophage activation, MHC class I and II expression, antigen processing and presentation ability	(Schroder *et al.*, 2004)
IL-4	Interleukin-4 is produced by mast cells, T lymphocytes and bone marrow stromal cells	Regulates the differentiation of naïve $CD4^+$ T cells into helper T_H2 cells	(Paul, 1991)
TGF-β1	Transforming growth factor-β1 is produced by almost every cell type	Multifunctional, regulates cell proliferation, growth and differentiation. Exists in active and latent forms.	(Annes *et al*., 2003)
Immunoglobulin			
IgA	Antibody produced by plasma cells, predominately at mucosal surfaces and in milk/colostrum	Contributes to protective immunity and mucosal homeostasis, neutralizes antigens in epithelial endosomes	(Pabst, 2012)
IgG	Antibody produced by plasma cells in blood stream and in tissues, bovine IgGs are divided into two subclasses	Generally involved in neutralization of antigen and interacts with complement	(Butler *et al*., 1971)
IgG_1	Most abundant subclass of IgG	Production induced by IL-4 and inhibited by IFN-γ, involved in neutralization and opsonization of antigens and interacts with complement	(Butler *et al*., 1971; Mix *et al*., 2006)
IgG_2	Second most abundant subclass of IgG, although most prevalent in bovine serum	Production induced by IFN-γ and inhibited by IL-4, involved in neutralization of antigens and interacts with complement	(Butler *et al*., 1971; Mix *et al*., 2006)

This table is continued on the next page.

Table 1-1 *continued.*

Immunoglobulin	Cellular Source	Cellular Target & Role	References
IgM	Plasma cells, predominately in the blood	First antibody isotype to be produced during an immune response, polyvalent due to its pentameric structure, involved in neutralization and opsonization of antigens and interacts with complement	(Ehrenstein and Notley, 2010)

Table 1-2. AIF Trial #7 days corresponding to the days since calving and days since KLH immunization.

Trial Day	Day Since Calving	Day Since KLH Immunization & Activity Completed
0	- 30	-----
30	0 → birthing day	-----
69	39	0 → baseline measure, primary immunization
70	40	1 → first day following immunization
76	46	7 → primary response measured
83	53	14 → booster dose, secondary immunization
90	60	21 → secondary response measured
120	90	51 → long-term response measured

Table 1-3. Serum IL-4 concentrations at selected time points after KLH immunization, and final measurements in systemic and mucosal tissue.

		IL-4[*]					
		Supplements			Statistics – *P* value		
Measure [**]	**Feed System**	**Control**	**Fish Oil**	**Microalgae**	**Feed System**	**Suppl.**	**Feed System × Suppl.**
Serum KLH d0	Pasture	1.5 ± 1.2	BLQ	1.5 ± 1.5	*n/a*	*n/a*	*n/a*
	TMR	BLQ	BLQ	BLQ			
Serum KLH d1	Pasture	4.6 ± 4.6	2.6 ± 2.6	BLQ	*n/a*	*n/a*	*n/a*
	TMR	BLQ	BLQ	BLQ			
Serum KLH d7	Pasture	BLQ	BLQ	BLQ	*n/a*	*n/a*	*n/a*
	TMR	BLQ	BLQ	BLQ			
Serum KLH d21	Pasture	BLQ	BLQ	BLQ	*n/a*	*n/a*	*n/a*
	TMR	BLQ	BLQ	2.0 ± 2.0			
Serum KLH d51	Pasture	14.1 ± 8.9	10.1 ± 10.1	7.2 ± 3.0	0.392	0.485	0.988
	TMR	9.4 ± 5.1	7.0 ± 4.5	2.3 ± 1.3			
Spleen KLH d51	Pasture	41.6 ± 20.8	61.7 ± 30.9	52.4 ± 26.2	0.661	0.702	0.621
	TMR	57.7 ± 28.8	56.3 ± 28.1	53.9 ± 26.9			
Caecum KLH d51	Pasture	16.3 ± 16.3	26.7 ± 22.1	0.0 ± 0.0	0.107	0.144	0.702
	TMR	50.2 ± 17.1	65.0 ± 32.9	484.4 ± 484.4			

[*] IL-4 concentrations (pg/mL in serum and pg/g in tissue) are presented as mean ± SEM. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA.
[**] $n = 4$ per supplement/feed management system, with the exception of KLH d1 which measured all cows in the trial; $n = 8$ on control, $n = 7$ on fish oil and $n = 8$ on microalgae in the pasture feed management system. $n = 9$ on control, $n = 8$ on fish oil and $n = 8$ on microalgae in the TMR feed management system.

Table 1-4. IFN-γ concentrations in serum at selected time points after KLH immunization, and final measurements in systemic and mucosal tissue.

		IFN-γ*					
		Supplements			Statistics – *P* value		
Measure **	**Feed System**	**Control**	**Fish Oil**	**Microalgae**	**Feed System**	**Suppl.**	**Feed System × Suppl.**
Serum KLH d0	Pasture	BLQ	BLQ	BLQ	*n/a*	*n/a*	*n/a*
	TMR	BLQ	BLQ	BLQ			
Serum KLH d1	Pasture	BLQ	BLQ	BLQ	*n/a*	*n/a*	*n/a*
	TMR	BLQ	BLQ	BLQ			
Serum KLH d7	Pasture	BLQ	BLQ	BLQ	*n/a*	*n/a*	*n/a*
	TMR	BLQ	BLQ	BLQ			
Serum KLH d14	Pasture	445.2 ± 76.5	389.1 ± 109.4	462.9 ± 105.3	0.953	0.263	0.578
	TMR	546.0 ± 31.5	333.5 ± 101.7	429.5 ± 43.2			
Serum KLH d21	Pasture	BLQ	BLQ	BLQ	*n/a*	*n/a*	*n/a*
	TMR	BLQ	BLQ	BLQ			
Serum KLH d51	Pasture	BLQ	BLQ	BLQ	*n/a*	*n/a*	*n/a*
	TMR	BLQ	BLQ	BLQ			
Spleen KLH d51	Pasture	67.4 ± 43.3	249.1 ± 123.7	346.0 ± 134.1	0.338	0.281	0.112
	TMR	138.9 ± 95.7	164.6 ± 81.6	167.5 ± 69.4			
Caecum KLH d51	Pasture	BLQ	293.2 ± 220.7	BLQ	0.338	0.281	0.112
	TMR	304.6 ± 304.6	94.2 ± 94.2	162.8 ± 162.8			

* IFN-γ concentrations (pg/mL in serum and pg/g in tissue) are presented as mean ± SEM. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA.

** $n = 8$ on control, $n = 7$ on fish oil and $n = 8$ on microalgae in the pasture feed management system. $n = 9$ on control, $n = 8$ on fish oil and $n = 8$ on microalgae in the TMR feed management system. $n = 4$ per supplement/feed management system in tissue analysis.

Table 1-5. Active TGF-β1 concentrations in serum at selected time points after KLH immunization, and final measurements in systemic and mucosal tissue.

		Active TGF-β1*					
		Supplements			Statistics – *P* value		
Measure **	**Feed System**	**Control**	**Fish Oil**	**Microalgae**	**Feed System**	**Suppl.**	**Feed System × Suppl.**
Serum KLH d0	Pasture$^{\alpha}$	9.9 ± 4.0	13.9 ± 5.3$^{\alpha}$	7.5 ± 2.7	0.015	0.644	0.197
	TMR$^{\beta}$	2.3 ± 2.0	0.8 ± 0.6$^{\beta}$	4.1 ± 3.1			
Serum KLH d1	Pasture	14.9 ± 7.6	13.6 ± 3.1	5.5 ± 2.6	0.731	0.302	0.973
	TMR	15.0 ± 7.4	16.3 ± 7.9	7.9 ± 3.8			
Serum KLH d7	Pasture	22.8 ± 4.7	18.2 ± 3.4	18.8 ± 4.6	0.319	0.955	0.629
	TMR	15.2 ± 2.9	17.4 ± 4.8	17.4 ± 3.0			
Serum KLH d14	Pasture	10.3 ± 5.7	4.2 ± 4.2	6.2 ± 4.3	0.666	0.296	0.934
	TMR	8.5 ± 6.0	1.2 ± 1.2	3.6 ± 2.8			
Serum KLH d21	Pasture$^{\alpha}$	29.7 ± 6.4$^{\alpha}$	21.2 ± 5.5	33.9 ± 6.1	0.006	0.269	0.701
	TMR$^{\beta}$	9.9 ± 3.5$^{\beta}$	11.9 ± 5.7	19.3 ± 8.8			
Serum KLH d51	Pasture$^{\alpha}$	49.8 ± 9.7$^{\alpha}$	36.8 ± 9.6	58.6 ± 10.4$^{\alpha}$	< 0.001	0.613	0.136
	TMR$^{\beta}$	27.8 ± 7.6$^{\beta}$	21.9 ± 4.2	15.1 ± 5.2$^{\beta}$			
Spleen KLH d51	Pasture$^{\alpha}$	BLQ	23.6 ± 8.9	29.2 ± 10.7	0.017	0.335	0.881
	TMR$^{\beta}$	49.0 ± 22.9	59.3 ± 27.9	87.0 ± 37.7			
Caecum KLH d51	Pasture$^{\alpha}$	118.6 ± 17.5$^{\alpha}$	190.4 ± 46.5$^{\alpha}$	126.2 ± 19.3$^{\alpha}$	< 0.001	0.551	0.159
	TMR$^{\beta}$	27.5 ± 21.0$^{\beta}$	5.4 ± 5.4$^{\beta}$	22.5 ± 22.5$^{\beta}$			

* Active TGF-β1 concentrations (pg/mL in serum and pg/g in tissue) are presented as mean ± SEM. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Different symbols denote significant differences between feed system overall or within a particular diet group as determined by Tukey's multiple comparison test.

** $n = 8$ on control, $n = 7$ on fish oil and $n = 8$ on microalgae in the pasture feed management system. $n = 9$ on control, $n = 8$ on fish oil and $n = 8$ on microalgae in the TMR feed management system. $n = 4$ per supplement/feed management system in tissue analysis.

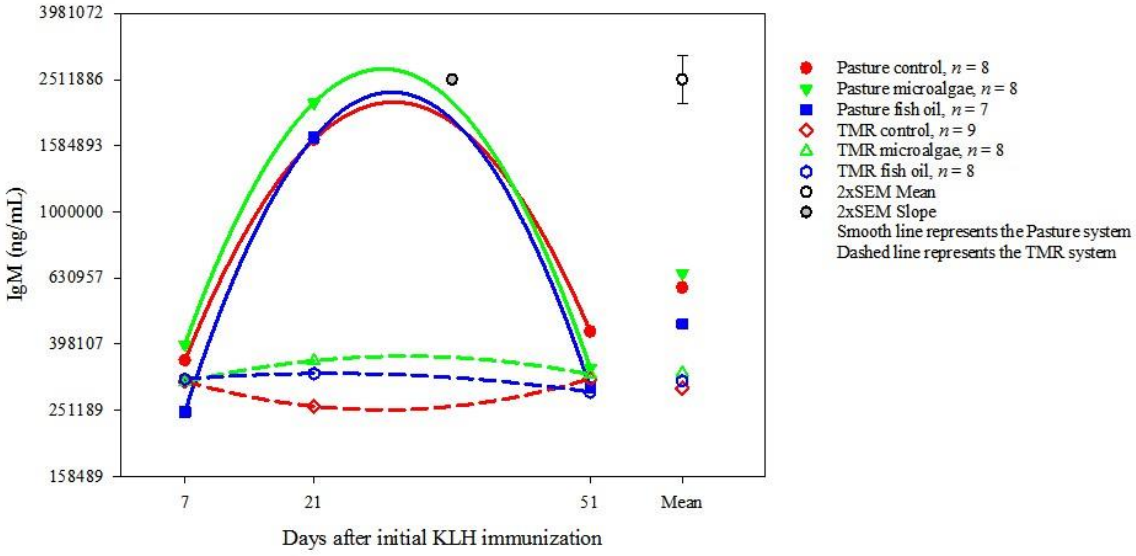
Table 1-6. Total TGF-β1 concentrations in serum at selected time points after KLH immunization, and final measurements in systemic and mucosal tissue.

		Total TGF-β1*					
		Supplements			Statistics – *P* value		
Measure **	**Feed System**	**Control**	**Fish Oil**	**Microalgae**	**Feed System**	**Suppl.**	**Feed System × Suppl.**
Serum KLH d0	Pasture$^{\alpha}$	1567.7 ± 307.3^{a}	2422.2 ± 620.2$^{a\alpha}$	1365.3 ± 292.4^{b}	0.046	0.021	0.096
	TMR$^{\beta}$	1778.6 ± 184.3$^{a\gamma}$	1259.0 ± 240.7$^{a\gamma\delta}$	776.0 ± 244.2$^{b\delta}$			
Serum KLH d1	Pasture$^{\alpha}$	1348.0 ± 259.8	1465.2 ± 477.9	959.6 ± 284.5	0.042	0.110	0.688
	TMR$^{\beta}$	1283.6 ± 288.1	654.8 ± 180.1	565.6 ± 183.6			
Serum KLH d7	Pasture	2312.1 ± 316.2	3218.4 ± 850.2	2268.3 ± 446.3	0.230	0.210	0.170
	TMR	2859.9 ± 261.9	2093.9 ± 481.2	1668.6 ± 313.4			
Serum KLH d14	Pasture	2218.9 ± 498.1	1870.5 ± 319.4	1848.9 ± 268.0	0.100	0.659	0.975
	TMR	1718.5 ± 448.1	1478.3 ± 417.9	1445.0 ± 417.6			
Serum KLH d21	Pasture$^{\alpha}$	2127.1 ± 387.9	2255.8 ± 313.2	3043.2 ± 593.4$^{\alpha}$	0.025	0.545	0.421
	TMR$^{\beta}$	1860.8 ± 266.3	1763.4 ± 231.7	1744.1 ± 238.7$^{\beta}$			
Serum KLH d51	Pasture$^{\alpha}$	3581.0 ± 1026.3	2187.1 ± 889.6	1757.9 ± 155.6$^{\alpha}$	0.009	0.165	0.543
	TMR$^{\beta}$	1580.3 ± 302.8	1468.1 ± 337.0	910.7 ± 167.2$^{\beta}$			
Spleen KLH d51	Pasture	12 112.4 ± 3843.5^{a}	10 116.1 ± 3314.5^{a}	25 260.9 ± 4471.4^{b}	0.749	0.007	0.498
	TMR	10 339.8 ± 2324.3^{a}	13 837.9 ± 965.6^{a}	20 446.5 ± 5048.2^{b}			
Caecum KLH d51	Pasture$^{\alpha}$	12 112.4 ± 3843.5$^{\alpha}$	10 116.1 ± 3314.5$^{\alpha}$	25 260.9 ± 4471.4$^{\alpha}$	< 0.001	0.925	0.841
	TMR$^{\beta}$	10 339.8 ± 2324.3$^{\beta}$	13 837.9 ± 965.6 $^{\beta}$	20 446.5 ± 5048.2 $^{\beta}$			

* Total TGF-β1 concentrations (pg/mL in serum and pg/g in tissue) are presented as mean ± SEM. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between supplements, and symbols denote significant differences between supplement overall or feed system overall as determined by Tukey's multiple comparison test.

** Sample *n* was the same as in the active TGF-β analysis (Table 1-5).

a)



b)

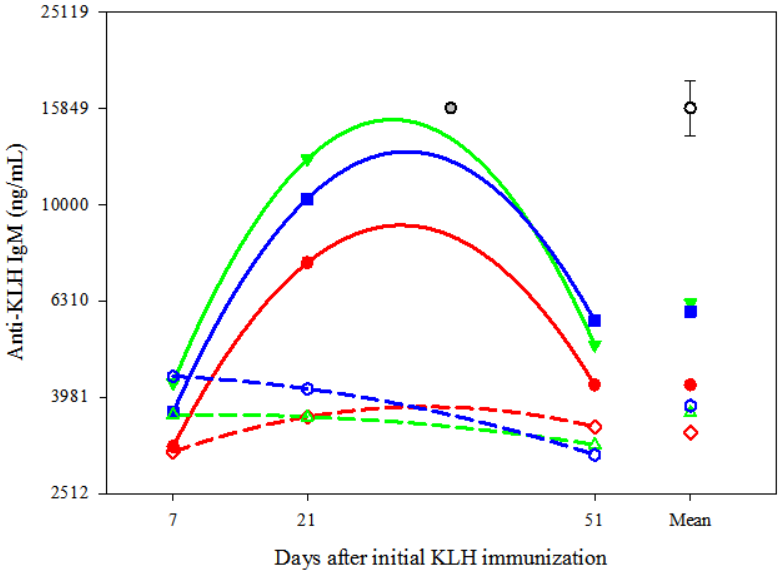
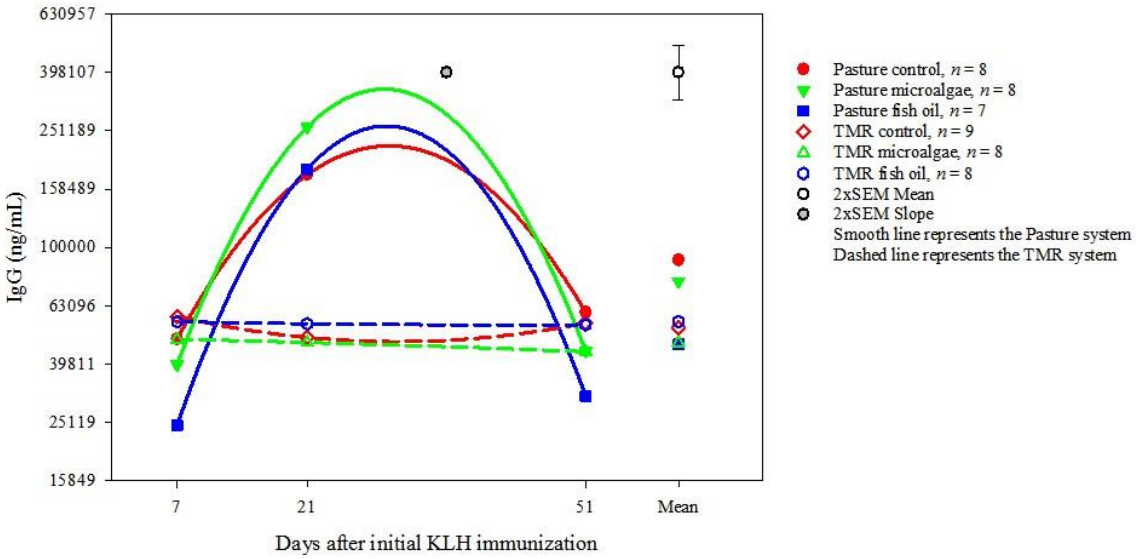


Figure 1-1. IgM concentrations quantified in serum following KLH immunization over primary, secondary and long-term time points in cattle on omega-3 dietary supplements and separate feed management systems. Significance ($P < 0.05$) for feed management system, supplement and interactions were determined using REML analysis, with Fisher's LSD multiple range test to determine differences between groups. **a**. Total IgM concentrations; the effect of feed system ($P < 0.001$) was significant. **b**. Anti-KLH IgM concentrations; the effect of feed system ($P < 0.001$), supplement ($P = 0.043$) and feed system × supplement interaction ($P = 0.049$) were significant.

a)



b)

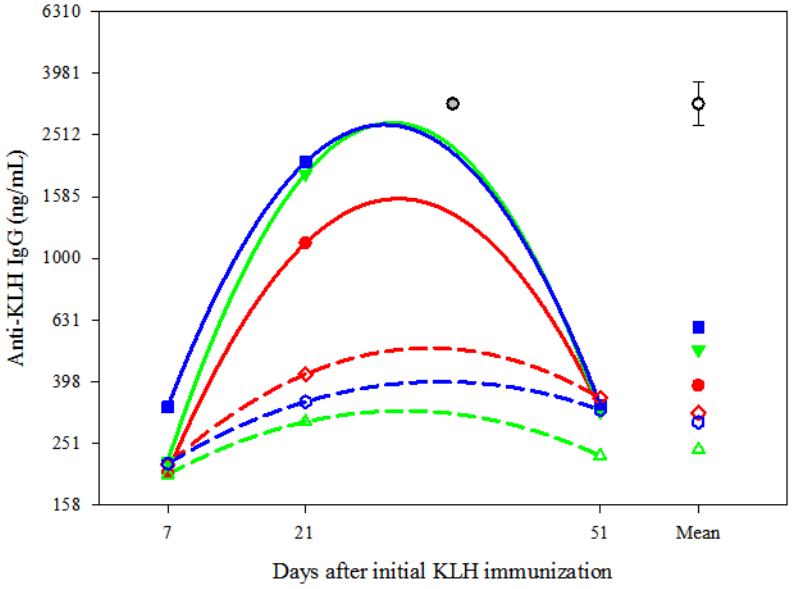
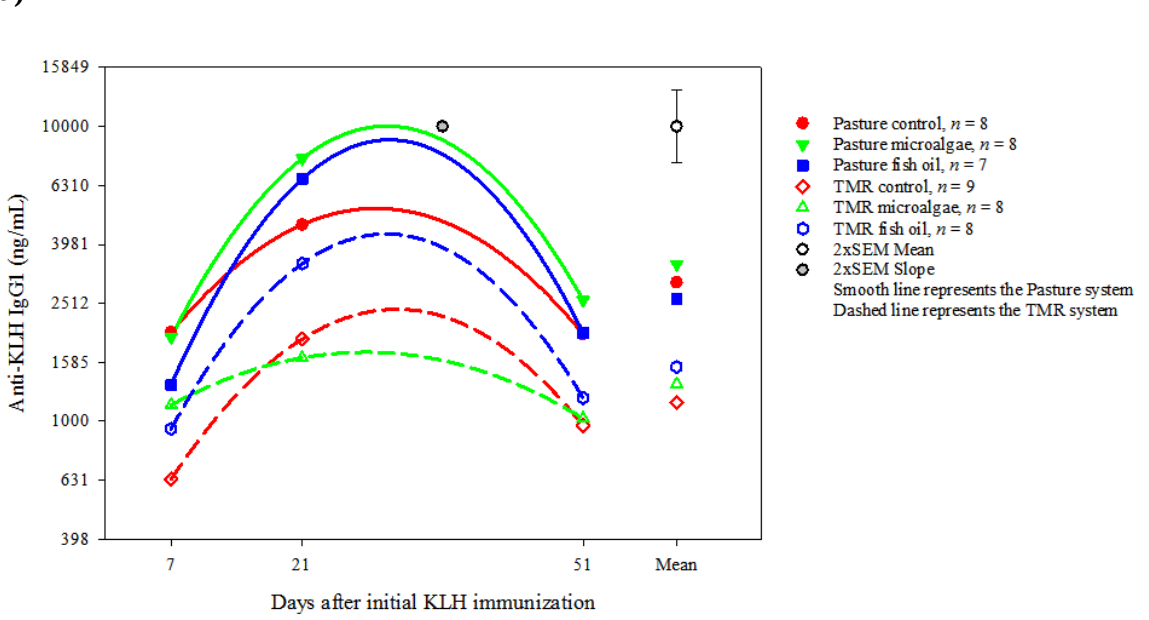


Figure 1-2. IgG concentrations quantified in serum following KLH immunization over primary, secondary and long-term time points in cattle on omega-3 dietary supplements and separate feed management systems. Significance ($P < 0.05$) for feed management system, supplement and interactions were determined using REML analysis, with Fisher's LSD multiple range test to determine differences between groups. **a**. Total IgG concentrations; differences between feed systems ($P < 0.001$), supplements ($P = 0.003$) and feed system × supplement interaction ($P = 0.008$) were significant. **b**. Anti-KLH IgG concentrations; the effect of feed system ($P < 0.001$), supplement ($P = 0.041$) and feed system × supplement interaction ($P = 0.044$) were significant.

a)



b)

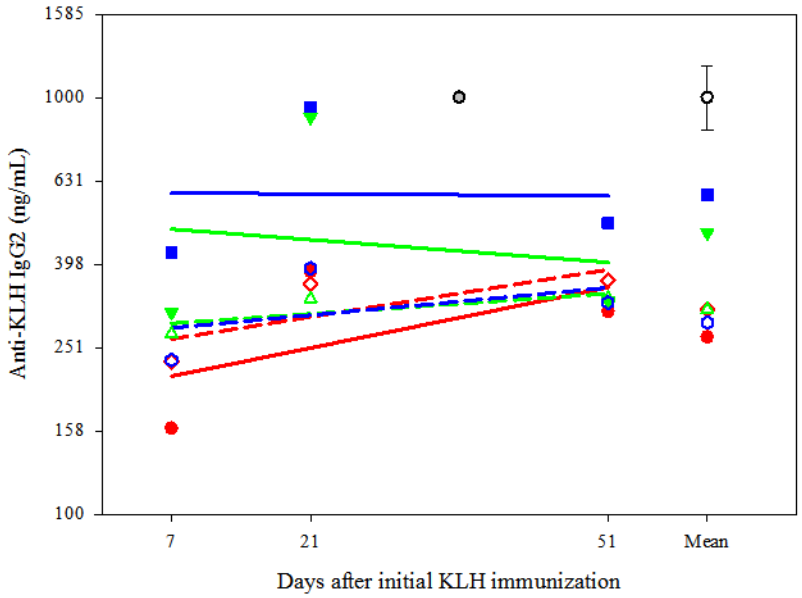
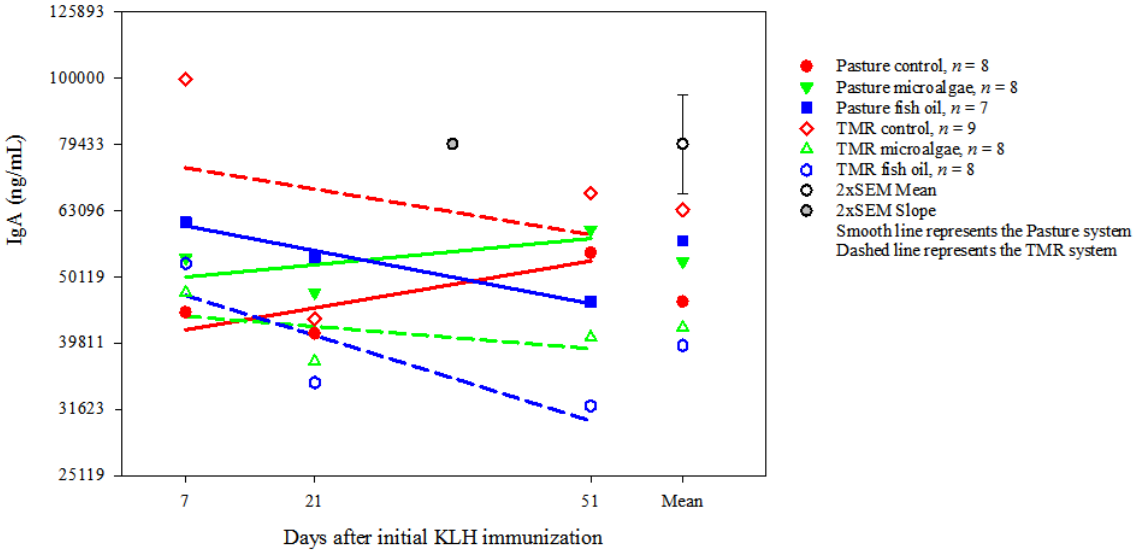


Figure 1-3. Anti-KLH IgG subclass concentrations quantified in serum following KLH immunization over primary, secondary and long-term time points in cattle on omega-3 dietary supplements and separate feed management systems. Significance ($P < 0.05$) for feed management system, supplement and interactions were determined using REML analysis, with Fisher's LSD multiple range test to determine differences between groups. **a**. Anti-KLH IgG_1 concentrations; the effect of feed system ($P < 0.001$) and feed system × supplement interaction ($P = 0.023$) were significant. **b**. Anti-KLH IgG_2 concentrations; the effect of feed system ($P = 0.047$) and feed system × supplement interaction ($P = 0.013$) were significant.

a)



b)

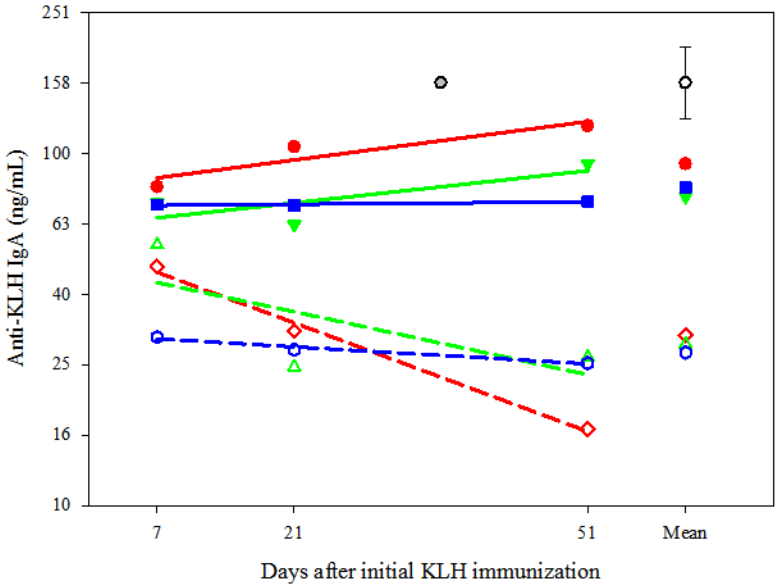


Figure 1-4. IgA concentrations quantified in milk following KLH immunization over primary, secondary and long-term time points in cattle on omega-3 dietary supplements and separate feed management systems. Significance ($P < 0.05$) for feed management system, supplement and interactions were determined using REML analysis, with Fisher's LSD multiple range test to determine differences between groups. **a**. Total IgA concentrations; the effect of feed system ($P = 0.003$) and supplement ($P = 0.003$) were significant. **b**. Anti-KLH IgA concentrations; the effect of feed system was significant ($P < 0.001$).

Chapter 2: Examining Effects of β2-1 fructans on the immune system in a human clinical trial

Introduction

The Intestinal Barrier and Mucosal Immunity

Mucosal surfaces of the body are in direct contact with the external environment and are protected by the mucosal immune system. The gastrointestinal (GI) tract is the largest mucosal surface in the body. Human intestines are colonized by commensal bacteria shortly after birth and the GI tract provides bacteria with nutrients from digested food (Honda and Takeda, 2009). Commensal bacteria of the intestinal tract are beneficial for the host since they aid in food digestion to supply essential nutrients and also prevent the invasion of pathogenic microbes (Honda and Takeda, 2009). Intestinal epithelial cells (IEC) form a physical barrier dividing the intestinal tissues from direct contact with the gut microbiota. Several mechanisms operate at the IEC level for separating the host from the microbiota, and these include tight junctions to prevent bacteria from moving between the epithelial cells, microvilli-covered surfaces to prevent bacterial attachment, and secretions from goblet cells which create mucous to prevent bacteria from penetrating the cells (Artis, 2008). Antimicrobial proteins such as defensins are also considered an immune mechanism in protecting the IEC as they aid in eliminating bacteria that penetrate the mucus layer (Hooper and Macpherson, 2010). The single monolayer of IEC has many important duties, including not only nutrient uptake, but also transmitting signals from luminal bacteria to immune cells such as macrophages and natural killer cells to respond to microbial threats (Vizoso Pinto *et al*., 2009). Due to these roles, IEC are often considered to be participants in the innate immune response.

The mucous membranes of the GI tract comprise the MALT and are essential in protecting the host from pathogenic bacteria. The synthesis of secretory immunoglobulin-A (sIgA) by B cells is a very important component in mucosal immunity. Mucosal IgA is involved in regulating the balance and composition of bacteria within the intestines and it has the ability to affect bacterial glycosylation (Peterson *et al*., 2007). sIgA produced by B cells is transcytosed across IEC into the lumen via the polymeric immunoglobulin receptor (pIgR), where it can bind to bacteria. sIgA thus creates an effective immunological barrier which may prevent infection by neutralization of pathogens (Leblanc *et al*., 2004), and therefore offers protection for the host since it maintains mucosal homeostasis (Johansen *et al*., 1999). The presence of sIgA often prevents enteropathogens from adhering to the intestinal epithelium walls and subsequent colonization or invasion (Leblanc *et al.*, 2004). In addition, sIgA has been shown to play an essential role in creating a "firewall" environment in the mesenteric lymph nodes, preventing the gut microbiota from penetrating further into the MALT and the lymphatic system (Macpherson and Uhr, 2004).

Dietary Fibre, Prebiotics and Bifidobacteria

Prebiotics are nondigestible oligosaccharides which selectively stimulate the growth of certain bacteria within the colon, and are believed to improve host health (Gibson and Roberfroid, 1995). The definition of a prebiotic demonstrates the fulfilment of three specific criteria for the classification of prebiotic fibre (Gibson *et al*., 2004). These criteria are that the fibre must be resistant to enzymatic digestion and gastrointestinal absorption, the fibre must be fermentable by the colonic microbiota, and the fibre must promote growth of intestinal bacteria which initiates a change towards a healthier microbiota balance (Gibson *et al*., 2004). Prebiotics have been widely claimed to be beneficial for the host, as

these fermentable materials have been reported to promote the activity of Bifidobacteria and Lactobacilli species (Brownawell *et al*., 2012; Kau *et al.*, 2011). Associations of these bacteria with potentially health-promoting qualities such as mucin production by IEC have been described in the literature (Gibson and Roberfroid, 1995). Bifidobacteria have been associated with accelerated colonic transit time (Marteau et al., 2002), synthesis of vitamins in the GI tract (Crittenden *et al*., 2003), and protection of the IEC barrier (Lievin *et al*., 2000). However, the effects of increased Bifidobacteria content in the gut may vary between individuals (Macfarlane *et al*., 2006), and while there are numerous reports of effects of Bifidobacteria on the immune system, there are also many questions surrounding the extent of their effects in healthy adults.

Much remains to be determined about the full extent of the effects of prebiotic fibres on the gut microbiota community structure. The degree to which the dietary fibre is fermented is characterized by the structure of the fibre as well as by the composition of the gut microbiota (Abnous *et al.*, 2009). An area of key interest is determining if shifts in gut microbiota composition induced by the fermentation of these dietary fibres impose a health benefit for the host (Abnous *et al.*, 2009). Until recently, most gut microbiota studies have relied on cultivation-based techniques to enumerate and identify the gut bacteria – an ineffective approach as many of these microbes are strict anaerobes and are not readily cultured. Current molecular approaches should reveal the true extent of prebiotic effects on the gut microbiota.

Effects of β2-1 Fructans on the Immune System

β2-1 Fructans include fructooligosaccharides (FOS) and inulin; they are among the most well-known fermentable materials which have been claimed to have effects on the gut microbiota. These substrates have been reported to stimulate the growth of Bifidobacteria *in vivo* (Bouhnik *et al.*, 2006) and *in vitro* (Langlands *et al.*, 2004), however the use of *Bifidobacterium* levels has been debated as a biomarker of gut health (Ouwehand *et al*., 2005). FOS is composed of short-length chains of 2-8 fructose units with a terminal glucose linked by β2-1 glycosidic bonds, whereas inulin is composed of 9-60 repeating fructose units (Roberfroid, 2005; Singh and Singh, 2010). β2-1 Fructans are rapidly and completely fermented in the proximal colon (Singh and Singh, 2010). Fermentation of β2-1 fructans leads to the production of lactate and short chain fatty acids (SCFA) (Looijer-van Langen and Dieleman, 2009).

The presence of SCFA in the gut has been reported to stimulate the attachment of Lactobacilli species to the intestinal mucosa and to deter pathogen adhesion (Bomba *et al.*, 2002). The terminal sugars of oligosaccharides inhibit the adherence of pathogenic bacteria and prevent colonization of the intestinal epithelium (Fukuda *et al*., 2011; Zopf and Roth, 1996). There is evidence to suggest that SCFA are important mediators in the interactions between gut microbiota on the immune system, as the SCFA butyrate has been shown to modulate the ubiquitination and degradation of Nuclear Factor-κB (NF-κB) inhibitor IκB-α (Kumar *et al*., 2009). The SCFA liberated from dietary fibre fermentation have also been associated with increased intestinal IgA concentrations in rats *(*Kudoh *et al.*, 1998; Lim *et al*., 1997). It has been hypothesized that the production of SCFA in the intestine may be involved in regulating a mucosal immune response (Looijer-van Langen and Dieleman,

2009), and it has been reported that these SCFA have involvement in immunomodulatory activities in the event of colonic inflammation (Kumar *et al.*, 2009). In murine models, dietary supplementation with SCFA has been shown to attenuate colitis in wild type mice through the enhancement of $FOXP3^{+}$ T_{reg} cell activity, however the same effect was not observed in *Gpr43*$^{-/-}$ mice, which lacked an important signaling receptor for SCFA (Smith *et al*., 2013).

β2-1 Fructans have been shown to prevent attachment of pathogenic strain *Clostridium difficile* using *in vitro* experiments (Hopkins and Macfarlane, 2003), and to stimulate the growth of protective bacteria including *Bifidobacterium spp*. and *Lactobacillus spp*. (Looijer-van Langen and Dieleman, 2009) suggesting an association with IEC defense mechanisms. Lactic acid produced by β2-1 fructan fermentation lowers the pH of the colon, and this has been suggested as a mechanism to exclude pathogens from the gut (Lomax and Calder, 2009). However, several contradictions regarding the protective nature of β2-1 fructan fermentation in the gut exist in the literature. For example, FOS has been shown to impair resistance to *Salmonella enteritidis* infection and increase intestinal permeability in rats (Ten Bruggencate *et al*., 2004; Ten Bruggencate *et al*., 2005), and also to decrease intestinal barrier function in healthy men (Ten Bruggencate *et al*., 2006).

Rodent models have been used to collect the majority of data on the physiological and immunological effects of β2-1 fructans (Bovee-Oudenhoven *et al.*, 2003; Ryz *et al*., 2009; Ten Bruggencate *et al*., 2005), and only a few studies to date have investigated the impact of β2-1 fructans on immune parameters in healthy human subjects in homeostatic conditions. A study investigating impacts on the immune system in healthy middle-aged

subjects who consumed β2-1 fructans for 4 weeks did not find alterations in immune cell populations of neutrophils or monocytes, NK cell activity, or circulating Ig concentrations (Lomax *et al*., 2012). A separate study investigating non-smoking men found decreased percentages of NK cells and immune cells expressing intercellular adhesion molecule-1 (ICAM-1) (Seidel *et al.*, 2007). In another study involving women with type 2 diabetes, circulating concentrations of LPS and TNF-α decreased after 8 weeks of β2-1 fructan consumption (Dehghan *et al*., 2014), suggesting an anti-inflammatory effect. The most consistent finding reported regarding β2-1 fructan supplementation in human subjects is that it increases faecal Bifidobacteria content (Bouhnik *et al*., 2006; Lomax *et al*., 2012), although it is not clear whether this generates an immunological or physiological benefit for the majority of healthy adults who have consumed it.

Functional parameters of systemic immunity should be analyzed in order to determine if gut microbiota shifts are beneficial for the host. TLRs are pattern-recognition receptors (PRR) which are expressed on innate immune cells such as macrophages and dendritic cells (DC). When TLRs bind microbial motifs, referred to as PAMPs, a signal transduction pathway is activated. TLR2 is activated by peptidoglycan or lipoteichoic acid while TLR4 is activated in the presence of LPS. Stimulated TLR2 and TLR4 receptors induce a signalling cascade through adaptor molecules including myeloid differentiation factor 88 (MyD88), and TLR4 can also induce signalling via the MyD88-indepent pathway involving TIR domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) (Abreu, 2010). These signalling cascades ultimately facilitate the activation of the NF-κB transcription factor and lead to production of proinflammatory cytokines (Abreu, 2010).

There have been suggestions in the literature that the gut microbiota influence TLR expression (Furrie *et al.*, 2005; Vizoso Pinto *et al*., 2009), and this is an aspect of prebiotic fibre delivery that has not been analyzed in detail. Changes in TLR expression or in responses to TLR agonists may provide a sensitive indicator of alterations in the innate immune response. The measurement of TLR expression on immune cell populations can be carried out through immunofluorescence with anti-TLR antibodies, and assessed functionally by stimulation of monocytes and DCs with TLR2 and TLR4 agonists (Schaaf *et al*., 2009; Versteeg *et al.*, 2009). Since systemic immunity has been shown to be influenced by the gut microbiota (Balmer *et al*., 2014; Clarke *et al.*, 2010), measuring responses to TLR agonists provides a novel method to determine effects of dietary FOS on the innate immune response.

Chapter 2 Study Objectives

A randomized, double blind, cross-over clinical trial was conducted to examine whether ingestion of β2-1 fructans could influence the immune system in healthy adults. Direct comparison of fermentable and non-fermentable dietary supplements was used to determine if consumption of β2-1 fructans influenced the gut microbiota community structure and whether such changes created detectable changes in immune activity. This investigation was a collaborative project with researchers at Health Canada (Ottawa, ON) and Agriculture and Agri-Food Canada (Guelph, ON and Kentville, NS), who performed analyses on the gut microbiota, and with researchers at McMaster University (Hamilton, ON), who performed analyses on gastrointestinal distress and parameters of well-being. My role in the trial was to determine the impact of β2-1 fructan supplementation on immune parameters in healthy subjects through examining its effects on the immune system using a range of immunological measures. It was hypothesized that dietary β2-1 fructan supplementation would have a pronounced impact on the immune system, and subjects would have higher levels systemic pro-inflammatory activity on the β2-1 fructan phase of the trial in comparison to placebo. The cytokines measured in determining the impact of β2-1 fructan supplementation on immune parameters are shown in **Table 2-1**.

Objective: to examine impacts of β2-1 fructans on immune activity in healthy subjects under steady-state conditions (in the absence of immune challenge, infection or disease).

Methods

Human Clinical Trial Subjects

Subjects were recruited for this investigation from Guelph, Ontario and its surrounding area by a newspaper advertisement. The trial involved 30 healthy adults (13 men and 17 women) who were not taking medication or other dietary supplements, and did not have a history of GI diseases or food allergies. The average age of the subjects was 28 years, and the ages ranged between 20-42 years. All subjects provided informed consent for their participation. Clinical visits took place at Nutrasource Diagnostics Inc. (Guelph, Ontario, Canada).

Human β2-1 Fructan Trial Design

The clinical β2-1 fructan trial began in January 2011 was designed as a double-blind, placebo-controlled, randomized crossover experiment. Since each participant consumed both dietary supplements, individuals served as their own control for each dietary supplement. The test supplement contained an oligofructose-enriched inulin, while the control diet was maltodextrin (did not contain β2-1 fructans). The trial subjects were randomized into groups based on the order in which they were to receive placebo or the β2-1 fructan supplement using an online randomization program (Seed no. 26285, Tufts University, Medford, MA, USA: www.tufts.edu/~ gdallal/random_block_size.htm). Subjects were instructed to consume the individually packaged 5 g supplement three times daily mixed in a beverage and taken with meals, for a total of 15 g of supplement each day. The trial participants consumed either the test or control dietary supplements for 28 days, followed by a 14 day wash out period, and continued with consuming the alternate

supplement for another 28 days. Fasting blood (12 hours) and faecal samples were collected at the beginning and end of each dietary supplement period for a total of four sample collection points. Subjects maintained daily diaries to record bowel movements, self-reported adverse events, or changes in their normal routine.

The test diet was Orafti®Synergy1 (Beneo-Orafti, Tienen, Belgium); a dietary supplement containing oligofructose-enriched inulin derived from chicory root. This supplement is currently available in European countries and is marketed as a prebiotic nutritional supplement with bone health properties (Holloway et al., 2007). The control supplement (placebo) was food grade maltodextrin (Beneo-Orafti, Tienen, Belgium); a soluble fibre which is digested rather than fermented in the GI tract. Maltodextrin is a common food additive used as a thickening or bulking agent (Oliveira et al., 2009). Subject compliance was calculated from the amount of returned (unconsumed) supplement packets. β2-1 fructans present in the background diet were estimated for each subject using a semi-quantitative food frequency questionnaire and data on the average portion sizes from the Canadian Community Health Survey (http://www.hc-sc.gc.ca/fn-an/surveill/nutrition/commun/cchs_focus-volet_escc-eng.php). The midpoint of the ranges reported was used in calculating the consumption of β2-1 fructan-containing foods (Moshfegh et al., 1999) to obtain a value representative of the average grams/day consumption of β2-1 fructans in the base diet.

Approval was received from the UOIT Research Ethics Board (REB) to commence the investigation in fall 2011. To have full involvement in the human clinical trial, I attended two Tri-Council Policy Statement (TCPS) training seminars to become informed

of the appropriate ethical conduct, procedures and limitations for research involving human subjects.

Measurement of Blood Parameters

Serum blood urea nitrogen (BUN) concentrations were measured at Health Canada using an ABX Pentra 400 automated clinical chemistry analyser and ABX Pentra Urea CP (urease-glutamate dehydrogenase) test kits (Horiba Canada Inc., Burlington ON, Canada). All other biochemical and haematological biomarkers were analyzed by LifeLabs Medical Services (Toronto, Ontario, Canada). These measures included standard haematological profiles, serum biochemistry (fasting glucose, creatinine, aspartate, γ-glutamyl-transferase, C-reactive protein (CRP), globulin, albumin and total blood protein) and lipid profiles (low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, total cholesterol and triglycerides.

Peripheral Immune Cell Population Analysis by Flow Cytometry

The flow cytometry laboratory technologist at Health Canada, Emily Chomyshyn, performed whole blood cell population analysis using flow cytometry with monoclonal antibodies to estimate population percentages of specific circulating immune cells. The list of antibodies used in the analysis are shown in **Table 2-3**. Peripheral populations analyzed included T cells and T cell subsets ($CD4^+$ and $CD8^+$), B cells, NK cells, plasmacytoid Dendritic cells (pDC) and myeloid DC (mDC), macrophages and granulocytes. Flow cytometry analysis was completed at the time of blood collection for the final day (d28) of each dietary supplement period. Blood samples were initially treated with Immunoprep™

Reagent system (Beckman Coulter, Indianapolis, IN, USA) to remove erythrocytes that could interfere with cell staining. Flow cytometry analysis was performed using a BD FACSCaliburr™ flow cytometer (BD Biosciences, San Jose, CA, USA), equipped with a blue laser (488 nm) and a red diode laser (488 nM). Forward scatter and side scatter gating techniques used during acquisition of 10 000 viable cells/lymphocytes were standard and similar to those outlined by Shingadia *et al.* (2001). Analysis of immune cell populations was conducted using BD FACSDIVA™ software (BD Biosciences, San Jose, CA, USA).

In addition to cell population analysis, a subset of the whole blood samples was stimulated with the TLR-4 agonist LPS (1 µg/mL; Sigma Aldrich, St. Louis, MO, USA) diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Aldrich, St. Louis, MO, USA) in order to investigate effects of β2-1 fructan supplementation on monocyte and mDC TLR-signaling activity. After a 1-hour incubation with the TLR4 agonist at 37°C and 5% CO_2, samples were treated with Brefeldin A (BFA; 10 µg/mL; Sigma Aldrich, St. Louis, MO, USA) in order to allow accumulation of intracellular cytokines. BFA is a known inhibitor of protein transport that prevents cytokine secretion (Della Bella et al., 2008), and BFA treatment is a standard technique to optimize intracellular cytokine detection. Samples were further incubated at 37°C and 5% CO_2 for an additional 2 hours. Following the incubation, immune cells were collected by centrifugation and were permeabilized for intracellular staining with anti-TNF-α and anti-IL-12p70. Once treated, the cells were analyzed by flow cytometry and intracellular production of TNF-α and IL-12p70 were measured.

Measurement of Circulating Cytokine Profiles

Initially, a BD™ Cytometric Bead Array (CBA) Human Flex set (BD Biosciences, San Jose, CA, USA) was used to quantify circulating cytokine concentrations. This assay system was customizable and utilized flow cytometry to detect soluble analytes, such as cytokines. The assay system was analogous to an ELISA, however the CBA was less time consuming than conventional ELISAs and offered high throughput capabilities since multiple cytokines could be measured in a single assay. The assay beads had a capture surface for specific proteins, and the cytokines were detected by the flow cytometer through the attachment of fluorescent antibodies. A collection of cytokines representing anti-inflammatory, pro-inflammatory, regulatory, T_H1 and T_H2 responses were measured to obtain a complex profile and include IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12p70.

Serum samples from all subjects at baseline and end point for each supplement were analyzed using the BD CBA Flex Set system on a BD LSRFortessa™ Flow Cytometer (BD Biosciences, San Jose, CA, USA). However, there were no serum samples containing measurable amounts of cytokines IFN-γ, IL-2, IL-4, IL-6 or IL-12p70. Out of all the tested samples, there were only two samples with detectable TNF-α, a single sample with IL-1β, and a single sample with IL-10. The only cytokine that was consistently detected in all serum samples was IL-8. Circulating cytokine concentrations are usually low in serum from healthy individuals, and the lack of detection of most cytokines may reflect the relative sensitivity of the multiplexed CBA approach compared to that of optimized ELISAs (Dossus *et al*., 2009; Young *et al*., 2008). For this reason, serum cytokines were quantified again using ELISA, and cytokine concentrations in keeping with those

anticipated in healthy adults were obtained with this format. The cytokine profile was expanded to include granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), IFN-γ, IL-1 receptor antagonist (IL-1Ra), IL-1β, IL-4, IL-6, IFN-γ-induced protein 10 (IP-10/CXCL10), soluble CD40 ligand (sCD40L/TNFSF5), all measured using DuoSet ELISA Development kits (R&D Systems, Minneapolis, MN, USA); IL-10, IL-12p70 and TNF-α using ELISA Max™ Sets (BioLegend, San Diego, CA, USA); and IL-8 was measured using a human IL-8 ELISA kit (Invitrogen, Carlsbad, CA, USA). Both active and total TGF-β1 concentrations were measured in plasma using the same methods as outlined in Chapter 1. In addition to the cytokines, LPS binding protein (LBP) was measured in serum using the HK315 Human LBP ELISA Kit (Hycult Biotech, Uden, The Netherlands).

ELISAs were carried out using 96-well high-binding half area microplates (Greiner Bio-One, Frickenhausen, Germany), using antibody concentrations recommended by the manufacturers protocols. Serum and plasma samples were pre-tested for appropriate assay dilutions. ELISA plates were coated with capture antibody diluted in phosphate buffered saline, and incubated overnight at 4°C. Following sample incubation, biotinylated detection antibody and horseradish peroxidase-conjugated streptavidin were added at separate steps, and its substrate TMB (Sigma Aldrich, St. Louis, MO, USA) was added. The colorimetric reaction was stopped with the addition of 1.8 N H_2SO_4, and the optical density of the reactions in the microplates were read at a wavelength of 450 nm on a Synergy HT multi-detection microplate reader using KC4 v3.4 analysis software (BioTek Instruments Inc, Winooski VT, USA).

Endotoxin Detection in Serum Samples

Initially, a HEK-Blue™ hTLR4 cell based assay (SEAP Reporter HEK293 cells expressing the human TLR4 gene; InvivoGen, San Diego, CA, USA) was tested to quantify the presence of LPS in plasma and serum samples. However, this assay system was difficult to use and results were inconsistent. The success of the assay depends on completely consistent cell culture growth rate and density, and is easily influenced by any biological contaminants (for example, selected cytokines and free fatty acids). The HEK293 cells used in this assay system retain expression of TLR3, TLR5 and NOD1 receptors in addition to increased expression of TLR4. Additionally, low concentrations of TNF-α are capable of activating NF-κB and lead to a colorimetric change in this assay, influencing the interpretation of LPS concentration that may be calculated. Many trial subjects had measurable concentrations of circulating TNF-α, so it was highly possible that this factor would have influenced LPS measurements in this assay system. After preliminary testing, it was decided to shift to a more established protocol to measure endotoxin concentrations, the Limulus Amebocyte Lysate (LAL) Assay.

Serum endotoxin concentrations were quantified using the LAL QCL-100 Assay (Lonza, Basel, Switzerland). All reagents and samples were brought to room temperature prior to reconstitution and use. Great care was used in minimizing potential sources of LPS contamination during this assay, and the entire experiment was carried under aseptic conditions. Only sterile, endotoxin-free consumables were used in this assay, such as endotoxin-free reagent reservoirs, sterile 96-well microplates suitable for cell culture, filter pipette tips and serological pipettes. The water used for preparing reagents and sample

dilutions was HyPure Cell Culture Grade Water, endotoxin-free < 0.005 EU/mL (Hyclone Laboratories, Logan, UT, USA).

To prepare for the assay, LAL was reconstituted immediately before use with endotoxin free water (3.0 mL/vial lysate) and vials were pooled to create one master LAL reagent. The LAL was gently swirled to avoid foaming and was protected from direct light. *E. coli* O111:B4 endotoxin was reconstituted by the addition of 1.0 mL endotoxin-free water, to obtain a stock concentration of 26 EU/mL. The endotoxin vial was shaken vigorously for 15 minutes on a vortex mixer to ensure even distribution. Endotoxin standards were then prepared in glass vials: 1.0, 0.5. 0.25, 0.1 EU/mL in endotoxin-free water. Each standard was mixed vigorously for 1 minute before use. The chromogenic substrate was reconstituted by the addition of 6.5 mL of endotoxin-free water to the vial, gently swirled and protected from light exposure. The LAL assay instructions suggested adjusting the pH of all samples to be within a biological range of 6.0 – 8.0 using 1N sodium hydroxide or 0.1N hydrochloric acid. In a subset of serum samples, the pH ranged from 7.6 – 7.78. Since these measurements were within a biological range, pH adjustments were not necessary.

To carry out the assay, a lidded 96-well sterile tissue culture grade plate was pre-warmed to 37°C using a dry heat block (VWR, Randor, PA, USA). To begin the assay, 50 µL of undiluted serum samples (run in duplicate), standards and blanks were dispensed in appropriate wells. All reagent additions and incubations were absolutely identical. At T = 0, 50 µL LAL was added to each well, and the plate was gently tapped to ensure thorough mixing before it was returned to the heating block. The samples were then incubated for 10 minutes at 37°C. At T = 10 minutes, 100 µL of pre-warmed chromogenic substrate

solution was added to each well, and the plate was gently tapped to ensure thorough mixing before it was returned to the heating block and incubated for an additional 6 minutes at 37°C. At T = 16 minutes, 100 µL of stop reagent (25% glacial acetic acid in endotoxin-free water v/v) was added to each well, and the plate was gently mixed by tapping. The absorbance of the microplate was then immediately read at 405 nm using a Synergy HT microplate reader (Bio-Tek, Winooski, VT, USA).

Since the serum samples had varying shades of colour ranging from pale wheat to amber, possibly due to red blood cell (RBC) lysis, the creation of serum sample blanks was necessary to ensure that background colour did not interfere with endotoxin quantification. Individual sample background blanks were created by mixing 50 µL sample, 150 µL endotoxin-free water and 100 µL stop reagent without incubation. Once the microplate was read at 405 nm and the assay background was subtracted from all samples, individual sample backgrounds were separately subtracted from each sample value before endotoxin concentrations were calculated. If sample endotoxin concentrations were above the standard curve, serum samples were diluted 1/5 and assayed again. A simplified procedure for endotoxin detection is shown in **Table 2.4**.

Measurement of Serum and Faecal Antibody Profiles

Resting-state serum antibody isotype profiles were measured using the Human Immunoglobulin Isotyping Magnetic Bead Panel (MILLIPLEX Map Kit; EMD Millipore Corp. Billerica, MA, USA) using a Luminex 200 analyzer (Austin, TX, USA). The immunoglobulin isotype profile quantified consisted of IgG_1, IgG_2, IgG_3, IgG_4, IgA and IgM. The Luminex assay format utilizes magnetic beads and detection antibodies

conjugated to phycoerythrin (PE), which is a fluorescent reporter signal used to quantify the proteins of interest. Advantages to this immunoassay format include customizable assays, an automated 96 well plate system that enables high throughput screening, and reduction of measuring inaccuracies since many samples can be measured all at once. Total IgG concentrations were not quantified by Luminex since the IgG subclasses cannot be measured at the same time (multiplexed) as total IgG, and this was a rather expensive assay to run with only one parameter. A random group of serum from 6 subjects over all four time points was retested by ELISA to confirm the results obtained by Luminex.

In order to quantify the resting-state faecal Ig concentrations, thawed faeces were prepared in a 1:5 (w/v) PBS solution containing 10 mg/mL bovine serum albumin (BSA) and 1% Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA) and incubated for 10 minutes at room temperature. Samples were then completely homogenized using a Brinkman Polytron PT3000 homogenizer (Kinematica, Luuzern Schweiz, Switzerland), centrifuged (4000 × *g* for 30 minutes at 20°C), and supernatants were collected and stored at -80°C until analysis. The faecal antibody isotype profiles were measured using the Human Ig ELISA Quantitation Set for IgA, IgG and IgM (Bethyl Laboratories Inc., Montgomery, TX, USA), and ELISA plates were read at a wavelength of 450 nm on a microplate reader (Infinite® 200 Pro Series; Tecan, Männedorf, Switzerland).

Measurement of ex vivo TLR-Induced Responses in Whole Blood Culture

Whole blood cultures from the end points of the β2-1 fructan and placebo phases of the trial were treated with TLR ligands to investigate responsiveness to TLR stimulation. Whole blood cultures which had been collected in sodium heparin blood collection tubes

were stimulated separately with TLR2 agonist Pam3Cys (P3C; 10 µg/mL; Novabiochem (EMD Millipore Corp.) and TLR4 agonist LPS (1 µg/mL; Sigma Aldrich, St. Louis, MO, USA) diluted in RPMI-1640 medium, in addition to an unstimulated control. Whole blood cultures were incubated at 37°C and 5% CO_2 in a humidified incubator. After 20 hours of incubation, blood assay tubes were centrifuged at 1300 × g for 5 minutes, and the supernatants were stored at -80°C until analysis.

Cytokine production profiles within the supernatants collected from the whole blood culture assay were measured using the BD CBA Human Flex set BD and acquired on a BD LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA, USA), equipped with blue (488 nm), red (640 nm) and violet (405 nm) lasers. Cytokine concentrations measured in the supernatants included anti-inflammatory, pro-inflammatory and regulatory cytokines – specifically IFN-α, TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70. These stimulated samples from the whole blood culture assay had higher cytokine concentrations and were above the concentration of the lowest assay standard, making it possible to use the CBA multiplex approach at this stage.

After the majority of the physiological, immunological, psychological, and microbiological data were collected and analyzed, the identities of the supplements as either placebo or β2-1 fructan test diet were revealed. Immediately following the supplement phase identification, statistical analyses were performed.

Statistical Analyses

The statistical program Statistica v12 (StatSoft Inc., Tulsa, OK USA) was used to analyze data collected during this trial. Repeated-measures ANOVA (rANOVA) was

utilized with time point (d0 and d28) and supplement (placebo and β2-1 fructan) as the repeated variables. In cases where there was only a single time point, as in the immune cell phenotype data, rANOVA were used with supplement as the repeated measure. A *P* value of < 0.05 was considered statistically significant. When significant values were obtained, rANOVA analysis was followed by Tukey's honest significant difference (HSD) test to determine differences within groups. For some measures, there were several values that were below the limit of quantification (LoQ). In these instances, the values were set to LoQ/2 (Armbruster *et al*., 1994) for analysis with Friedman's test (non-parametric) followed by a Wilcoxon's matched-pairs signed-rank test. Data was normalized using Box-Cox transformations when necessary. All results in tables are presented as the mean ± SEM.

Results

Characteristics of the Trial Subjects

The baseline demographic data of the individual subjects at the study baseline is shown in Appendix B, Table A1. All subjects were found to have a healthy status before the trial began. The average participant age was 27 and the average BMI was 24.2 kg/m^2. Daily intakes of β2-1 fructans in the base diet were estimated (**Table 2-5**), and no significant difference ($P = 0.150$) was found between the placebo (1.09 g/day) and β2-1 fructan (0.96 g/day) supplement phases in the regular diets of the subjects. Supplement receiving order was analyzed for every measured immune parameter in the study as a factor in a rANOVA, however no differences were attributable to the order in which subjects entered the supplement phases. Subject compliance was calculated by the number of supplements consumed/total amount provided for each phase of the trial; the placebo phase compliance was 97.6 ± 0.5 %, and the β2-1 fructan phase compliance was 99.9 ± 6.9 %.

Assessment of Blood Parameters

Supplementation with either β2-1 fructan or placebo had no impact on BMI, BUN, standard blood biochemistry including lipid profiles, or haematological profiles (**Table 2-6**).

Analysis of Peripheral Immune Cell Populations and mDC/Monocyte Cytokine Activity

Immune cell populations were examined to determine if the consumption of β2-1 fructans had an impact on circulating population profiles. Percentages of $CD282^+/TLR2^+$

mDC significantly increased after β2-1 fructan supplementation ($P = 0.008$; **Table 2-7**). A tendency of higher $CD284^+/TLR4^+$ mDC ($P = 0.060$) and $CD284^+/TLR4^+$ granulocyte ($P = 0.050$) percentages was also found after β2-1 fructan supplementation. No other differences were found between placebo and β2-1 fructan supplementation for percentages of mDC, pDC, monocytes, granulocytes, neutrophils, NK cells, B cells, T cells or T cell subsets.

Following LPS stimulation of whole blood samples, intracellular cytokine production was measured in mDC and monocytes using flow cytometry to further investigate responses to TLR4 activation at a cell-specific level. In a subgroup of six subjects, accumulation of TNF-α and IL-12 in mDC and monocytes was measured at the supplement phase end points (**Table 2-8**). The percentages of mDC and monocytes expressing TNF-α and IL-12 did not differ between supplement phases in unstimulated controls or after LPS stimulation. Percentages of mDC and monocytes expressing TNF-α did increase after LPS stimulation, however this was not linked with supplementation. Percentages of mDC and monocytes expressing IL-12 did not change after LPS stimulation, suggesting that the 1 μg/mL stimulation may not have been strong enough to induce a response.

Assessment of Circulating Cytokine Profiles, Serum Endotoxin and LBP

Circulating cytokine profiles, LPS and LBP concentrations were measured to determine if β2-1 fructan supplementation influenced systemic immune activity. The mean serum concentrations are presented in **Table 2-9a**, and the median values with the 25^{th}-75^{th} percentiles are presented in **Table 2-9b** to show the natural variation in these

measures in healthy adult subjects. By the end of the β2-1 fructan phase, subjects had lower concentrations of regulatory cytokine IL-10 ($P < 0.001$), and increased concentrations of T_H2 cytokine IL-4 ($P < 0.001$) and proinflammatory cytokine GM-CSF ($P = 0.002$). Concentrations of TNF-α were higher on day 28 of the placebo phase than day 0 ($P < 0.001$), however the day 28 placebo value was not different from either time point measured during the β2-1 fructan phase. IP-10 (CXCL10) concentrations were lower on day 28 of the β2-1 fructan phase than day 0 ($P = 0.010$), however this value was not different from the values obtained during the placebo phase. No effects due to supplement or phase day were found for serum concentrations of sCD40L, G-CSF, IFN-γ, IL-1β, IL-1Ra, IL-6, IL-8 and IL-12p70, or for plasma concentrations of active and total TGF-β1.

Serum LPS concentrations were significantly higher after 28 days of β2-1 fructan supplementation than any other time point measured in the trial ($P < 0.03$). Serum LBP concentrations did not differ significantly within or between supplement phases.

Assessment of Circulating and Faecal Ig Profiles

Resting-state antibody profiles were measured to determine if supplement had an impact on adaptive immunity in the absence of a defined challenge. Although there were several differences between serum Ig concentrations over the time points measured, these differences did not correlate with treatment phase (**Table 2-10**). Concentrations of IgA, IgM and all subclasses of IgG (IgG_1, IgG_2, IgG_3, IgG_4) were significantly different between day 0 and day 28 of the placebo phase, however the d-28 values did not differ from the values measured in the β2-1 fructan phase. Serum IgA was lower on day 28 of

the β2-1 fructan phase, and it was not different from the values measured in the placebo phase. Serum IgG_1 and IgG_2 were lower on day 28 of the β2-1 fructan phase than day 0 and day28 of the placebo phase.

β2-1 Fructan supplementation did not appear to impact faecal Ig profiles either; the only difference was found for IgM concentrations between day 0 and day 28 during the placebo phase ($P < 0.005$), and the values on placebo at day 28 were not different from either time point measured in the β2-1 fructan phase.

Assessment of Responses to ex vivo TLR2 and TLR4 Stimulation in Whole Blood Cultures

The *ex vivo* whole blood TLR stimulation assay was utilized to examine differences in TLR responsiveness to microbial-derived agonist stimulation between supplement phases and was analyzed by measuring cytokine production. There were no significant differences in cytokine production in the unstimulated or LPS stimulated blood cultures (**Table 2-11**). P3C-induced IL-10 concentrations significantly increased at the end of the β2-1 fructan phase ($P = 0.016$). TNF-α concentrations were higher after stimulation with LPS than P3C, however these differences were not associated with a supplement phase. IFN-α was below the level of detection at all time points measured, and only one individual displayed IL-12p70 production after P3C stimulation on day 28 of the β2-1 fructan phase.

Discussion

After four weeks of supplementing the diet of healthy adults with β2-1 fructans, several impacts on host immune activity were identified. However, supplementation with either β2-1 fructan or placebo had no impact on BMI, BUN, routine blood biochemistry including lipid profiles, or haematological profiles. The subjects who participated had a healthy status entering the trial, so the unchanged blood chemistry and haematological profiles according to phase provided assurance that supplements did not change these clinical biomarkers. Several studies have suggested β2-1 fructan consumption may be associated with lowering blood lipid profiles and total cholesterol concentrations. Serum triglyceride concentrations decreased after 20 g per day of β2-1 fructan consumption over three weeks in hypercholesterolemic men (Causey *et al*. 2000), and HDL-cholesterol concentrations increased after 10 g per day of β2-1 fructan and soy supplementation in hyperlipidemic adults (Wong *et al*., 2010). No alterations in blood lipids were found during this trial, which is similar to a study in healthy women who consumed 14 g of β2-1 fructans over a four-week period, where no differences were found in plasma concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol or triacylglycerol after supplementation (Pedersen *et al*., 1997). The differences in findings from these studies suggest β2-1 fructans may impact blood lipids differentially in healthy adults versus those with conditions of high blood lipids.

An interesting topic regarding β2-1 fructans in the literature is their potential use in promoting satiation. In a study of healthy females consuming 6 g of inulin daily with their breakfast meal for a period of eight days, it was found that inulin consumption lowered the desire to eat and prospective food consumption, but did not influence hunger or overall energy intake (Heap *et al.*, 2016). Another study involving both males and females who

had consumed 22.4 g of inulin per day for a seven-day period found no changes in qualitative or quantitative appetite measures (Darzi *et al*., 2016). These indices of appetite were not evaluated in the current trial, although it is unlikely that this β2-1 fructans supplement had long-term influences on appetite since the BMI or total weight did not fluctuate between phases.

Serum and faecal antibody concentrations were measured since Ig concentrations and isotype profiles can serve as a biomarker for changes in host adaptive immune activity (Albers *et al*., 2005; Cassidy *et al*., 1974). After 4 weeks of consuming β2-1 fructans at a dose of 15 g per day, there were no associations found between β2-1 fructan supplementation and Ig concentrations or isotype profiles, which was in common with another study of healthy middle-aged adults in which subjects consumed 8 g of β2-1 fructans daily for 4 weeks (Lomax *et al*., 2012). A considerable degree of variation was observed in both serum and faecal Ig concentrations over the course of the trial, which occurred independently of placebo or β2-1 fructan supplementation. Circulating Ig concentrations are subject to short-term periodic fluctuations; serum IgA and IgG concentrations may change by 20% on a bi-weekly basis in healthy individuals (Veys *et al*., 1977). Ig concentrations can change in response to seasonality, with greatest variation observed during fall and winter months, markedly between mid-August to mid-February (Nordby and Cassidy, 1983). Since this trial took place from January to April, it is possible that some of the Ig fluctuations could be attributed to seasonality, as treatment order analysis did not reveal any differences in Ig concentrations. Natural variations in concentrations of serum Ig exist between healthy individuals due to effects of gender, ethnicity and age (Cassidy *et al*., 1974; Maddison *et al*., 1975), menstrual cycles (Vellutini

et al., 1997), alcohol consumption and cigarette smoking (Gonzalez-Quintela *et al*., 2008), adding complexity to the interpretation of changes in total serum Ig in this type of study.

In a trial involving infants, faecal sIgA concentrations significantly increased after 16 weeks of consumption of a β2-1 fructan-supplemented formula in comparison to standard formula-fed infants (Bakker-Zierikzee *et al.*, 2006). A separate study following infants for the first 26 weeks after birth found that a β2-1 fructan-supplemented formula-fed infants had higher faecal sIgA than infants on a control formula (Scholtens *et al.*, 2008). It is not clear in the literature how β2-1 fructans influence faecal Ig concentrations in healthy adults. Positive correlations have been found between dietary fibre consumption and intestinal IgA concentrations in rat models (Kudoh *et al*., 1998; Lim *et al*., 1997), which has led to suggestions that dietary fibre or the products of its fermentation may support B cell responses in the gut and thus influence IgA production (Kim *et al*., 2016). While β2-1 fructans had no clear impact on serum or faecal Ig concentrations or isotype profiles in this study, these determinations were carried out under resting conditions and it remains a possibility that differences might be apparent following an immune challenge such as an infection or immunization.

β2-1 fructans have been shown to modulate adaptive immunity in response to immunization. Infants between 7-9 months of age who had consumed a β2-1 fructan-supplemented cereal (0.2 g β2-1 fructan per kg body weight per day) for 10 weeks had higher serum antibody concentrations of anti-measles IgG after vaccination to protect against measles (Firmansyah *et al.*, 2001; Saavedra and Tschernia, 2002). In a recent study involving healthy middle-aged adults consuming β2-1 fructans (8 g per day) subjects were immunized with a trivalent seasonal influenza vaccine after 4 weeks of supplementation.

Concentrations of serum vaccine-specific IgG_1 antibodies and serum antibody titers against influenza H3N2 were higher in those consuming β2-1 fructan two weeks post-vaccination (Lomax *et al.*, 2015). These studies suggest that β2-1 fructan supplementation may enhance responses to immunization, however these small sample sizes of infants and middle-aged adults may not be representative of effects on larger cohorts of adults.

There were several results of this study which suggest that immune cells had increased exposure to microbial components during the β2-1 fructan phase of the trial. Peripheral percentages of $CD282^+/TLR2^+$ mDC significantly increased and $CD284^+/TLR4^+$ mDC and $CD284^+/TLR4^+$ granulocyte percentages tended to be higher following β2-1 fructan supplementation. Increased exposure to TLR ligands has been shown to increase TLR2 and TLR4 expression (Juarez *et al.*, 2010; Visintin *et al.*, 2001), suggesting the increase in $TLR2^+$ and $TLR4^+$ mDC populations reflect increased contact with microbial components. DC in the gut may interact with bacteria through epithelial tight junctions, as they are capable to extending dendrites to sample the gut lumen, they may encounter bacterial components within the lamina propria, or they may indirectly interact with bacterial components through M cells (Bekiaris *et al.*, 2014; Farache *et al.*, 2013). In a study investigating rectal biopsies of Crohn's disease patients, increased percentages of intestinal DC expressing TLR2 and TLR4 were found after β2-1 fructan supplementation, in addition to increased percentages of IL-10 producing DC (Lindsay *et al.*, 2006). This study demonstrated impacts of β2-1 fructan supplementation on immune cells in local gut tissue, however peripheral immune cells percentages were not examined to determine if the supplement had a systemic impact. In the current investigation, it was

not possible to biopsy gut tissue, although it is speculated that there may have been impacts at the local gut level since effects on immune cells had manifested systemically.

There were no alterations in lymphocyte populations observed in this trial, in keeping with another β2-1 fructan study investigating impacts on immune parameters in middle-aged healthy adults (Lomax *et al*., 2012). However, a study utilizing elderly subjects found that β2-1 fructan supplementation increased percentages of total T lymphocytes and subsets $CD4^+$ and $CD8^+$ T cells (Guigoz *et al*., 2002). Taken together, these findings suggest that β2-1 fructan supplementation may differentially modulate immune parameters in separate age categories.

β2-1 fructans had an impact on circulating cytokines, as concentrations of T_H2 cytokine IL-4 and proinflammatory cytokine GM-CSF increased, and regulatory cytokine IL-10 concentrations decreased. Higher concentrations of IL-4 would lead to increased B and T cell proliferation, higher GM-CSF concentrations would lead to increased macrophage, neutrophil and DC activity, and lower IL-10 concentrations implies decreased ability to maintain immune homeostasis, however interpreting the outcome of cytokine concentration is context dependent. Circulating concentrations of LPS were significantly higher after the β2-1 fructan phase. Measuring circulating LPS is an indirect measure of assessing gut permeability (Albers *et al*., 2005; Bala *et al*., 2014; Bischoff *et al*., 2014), and increased LPS concentrations insinuate that microbial contents were translocated across the IEC barrier. A study of healthy men consuming β2-1 fructans for 2 weeks suggested that β2-1 fructans irritate the gut mucosa since increased faecal mucin excretion and intestinal discomfort were observed (Ten Bruggencate *et al*., 2006), effects likely stemming from rapid β2-1 fructan fermentation and liberation of SCFA which decrease

lumen pH. This study of healthy men had a similar β2-1 fructan dosage to the current trial (20 g per day versus 15 g day), and it was only half of the duration (two weeks versus four weeks), so it is probable that β2-1 fructan may have had a greater impact on gut mucosa irritation with the increased length of supplementation. In rat models, β2-1 fructan consumption has been associated with increased intestinal permeability and also increased translocation of *S. enteritidis* (Ten Bruggencate *et al.*, 2004; Ten Bruggencate *et al*., 2005). Heightened potential for microbial components to cross the IEC barrier could explain the alterations observed in circulating cytokine profiles, and also the increase in TLR2 and TLR4-expressing peripheral immune cells. However, LBP concentrations did not differ between phases, and neither did CRP, a measure of inflammation (Pagana and Pagana, 2002), suggesting that changes to IEC barrier integrity were non-acute.

The *ex vivo* whole blood culture assay allowed for determination of effects of β2-1 fructan supplementation on responses to TLR agonist stimulation, as TLR-expressing cells in blood samples respond by producing and releasing cytokines. This assay provided a means to determine the effects of β2-1 fructan fermentation on this aspect of the immune response at the systemic level. The profile and cytokine production amount provided a measure of the strength of the TLR-induced response, and some insight into the responding cell types, as certain cytokines are produced by distinct cell types. The cytokines analyzed in plasma included IFN-α, the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-8 and IL-12p70, and the regulatory cytokine IL-10. IFN-α is produced by pDCs in response to TLR stimulation; monocytes and macrophages produce IL-6, IL-8 IL-10, IL-12p70 and TNF-α; T lymphocytes produce IL-6 and IL-10 and T_H1 cells produce TNF-α; B cells

produce IL-10, IL-12p70 and TNF-α; and mDC produce IL-1β, TNF-α and IL-12p70 (Della Bella *et al*., 2008).

β2-1 Fructans may also directly influence immune activity through interaction with TLRs. Previous *in vitro* studies have shown TLR2 activation by FOS in human T84 IECs (Vogt et al., 2014), predominant TLR2 activation and mild TLR4, 5, 7, 8 and nucleotide-binding oligomerisation domain-containing protein (NOD) 2 activation in reporter cell lines, and stimulation of cytokine production by human PBMCs (Vogt *et al*., 2013). Cross talk between immune cells and gut microbiota through PPRs including TLRs may influence MyD88 signaling, which is necessary for NF-κB activation and subsequent proinflammatory cytokine production. Although the impact of the gut microbiota on TLR expression may vary with specific type of TLR (Brandao *et al*., 2015). Microbiota depletion via antibiotics has been shown to influence TLR expression in the ileum and in the colon in a murine model; however, increases or decreases in TLR expression depended on which TLR was examined (Grasa *et al*., 2015).

Analysis of the cytokine profiles produced in the *ex vivo* whole blood culture assay revealed that IL-10 concentrations increased after stimulation with TLR2 agonist P3C in whole blood collected from the endpoint of the β2-1 fructan phase. This finding suggests increased sensitivity to a TLR2 agonist, which may influence the host's ability to respond to infection since TLRs and the innate immune system are the primary line of defence (Barreiro *et al*., 2009; Turvey and Hawn, 2006). The increased sensitivity to a TLR2 agonist is also consistent with the increased peripheral mDC $CD282^{+}/TLR2^{+}$ populations, and could be indicative of increased microbial component exposure during the β2-1 fructan phase. Stimulation with TLR4 agonist LPS did not change the cytokine profiles between

supplement phases. Since circulating LPS concentrations increased during the β2-1 fructan phase, peripheral immune cells had contact with this bacterial motif. The diminished TLR response to LPS stimulation could be interpreted in two ways, either TLR synergy or the phenomenon of LPS tolerance. TLR synergy implies that costimulation with both TLR2 and TLR4 agonists will amplify cytokine production (Cao, 2016; Sato *et al.*, 2000), and the increased LPS concentrations in the blood combined with P3C stimulation may have synergized to increase the production of IL-10. LPS tolerance may have also occurred, which is decreased surface expression of TLR4 due to continuous exposure to LPS (Cao, 2016; Sato *et al*., 2000; Ziegler-Heitbrock, 1995). The latter reason may be less probable, since mDC $CD284^{+}/TLR4^{+}$ tended to increase at the end of the β2-1 fructan phase, however TLR4 expression was not measured on all immune cell populations. Cigarette smoking may influence TLR responsiveness in whole blood assay cytokine production after stimulation with LPS and P3C, and also TLR2 and TLR4 expression on monocytes (Versteeg *et al*., 2009), however it was not known if any of the subjects in the trial were active smokers as it was not a factor in trial exclusion criteria or in the initial health assessment.

Immune responses can vary between sexes, and males have been shown to have higher macrophage TLR4 expression and macrophage proinflammatory cytokine production than females (Klein and Flanagan, 2016). Additionally, IL-10 production stimulated by TLR9 and TNF-α production by LPS is greater in PBMCs isolated from males than females (Asai *et al.*, 2001; Klein and Flanagan, 2016). A study in a rat model has shown differences in concentrations of colonic and liver IL-10 and caecal and liver IL-6 between males and females after β2-1 fructan feeding (Shastri *et al*., 2015b). It is highly

probable that sex differences in immune responses may have been apparent in the peripheral immune cell population analysis and in the cytokine production of the TLR agonist *ex vivo* whole blood culture assay. Since this current trial was not designed to investigate differences between males and females, the sample numbers for sex were not evenly matched, as there were 13 males and 17 females. In separating the data according to sex, there were several pronounced differences found in mDC $CD282^+$ and $CD284^+$ subpopulations, circulating cytokine and LPS concentrations, serum and faecal Ig concentrations, and *ex vivo* TLR-induced cytokine production (**Appendix B**, **Figure B1**, **Table B2**, **Table B3**, and **Table B4**), suggesting that sex differences would be an interesting consideration in future dietary supplement clinical trial design.

In this human clinical trial, the amount of blood volume per subject was limited to 20 mL for each sampling time point; higher volumes were considered to be above the safe blood draw limit for healthy volunteers. This blood volume limited the number of TLR agonists that could be used in the *ex vivo* whole blood culture assay, and also limited the number of samples available for the mDC and monocyte TLR-signaling activity assay involving intracellular cytokine production measurements. Vaccination with a seasonal influenza preparation would be interesting in determining impacts at a systemic level and to further understand the role of β2-1 fructans in adaptive immunity. It was not possible to directly explore mucosal responses to β2-1 fructan supplementation since tissue samples could not be taken from these healthy volunteers. Measurement of the mucin content in faeces using newly available ELISA formats may provide information in determining if alterations in mucin excretion exist after continuous β2-1 fructan consumption, as increased mucin sloughing has been suggested as a result of mucosal irritation induced by

β2-1 fructans (Ten Bruggencate *et al.*, 2006), although this effect has varied between studies of healthy human (Scholtens *et al*., 2006). Lastly, measuring the urinary excretion of chromium-labeled EDTA (Ten Bruggencate *et al.*, 2006) could provide a way to confirm the suggestions made in this study regarding β2-1 fructan consumption compromising the integrity of the IEC barrier.

Several non-immunological impacts of β2-1 fructans on host well-being and gut microbiota structure were discovered by other researchers involved in the trial, and these findings have been published in Clarke *et. al* (2016). Dr. Premsyl Bercik and Dr. Christian Avila (McMaster University, Hamilton, ON) investigated measures of well-being and gut symptomology. It was found that β2-1 fructan consumption significantly increased indigestion and these subjects and had a tendency of increased abdominal pain. β2-1 fructan supplementation was also associated with increased headaches, gas, bloating and cramping. Dr. Martin Kalmokoff (AAFC, Kentville, NS) and Dr. Stephen Brooks (Health Canada, Ottawa, ON) investigated the impacts of β2-1 fructans on faecal Bifidobacteria populations from subjects in this study. It was determined that β2-1 fructan supplementation increased the content of faecal Bifidobacteria 16S rRNA genes, in keeping with findings from other human clinical trials (Lindsay *et al*., 2006; Lomax *et al*., 2012). However, there was no effect on the frequency of daily bowel movements, a claim that has been frequently contradicted in previous human trials (Dahl *et al*., 2005; Marteau *et al*., 2011; Slavin and Feirtag, 2011).

In conclusion, supplementation with β2-1 fructans had some impact on immune activity in healthy subjects under steady-state conditions. β2-1 fructan supplementation significantly increased serum GM-CSF and IL-4 concentrations, while IL-10

concentrations decreased. Circulating population percentages of $CD282^+/TLR2^+$ mDC increased, and there was a tendency toward higher $CD284^+/TLR4^+$ mDC and $CD284^+/TLR4^+$ granulocyte population percentages. β2-1 fructan supplementation was associated with higher serum LPS concentrations, suggesting heightened microbial contact during this phase. Increased IL-10 production was observed in response to TLR2 stimulation with P3C in an *ex vivo* whole blood culture assay, implying increased sensitivity to TLR2 agonists. While TLR cross talk patterns can be complex, heightened sensitivity to one TLR ligand can occur as a result of prior exposure to another TLR ligand (Cao, 2016). Whether this sensitivity extended to other TLR agonists would require further testing of a range of TLR agonists, which could not be performed in this study due to sample volume limitations. Collectively, the findings of this study suggest that the innate immune system had increased contact with microbial-derived stimuli during the β2-1 fructan phase of the trial, possibly due to alterations in gut barrier integrity, and demonstrate that dietary supplementation with β2-1 fructans can, to an extent, influence immune activity at the systemic level in healthy adults.

Table 2-1. Cytokines measured in subjects receiving β2-1 fructan supplementation or placebo.

Cytokine /Chemokine	Cellular Source	Cellular Target & Role	References
sCD40L	Cell surface protein released from B cells, monocytes, dendritic cells and T lymphocytes	Involved in proliferation and differentiation of B cells, and Ig class switching	(Huang *et al*., 2012)
G-CSF	Macrophages, fibroblasts, endothelial cells and bone marrow stroma	Stimulates the development of progenitor cells to neutrophils	(Demetri and Griffin, 1991)
GM-CSF	Monocytes, T-lymphocytes, fibroblasts and endothelial cells	Stimulates development of neutrophils, dendritic cells and macrophages, promotes eosinophil proliferation and development	(Caux *et al.*, 1992)
IFN-α	Peripheral blood leukocytes and lymphoblastoid cells	Influences cell proliferation, modulates immune responses, promotes resistance against viral infections and inhibits release of eosinophil granule proteins	(Aldebert *et al*., 1996)
IFN-γ	Activated NK cells, $CD4^+$ and $CD8^+$ T lymphocytes	Promotes macrophage activation, MHC class I and II expression, antigen processing and presentation ability; T_H1 signature cytokine.	(Schroder *et al*., 2004)
IL-1β	Monocytes, tissue macrophages and dendritic cells	Proinflammatory cytokine, promotes IL-2 release, B-cell maturation and proliferation	(Dinarello, 1989)
IL-1Ra	Monocytes and macrophages	Competitively inhibits the activity of IL-1β	(Arend and Gabay, 2000)
IL-2	T lymphocytes	Promotes signaling required for T-cell proliferation	(Popmihajlov *et al*., 2012)
IL-4	Mast cells, T lymphocytes and bone marrow stromal cells	Regulates the differentiation of naïve $CD4^+$ T cells into helper T_H2 cells; T_H2 signature cytokine	(Paul, 1991)

This table is continued on the next page.

Table 2-1 *continued*.

Cytokine /Chemokine	Cellular Source	Cellular Target & Role	References
IL-6	T lymphocytes, monocytes, macrophages, fibroblasts and endothelial cells	Proinflammatory cytokine and also considered an adipokine, regulates immune responses and stimulates antibody production	(Mauer *et al*., 2015)
IL-8 /CXCL8	Monocytes, macrophages and endothelial cells, and is considered a chemokine	Proinflammatory, chemoattractant of neutrophils and T cells	(Hersh *et al*., 1998)
IL-10	Monocytes, macrophages (M2), T lymphocytes, B cells and keratinocytes	Regulatory, inhibits T_H1 cell activity, prevents macrophage cytokine release; T_{reg} signature cytokine.	(Borish, 1998)
IL-12p70	Activated monocytes, macrophages and B cells	Induces IFN-γ production by NK and T cells, promotes growth of NK cells and T_H cells	(Vignali and Kuchroo, 2012)
IP-10 /CXCL10	Interferon and LPS stimulated cells in the thymus, spleen and lymph nodes	Chemoattracts monocytes and T_H1 lymphocytes, stimulates adhesion of activated T cells and NK cells to endothelial cells	(Dufour *et al*., 2002)
TGF-β1	Produced by almost every cell type	Immunoregulatory, regulates cell proliferation, growth and differentiation, involved in class switching to IgA	(Annes *et al*., 2003)
TNF-α	T_H1 cells, adipocytes, B cells, activated monocytes and macrophages	Proinflammatory, cytotoxic, activates macrophages and induces nitric oxide production	(Bradley, 2008)

Table 2-2. Description of standard biochemistry and haematological markers analyzed in subjects receiving β2-1 fructan supplementation or placebo.

Clinical Indicator	Clinical Marker
Diabetic/pre-diabetic/insulin resistant	fasting glucose
Renal function	creatine, glomerular filtration rate, globulin, albumin, total protein, blood urea nitrogen
Liver function	aspartate aminotransferase, gamma-glutamyltransferase
Inflammation	C-reactive protein
Anemia	Haemoglobin
Complete Blood Cell Counts	Haematocrit, white blood cell count, red blood cell count, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin
Differential Blood Cell Counts	Neutrophil, lymphocyte, monocyte, eosinophil and basophil counts
Serum lipids	Total cholesterol; LDL-C – Low density lipoprotein cholesterol; HDL-C - High density lipoprotein cholesterol; T/HC – TC/HDL-C ratio; TG – Triacylglycerol

Table 2-3. Flow cytometry antibodies used in peripheral cell population analysis.

Marker	Description	Clone	Conjugate[1]	Manufacturer[2]
Control	Isotype – IgG1	T1B9	PE	Invitrogen
Control	Isotype – IgG1	T1B9	FITC	Invitrogen
CD3[3]	T lymphocytes	SK7	FITC	BD Biosciences
CD4[3]	T_H1 and T_H2 T cells, monocytes, macrophages	SK3	APC	BD Biosciences
CD8[3]	cytotoxic T cells	SK1	PE	BD Biosciences
CD11c	myeloid DC	S-HCL-3	APC	BD Biosciences
CD14	monocytes	M5E2	APC	BD Biosciences
CD16	NK cells and granulocytes	3G8	AF647	BD Biosciences
CD16[3]	NK cells and granulocytes	B73.1	PE	BD Biosciences
CD19[3]	B cells	SJ25C1	APC	BD Biosciences
CD45	leukocytes	2D1 (HLe-1)	FITC	BD Biosciences
CD45[3]	leukocytes	2D1 (HLe-1)	PerCP	BD Biosciences
CD56[3]	NK cells	NCAM 16.2	PE	BD Biosciences
CD86	monocytes, activated B cells, DC	2331 (FUN-1)	PE	BD Biosciences
CD123	plasmacytoid DC	9F5	PE	BD Biosciences
CD282	TLR-2	TLR2.1	PE	BioLegend
CD284	TLR-4	HTA125	PE	BioLegend
HLA-DR	MHC II, B cells, monocytes, activated T cells	L243	PerCP	BD Biosciences
IL-12	intracellular IL-12	C8.6	FITC	Invitrogen
lin1	T lymphocytes, monocytes, macrophages, neutrophils, eosinophils, neutrophils, NK cells, B lymphocytes	SK7, 3G8, SJ25C1, L27, M Pφ9, NCAM16.2	FITC	BD Biosciences
TNF-α	intracellular TNF-α	6401.1111	PE	BD Biosciences

[1] AF647, Alexa Fluor 647; APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin–chlorophyll–protein complex.
[2] Location of manufacturers: BD Biosciences, San Jose CA; BioLegend, San Diego CA, USA; Invitrogen, Carlsbad CA, USA.
[3] Part of a BD Multitest™ kit.

Table 2-4. Simplified LAL assay procedure.

Procedure	Sample	Blank
Test sample or standard	50 µL	------
LAL reagent water	------	50 µL
LAL	50 µL	50 µL
Mix and incubate at 37°C	10 minutes	10 minutes
Substrate solution at 37°C	100 µL	100 µL
Mix and incubate at 37°C	6 minutes	6 minutes
Stop Reagent	100 µL	100 µL
Mix immediately		
Read plate	405 nm	405 nm

Table 2-5. Estimated daily background dietary β2-1 fructan intakes in subjects during placebo and β2-1 fructan phases.

ID[1]	Placebo Phase (g β2-1 fructan)[2]	β2-1 Fructan Phase (g β2-1 fructan)
001	1.29	0.52
002	0.83	1.14
003	1.86	2.40
004	0.58	0.94
005	1.45	0.92
006	1.10	0.50
007	0.69	0.77
008	1.31	1.39
009	1.17	0.35
010	0.80	0.68
011	1.06	1.13
012	1.82	2.13
013	0.49	0.42
015	2.36	0.74
016	1.18	1.48
018	0.22	0.52
022	2.04	1.53
023	0.47	0.30
024	0.74	0.65
025	1.40	0.98
026	1.28	0.92
027	0.32	0.22
028	1.13	0.70
029	2.29	2.05
030	1.74	2.12
031	0.13	0.42
032	0.95	0.95
033	1.24	0.69
034	0.17	0.13
035	0.46	1.03
Estimated β2-1 fructan intake (g)	1.09	0.96

[1] $n = 30$.

[2] Data is represented as midpoint values of reported ranges.

Table 2-6. BMI, BUN and routine biochemical and haematological biomarkers at baseline and endpoint of placebo and β2-1 fructan phases[1].

Marker[2]	Placebo day 0[3]	Placebo day 28	β2-1 Fructan day 0	β2-1 Fructan day 28
BMI	24.1 ± 0.5	24.2 ± 0.5	24.1 ± 0.5	24.2 ± 0.5
BUN	5.0 ± 0.2	4.7 ± 0.3	4.9 ± 0.3	4.8 ± 0.3
fast Glu	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.1
Creat	77.1 ± 2.7	76.7 ± 0.1	75.8 ± 2.4	75.4 ± 2.3
eGFR	91.0 ± 2.5	90.8 ± 2.2	91.4 ± 2.4	92.5 ± 2.2
AST	24.1 ± 1.6	23.4 ± 1.2	23.8 ± 0.9	24.1 ± 1.0
GGT	18.9 ± 1.7	20.2 ± 1.8	19.8 ± 2.2	20.4 ± 1.8
Glob	26.6 ± 0.5	27.2 ± 0.5	26.5 ± 0.5	27.2 ± 0.6
T-Pr	71.9 ± 0.5	72.9 ± 0.5	71.9 ± 0.5	72.7 ± 0.6
Alb	45.3 ± 0.5	45.7 ± 0.5	45.7 ± 0.5	45.5 ± 0.4
hs CRP	2.7 ± 0.8	3.4 ± 1.3	3.0 ± 1.0	2.5 ± 0.9
TC	4.6 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	4.7 ± 0.1
LDL	2.6 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1
HDL	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
TC/HDL	3.3 ± 0.2	3.1 ± 0.1	4.0 ± 0.9	3.2 ± 0.2
TG	1.0 ± 0.1	1.3 ± 0.3	1.1 ± 0.1	1.2 ± 0.1
Hgb	138.0 ± 2.2	137.9 ± 2.4	137.1 ± 2.0	138.7 ± 0.6
HCT	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
WBC	6.4 ± 0.4	6.4 ± 0.4	6.5 ± 0.3	6.3 ± 0.3
RBC	4.7 ± 0.1	4.7 ± 0.1	4.6 ± 0.1	4.7 ± 0.1
MCV	89.0 ± 0.6	89.1 ± 0.7	88.9 ± 0.7	88.9 ± 0.7
MCH	29.6 ± 0.3	29.6 ± 0.3	29.5 ± 0.3	29.8 ± 0.3
MCHC	333.3 ± 1.9	332.6 ± 1.7	332.5 ± 1.7	334.7 ± 1.8
RDW	13.0 ± 0.1	13.0 ± 0.1	13.0 ± 0.1	13.1 ± 0.1
Pl.Ct.	257.9 ± 14.3	264.0 ± 13.6	270.4 ± 13.6	264.2 ± 13.5

[1] Values expressed as mean ± SEM (n = 30). rANOVA analysis did not find differences between time points or phases for any marker.

[2] Alb, albumin; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; Creat, creatinine; eGFR, calculated glomerular filtration rate; fast GLU, fasting glucose; GGT, gamma glutamyltransferase; Glob, globulin protein; HCT, haematocrit; Hgb, haemoglobin; HDL, high-density lipoprotein; hs CRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; Pl.Ct., platelet count; RBC, red blood cell count; RDW, red cell distribution width; T-Pr, total protein; TC, total cholesterol; TC/HDL, total cholesterol/high-density lipoprotein ration; TG, triglycerides; WBC, white blood cell count.

Table 2-7. Peripheral immune cell populations on day 28 of placebo and β2-1 fructan phases.

Cell Phenotype[1]	Placebo day 28	β2-1 fructan day 28	rANOVA *P* value
lymphocytes[2]	34.2 ± 1.7	34.9 ± 1.1	NS
$CD3^+$ T cells[3]	74.2 ± 1.2	73.9 ± 1.2	NS
$CD3^+CD4^+$ T_H cells[3]	45.5 ± 1.5	45.6 ± 1.4	NS
$CD3^+CD8^+$ cytotoxic T cells[3]	27.5 ± 1.2	27.0 ± 1.2	NS
$CD3^-CD19^+$ B cells[3]	12.6 ± 0.7	13.0 ± 0.7	NS
$CD3^+CD16^+CD56^+$ NK cells[3]	12.4 ± 1.1	12.2 ± 1.1	NS
$CD3^+CD4^-CD8^-$ γδ T cells[3]	3.0 ± 0.3	2.9 ± 0.3	NS
$CD3^-14^+$ monocytes[4]	7.2 ± 0.4	7.0 ± 0.3	NS
$CD3^-16^+$ neutrophils and granulocytes[4]	47.3 ± 1.6	45.3 ± 1.7	NS
$lin1^-$HLA-DR^+CD123^+ pDCs[5]	10.4 ± 1.3	10.8 ± 1.1	NS
$lin1^-$HLA-DR^+CD11c^+ mDCs[5]	23.2 ± 1.3	22.2 ± 1.9	NS
$CD86^+$ mDCs[5]	13.5 ± 1.0	13.3 ± 1.2	NS
$CD282^+$/$TLR2^+$ mDCs[5]	8.8 ± 0.8	11.2 ± 1.2	0.008
$CD284^+$/$TLR4^+$ mDCs[5]	9.7 ± 1.0	11.4 ± 1.3	0.060
HLA-DR^+CD14^+ monocytes[4]	5.5 ± 0.3	5.3 ± 0.3	NS
$CD282^+$/$TLR2^+$ monocytes[4]	6.5 ± 0.8	7.8 ± 0.9	NS
$CD284^+$/$TLR4^+$ monocytes[4]	7.3 ± 0.9	8.8 ± 1.1	NS
HLA-DR^-CD16^+ granulocytes[4]	28.1 ± 1.8	30.2 ± 1.6	NS
$CD282^+$/$TLR2^+$ granulocytes[4]	0.6 ± 0.2	1.6 ± 0.5	NS
$CD284^+$/$TLR4^+$ granulocytes[4]	1.0 ± 0.2	2.7 ± 0.8	0.050

[1] Data is shown as the percentage of immune cells expressing the surface markers indicating the cell type ± SEM (n = 30).
[2] Determined by white blood cell differential count on a Beckman Coulter instrument.
[3] Expressed as % of lymphocytes.
[4] Expressed as % of leukocytes.
[5] Expressed as % of $lin1^-$HLA-DR^+ cells.

Table 2-8. Peripheral mDC and monocyte intracellular cytokine production after placebo and β2-1 fructan supplementation.

Unstimulated (control) or LPS Stimulation[1] Cell Population %		**Placebo day 28**	**β2-1 Fructan day 28**	**Phase *P* value[2]**	**Stimulation *P* value[3]**
Mean % mDC	Control	25.13 ± 1.38	22.10 ± 1.76	0.086	0.270
	LPS	23.92 ± 0.77	20.67 ± 2.67		
% Actual TNF-α$^+$ mDC	Control	1.68 ± 1.39	3.36 ± 2.70	0.938	0.012
	LPS	17.68 ± 6.85	15.10 ± 4.22		
% Actual IL-12$^+$ mDC	Control	6.40 ± 2.08	7.92 ± 3.25	0.375	0.953
	LPS	5.55 ± 1.68	10.76 ± 5.27		
Mean % monocytes	Control	5.78 ± 0.63	4.86 ± 0.72	0.179	0.351
	LPS	6.18 ± 0.82	4.96 ± 0.52		
% Actual TNF-α$^+$ monocytes	Control	0.00 ± 0.00	7.47 ± 4.52	0.590	0.034
	LPS	30.49 ± 6.66	25.78 ± 6.02		
% Actual IL-12$^+$ monocytes	Control	3.99 ± 1.94	8.36 ± 3.18	0.780	0.571
	LPS	7.95 ± 2.34	7.90 ± 1.91		

[1] Data is shown as the percentage of immune cells expressing the surface markers indicating the cell type and the actual percentage of the specific cell type producing cytokines ± SEM (n = 6).
[2] rANOVA *P* value according to phase.
[3] rANOVA *P* value according to stimulation with LPS.

Table 2-9a. Mean values for serum LPS, LBP and cytokine/chemokine concentrations from participants receiving placebo or β2-1 fructan supplements.

Marker[1]	**Placebo**		**β2-1 Fructan**	
	day 0	**day 28**	**day 0**	**day 28**
LPS (EU/mL)	0.52 ± 0.09	0.53 ± 0.09	0.53 ± 0.07	0.62 ± 0.10[2]
LBP (μg/mL)	12.8 ± 1.3	15.2 ± 1.7	13.8 ± 1.5	14.2 ± 1.4
sCD40L (ng/mL)	6.6 ± 4.7	6.7 ± 4.9	6.2 ± 4.8	5.5 ± 4.0
G-CSF (pg/mL)	11.7 ± 4.20	14.4 ± 3.63	2.9 ± 1.6	5.4 ± 2.5
GM-CSF (pg/mL)	223.5 ± 172.2	238.2 ± 176.6	220.4 ± 173.9	242.0 ± 178.2[3]
IFN-γ (pg/mL)	BLQ	2.7 ± 2.1	15.2 ± 7.9	66.5 ± 22.9
IL-1β (pg/mL)	164.4 ± 104.7	162.3 ± 104.9	165.1 ± 105.1	169.5 ± 114.2
IL-1Ra (pg/mL)	81.2 ± 40.4	107.1 ± 51.4	71.0 ± 41.6	573.5 ± 413.2
IL-4 (pg/mL)	13.0 ± 6.45	11.6 ± 6.35	10.4 ± 5.5	14.0 ± 7.23[4]
IL-6 (pg/mL)	93.3 ± 57.7	78.6 ± 48.7	46.6 ± 31.1	82.9 ± 51.1
IL-8 (pg/mL)	18.4 ± 5.00	23.1 ± 8.39	26.8 ± 8.3	30.3 ± 9.5
IL-10 (pg/mL)	71.5 ± 36.2	79.8 ± 42.15	51.9 ± 28.0	41.2 ± 29.0[4]
IL-12p70 (pg/mL)	215.7 ± 153.1	199.8 ± 139.0	153.9 ± 118.4	206.7 ± 161.8
IP-10 (pg/mL)	386.7 ± 261.1	487.4 ± 282.0	480.9 ± 263.2	453.7 ± 275.2[5]
TNF-α (pg/mL)	61.4 ± 39.3	123.0 ± 85.5[6]	106.7 ± 83.1	123.5 ± 100.9
Active TGF-β1 (pg/mL)	2.5 ± 1.2	3.4 ± 1.2	3.0 ± 1.3	5.4 ± 2.1
Total TGF-β1 (pg/mL)	4027.8 ± 318.3	3763.0 ± 374.6	3992.0 ± 379.5	3724.1 ± 376.6

[1] Concentrations are shown as mean ± SEM (n = 30).
[2] Value was significantly different from all other time points (rANOVA with LSD test $P < 0.03$).
[3] Value was significantly different from all other time points (rANOVA with LSD test, $P = 0.002$).
[4] Values were significantly different from all other time points (rANOVA with LSD test, $P < 0.001$).
[5] Value was significantly different from fructan d0 (rANOVA with LSD test, $P = 0.01$).
[6] Value was significantly different from placebo d0 (rANOVA with LSD test, $P < 0.001$).

Table 2-9b. Median values for serum LPS, LBP and cytokine/chemokine concentrations from participants receiving placebo or β2-1 fructan supplements.

Marker[1]	**Placebo**				**β2-1 Fructan**			
	day 0		**day 28**		**day 0**		**day 28**	
	Median	**25th-75th percentiles**	**Median**	**25th-75th percentiles**	**Median**	**25th-75th percentiles**	**Median**	**25th-75th percentiles**
LPS EU/mL	0.27	0.17-0.89	0.28	0.16-1.08	0.37	0.12-0.90	0.36	0.27-0.83
LBP µg/mL	10.7	9.7-32.0	11.9	9.1-19.3	11.7	9.9-15.1	12.5	9.3-15.1
sCD40L pg/mL	700.4	424.3-1085.0	454.0	224.1-916.1	500.3	331.1-706.3	449.1	209.6-646.7
G-CSF pg/mL	0.6	0.6-11.8	0.6	0.6-31.0	0.6	0.6-0.6	0.6	0.6-0.6
GM-CSF pg/mL	3.7	0.2-39.0	6.2	0.2-28.0	5.1	0.2-23.2	0.2	0.2-27.8
IFN-γ pg/mL	4.1	4.1-4.1	4.1	4.1-4.1	4.1	4.1-4.1	4.1	4.1-101.5
IL-1β pg/mL	1.3	0.3-32.9	5.4	0.3-24.6	0.3	0.3-21.2	0.3	0.3-11.1
IL-1Ra pg/mL	5.0	5.0-32.3	5.0	5.0-127.9	5.0	5.0-61.5	5.0	5.0-47.0
IL-4 pg/mL	0.1	0.1-5.8	0.1	0.1-4.7	0.1	0.1-2.9	1.5	0.6-7.5
IL-6 pg/mL	2.9	0.1-18.9	0.4	0.1-13.6	1.3	0.1-12.7	0.1	0.1-14.4
IL-8 pg/mL	8.6	7.8-28.2	6.7	5.6-11.8	8.4	6.8-17.0	8.1	4.4-23.7
IL-10 pg/mL	4.1	1.6-33.3	8.3	3.9-57.0	7.4	3.9-20.8	2.5	0.5-29.1
IL-12p70 pg/mL	2.9	2.9-24.3	2.9	2.9-19.9	2.9	2.9-19.2	2.9	2.9-31.3
IP-10 pg/mL	54.8	38.7-114.7	71.1	41.2-104.2	71.1	43.6-178.6	60.3	37.5-116.5
TNF-α pg/mL	0.2	0.2-8.4	1.5	0.2-17.1	0.2	0.2-16.0	0.2	0.2-9.1
Active TGF-β1 pg/mL	1.0	1.0-1.0	1.0	1.0-1.0	1.0	1.0-1.0	1.0	1.0-1.0
Total TGF-β1 pg/mL	3968.2	2582.4-5133.3	3480.2	2122.6-4528.8	3732.2	2253.6-5023.8	3387.5	1980.6-4938.0

[1] Concentrations are shown as the median (n = 30).

Table 2-10. Concentrations of serum and faecal Ig at baseline and endpoint of placebo and β2-1 fructan phases.

Sample[1]	**Class**	**Placebo**		**β2-1 Fructan**	
		day 0	**day 28**	**day 0**	**day 28**
Serum (ng/mL)	**IgA**	698.4 ± 75.6	1490.8 ± 236.7[2]	1389.9 ± 234.2	911.7 ± 138.2[3]
	IgM	403.8 ± 33.3	821.3 ± 138.3[2]	776.6 ± 124.8	485.8 ± 66.5
	IgG_1	2746.9 ± 217.6	7196.4 ± 1257.2[2]	5148.6 ± 797.7	2878.6 ± 214.5[3,4]
	IgG_2	1655.0 ± 130.9	20 759.1 ± 6911.7[2]	9659.1 ± 2348.5	1595.6 ± 181.4[3,4]
	IgG_3	10 452.2 ± 1237.7	28 181.8 ± 3644.5[2]	28 647.1 ± 2913.6	30 560.7 ± 3355.6
	IgG_4	677.1 ± 122.6	2203.7 ± 472.5[2]	1767.1 ± 364.5	2148.0 ± 440.2
Faecal (ng/g)	**IgA**	95 076.4 ± 17 617.1	92 189.3 ± 15 075.2	84 686.0 ± 15 752.7	87 428.8 ± 12 137.8
	IgM	6009.3 ± 2581.5	13 399.3 ± 2955.4[2]	22 513.8 ± 7029.3	14 108.7 ± 4834.1
	IgG	2704.2 ± 1538.6	918.5 ± 261.0	697.2 ± 154.1	2406.9 ± 983.6

[1] $n = 30$ for serum Ig, $n = 30$ for faecal Ig at time points except for placebo d0, $n = 29$, values expressed as mean ± SEM.
[2] Value was significantly different between placebo d-0 and d-28 (rANOVA with LSD test, $P < 0.005$).
[3] Value was significantly different between placebo d-28 and β2-1 fructan d-28 (rANOVA with LSD test, $P < 0.005$).
[4] Value was significantly different between β2-1 fructan d-0 and β2-1 fructan d-28 (rANOVA with LSD test, $P < 0.005$).

Table 2-11. *Ex vivo* cytokine production in unstimulated and TLR agonist-stimulated whole blood cultures from subjects at the endpoints of placebo and β2-1 fructan phases.

Cytokine	Unstimulated[1] Placebo day 28	Unstimulated β2-1 Fructan day 28	LPS Placebo day 28	LPS β2-1 Fructan day 28	P3C Placebo day 28	P3C β2-1 Fructan day 28
IFN-α (pg/mL)	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
IL-1β (ng/mL)	BLQ	0.03 ± 0.0	8.2 ± 1.1	9.2 ± 1.6	0.3 ± 0.1	0.5 ± 0.1
IL-6 (ng/mL)	0.1 ± 0.0	0.4 ± 0.3	34.4 ± 1.4	36.6 ± 1.7	8.5 ± 0.9	11.5 ± 1.9
IL-8 (ng/mL)	5.3 ± 1.3	17.8 ± 8.1	64.8 ± 1.9	72.7 ± 2.3	57.0 ± 3.2	60.7 ± 4.9
IL-10 (pg/mL)	BLQ	21.14 ± 21.1	2044.0 ± 147.0	2001.0 ± 188.0	16.0 ± 7.2	52.0 ± 18.5[3]
IL-12p70[2] (pg/mL)	BLQ	BLQ	BLQ	BLQ	BLQ	3.3 ± 3.3
TNF-α (ng/mL)	BLQ	0.1 ± 0.1	2.6 ± 0.3	3.0 ± 0.3	0.2 ± 0.0	0.2 ± 0.0

[1] n = 30 for unstimulated samples and LPS stimulation; n = 24 for P3C stimulation; concentrations expressed as mean ± SEM.
[2] IL-12p70 was detected in only 1 subject after P3C stimulation in whole blood culture collected at the end of the β2-1 fructan phase.
[3] Value was significantly different from the placebo group (rANOVA with LSD test, P = 0.016).

Chapter 3: Effects of Dietary Fermentable Material and a High-Energy Diet on Obesity-Associated Immune Biomarkers in Sprague Dawley Rats

Introduction

Obesity, High Energy Diets and Inflammation

Interactions between metabolic parameters and the immune system are evident in obesity and metabolic syndrome disorders (Iyer *et al*., 2015; Matarese and La Cava, 2004). Obesity has been associated with continuous low grade systemic inflammation (Barbarroja *et al*., 2010). In metabolic syndrome, gut level inflammation increases with high energy (HE) diets (Hotamisligil *et al*., 1995), which further perpetuate systemic inflammation. Obese individuals have been shown to have higher expression of proinflammatory cytokines TNF-α, IL-1β, IL-6, and also have greater macrophage infiltration in visceral adipose tissue when compared to lean subjects (Barbarroja *et al*., 2010). Greater expression of TNF-α has been positively correlated to adipose mass in obese subjects (Hotamisligil *et al.*, 1995). The increase in expression of proinflammatory cytokines has also been observed in HE feeding trials involving obese animal models, including Wistar (Stemmer *et al*., 2012; Zhu *et al.*, 2014) and Sprague Dawley rats (Boonloh *et al.*, 2015; Barbier de La Serre *et al*., 2010). Continuous and dysregulated expression of proinflammatory cytokines contribute to the metabolic dysfunction observed in obese subjects (Weisberg *et al*., 2003).

Peroxisome proliferator-activated receptors (PPAR) are a family of transcription factors (PPARα, PPARβ/δ & PPARγ) which regulate inflammation and also have involvement in energy homeostasis (Stienstra *et al*., 2007). Activation of PPARα prevents hepatic fat storage through stimulating hepatic fatty acid oxidation (Kersten *et al*., 1999),

and also inhibits inflammatory gene expression through blocking NF-κB activation of IL-6 transcription (Delerive *et al*., 1999). PPARβ/δ activation has also been associated with decreased fat storage in the liver and reduced production of IL-6 and TNF-α (Nagasawa *et al*., 2006). Activation of PPARγ reduces macrophage accumulation and inflammatory gene expression, thereby reducing production of nitric oxide, TNF-α, IL-1β and IL-6 (Weisberg *et al*., 2003). Both obese humans and animal models typically have decreased activation of PPAR transcription factors, which may in part account for obesity-induced inflammation (Stienstra *et al*., 2007).

Proinflammatory cytokines TNF-α, IL-1β and IL-6 are of special interest in obesity studies since these cytokines are continuously produced by macrophages and adipocytes residing in adipose tissue, and continuous and dysregulated production of these proinflammatory cytokines may lead to insulin resistance (Aroor *et al*., 2013) and contribute to metabolic dysfunction (Weisberg *et al*., 2003). Within adipose tissue, macrophages and adipocytes are involved in local cross-talk which propagates continuous proinflammatory cytokine release (Schaffler *et al*., 2007). Macrophages produce TNF-α, which activates the TNF-α receptor on adipocytes and induces NF-κB activation. Activation of TLR4 by LPS on both macrophages and adipocytes will also induce NF-κB activation, leading to the production of proinflammatory cytokines (Suganami *et al*., 2007). Additionally, NF-κB activation induced by TNF-α leads to lipolysis in adipocytes (Suganami *et al*., 2005), which releases free fatty acids that are capable of activating TLR4 on macrophages (Schaeffler *et al*., 2009; Suganami *et al*., 2005; Suganami *et al*., 2007). The continuous cytokine and free fatty acid release by macrophages and adipocytes leads to a steady state of inflammation in the adipose tissue. To further propagate inflammation,

consumption of a high fat diet increases LPS absorption across the intestinal barrier and leads to increased serum LPS concentrations; this effect has been observed in rodents (Cani *et al*., 2007; Barbier de La Serre *et al*., 2010) and in humans (Erridge *et al*., 2007).

Macrophages may be activated into distinct states, including the classically activated M1 macrophage phenotype or the alternatively activated M2 macrophage phenotype (Gordon and Taylor, 2005; Mantovani *et al*., 2002). M1 macrophages may be polarized by T_H1 cytokine activity involving IFN-γ or TNF-α, and also through TLR4 activation by LPS (Martinez and Gordon, 2014). The classically activated macrophage phenotype will promote T_H1 responses, has involvement in antigen presentation, and contributes to high levels of proinflammatory activity leading to tissue damage (Mantovani *et al.*, 2002). The M1 macrophage cytokine profile has been identified as having high IL-12 and IL-23 expression and low IL-10 expression (Mantovani *et al*., 2004). M2 macrophages are polarized by IL-4, a signature T_H2 cytokine, and this macrophage phenotype is typically involved in tissue remodelling and anti-inflammatory activity (Biswas and Mantovani, 2010; O'Neill and Pearce, 2016). The M2 macrophage cytokine profile has been identified as having high expression of IL-10 and IL-1Ra, and low expression of IL-12 (Mantovani *et al*., 2002), although the alternatively activated macrophages have been further classified into a spectrum of M2 macrophages, including M2a, M2b and M2c (Mantovani *et al*., 2004). These subsets are defined by differences in cytokine production and cell surface molecule expression, although these differences are not as clear-cut in their identification and overlap one another. A mouse model of diet-induced obesity has shown macrophage polarization to the M1 phenotype in adipose tissue, which contributed to greater inflammation and insulin resistance (Lumeng *et al*., 2007).

However, there remains debate regarding the appropriate cellular markers and cytokine measurements to categorize macrophage phenotypes, as well as differences between humans and rodents.

Key adipokines of interest in studying the onset of obesity and metabolic disease include leptin, adiponectin and resistin, and these are produced by adipocytes in adipose tissue deposits. Leptin in particular has been shown to be a mediator involved in innate and adaptive immune responses (Mackey-Lawrence and Petri, 2012), and increases in leptin concentrations have been associated with autoimmune disorders (Fernandez-Riejos et al., 2010; Matarese and La Cava, 2004). Since the primary production source of leptin is adipocytes, obese subjects are more prone to leptin-resistance and dysregulation of appetite and energy expenditure (Villanueva and Myers, 2008), and to interruptions in lymphocyte homeostasis, such as an altered balance between T_H1-T_H2 activity (Matarese *et al*., 2002). Leptin also functions as a hormone in that it decreases appetite while increase energy expenditure (Friedman, 2010). Adiponectin has a role in energy metabolism as it regulates blood glucose and fatty acids, and also plays a role in inflammation (Cheng *et al*., 2012). Adiponectin has been shown to suppress TNF-α and IL-6 production while increasing IL-10 production by macrophages stimulated with LPS (Wulster-Radcliffe *et al*., 2004). Resistin has been shown to promote TNF-α, IL-6 and IL-8 production (Dong *et al.*, 2013), and also has a role in energy metabolism. Dysregulation of resistin production impairs glucose tolerance (Flier, 2001) and may lead to insulin resistance (Aroor *et al*., 2013).

Peptide hormones of interest in studying metabolic disease include insulin and glucagon, and these are produced in the pancreas and function to regulate blood glucose levels. Insulin is an important peptide hormone that functions to regulate and reduce blood

glucose levels through facilitating glucose absorption by muscle, liver or fat cells. Insulin's activity may be impacted by adipokines and dysregulated inflammation, such as the continuous release of TNF-α and IL-6 in adipose tissue, which could mediate the onset of insulin resistance (Aroor *et al.*, 2013). Glucagon functions in a manner opposite to insulin, raising glucose levels in instances when blood glucose is low, and has a role in appetite control and energy intake (Flint *et al.*, 1998). Glucagon production increases in the presence of inflammatory cytokine IL-6 (Barnes *et al.*, 2014), and also after LPS injection in rodents (Nguyen *et al*., 2014). Circulating concentrations of sICAM-1/sCD54 are elevated in obese animal models (Matarese *et al*., 2002) and increased concentrations of this marker correlate with increased risk of cardiovascular disease (Albers *et al.*, 2005). Dysregulation of sICAM-1 release has been shown to impair neutrophil emigration (Sligh *et al.*, 1993) and wound healing, to influence disease progression (van de Stolpe and van der Saag, 1996; Witkowska, 2005), and has been associated with insulin and leptin resistance (Kent *et al*., 2004). Adipokines and peptide hormones are intricately connected with the immune system and dysregulated in obesity; leptin and resistin concentrations increase in obesity while adiponectin and glucagon concentrations are lower, and insulin-resistance is common.

Gut Microbiota and Inflammation

It has been previously suggested that alterations in the gut microbiota structure affect energy homeostasis since certain bacterial communities may extract or store energy more efficiently (Ley *et al.*, 2005), and changes with the gut bacterial community have been linked with obesity (Ley, 2010). These statements have insinuated that shifts in the

presence and abundance of communities within the microbiota could therefore alter the energy balance so that more energy could be potentially obtained from food. Gut microbiota shifts have been shown in obese mice, and these shifts created a reduction in *Bacteroidetes* and increased the abundance of *Firmicutes* when compared to lean counterparts (Ley *et al*., 2005). However, the gut microbiota shifts that have occurred in mouse models are not always indicative of shifts that may occur in other animal models or in humans. A meta-analysis investigation has shown that signature microbiome structures of lean and obese individuals are inconsistent between studies, even when similar methods of analysis are employed (Walters *et al.*, 2014). A separate meta-analysis investigation has also determined that variability between studies is greater than the differences found in lean and obese individuals of the same study (Finucane *et al*., 2014). Investigators involved in these meta-analyses have concluded that the low *Bacteroidetes*/*Firmicutes* is not an accurate representation of every obese individual, the ratio is frequently inconsistent, and that cohort selection, methodology, and statistical analysis strategies will need to mirror each other for appropriate comparisons (Finucane *et al*., 2014; Walters *et al*., 2014).

Although the exact alterations of gut microbiome structure of obese models have been debated, HE diets have been shown to shift microbiota profiles and increase systemic inflammation. Studies in mice have shown that diets high in saturated fats altered the gut microbiota profile and increased gut permeability compared to control diets (Lam *et al*., 2012), which suggests that HE diets may further increase systemic inflammation in an obese-prone model. Studies in obese rat models have also shown shifts in gut microbiota composition after consumption of HE diets, and that the fluctuation may have been associated in the development of systemic inflammation (Barbier de La Serre *et al*., 2010).

Specifically, obese rats had higher plasma LPS concentrations, and this was likely due to intestinal inflammation diminishing the epithelial barrier integrity (Barbier de La Serre *et al*., 2010). Further studies are required in obese models to determine if gut microbiota shifts introduced through dietary fermentable material (DFM) consumption alter the inflammation that is typically observed in obesity.

Dietary Fibre and Inflammation

Acetic acid, propionic acid and butyric acid are SCFA liberated after fermentation of dietary fibre by the gut microbiota. SCFA can significantly decrease the pH of the intestinal tract and cause mucosal injury in otherwise healthy humans (Ten Bruggencate *et al*., 2006) and in rats (Lin *et al*., 2002). High concentrations of butyrate can inhibit proliferation and induce apoptosis of the human IEC line Caco-2 (Peng *et al*., 2007), suggesting one route through which butyrate could influence epithelial integrity. In addition, increased permeability in the colon after acute exposure to butyrate has been reported in normal rat colonic mucosa (Mariadason *et al*., 1999). Butyrate has also been reported to cause cell necrosis and reduction of functional tight junctions between IEC (Mariadason *et al.*, 1999), which would result in increased permeability in intestinal mucosa. Intestinal trefoil factor (ITF) is expressed in goblet cells within the intestinal tract and has involvement in mucosal defense (Lin *et al.*, 2005). An investigation of mucosal injury in newborn rats has shown that ITF gene expression is down-regulated in the presence of SCFA (Lin *et al*., 2005). This down-regulation may indicate that the goblet cells are among the first to perish during mucosal injury. With decreases in goblet cell populations, there would also be decreases in mucin secretion, which combined with IEC

death and the accompanying decrease in IgA translocation, would further diminish mucosal defense, suggesting a negative role for SCFA production by the gut microbiota. However, the role of SCFA is not easily classified as completely detrimental or beneficial. Interestingly, acetate production by *Bifidobacterium* has been reported to inhibit IEC uptake of Shiga-like toxin during infection by *Escherichia coli* O157:H7, suggesting some beneficial effects of SCFA in the context of host defence (Fukuda *et al*., 2011). These contradictory roles of SCFA highlight the need for further study on its effects on the immune system.

The effects of fructooligosaccharides (FOS, 2-8 repeating fructose units) and inulin (9-60 repeating fructose units) during intestinal infections have been studied in rats (Ten Bruggencate *et al.*, 2004; Ten Bruggencate *et al.*, 2005). It has been determined that dietary FOS and inulin impair resistance to *Salmonella enteritidis* due to increases in intestinal permeability (Ten Bruggencate *et al*., 2004; Ten Bruggencate *et al*., 2005). The altered permeability would subsequently increase translocation of *Salmonella* to sites beyond the intestinal tract enhancing opportunities for systemic infection. Feeding upon FOS and inulin also increased mucin production in rat colonic contents (Ten Bruggencate *et al*., 2005). Increases in mucin secretion may actually be a protective action for IEC to prevent damage from the SCFA produced as a result of FOS and inulin fermentation. Since increased mucin production is also considered to be a basic mechanism of host defence, it appears that the effects of FOS in the context of infection are not clear cut. However, the observations during intestinal infection with *Salmonella* do reveal how dietary FOS and inulin could potentially increase systemic inflammation.

The SCFA profile differs during the fermentation of specific fibres, as does the context in which DFM may influence bacterial community structure and immune activity. A study analyzing the faecal community composition of BioBreeding rats found increased bacterial community richness in animals which received either wheat bran or oat bran compared to the control diet (Abnous *et al.*, 2009). BioBreeding rats on diets supplemented with wheat bran have been previously shown by Shastri *et al*. (2016) to have decreased T cell and $CD4^{+}T_{H}$ cell percentages in the mesenteric lymph nodes (MLN), while having increased B cell percentages in the spleen in comparison to rats on a control diet. In this same study, rats on oat bran and resistant starch (RS)-supplemented diets displayed increased concentrations of TGF-β in the liver (Shastri *et al*. 2016), indicating impact at the systemic level. A study which investigated effects of dietary supplementation with wheat bran, oat bran, RS and FOS on colon tumor formation in male Fischer 344 rats found that tumor burden was significantly reduced in rats on the wheat bran diet (Raju *et al*., 2016). RS-supplemented diets have led to increased faecal SCFA concentrations in BioBreeding rats (Kalmokoff *et al*., 2013); however, RS diets have also been shown to decrease faecal and caecal bacterial community diversity compared to control diets in this same rat strain (Kalmokoff *et al*., 2015). FOS has been previously shown to interact directly with TLRs (Vogt *et al.*, 2013; Vogt *et al.*, 2014); RS may also directly influence immune activity through TLR activation. A study by Bermudez-Brito *et al*. (2015) has shown that RS binds to TLR2 on human DC and modulates immune activity through increased chemokine production. With the results of the above trials taken together, it seems that different DFM distinctly influence immune activity and may have compartmental effects on either systemic or mucosal immunity.

An Obesity Model to Study Inflammation and Fibre Supplementation

Sprague Dawley rats are an outbred multipurpose albino rat breed which are frequently used in medical and metabolic studies. When Sprague Dawley rats are fed HE diets, they can be readily separated into diet-induced obesity prone or obesity resistant phenotypes based on weight gain after 2 weeks of feeding diets higher in fat and energy density than regular rodent diets (Aziz *et al*., 2009; Barbier de La Serre *et al.*, 2010). Under these conditions, some of the rats will gain significantly more weight than others, an outcome that parallels the human situation. While roles for the gut microbiota in the development of obesity and systemic low-grade inflammation have been proposed (Bleau *et al.*, 2015), much remains to be determined about the nature of gut microbiota interactions with the immune system, outcomes with respect to immunometabolism and the extent to which DFM can influence these outcomes.

Chapter 3 Study Objectives

A trial was designed to evaluate the effects of DFM on inflammation and obesity in the context of a high fat diet using a rat model. The purpose of the experiment was to investigate interactions of DFM and a HE diet on obesity-associated immune biomarkers in Sprague Dawley rats. The trial took place from July to November 2014. DFM were selected to include a range of structural properties and degree of fermentability, and included wheat bran, oat bran, high amylose corn starch (resistant starch type II: RS) and FOS. These DFM have been previously shown to differ in their effects on gut microbiota diversity, short and branched chain fatty acid production, gut mucosal cytokine profiles, and in the ability to influence cytokine profiles at the mucosal and systemic level in rat models (Kalmokoff *et al*. 2015; Shastri *et al*. 2015b, 2016). A preliminary phase of the trial was utilized for the development of obese and non-obese phenotypes while rats consumed a HE diet. Immediately following the segregation of phenotypes, rats consumed HE diets supplemented with DFM for 11 to 12 weeks. It was hypothesized that the obese rats would have higher levels of inflammation than the non-obese rats, and that rats on the HE FOS diet would have increased concentrations of proinflammatory cytokines than rats on the other diets. The fermentation properties of the DFM utilized in this study are shown in **Table 3-1**, and the cytokines and chemokines measured while investigating immune activity are shown in **Table 3-2**.

Objective 1: to evaluate associations between diet-induced obesity, inflammation and associated immune parameters after consumption of a HE diet for a 2-week period (the preliminary phase of the trial).

Objective 2: to evaluate inflammation and immune parameters associated with obesity after an 11 to 12-week period on selected HE DFM diets, while monitoring differences between obese and non-obese phenotypes which diverged during the preliminary trial.

Methods

Trial Design

Sprague Dawley rats display a continuum of weight gain when exposed to a moderately high-fat/HE diet. This diet was based on the standard rat chow, the American Institute of Nutrition 93G growth purified diet (AIN-93G; ingredients and nutritional profile is provided in **Appendix C**, **Table C1**), with increased fat content. Feeding Sprague-Dawley rats high fat diets is a common method for promoting the exhibition of obesity-prone or obesity resistant phenotypes (Barbier de La Serre *et al*., 2010; Levin *et al.*, 1997). In this model, obese rats (Diet-Induced Obesity, DIO) are identified as belonging to the upper 40% of weight gainers and resistant rats (Diet-Induced Obesity Resistant, DR) are identified as the lower 40% of weight gainers.

Initially, 176 weanling male Sprague Dawley rats were obtained from Charles River (Saint Constant, QC) in July 2014. The animals were 28-32 days old upon arrival at the Animal Research Division at the Banting Research Centre, Health Canada (Ottawa, ON). Animals were numbered (the odd-numbered rats received a black marking on their upper tail) and paired-housed in Sealsafe® PLUS Rat IVC Green Line cages with wire mesh bottoms separating the rats from the corn cob bedding (Bed-o'Cobs, The Andersons Lab Bedding Products, Maumee, OH, USA). This separation was necessary to prevent coprophagia and ingestion of bedding material, which could influence the study outcome (Le Leu *et al*., 2015). The environmental enrichment provided included sanitized nylon gnawing sticks, a flat stainless steel sheet for resting, a covered tunnel, and continuous music from a radio (French-Canadian Radio). Water and AIN-93G rat chow were provided *ad libitum* in self-feeding hoppers. The animal room was programmed with a 12-hour

light/dark cycle, and temperature was held constant at 22°C. Animals were assessed daily by the Animal Research Division staff for signs of distress (reduced feed consumption, sensitivity to handling, sensitivity to noise or light, and listlessness). The protocol for animal care procedures was approved by the Health Canada Animal Care Committee and the University of Ontario Institute of Technology's Animal Care Committee. In order to participate in the trial sample collections and necropsies, I completed the Canadian Council on Animal Care in Science's Institutional Animal User Training Program and received additional training in animal handling and sample collection procedures.

After animals experienced an acclimatization period of seven days, a HE diet consisting of 16% dietary fat (HE AIN-93G) was introduced for two weeks to identify DIO and DR phenotypes. The DIO rats were the 40% highest weight gainers and the DR rats were the 40% lowest weight gainers, with 70 animals per group, and the remaining 36 rats were designated as intermediate responders (MID). At this time, the 10 highest weighing DIO and 10 lowest weighing DR rats were autopsied (August 2014) to determine effects of the HE diet alone on physiological measures in both groups. After the initial two-week feeding trial, a tail vein blood sample was obtained from the remaining 60 DIO and 60 DR rats for immune and metabolic measures. At this same time, faecal samples were collected to establish baseline structures of the bacterial community before fibre diets were introduced. Animals were then re-paired if necessary to match DIO, DR and MID rats together in cages, and the odd-numbered rats received ear notches to reinforce their markings. Rat pairs were randomly assigned to receive one of six diet groups (ten rats per diet group for DIO and DR) for a further 11-12 weeks. These diets were formulated from modifications of the standard AIN-93G rat chow, and include control (AIN-93G), HE

control (AIN-93G modified with high fat content) and HE DFM-supplemented diets (AIN-93G modified with high fat content and DFM) including HE wheat bran, HE oat bran, HE high amylose corn starch resistant starch type II (RS), and HE FOS. The diet formulations were created by Dr. Stephen PJ Brooks (Bureau of Nutritional Sciences, Health Canada) and are shown in **Table 3-3**. Each rat in the trial had their weight recorded on a weekly basis.

Upon trial completion, necropsies were performed on all animals. Euthanasia by exsanguination was carried out under isoflurane anesthesia. Pathologist Dr. Don Caldwell (Scientific Services Division, Bureau of Chemical Safety Food Directorate, Health Products and Food Branch, Health Canada) inspected all rats for abnormalities and performed tissue sectioning. Tissues were collected, rinsed in ice-cold PBS, weighed and immediately cryopreserved in liquid nitrogen to examine effects on cytokine/adipokine production, and included systemic (serum, liver, spleen), mucosal (ileum, ileal Peyer's patches (PP), caecum, proximal colon, distal colon, MLN), and adipose tissue (epididymal fat and mesenteric fat). Tissues were then transferred to -80°C storage pending homogenization. Caecal and faecal contents were also collected cryopreserved to determine bacterial community structures and SCFA analysis.

In order to conduct histological scoring of the gut tissue and examination of immune cells present in the gut wall, additional intestinal tissues were preserved in 1.0 cm cylindrical sections in 10% neutral buffered formalin in 20 mL glass scintillation vials. These tissues included duodenum, mid jejunum, ileum, proximal colon and mid colon.
Blood samples were divided into lavender cap BD Vacutainer K_2EDTA blood collection tubes (#367861, BD, Franklin Lakes, NJ, USA) for whole blood TLR assay (4.0 mL),

lavender cap BD microtainer K_2EDTA tubes (#365974, BD, Franklin Lakes, NJ, USA) for hematology (0.2 mL), orange cap BD Vacutainer serum separation tubes (SST; #367983, BD, Franklin Lakes, NJ, USA) for serum immunological analysis (3.5 mL), and yellow cap BD microtainer SST (#365967, BD, Franklin Lakes, NJ, USA) for BUN and glucose measurements. Anti-coagulated whole blood samples collected in lavender cap BD Vacutainer K_2EDTA was used immediately following necropsies for hematological measures and the TLR agonist assay. Once blood collected in the orange and yellow cap BD Vacutainer SST was separated into serum, samples were stored at -80°C until analysis.

Histology

Dr. Don Caldwell (Scientific Services Division, Bureau of Chemical Safety Food Directorate, Health Products and Food Branch, Health Canada) performed histological scoring of gut tissue collected during the preliminary and final necropsies. Samples fixed in neutral buffered formalin were trimmed, processed and embedded into 4 µM thick H and E stained slides. Pathological examination was carried by light microscopy on duodenum, jejunum, ileum, proximal and mid colon samples from three rats of each diet group, using an assessment protocol adapted from Dickson *et al.* (2006). Attention was focused on the following: mucosal inflammation, villous architecture and ratio, crypts, lamina propria, muscularis mucosae, enterocytes, the composition of and numbers of inflammatory cells in the lamina propria and lining enterocytes, the brush border and the lumen border. Intraepithelial lymphocytes (IELs) were counted per 100 villous enterocytes for 5 villi per section in the small bowel.

Immune Cell Population Analysis by Flow Cytometry

Hematology measures were used to investigate circulating cell populations from blood collected immediately following both preliminary and final necropsies. Hematological measures were performed by Dominique Patry (Hematology & Biochemistry lab, Animal Research Division, Health Canada) using the AcT™ 5diff Cap Pierce hematology analyzer (Beckman Coulter, Brea, CA, USA). This same cell counter was used to determine cell concentrations for flow cytometry analysis.

Analysis of cell population patterns allows determination of DFM effects on total leukocytes, B and T lymphocytes (including T helper (T_H) cells, cytotoxic T cells, γδ-T cells, and $Foxp3^+$ regulatory T cells which will be referred to as Treg cells), T cell homing receptor expression (referred to as memory T cells (Obhrai et al., 2006)), Dendritic cells (DC), Natural Killer (NK) cells and macrophages (total macrophages and M1/M2 subsets). At the final necropsy, immunofluorescence and flow cytometry were utilized to examine immune cell populations (n = 5 per diet/phenotype/tissue) in systemic tissue (spleen) and mucosal tissues (MLN and ileal PP). To prepare cells for staining, tissues were placed on a 70 µm cell strainer in a 50 mL Falcon tube. Cells were strained through using a syringe plunger and 5 mL of ice-cold RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA). Cell suspensions were centrifuged at 340 × g for 6 minutes, and re-suspended in phosphate buffered saline to determine concentration. The immune cell markers were divided into three separate panels and details on all antibodies and conjugated fluorochromes may be found in **Table 3-4**. The same panels were used to enumerate immune cell population percentages in the spleen, MLN and ileal PP. To determine concentrations for optimal detection, fluorescent antibodies against

standard cell markers were titrated and tested. A viability stain was used to irreversibly label dead cells prior to permeablization and staining procedures to ensure that viable cells were gated separately, and an isotype control was used to assess non-specific binding in $Foxp3^+$ T cells. Cells were diluted to 1×10^7 cells/mL for viability staining, and a minimum of 1×10^6 cells/mL was required for antibody staining.

Flow cytometry was performed using a BD LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA, USA), equipped with blue (488 nm), red (640 nm) and violet (405 nm) lasers. The forward scatter and side scatter gating techniques used during acquisition of 10 000 viable cells/lymphocytes were standard and similar to those outlined by Shingadia *et al.* (2001). Initial analysis of immune cell populations was conducted using BD FACSDIVA™ software (BD Biosciences, San Jose, CA, USA), and additional analysis was conduced on cell populations with improved gating using FlowJO 10.0.8 software (Ashland, OR, USA) with assistance from Jason Fine, flow cytometry technician with Animal Research Division at Health Canada.

An ex vivo Whole Blood TLR Stimulation Assay

Direct measurement of TLR expression on rat immune cell populations using immunofluorescence with anti-TLR antibodies was not possible due to the unavailability of anti-rat CD282 and CD284 antibodies suitable for flow cytometry. However, TLR activity may be measured functionally by stimulating monocytes and DCs with TLR ligands, such as peptidoglycan (TLR2), LPS (TLR4), Flagellin (TLR5) and CpG DNA (TLR9). This whole blood stimulation method allows for determination of effects on

responses to TLR agonist stimulation, as TLR-expressing cells in blood samples will respond by releasing cytokines.

Blood collected from 20 animals at the preliminary necropsy was stimulated with the following TLR agonists; P3C (TLR2, Calbiochem, EMD Millipore, Etobicoke, ON, Canada), LPS (TLR4, Sigma Aldrich, St. Louis, MO, USA), Flagellin (TLR5, Alexis Biochemicals, Enzo Life Sciences, Farmingdale, NY, USA), *Escherichia coli* ssDNA (TLR9, InvivoGen, San Diego, CA, USA), CpG ODN 2006 DNA (TLR9, Imgenex, Novus Biologicals, Littleton, CO, USA), and RPMI-1640 medium (unstimulated control, Sigma Aldrich, St. Louis, MO, USA). The TLR agonist stimulation concentrations tested in the preliminary trial are listed in **Table 3-5**, are were selected based on other studies using this approach (Gillaux et al., 2011; Li et al., 2012; Liu et al., 2012). All TLR agonists were diluted in RMPI-1640 medium, and stock concentrations were prepared in volumes which made it possible to add 12 µL of agonist to 1.2 mL of whole blood to achieve the desired concentration.

To begin the assay, lavender cap BD Vacutainer K_2EDTA (#367861, BD, Franklin Lakes, NJ, USA) blood collection tubes were gently inverted five times to ensure mixing, and three 1.2 mL aliquots were prepared from each rat in 5 mL polypropylene round-bottom tubes (#352063, BD, Franklin Lakes, NJ, USA). In an effort to maintain aliquot reproducibility and reduce sample retention, Axygen Maxymum Recovery Universal Fit filter pipette tips (Union City, CA, USA) were used. From each rat, two aliquots were used for stimulation with two different TLR agonists, and the third aliquot was used as an unstimulated control. Once 12 µL of the appropriate TLR agonist was added to whole blood, the tube was lightly vortexed to ensure the agonist was evenly distributed in the

sample. Blood tubes were subsequently placed in a 5% CO_2 humidified incubator set at 37°C on a 5° slant with loose caps to prevent evaporation but allow for gas exchange. After 20 hours of incubation, blood assay tubes were centrifuged at 1300 × g for 5 minutes. The supernatants were carefully transferred into microtubes (approximately 2 × 200 µL) and frozen at -80°C until analysis. Cytokines analyzed in these samples included pro-inflammatory cytokines CINC-1/CXCL1, CINC-2αβ/CXCL3, IFN-γ, IL-1β, IL-6 and TNF-α, and the regulatory cytokine IL-10. All cytokines were measured using rat DuoSet ELISA Development kits according to manufacturer's procedures (R&D Systems, Minneapolis, MN, USA). After the preliminary trial samples were analyzed, it became clear that whole blood stimulation with a TLR-9 agonist would not be possible at the end of the trial due to cost. The optimal stimulating concentrations determined for each agonist at the final necropsy were 10 µg/mL for P3C, 5 µg/mL for LPS, and 100 ng/mL for Flagellin.

Whole blood collected from DIO and DR animals at the final necropsy (n = 60) was stimulated with TLR2, TLR4 and TLR5 agonists using the same protocol as outlined for the preliminary trial. At the end of the trial, the concentrations of cytokines measured included CINC-1, IL-1β, IL-6, IL-10 and TNF-α.

Detection of Serum Endotoxin

Serum endotoxin was quantified in order to determine whether circulating concentrations of this gut microbial component differed between HE DFM-supplemented diets. Serum endotoxin levels were analyzed from blood drawn at the preliminary necropsy (n = 20) and at the end of the trial for DR (n = 60) and DIO rats (n = 60), using the LAL

Assay QCL-100 (Lonza, Basel, Switzerland) following the procedure as described in Chapter 2. In a subset of serum samples, the pH ranged from 7.63 – 7.69. Since these measurements were within a biological range, pH adjustments were not necessary. There were several amendments made to the LAL assay during pre-testing with rat serum. This time, serum samples were diluted 1/5 in endotoxin-free water, heat-inactivated for 10 minutes at 70°C in a water bath (Cani *et al*., 2008), and Pyrosperse Dispersing Reagent F188 (Lonza, Basel, Switzerland) was added to samples at a 1/200 final dilution prior to beginning the LAL assay (Dehghan *et al*., 2014). Pyrosperse is a metallo-modified polyanionic dispersant, and it is used to reduce interference during the endotoxin assay. The heating and Pyrosperse steps were added to reduce and disperse lipid micelles which may have been introduced by the high fat diet in an effort to maintain LAL assay sensitivity. An alternate stop reagent, sodium dodecyl sulfate (10% w/v) was necessary for use with the Pyrosperse Dispersing Reagent.

Assessment of Blood Glucose and Blood Urea Nitrogen

Non-fasting blood glucose and blood urea nitrogen (BUN) were measured from serum collected at the preliminary necropsy and from all animals (DR, DIO and middle gainers) at the end of the trial to determine if HE and selected fibre diets altered glucose or nitrogen metabolism. Fasting measurements were not possible since this would have diminished the available fresh caecal and feceal sample collections, which were needed for bacterial community analysis and to measure SFCA content. Serum BUN and glucose concentrations were measured at Health Canada using a VITROS® 5,1 FS automated

clinical chemistry analyser with VITROS® urea/BUN and glucose test kits (Ortho-Clinical Diagnostics, Markham ON, Canada).

Tissue Homogenization

Tissue homogenates were prepared in an immunoprecipitation buffer adapted from Burgos-Ramos *et al.* (2012; 50 mM NaH_2PO_4, 100 mM Na_2PO_4, 0.1% sodium dodecyl sulfate, 0.5% NaCl, 1% Triton X-100, 5 mg/mL sodium deoxycholate) with the addition of 1% Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA). The tissues were suspended in immunoprecipitation buffer at a ratio of 1:5 (wt/vol) and then homogenized using a VWR®200 homogenizer (VWR International, Radnor PA, USA) and centrifuged (17 200 × g for 30 min at 4°C). Supernatants were collected and stored at -80°C until cytokine analysis. Only MLN tissue homogenates required a second round of centrifuging due to high lipid levels. A small section from the left lateral lobe of the liver was homogenized in place of the entire organ.

Before tissue homogenates were analyzed for cytokine, chemokine and adipokine profiles, Bradford assays were carried out to ensure consistent protein concentrations among tissue homogenates. Coomassie Plus Protein Assay Reagent and albumin standards (Thermo Fisher Scientific, Waltham, MA, USA) were used in this assay. Samples were diluted 1/15 in water, and standards were diluted in a 1/15 solution of homogenization buffer to account for background. Microplates were read at a wavelength of 595 nm to determine protein concentration, and values are expressed as protein (mg) per gram (g) tissue.

Assessment of Cytokine Profiles

Cytokine quantifications allow insight into the effects of each diet on gut-associated lymphoid tissue, and more specifically the T cell subsets. Cytokines profiles analyzed in serum and tissue homogenates collected from both preliminary (n = 10 per diet) and final necropsies (n = 10 per diet/phenotype) included both regulatory and proinflammatory cytokines/chemokines. Regulatory cytokines included in the analysis were active and total TGF-β1, IL-10 and IL-22; proinflammatory cytokines and chemokines included CINC-1, CINC-2αβ, CINC-3, GM-CSF, soluble ICAM-1/CD54, IFN-γ, IL-1β, IL-4, IL-6, IL-17 and TNF-α. Cytokine/chemokine profiles were examined in serum and both mucosal and systemic immune tissue homogenates (spleen, liver, epididymal fat, mesenteric fat, MLN, ileum, ileal PP, caecum, distal colon and proximal colon). All cytokines in serum and tissues were quantified using rat DuoSet ELISA Development kits according to manufacturer's procedures (R&D Systems, Minneapolis, MN, USA). ELISAs were carried out using 96-well high-binding half area microplates (Greiner Bio-One, Frickenhausen, Germany). The optical density of the reactions in the ELISA plates were read at wavelength of 450 nm on a Synergy HT multi-detection microplate reader (BioTek Instruments Inc, Winooski, VT, USA) using KC4 v3.4 analysis software (BioTek Instruments Inc., Winooski, VT, USA). In addition to the cytokine analysis, CRP was quantified in serum and liver homogenates via ELISA (R&D Systems, Minneapolis MN, USA).

For quantification of IL-22 in ileum, caecum, distal colon and MLN homogenates collected from the preliminary trial (n = 3 per diet/phenotype), a Mouse/Rat IL-22 Quantikine® ELISA kit was used (R&D Systems, Minneapolis MN, USA). This same IL-22 ELISA format was utilized again for distal colon and caecal homogenates collected

from the end of the trial (n = 10 per diet/phenotype). Due to the expensive cost of this assay, it was not possible to expand the IL-22 measurements to other tissues.

MLN and ileal PP of DIO and DR animals (n = 5 per diet group/phenotype/tissue) at the final necropsy were analyzed separately for CINC-2αβ, CINC-3, GM-CSF, sICAM-1/sCD54, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10 and TNF-α using the Rat Magnetic Luminex® Screening Assay (R&D Systems, Minneapolis, MN, USA) on a Luminex 200 analyzer (Austin, TX, USA).

Assessment of Peptide Hormone and Adipokine Profiles

Peptide hormone and adipokine concentrations (non-fasting) were measured to determine if the selected diets created alterations in insulin signaling and metabolism. Leptin was quantified in mesenteric fat, epididymal fat, distal colon homogenates and serum collected during the preliminary trial (n = 3 per phenotype/tissue) using the Mouse/Rat Leptin ELISA kit (B-Bridge International Inc., Santa Clara, CA, USA). Leptin, insulin and glucagon were measured in final necropsy serum samples (n = 5 per diet/phenotype) using the MILLIPLEX Map Kit Rat Metabolic Magnetic Bead Panel (EMD Millipore Corp. Billerica, MA, USA) on a Luminex 200 analyzer (Austin, TX, USA). Adiponectin was measured in serum and epididymal fat tissue homogenates (n = 10 per diet/phenotype) via Rat Adiponectin/ARP30 ELISA (R&D Systems, Minneapolis MN, USA), since this is the most prevalent adipokine and is difficult to multiplex accurately. Leptin was also measured in distal colon homogenates (n = 6 per diet/phenotype) using the Mouse/Rat Leptin Quantikine® ELISA (R&D Systems, Minneapolis, MN, USA). Again,

due to the expensive cost of this assay, it was not possible to expand leptin measurements to other tissues.

Statistical Analyses

The statistical program SigmaPlot v13.0 (Systat Software Inc., San Jose, CA, USA) was used to analyze data collected during this trial. In the preliminary trial analysis, unpaired *t*-tests (Student's *t*-tests) were carried out to determine differences between DR and DIO phenotypes, and the Mann-Whitney *U* test was utilized when data were not normally distributed. For data collected at the end of the trial, two-way ANOVA was used to examine differences between DR and DIO phenotypes, overall effects of diet and interactions (Phenotype × Diet). ANOVA analysis was followed by Tukey pairwise multiple comparison tests. Data was transformed if it did not pass normality (Shapiro-Wilk) or equal variance (Brown-Forsythe) tests, using common transformations available in the software program. A P value of < 0.05 was considered statistically significant. For some measures, there were several values that were below the LoQ. In these instances, the values were set to LoQ/$\sqrt{2}$ (Armbruster *et al.,* 1994) for analysis.

All results in tables are presented as the mean ± SEM. Figures were created using GraphPad Prism v7.01 software (La Jolla, CA, USA). The figures in this chapter are box and whisker plots; the ends of the boxes represent the upper and lower quartiles, the line in the centre of the box indicates the median value, and the whiskers display the maximum and minimum values.

Results

Preliminary Trial Analysis

The preliminary trial was considered to be the first two weeks after the acclimatization period, during which time all rats consumed the modified HE AIN-93G diet, referred to as the HE control diet. The average weights of the rats at this time point were as follows; 290.2 ± 0.7 g for DIO rats, 277.4 ± 0.2 g for MID rats, and 263.0 ± 0.7 g for DR rats. The amount of weight gained during the two week HE feeding period is displayed in **Figure 3-1a**, and there was a distinct and significant separation between the weights of DR, MID and DIO rats ($P < 0.001$). Tissues were collected from ten of the lightest DR rats and ten of the heaviest DIO rats at the end of this period. The average amount of food consumed each day during the preliminary trial is displayed in **Figure 3-1b**, and DIO rats ($P < 0.001$) and MID rats ($P = 0.003$) consumed more food each day than DR rats. The main goals of the preliminary trial were to determine effects of the HE diet alone on immunological and physiological measures in DR and DIO groups, and to pre-test several assays so that optimized procedures were in place for analysis of samples collected at the end of the trial.

The first analyses were performed on blood samples using standard hematology measures. Hematological analysis did not reveal any differences in peripheral immune cell populations between groups; however, DIO rats had higher percentages of red blood cell (rbc) distribution width ($P = 0.032$), higher platelet counts ($P = 0.028$) and higher mean platelet volumes ($P = 0.013$) than DR rats (**Table 3-6**). DR and DIO phenotypes did not reveal differences in serum measures, including cytokine/chemokine, CRP, LPS, BUN or blood glucose concentrations (**Table 3-7**). The whole blood TLR stimulation assay

performed after the two-week preliminary HE feeding was mainly used to test a panel of TLR agonists and stimulatory concentrations in preparation for the end of the trial (**Table 3-8**). Sample numbers were small since two concentrations per agonist were tested, making it difficult to determine if differences were apparent between phenotypes in responsiveness to TLR stimulation. Based on this preliminary testing, the following agonist stimulation concentrations were selected: 10 µg/mL for P3C (TLR2), 5 µg/mL for LPS (TLR4) and 100 ng/mL for Flagellin (TLR5). Responses to TLR9 stimulation with CpG ODN 2006 at 10 µg/mL were measurable, however this agonist was very costly and it was decided not to continue with it for analysis at the end of the trial. Overall, hematology and TLR agonist based measures did not reveal many immunological or physiological differences between phenotypes in the preliminary trial.

Examination of gut mucosal tissues was then conducted. Histological scoring was used to assess the presence of gut-level inflammation in sections of the small and large bowel, and there were no differences in morphology found between the DIO and DR groups after the initial two-week HE diet feeding. There were no differences in IEL counts in the duodenum, and no IELs were found in the jejunum of DR or DIO rats (**Table 3-9**). In ileal tissue, DR rats displayed a trend toward higher concentrations of total TGF-β1 than DIO rats ($P = 0.051$, **Table3-10**). The opposite effect was noted in the caecum, where DIO rats displayed a trend toward higher total TGF-β1 concentrations than DR rats ($P = 0.061$, **Table 3-11**). In the proximal colon (**Table 3-12**), higher concentrations of all measured cytokines were highest in DIO rats, however only IL-10 ($P = 0.032$) and TNF-α ($P = 0.016$) were significantly higher in DIO rats, and IL-17F trended to be higher in DIO rats ($P = 0.070$). Cytokine concentrations in distal colon samples were also highest in the DIO

phenotype (**Table 3-13**). Although the differences were not statistically significant in this tissue, IL-17F ($P = 0.061$), IL-22 ($P = 0.059$), and total TGF-β1 ($P = 0.072$) all trended to be higher in DIO rats. Overall, the pattern in the intestinal tissue was that DIO rats displayed higher concentrations of inflammatory cytokines and of total TGF-β1, a regulatory cytokine.

Cytokine profiles were also analyzed in the liver, spleen, MLN, and ileal PP to examine impact of diet on immune tissues at systemic and local levels. In liver homogenates, CINC-1 was significantly higher in DIO rats ($P = 0.017$, **Table 3-14**). Active and total TGF-β1 concentrations were nearly matching, indicating that all TGF-β1 in the liver was in its active form. In spleen homogenates, very low IL-10 concentrations, just at the level of detection, were present in both phenotypes. DIO rats displayed higher splenic TNF-α concentrations ($P = 0.049$) than DR rats (**Table 3-15**). Examination of the MLN revealed that DR rats had higher concentrations of sICAM-1/sCD54 ($P = 0.018$) than DIO rats (**Table 3-16**). No differences in cytokine or chemokine concentrations or profiles were found in the ileal PP (**Table 3-17**). Overall, there were relatively minor differences found in cytokine profiles at either systemic or local immune locations.

The final group of tissue analyzed was adipose tissue. In mesenteric fat, no differences in cytokine or chemokine concentrations were detected, other than a trend toward higher CINC-2α/β concentrations in DR rats ($P = 0.053$, **Table 3-18**). Although every effort was made to ensure that all MLN were dissected out of the mesenteric fat, it remains possible that some MLN could have been present during mesenteric fat homogenization, and this could have influenced cytokine measurements. Since no major differences were found in the mesenteric fat, it was decided not to pursue analysis of this

tissue at the end of the trial. In epididymal fat, DIO rats displayed somewhat elevated CINC-2αβ, CINC-3, IL-6 and TNF-α concentrations in comparison to DR rats, although statistically significant differences were not found (**Table 3-19**). It was interesting that DIO rats had reduced IL-10 concentrations (a regulatory cytokine) in comparison to DR rats. Analysis of mesenteric and epididymal fat revealed opposite patterns, with DR rats having higher cytokine concentrations in mesenteric fat, and lower concentrations in the epididymal fat.

Leptin was the adipokine chosen for the preliminary trial analysis. Concentrations of leptin were measured in serum and adipose tissue (mesenteric and epididymal), as well as distal colon for a mucosal measure (**Figure 3-2**). DIO rats had consistently higher concentrations of leptin than DR rats overall, with significantly higher concentrations in the distal colon ($P < 0.001$) and a trend of heightened leptin concentrations in serum ($P = 0.077$) and mesenteric fat ($P = 0.067$).

After the preliminary two-week HE control diet, DIO and DR phenotypes did segregate at a few immunological measures, however biochemical measures seemed to be unaffected at this point. Overall, measurements in intestinal gut tissue revealed higher concentrations of inflammatory cytokines and regulatory total TGF-β1 in DIO rats, minor differences were detected at systemic and local immune tissues, and epididymal fat of DIO rats had higher inflammatory cytokine concentrations. A continuous pattern of higher leptin concentrations was found in all locations measured in DIO rats, although it was only statistically higher in the distal colon. Data collected from assays pre-tested in the preliminary trial were used in determining the profiles of cytokines, chemokines and adipokines measured in samples collected at the end of the trial.

End of Trial Analyses

The feeding trial concluded after consumption of the AIN-93G modified diets for 11-12 weeks. On the morning of necropsy, the animals' final weights were recorded, and the average weight was 533.0 ± 4.1 g for DIO rats and 479.3 ± 3.6 g for DR rats. The total weight gained during the 11-12 weeks on the AIN-93G modified diets is displayed in **Figure 3-3a/b.** An overall phenotype effect was observed in the weight gain values ($P < 0.001$), DIO rats had higher weight gain than DR rats on the HE control ($P < 0.001$), HE oat bran ($P = 0.008$), HE RS ($P = 0.002$) and HE FOS ($P = 0.007$) diets. An overall diet effect was also observed in the weight gain values ($P < 0.001$). Rats on the HE oat bran diet had higher weight gain that rats on the control ($P < 0.001$), HE control ($P < 0.001$), HE wheat bran ($P = 0.031$) and HE RS ($P = 0.005$) diets. Additionally, rats on HE wheat bran ($P = 0.007$), HE RS ($P = 0.041$) and HE FOS ($P = 0.001$) diets had higher final weights than those on the control diet. Within the DR phenotype, rats on the HE oat bran diet had higher weight gain than those on the HE control ($P = 0.005$) and control ($P < 0.001$) diets. Within the DIO phenotype, rats on the HE oat bran ($P < 0.001$), HE FOS ($P = 0.012$) and HE control ($P = 0.036$) diets had higher weight gain than those on the control diet.

The average amount of food consumed each day during the trial is displayed in **Figure 3-4a/b**. Rats in each diet group had significantly different daily average food intakes from the other diet groups ($P = 0.036$ or less) with the following exceptions: rats on the control diet did not consume more than rats on the HE wheat bran or HE FOS diets, rats on the HE control did not consume more than rats on the HE RS or HE FOS diets, and rats on HE RS did not consume more than rats on HE FOS. Overall, DIO rats consumed significantly more food each day than DR rats ($P < 0.001$). It is important to note that the

recorded daily intakes for the HE oat bran diet may be higher than the amount rats on this diet actually consumed, since it was found that this particular kibble diet was brittle, and increased the possibility of food spillage.

Analysis of Peripheral and Tissue Immune Cell Populations

Hematological analysis revealed an effect on white blood cell (wbc) counts according to phenotype, with DR rats having lower concentrations overall than DIO rats ($P = 0.008$, **Table 3-20a/b**). On the HE oat bran diet, DR rats had lower wbc counts than DIO rats ($P = 0.014$). An overall diet effect was observed for rbc counts ($P = 0.017$); rats on the HE FOS diet had higher counts than rats on the control diet ($P = 0.020$), regardless of phenotype. Hematocrit levels were lower in DR rats on the HE oat bran diet than in DIO rats ($P = 0.036$). On the control diet, DIO rats had higher neutrophil counts than DR rats ($P = 0.028$). An overall phenotype effect was observed for percentages of circulating monocytes, with DIO rats having higher percentages than DR rats ($P = 0.022$). Within the DR phenotype, monocyte percentages for rats on the HE oat bran diet were higher than those on the control diet ($P = 0.031$), and the same pattern was present within the DIO phenotype ($P = 0.026$). Additionally, a diet effect ($P < 0.001$) was observed on the monocyte population. Overall, circulating monocyte percentages for rats on the HE control ($P = 0.014$), HE wheat bran ($P = 0.016$), HE oat bran ($P < 0.001$) and HE FOS ($P = 0.015$) diets were higher than those on the control diet.

Immune cell populations investigated within the spleen are shown in **Table 3-21a/b**. Within the DIO phenotype, rats on the HE RS diet displayed lower $CD3^+$ T lymphocyte percentages than those on the control diet ($P = 0.043$). Within $CD3^+$ T

lymphocyte populations, there was an overall effect due to diet, with rats on the HE FOS diet displaying higher $CD3^+$ percentages than those on the control diet (P = 0.019), regardless of phenotype. There was an effect on $CD3^+CD62L^+$ memory T cells due to diet within the DR phenotype; rats on HE RS (P = 0.034) and HE FOS (P = 0.002) diets had higher percentages than those on the control diet. Within the DIO phenotype, rats on the HE RS diet had higher $CD3^+CD62L^+$ percentages than those on the HE control (P = 0.006) and control (P = 0.004) diets, and rats on the HE FOS diet had lower $CD3^+CD62L^+$ percentages than those on the HE control (P = 0.003) and control (P = 0.002) diets. A diet effect was observed for $CD3^+CD62L^+$ memory T cells (P < 0.001), with combined phenotypes on the HE oat bran (P = 0.030), HE RS (P < 0.001) and HE FOS (P < 0.001) diets having higher $CD3^+CD62L^+$ memory T cell percentages than those on the control diet. Combined phenotypes on the HE RS (P = 0.003) and HE FOS (P < 0.001) diets had higher $CD3^+CD62L^+$ percentages than those on the HE control diet, while rats consuming the HE wheat bran displayed higher $CD3^+CD62L^+$ percentages than those on the HE FOS diet (P = 0.019). In splenic $CD3^+CD8a^+$ cytotoxic T cell populations, DIO rats on the HE FOS diet had lower percentages than those on the control diet (P = 0.044). With combined phenotypes, a diet effect was also observed (P = 0.005), with rats on the HE RS (P = 0.042) and HE FOS (P = 0.010) diets having lower $CD3^+CD8a^+$ cytotoxic T cell percentages than those on the control diet. DIO rats on the HE RS (P = 0.015) and HE FOS (P = 0.005) diets had lower percentages of $CD3^+CD45^+$ leukocytes than those on the control diet. There was also an overall diet effect on $CD3^+CD45^+$ leukocyte populations in the spleen (P < 0.001), with combined phenotypes on the HE RS diet having higher percentages than rats on the control diet (P = 0.010), and rats on the HE FOS diet having lower percentages than rats

on the control diet ($P < 0.001$). An effect due to diet was observed on splenic $CD3^-CD45RA^+$ B cell populations ($P = 0.015$). All rats on the HE FOS diet had lower B cell percentages than those on the control diet ($P = 0.042$), regardless of phenotype. A mix of NK cells and activated monocytes are present in the $CD3^-CD161a^+$ populations. Within the DIO phenotype, rats on the HE FOS diet had lower percentages of NK cells/activated monocytes than rats on the control diet ($P = 0.001$), and rats on the HE oat bran diet had higher percentages than rats on the HE FOS diet ($P = 0.029$). Within this population of splenic $CD3^-CD161a^+$ cells, an overall diet effect was observed ($P = 0.006$), with all DR and DIO rats combined on the HE FOS diet having lower percentages than rats on the control diet ($P = 0.006$), and higher percentages than rats on the HE oat bran diet ($P = 0.025$). In the $CD3^-CD4^-CD161a^+$ NK cell population (activated monocytes are excluded) an overall effect due to diet was observed ($P = 0.045$). Within the DIO phenotype, rats on the HE FOS diet had lower $CD3^-CD4^-CD161a^+$ NK cell percentages than rats on the control diet ($P = 0.022$). Within the macrophage population defined by $CD3^-His36^+$ cells, DR rats on the HE FOS diet had elevated percentages in comparison to DIO rats on the same diet ($P = 0.016$). No differences between diets or phenotypes were observed in percentages of $CD3^+CD4^+$ T_H cells, $CD3^+\gamma\delta TCR^+$ T cells, $CD3^+CD4^+Foxp3^+CD25^+$ T_{reg} cells or $CD3^-CD103^+$ DCs.

In summary, the diets which appeared to impact splenic immune cell population percentages most frequently in this study were the HE RS and HE FOS diets. Rats on the HE RS diet had decreased percentages of T lymphocytes and cytotoxic T cells, and increased memory T cells and leukocytes relative to rats on the control diet. Rats on the HE FOS diet displayed lower splenic cell population percentages of cytotoxic T cells,

leukocytes, B cells and NK cells, and higher percentages of T lymphocytes and memory T cells relative to rats fed the control diet.

Immune cell populations investigated within the MLN are shown in **Table 3-22a/b**. On the control diet, DR rats had higher percentages of $CD3^+CD4^+$ T_H cells than DIO rats ($P = 0.041$). On the HE control diet, DIO rats had higher $CD3^-CD45RA^+$ B cell percentages than DR rats ($P = 0.028$). An effect due to diet was observed on MLN B cell populations ($P = 0.045$); combined phenotypes on the HE oat bran diet had higher percentages than rats on the HE FOS diet ($P = 0.030$). A phenotype × diet interaction was observed in the $CD3^-CD103^+$ DC population ($P = 0.002$). Within the DIO phenotype, rats on the HE wheat bran diet displayed higher $CD3^-CD103^+$ DC percentages than rats on the HE FOS diet ($P = 0.020$). On the HE wheat bran diet, DIO rats had higher $CD3^-CD103^+$ DC percentages than DR rats ($P = 0.002$). On the HE FOS diet, DR rats had higher $CD3^-CD103^+$ DC percentages than DIO rats ($P = 0.024$). Within the MLN $CD3^-His36^+$ macrophage population, DR rats on the HE RS diet had elevated percentages in comparison to DIO rats on the same diet ($P = 0.019$). Within the DIO phenotype, rats on the HE RS diet had higher MLN $CD3^+\gamma\delta TCR^+$ T cell percentages than rats on the HE control diet ($P = 0.041$). On the HE control diet, DR rats had higher $CD3^+\gamma\delta TCR^+$ percentages than DIO rats ($P = 0.017$). Upon examining T_{reg} cell populations in the MLN, an overall diet effect was observed ($P = 0.043$). Rats of both phenotypes on the HE oat bran diet had lower $CD3^+CD4^+Foxp3^+CD25^+$ T_{reg} cell percentages ($P = 0.023$) than rats on the HE RS diet. In the DR phenotype, rats on the HE oat bran diet had lower percentages of $CD3^+CD4^+Foxp3^+CD25^+$ cells ($P = 0.031$) than rats on the HE RS diet. No differences between diets or phenotypes were observed in

percentages of $CD3^+$ T lymphocytes, $CD3^+CD62L^+$ memory T cells, $CD3^+CD8a^+$ cytotoxic T cells, $CD3^+CD45^+$ leukocytes, $CD3^-CD161a^+$ or $CD3^-CD4^-CD161a^+$ NK cells.

To summarize the MLN immune cell population percentages, rats on the HE oat bran diet had higher B cell percentages than those on HE FOS and higher T_{reg} cell percentages than those on HE RS, and rats of the DIO phenotype on HE wheat bran had higher DC percentages than those on HE FOS, and DIO rats on HE RS had higher $CD3^+\gamma\delta TCR^+$ T cell percentages than those on the HE control diet.

Immune cell populations investigated within the ileal PP are shown in **Table 3-23a/b**. On the control diet, DR rats had higher percentages of $CD3^+$ T lymphocyte populations than DIO rats ($P = 0.016$). A similar pattern was observed in $CD3^+CD4^+$ T_H cell populations where DR rats on the control diet had higher percentages than DIO rats ($P = 0.018$). Within the DR phenotype, rats on the HE RS ($P = 0.034$) and HE FOS ($P = 0.031$) diets had lower $CD3^+CD8^+$ cytotoxic T cell percentages than those on the control diet. There was an overall diet effect on ileal PP $CD3^+CD8^+$ cytotoxic T cell percentages ($P = 0.021$), with DIO rats having lower percentages than DR rats on the control diet ($P = 0.006$). An overall diet effect on $CD3^+CD45^+$ leukocytes was also present ($P = 0.022$), and rats from both phenotypes on the HE wheat bran diet had higher percentages than those on the HE FOS diet ($P = 0.046$). In populations of $CD3^-CD45RA^+$ B cells within the DR phenotype, rats on the HE RS diet had lower percentages than rats on the HE oat bran ($P = 0.039$) and control ($P = 0.046$) diets. Within the DIO phenotype, rats on the HE FOS diet had lower $CD3^-CD45RA^+$ B cell percentages than rats on the HE wheat bran ($P = 0.008$), HE oat bran ($P = 0.034$) and HE control ($P = 0.006$) diets. Also in the DIO phenotype, rats on the HE control diet had higher $CD3^-CD45RA^+$ percentages than those on the control

diet (P = 0.043). An overall diet effect on $CD3^-CD45RA^+$ B cells (P = 0.039) was present; rats on HE FOS diet had lower percentages than rats on the HE control diet (P = 0.009). In populations of $CD3^-CD161a^+$ NK cells with activated monocytes, DIO rats had higher percentages than DR rats within HE control diet group (P = 0.015). Within the DIO phenotype, rats the HE FOS diet had lower $CD3^-CD161a^+$ NK cell percentages than those on the HE control diet (P = 0.022). A phenotype × diet interaction was observed in the $CD3^-CD4^-CD161a^+$ NK cell population (P = 0.040). Within the DIO phenotype, rats on the HE FOS diet had lower $CD3^-CD4^-CD161a^+$ cell percentages than rats on the HE control diet (P = 0.015). Overall, the DIO phenotype had higher $CD3^-CD4^-CD161a^+$ cell percentages than DR rats on the HE RS (P = 0.039) and HE control (P = 0.006) diets. In populations of ileal PP $CD3^-CD103^+$ DCs, DIO rats had higher percentages than DR rats on the HE RS diet (P = 0.043). DR rats displayed higher percentages of $CD3^-His36^+$ macrophages than DIO rats on the HE RS diet (P = 0.018). No differences between diets or phenotypes were observed in percentages of $CD3^+CD62L^+$ memory T cells, $CD3^+\gamma\delta TCR^+$ T cells, or $CD3^+CD4^+Foxp3^+CD25^+$ T_{reg} cells in the ileal PP.

In summary of the ileal PP immune cell population percentages, T lymphocytes, $CD4^+$ T_H cells and cytotoxic T cell percentages were lower in DIO rats than DR rats on control. Leukocytes percentages were higher in rats on the HE wheat bran diet than those on the HE FOS diet. Effects of diet on B cell population percentages differed between phenotypes; DR rats on HE RS had lower percentages than those on HE oat bran or control diets, and DIO rats on the HE FOS diet had lower percentages than those on HE wheat bran, HE oat bran and HE control diets, while those on HE control diet had higher

percentages than those on the control diet. In NK cell populations, DIO rats had lower percentages on the HE FOS diet compared to the HE control.

Due to unfortunate circumstances, the flow cytometry laboratory at Health Canada was unavailable during the timing of the preliminary necropsy, preventing pre-testing of antibodies. During the flow cytometry acquisition at the end of the trial, the intracellular pan macrophage marker $CD68^+$ was not effectively measured, and subsequent macrophage markers in the same panel for $CD197^+$ M1 macrophages and $CD163^+$ M2 macrophages were subsequently omitted from the analyses of immune cell populations in the spleen, MLN and ileal PP tissues.

Ex vivo Analysis of TLR Agonist Induced Responses of Peripheral Blood Cells

The *ex vivo* whole blood culture assay involving stimulation of peripheral immune cells with TLR agonists was utilised to investigate and compare systemic innate immune responses between phenotype and diet groups. At trial completion, the following cytokines were measured in supernatants collected from whole blood cultures that had been stimulated separately with TLR2 (P3C), TLR4 (LPS) or TLR5 (Flagellin) agonists as well as an unstimulated control: CINC-1, IL-1β, IL-6, IL-10 and TNF-α.

CINC-1 concentrations measured from the whole blood TLR assay are in **Table 3-24a/b**. In unstimulated whole blood collected from rats on the HE FOS diet, DIO rat blood had higher CINC-1 concentrations than DR rat blood (P = 0.039). In TLR4 stimulated whole blood cultures from rats on the HE wheat bran diet, CINC-1 concentrations were higher in DR rats than in DIO rats (P = 0.026). A phenotype × diet interaction was found in TLR5 stimulated whole blood (P = 0.037). Whole blood cultures from DR rats on the

HE wheat bran diet had higher CINC-1 concentrations than DIO rats (P = 0.042), and within the HE FOS diet, blood collected from DIO rats had higher CINC-1 concentrations than DR rats (P = 0.008). No differences were detected between diets or phenotypes for CINC-1 production from whole blood stimulated with TLR2.

IL-1β concentrations measured from the whole blood TLR assay may be found in **Table 3-25a/b**. Within unstimulated whole blood samples collected from rats on the HE oat bran diet, DR rats produced higher IL-1β concentrations than DIO rats (P = 0.015). A phenotype × diet interaction was found in TLR2 simulated whole blood cultures (P = 0.040). In whole blood collected from DR rats and stimulated with TLR2, rats on the HE oat bran diet had higher IL-1β concentrations in than rats on the control (P = 0.017), HE control (P = 0.034), HE wheat bran (P = 0.028), HE RS (P = 0.010) and HE FOS (P = 0.010) diets. Additionally, DR rats on the HE oat bran diet had higher IL-1β concentrations in blood cultures stimulated with TLR2 than DIO rats on the same diet (P = 0.001). No differences were detected between diets or phenotypes for IL-1β production from whole blood stimulated with TLR4 or TLR5.

IL-6 concentrations measured from the whole blood TLR assay are in **Table 3-26a/b**. Within the control diet, TLR2 stimulated whole blood cultures produced higher IL-6 concentrations from the DR phenotype than the DIO phenotype (P = 0.009). Within the HE control diet, TLR4 stimulated samples produced higher IL-6 concentrations in blood collected from the DR phenotype than DIO (P = 0.047). No differences were detected between diets or phenotypes for IL-6 production from whole blood stimulated with TLR5 or in the unstimulated control.

IL-10 concentrations measured from the whole blood TLR assay may be found in **Table 3-27a/b**. An overall diet effect was observed for TLR4 stimulated samples (P = 0.019), regardless of phenotype. IL-10 concentrations of blood cultures from all rats on the HE RS diet were higher than rats on the control (P = 0.018), HE bran (P = 0.042) and HE FOS (P = 0.034) diets. No differences were detected between diets or phenotypes for IL-10 production from whole blood cultures stimulated with TLR2 or TLR5, or in the control.

TNF-α concentrations measured from the whole blood TLR assay are in **Table 3-28a/b**. Within the DIO phenotype, unstimulated whole blood cultures collected from rats on the HE FOS diet had lower TNF-α concentrations than those on the control diet (P = 0.010), but higher TNF-α concentrations than those on the HE control (P = 0.027) and the HE oat bran (P = 0.027) diets. Within the HE FOS diet, unstimulated blood collected from DIO rats had higher TNF-α concentrations than blood collected from DR rats (P = 0.002). A phenotype × diet interaction was found in TLR4 stimulated whole blood (P = 0.026). Within whole blood cultures collected from DIO rats and stimulated with TLR4, rats from the HE oat bran diet displayed higher TNF-α concentrations than those on the HE RS diet (P = 0.027). In TLR4 stimulated blood cultures from rats on the HE control diet, DIO rats had higher TNF-α concentrations than DR rats (P = 0.041). Additionally, DIO rats on the HE RS diet had higher TNF-α concentrations than DR rats (P = 0.027) in the TLR4 stimulated whole blood cultures. No differences were detected between diets or phenotypes for TNF-α production from whole blood cultures stimulated with TLR2 or TLR5.

To encapsulate the main findings of the *ex-vivo* whole blood TLR agonist assay, there were no overall differences due to phenotype. The only overall difference due to diet was found in TLR4-stimulated IL-10 production, where blood collected from rats on the

HE RS diet produced higher concentrations than those on HE oat bran, HE FOS and control diets. A consistent pattern of change across the measured cytokines and TLR agonists could not be established for phenotype or for a specific diet.

Assessment of Serum Cytokines, Chemokines, CRP, LPS, BUN and Glucose

Serum measures are displayed in **Table 3-29a/b.** Within the HE FOS diet, DIO rats had significantly higher CINC-1 concentrations than DR rats ($P = 0.006$). DIO rats on the HE RS diet displayed significantly higher CINC-2 concentrations than DR rats on the same diet ($P = 0.042$). A phenotype × diet interaction was found in CINC-3 concentrations ($P = 0.036$); DIO rats on the HE oat bran diet had lower CINC-3 concentrations that DR rats on the same diet ($P = 0.047$), and DIO rats on the HE RS diet had significantly higher CINC-3 concentrations than DR rats on the same diet ($P = 0.015$). A phenotype × diet interaction was also found in examining sICAM-1/sCD54 concentrations ($P = 0.044$); DIO rats on the HE control diet had higher sICAM-1/sCD54 concentrations that DR rats ($P < 0.001$). Within the control diet, DR rats displayed higher concentrations of active TGF-β1 than DIO rats ($P = 0.045$). Within the DR phenotype, rats on the HE control diet had significantly higher CRP concentrations than rats on the HE RS diet (P = 0.020). Within the DIO phenotype, rats on the control diet had significantly higher CRP concentrations than rats on the HE RS diet ($P = 0.016$). Additionally, an overall diet effect was observed with CRP concentrations ($P = 0.043$), with rats on the control ($P = 0.005$), HE control ($P = 0.011$) and HE wheat bran ($P = 0.027$) diets having higher CRP concentrations than rats on the HE RS diet.

Analysis of serum samples collected at the final necropsy revealed a diet effect ($P < 0.001$) on BUN concentrations, and the highest concentrations were present in the animals which consumed the control diet, regardless of phenotype. The HE diet appeared to decrease BUN concentrations. Within the DR phenotype, rats on the HE RS diet had lower BUN concentrations than rats on the control diet ($P = 0.037$). Within the DIO phenotype, rats on the HE control ($P = 0.007$), HE oat bran ($P = 0.001$), HE RS ($P = 0.006$) and HE FOS ($P < 0.001$) diets had lower BUN concentration than rats on the control diet. Combining phenotypes, all rats on HE control ($P = 0.020$), HE wheat bran ($P = 0.003$), HE oat bran ($P < 0.001$), HE RS ($P < 0.001$) and HE FOS ($P < 0.001$) diets displayed lower BUN concentrations than rats on the control diet. There were no differences found between phenotypes or diet groups in blood glucose or serum LPS or total TGF-β1 concentrations.

Assessment of Immune Activity in Gut Tissues

Histological scoring was performed to assess the presence of gut-level inflammation in sections of the small and large bowel collected from DIO rats at the end of the trial to determine if the addition of DFM to a HE diet produced any visible morphological change in intestinal tissues. After the examination of duodenum, jejunum, ileum, proximal and mid colon sections, pathological assessment did not reveal any differences in bowel morphology between the fibre supplemented diets in DIO rats. Furthermore, there were no differences in IEL counts in the duodenum or jejunum between diets (**Table 3-30**).

In the analysis of ileal tissue, concentrations of both proinflammatory and regulatory cytokines were measured (**Table 3-31a/b**). No differences were apparent in IL-

1β, IL-6, IL-10, IL-17F, active and total TGF-β1 concentrations in the ileum between phenotype or any of the diet groups after a multiple comparison test.

The cytokine/chemokine profile used to examine caecal tissue is in **Table 3-32a/b**. A diet effect on CINC-2αβ concentrations was evident ($P = 0.014$). In the DR phenotype, rats on the HE wheat bran ($P = 0.035$) and HE RS ($P = 0.027$) diets had lower CINC-2αβ concentrations than those on the control diet, rats on the HE wheat bran ($P = 0.017$), HE RS ($P = 0.013$) and HE FOS ($P = 0.044$) diets had lower CINC-2αβ concentrations than those on the HE control diet, and rats on the HE oat bran diet had higher CINC-2αβ concentrations than those on HE wheat bran ($P = 0.038$) and HE RS ($P = 0.029$) diets. Within the DR phenotype, rats on the HE RS diet had lower IL-6 concentrations than rats on the HE control ($P = 0.044$), HE wheat bran ($P = 0.040$) and HE FOS ($P = 0.003$) diets. An overall diet effect was also observed for IL-6 concentrations in caecal tissue ($P = 0.003$) when combining phenotypes; rats on the HE RS diet had lower IL-6 concentrations than those on the control ($P = 0.010$), HE control ($P = 0.037$) and HE FOS ($P = 0.002$) diets. A phenotype effect was observed for total TGF-β1 concentrations ($P = 0.047$), with DIO rats having higher total TGF-β1 concentrations overall than DR rats. No differences were apparent between phenotypes or diets for CINC-1, CINC-3, GM-CSF, IL-10, IL-17F, IL-22, or active TGF-β1 concentrations in the caecum. During necropsy, the caeca collected from rats of both phenotypes on the HE RS and HE FOS diets were visibly different from rats on other diets. The caeca from rats on the HE RS diet were wrinkled and shriveled in appearance, and the caeca from rats on the HE FOS dies were notably larger and distended.

The cytokine/chemokine profile used to examine the proximal colon is in **Table 3-33a/b**. A phenotype × diet interaction was found in examining IL-6 concentrations ($P =$

0.016). Within the DIO phenotype, rats on the HE oat bran (P = 0.050) and HE FOS (P = 0.006) diets had lower IL-6 concentrations than rats on the control diet. A trend was observed for a diet effect (P = 0.060), and DIO rats on the control diet had higher IL-6 concentrations than DR rats on the same diet (P = 0.003). Within the DIO phenotype, rats on the HE FOS diet had significantly lower IL-17F concentrations than rats on the control diet (P = 0.012). When examining TGF-β1, DIO rats on the HE RS diet had higher concentrations of active TGF-β1 (P = 0.040) and total TGF-β1 (P = 0.038) than DR rats on the same diet. No differences were apparent between phenotypes or diets for IL-10 or TNF-α concentrations in the proximal colon.

The cytokine/chemokine profile used to examine the distal colon is in **Table 3-34a/b**. Within the HE control diet, DR rats had higher IL-10 concentrations than DIO rats (P = 0.037). DR rats displayed higher IL-22 concentrations on the HE RS diet than DIO rats (P = 0.041). When examining total TGF-β1 concentrations on the control diet, DIO rats had higher concentrations than DR rats (P = 0.022). No differences were apparent between phenotypes or diets for IL-6, IL-17F, TNF-α or active TGF-β1 concentrations in the distal colon.

After the assessment of immune activity within gut tissues, it was clear that there were no lasting impacts of diet on ileal tissue, and no morphological differences were apparent between phenotype or diet groups after histological examination of the small and large bowel. Rats on HE RS and HE FOS diets had caeca that appeared remarkably different from rats on the other diets, however, the caeca collected from rats on the HE RS and HE FOS diets had lower pro-inflammatory cytokine and chemokine concentrations than rats on the control diets. In the proximal colon, DIO and DR phenotypes differed at

active and total TGF-β1 concentrations, and in the distal colon, DIO rats displayed higher IL-6 concentrations than DR rats, while DR rats displayed higher IL-10 concentrations than DIO rats.

Assessment of Immune Activity in Systemic and Local Tissues

The cytokine/chemokine profile examined in the liver is shown in **Table 3-35a/b**. Within the DR phenotype, rats on the HE control ($P = 0.008$), HE oat bran ($P = 0.001$) and HE RS (trend at $P = 0.058$) diets had higher CINC-2αβ concentrations than those on the control diet. On the control diet, DIO rats had higher CINC-2αβ concentrations than DR rats ($P = 0.035$). An overall diet effect was observed for CINC-2αβ concentrations ($P = 0.005$); rats combined from DIO and DR phenotypes on the HE control ($P = 0.039$), HE oat bran ($P = 0.010$) and HE RS ($P = 0.032$) diets had higher CINC-2αβ concentrations than rats on the control diet. Within the DIO phenotype, rats on the HE RS diet had lower liver CRP concentrations that rats on the control diet ($P = 0.030$). On the control diet, DIO rats had higher CRP concentrations that DR rats ($P = 0.038$). An overall diet effect was revealed ($P = 0.046$) in the analysis of liver IL-1β concentrations, and rats on the HE oat bran diet had higher concentrations than rats on the control diet ($P = 0.025$). No differences were apparent between phenotypes or diets for CINC-1, CINC-3, IFN-γ, IL-4, IL-6, IL-10, TNF-α, active or total TGF-β1 concentrations in the liver. As previously noted in the preliminary trial results, concentrations of active and total TGF-β in the liver nearly matched, indicating all TGF-β was in its active form.

The cytokine/chemokine profile examined in the spleen is in **Table 3-36a/b**. On the HE control diet, DR rats had higher CINC-3 concentrations than DIO rats ($P = 0.018$).

Within the DIO phenotype, rats on the HE wheat bran diet had much lower GM-CSF concentrations than rats on the HE oat bran diet ($P = 0.015$). A diet effect was observed in the analysis of splenic GM-CSF concentrations ($P = 0.017$); overall, rats on the HE wheat bran diet had lower concentrations than rats on the HE oat bran diet ($P = 0.047$). No differences were apparent between phenotypes or diets for CINC-1, CINC-2αβ, IFN-γ, IL-1β, IL-4, IL-6, IL-10, TNF-α, active or total TGF-β1 concentrations in the spleen.

A profile of eleven cytokines/chemokines was analyzed in MLN homogenates (**Table 3-37a/b**) using Luminex technology. On the control diet, CINC-2αβ concentrations were higher in DIO rats than in DR rats ($P = 0.017$). On the HE control diet, DIO rats had lower CINC-3 concentrations than DR rats ($P = 0.003$). A phenotype effect was also observed within CINC-3 concentrations, and DIO rats overall had lower concentrations than DR rats ($P = 0.005$). On the HE FOS diet, DR rats had higher IFN-γ concentrations than DIO rats ($P = 0.046$). Within the DIO phenotype, rats on the HE RS diet had lower IL-2 ($P = 0.029$) and lower IL-1β ($P = 0.018$) concentrations in the MLN than rats on the control diet ($P = 0.018$). On the HE RS diet, DR rats had higher IL-1β concentrations than DIO rats ($P = 0.019$). Within the DIO phenotype, rats on the HE wheat bran diet had lower IL-10 concentrations than rats on the control diet ($P = 0.047$). On the control diet, DIO rats had higher IL-10 concentrations in the MLN than DR rats ($P = 0.021$). No differences were apparent between phenotypes or diets for GM-CSF, sICAM-1/sCD54, IL-4, IL-6, or TNF-α concentrations in the MLN.

The same profile of eleven cytokines/chemokines analyzed in the MLN was also used examined in the ileal PP homogenates (**Table 3-38a/b**) with Luminex technology. A phenotype effect was present, and DIO rats had significantly higher CINC-2αβ

concentrations than DR rats (P = 0.038). Within the DIO phenotype, rats on the HE oat bran diet had higher CINC-2αβ concentrations than rats on the HE control diet (P = 0.047). An overall diet effect was also present (P = 0.007), with combined phenotypes on the HE oat bran diet having higher CINC-2αβ concentrations than rats on the HE control (P = 0.017) and HE FOS (P = 0.007) diets. After multiple comparison tests, no differences were apparent between phenotypes or diets for CINC-3, GM-CSF, sICAM-1/sCD54, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10 or TNF-α concentrations in the ileal PP.

In summary, cytokine profile analyses of systemic tissues indicated that HE diets increased liver CINC-2αβ concentrations relative to the control diet, the HE RS diet lowered liver CRP concentrations in the DIO phenotype, and rats on the HE wheat bran diet had lower splenic GM-CSF concentrations than those on the HE oat bran diet. In the assessment of cytokine profiles within local tissues, it was found that DIO rats had lower MLN CINC-3 concentrations than DR rats, DIO rats on the HE RS diet had lower MLN IL-1β and IL-2 concentrations in comparison to the control diet, DIO rats had higher ileal PP CINC-2αβ concentrations than DR rats, and rats on the HE oat bran diet had higher CINC-2αβ concentrations in the ileal PP than rats on the control diet. Overall, there were relatively few patterns in immune activity according to diet and phenotype noted, given how many measurements were performed on systemic and local immune tissues.

Assessment of Immune Activity in Adipose Tissue

The cytokine profile examined in epididymal fat is present in **Table 3-39a/b**. Rats on the HE wheat bran diet displayed lower CINC-2αβ concentrations than those on the control diet (P = 0.042), and DR rats on the HE wheat bran diet had higher CINC-2αβ

concentrations than DIO rats on the same diet (P = 0.012). Although TNF-α concentrations were relatively low in this tissue, DIO rats had higher concentrations than DR rats on the control diet (P = 0.030). An overall diet effect was observed on epididymal fat concentrations of active TGF-β1 (P = 0.012); rats on the HE wheat bran (P = 0.040), HE RS (P = 0.027) and HE FOS (P = 0.012) diets had lower concentrations than rats on the HE control diet. DR rats on the HE wheat bran (P = 0.012) and control (P = 0.047) diets had lower active TGF-β1 concentrations than those on the HE control diet, and DR rats had higher active TGF-β1 concentrations than DIO rats on the HE FOS diet (P = 0.044). An overall diet effect was also observed in epididymal fat total TGF-β1 concentrations (P = 0.026); rats on the HE wheat bran (P = 0.048) and HE oat bran (P = 0.040) diets had lower total TGF-β1 concentrations than rats on the HE control diet. No differences were apparent between phenotypes or diets for CINC-1, CINC-3, IL-1β, IL-6, or IL-10 concentrations in the epididymal fat.

Assessment of Peptide Hormones and Adipokines

Serum concentrations of peptide hormones glucagon and insulin, as well as adipokines leptin and adiponectin were measured. There was an overall diet effect on glucagon concentrations (P = 0.032, **Figure 3-5a/b**); rats on the HE FOS diet had higher glucagon concentrations than rats on the HE oat bran diet (P = 0.036). A phenotype effect was present in insulin concentrations (P = 0.013, **Figure 3-6a/b**); overall, DIO rats had higher serum insulin concentrations than DR rats. On the HE oat bran diet, DIO rats displayed higher insulin concentrations than DR rats (P = 0.032). A phenotype effect was also present for serum leptin concentrations (P = 0.034, **Figure 3-7/b**); DIO rats had higher

concentrations overall than DR rats. On the control diet, DIO rats had higher leptin concentrations than DR rats (P = 0.004). After multiple comparison tests, no differences were apparent between phenotypes or diets for adiponectin concentrations in the serum (**Figure 3-8a/b**).

In epididymal fat tissue, adiponectin concentrations were quantified. (**Figure 3-9a/b**). Like the serum concentrations, no differences were apparent between phenotypes or diets for adiponectin concentrations in the epididymal fat. In the distal colon, leptin concentrations were measured (**Figure 3-10a/b**). Within the DR phenotype, rats on the HE oat bran diet had higher leptin concentrations in the distal colon that rats on the control (P = 0.013) and HE RS (P = 0.011) diets. There was an overall diet effect on leptin concentrations (P = 0.007); rats from both phenotypes on the HE oat bran diet had higher leptin concentrations than those on the control (P = 0.029) and HE RS (P = 0.026) diets.

Overall, in examining peptide hormones and adipokines, DIO rats had higher concentrations of serum insulin and leptin than DR rats. Additionally, rats on the HE FOS diet displayed higher serum glucagon concentrations than rats on the HE oat bran diet, and rats on the HE oat bran diet had higher distal colon leptin concentrations that rats on the HE RS and control diets.

Discussion

After consumption of a HE diet for only two weeks, Sprague Dawley rats displayed two distinct phenotypes, DR or DIO, based on the amount of weight the animals gained. There were distinct differences in weight gain between DR, MID and DIO rat groups; the average weight of DR rats was 263 g and the average weight of DIO rats was 290 g. The trial continued for another 11-12 weeks with 60 DR animals and 60 DIO animals, allowing for an investigation of the impact of selected DFM-containing HE diets on immune biomarkers, gut inflammation and systemic inflammation. At the end of the trial, the distinct DR and DIO phenotypes remained, further indicating the utility of the Sprague Dawley rat strain as a model for diet-induced obesity (Abnous *et al*., 2009; Barbier de la Serre *et al*., 2010). The final average weights were 479 g for DR rats and 533 g for DIO rats, and it was not surprising to find that DIO rats consumed significantly more food each day than DR rats. Rats on the HE diets displayed higher weight gain overall than those on the control diet, although this did not necessarily mirror the pattern in the daily amount of food consumed. Rats of both phenotypes had the highest weight gain on the HE oat bran diet. The HE oat bran diet had slightly more fat (1.2 – 1.8%) than the other HE diets, providing more energy available from fat in the diet. The separation of DR and DIO phenotypes according to weight provided non-obese and obese states to study the effects of DFM-supplemented HE diets and to compare differences in immune and obesity-associated biomarkers.

Hematological analysis was performed to assess whether DFM-supplemented HE diets altered standard blood cell measures. After the preliminary trial, there were relatively minor hematological differences between DR and DIO rats due to rbc distribution width, platelet counts and mean platelet volume. Red blood cell dysfunction may have been

suspected if alterations were present in either the rbc count or rbc distribution width. Red blood cell dysfunction can lead to increased interaction with macrophages leading to enhanced macrophage adhesion to endothelial cells, and has been proposed as a contributor to atherosclerosis in a mouse model of diet-induced obesity on high-fat diet (Unruh *et al*., 2015). However, the rbc component differences between phenotypes found after the preliminary trial dissipated by the end of the trial, and only rats on the HE FOS diet had higher rbc counts than rats on the control diet at the trial completion. There were diet and phenotype effects observed at the end of the trial on hematocrit levels and neutrophil percentages. DR rats had lower hematocrit levels than DIO rats on the HE oat bran diet, and DIO rats had higher neutrophil percentages than DR rats on the control diet. While these differences, as those observed in the preliminary trial, were relatively minor, other hematological differences observed at the end of the trial between circulating monocytes and wbc counts may have more significance in the context of this study. Rats of both phenotypes had higher circulating monocyte percentages on the HE diets compared to those on the control diet. DIO rats displayed higher total wbc counts than DR rats, and this population includes lymphocytes, monocytes, neutrophils, basophils and eosinophils. Although higher wbc counts would potentially be in keeping with higher circulating cytokine and chemokine concentrations, this was not found after serum cytokine analysis. Increased numbers of immune cell populations are not necessarily connected to higher cytokine or chemokine production levels unless there is involvement of an immune stimulus. A study performed by Shabbir *et al*. (2015) found that increased wbc counts coincided with increased serum IL-6 concentrations in obese Sprague Dawley rats on an HE diet. However, the serum proinflammatory cytokines measured in the current trial did

not increase in a pattern that would reflect heighted monocyte activity in rats on the HE diets or wbc activity in DIO rats.

The diets with the greatest impact on immune cell populations in the spleen were HE RS and HE FOS. Rats on the HE RS diet had higher percentages of total leukocytes and memory T cells but lower percentages of cytotoxic T cells compared to rats on the control diet, and DIO rats on HE RS had lower total T cell percentages than DR rats. Rats on the HE FOS diet had the lowest percentages of T lymphocytes, memory T cells, cytotoxic T cells, leukocytes, B cells and NK cells relative to those on the control diet, and DIO rats on the HE FOS diet had lower percentages of macrophages than DR rats. Rats on FOS-supplemented diets have been previously shown to have decreased percentages of splenic T cells (Shastri *et al*., 2015b), which demonstrates that FOS diets influence systemic immune cell populations. Since rats consuming the HE FOS diet had lower splenic lymphocyte percentages (NK cells, B cells and T cells), it is possible that the rats on this diet could have an impaired immune response should infection occur, since few lymphocytes would available to migrate from the spleen. However, further testing would be required to determine the extent of the outcome of these population changes in the context of an immune challenge.

In the MLN, rats on the HE oat bran diet had higher B cell percentages than rats on the HE FOS diet and higher T_{reg} cell percentages than those on the HE RS diet. DIO rats on the HE wheat bran had higher DC percentages than those on HE FOS, and DIO rats on HE RS had higher $\gamma\delta TCR^+$ T cell percentages than those on HE FOS. Since rats consuming the HE FOS diet had decreased percentages of B cells, T_{reg} cells, DC and $\gamma\delta TCR^+$ T cells in the MLN compared to other diet groups, it is possible that the HE FOS diet may influence

B and T cell migration to the gut, an effect that would have a negative impact in the event of an infection.

Percentages of T lymphocyte populations in the ileal PP were impacted by phenotype on the control diet, as DIO rats had lower percentages of total T cells, $CD4^+$ T_H cells and cytotoxic T cells than DR rats. Rats on the HE wheat bran diet had higher leukocyte percentages than rats on the HE FOS diet. B cell percentages in the ileal PP fluctuated between diets and phenotypes, and a consistent pattern of change was not established other than that rats on the HE FOS diet had the lowest B cell population percentage. In examining NK cell populations, DIO rats had higher percentages than DR rats on the HE control diet, and rats on the HE FOS diet had lower percentages than those on the HE control diet. On the HE RS diet, DIO rats had higher DC percentages than DR rats, and this pattern was reversed for macrophage percentages on the same diet, as DR rats had higher macrophage percentages than DIO rats. The ileal PP are considered part of the gut-associated lymphoid tissue (GALT), and the immune cells contained in these organized lymphoid nodules are the first to encounter antigens present in the lower ileum. These results suggest that DIO rats may have altered immune responses due to lower percentages of T lymphocytes, $CD4^+$ T_H cells and $CD8^+$ cytotoxic T cells compared to DR rats, which would ultimately be anticipated to result in decreased cytokine production, T cell help and killing of virus-infected cells during infection.

Literature suggests that the gut microbiota (Furrie *et al*., 2005) and high-fat diets (Jialal *et al*., 2014; Suganami *et al.*, 2005) influence TLR expression. A change in response to TLR agonists is a sensitive indicator of alterations in the innate immune response (Della Bella *et al*., 2008; Ida *et al*., 2006), and we have previously used this technique to assess

effects of DFM on human and rat innate immune activity (Clarke *et al*., 2016; Shastri, 2015a). A whole blood TLR-agonist assay was performed to assess parameters of systemic immunity and to determine the effects of dietary fibre fermentation by gut microbiota on immune responses. This *ex vivo* whole blood stimulation method allows for determination of effects on responses to TLR2, 4 and 5 agonist stimulation, as TLR-expressing immune cells in blood samples will respond by releasing cytokines. The cytokine concentrations and the profile produced provide a measure of the strength of the TLR-induced response, and insight into the responding cell types, as certain cytokines are produced by distinct cell types. The cytokines measured in this assay were CINC-1, IL-1β, IL-6, IL-10 and TNF-α. Circulating monocytes produce all of these cytokines; macrophages produce CINC-1, IL-6 and TNF-α, and M2 macrophages produce IL-10; T lymphocytes produce IL-6 and IL-10 and T_H1 cells produce TNF-α; B cells produce IL-10 and TNF-α; and mDC produce IL-1β.

CINC-1 concentrations produced in the *ex vivo* assay mainly differed according to phenotype. With TLR4 and TLR5 stimulation, DR rats on the HE wheat bran diet produced higher CINC-1 concentrations than DIO rats on the same diet, and DIO rats on HE FOS had higher CINC-1 production than DR rats after TLR5 stimulation. IL-1β production after TLR2 stimulation was higher in DR rats than in DIO rats on the HE oat bran diet, and DR rats on the HE oat bran diet produced the highest IL-1β concentrations compared to DR rats on the other diets. IL-6 production was higher in DR rats than DIO rats after TLR2 stimulation on the control diet and after TLR4 stimulation on the HE control diet. IL-10 production after TLR4 stimulation was higher in DR rats on the HE RS diet than DIO rats, and DIO rats produced higher IL-10 concentrations than the DR rats on the HE FOS diet.

After TLR4 stimulation with LPS, rats on the HE RS diet had the highest IL-10 response overall. TNF-α production in unstimulated whole blood cultures differed between diets in the DIO phenotype; rats on the control diet had the highest TNF-α concentrations in comparison to rats on all HE diets. After TLR4 stimulation, DIO rats had higher TNF-α concentrations than DR rats on the HE control and HE RS diets. A consistent pattern of change in the cytokines produced after TLR activation by various agonists was not established for phenotype or diet. The greatest amount of cytokine production was induced by TLR4 stimulation, and the cytokine profiles were indicative of monocyte, macrophage, M2 macrophage, B cell activity.

The most robust TLR agonist of this assay was LPS, activating TLR4, since the highest levels of cytokine production were detected after LPS stimulation. Cytokine profiles induced by TLR5 stimulation with flagellin had fewer differences due to phenotype or diet in cytokine production than did TLR2 and TLR4 activation with P3C and LPS respectively. This *ex vivo* assay could be expanded in future experiments to include different agonists for TLR2 and TLR4 stimulation, such as a fungal-derived ligand in addition to the microbial-derived ligands. The addition of *Candida albicans* to PBMC *in vitro* has been shown to activate TLR2 and TLR4, the addition of *Saccharomyces cerevisiae* activates TLR2, and both strains enhance cytokine production by monocytes (Rizzetto *et al*., 2016). Another option could be the use of zymosan, a cell wall component of *S. cerevisiae*, as a TLR2 ligand (Underhill *et al.*, 1999). Inclusion of a fungal agonist to activate pattern recognition receptors (PPR) in the *ex vivo* whole blood assay would encompass possible stimulus from the mycobiome, a relatively unexplored area in examining impacts of DFM and in obesity. It would have also been interesting to further

explore the TLR9 activation with unmethylated CpG DNA as an agonist, since TLR9 is internal and cells must endocytose antigen for its activation, whereas TLR2, TLR4 and TLR 5 are expressed on the outer cell membrane.

After the preliminary trial, there were no significant differences found in blood glucose or BUN between DIO and DR rats, so it is unlikely that the two week HE diet altered the physiological parameters to a point where glucose and nitrogen metabolism were affected. By the end of the trial, blood glucose concentrations did not differ between phenotypes or diet groups, but the BUN concentration was significantly reduced in rats on all HE diets compared to the control diet. Rats on HE RS had the lowest BUN concentrations in the DR phenotype, and this effect on BUN in association with a RS supplemented diet has been observed before in rats (Kalmokoff *et al*., 2013). Through fermentation of dietary or endogenous protein in the gut, ammonia may be derived from the breakdown of urea (Smith and MacFarlane, 1998). Rats consuming RS diets have been shown to have reduced ammonia content in the caecum (Younes *et al.*, 1995). It has been previously proposed that gut bacteria may be utilizing more ammonia derived from endogenous urea due to changes in the diet, making less urea available for the host and thereby reducing circulating BUN concentrations (Kalmokoff *et al.*, 2013). There have been examples of influence on BUN concentrations following consumption of diets with varied carbohydrate, fat and protein proportions (Alford *et al*., 1990) and due to diets containing rapidly fermented fibres (Kalmokoff *et al*., 2013). However, less is known about the fluctuation of BUN concentrations in the context of a HE diet, or about the impact of this effect on nitrogen utilization on the immune system.

Serum LPS concentrations were quantified after the preliminary 2-week HE diet and at the end of the trial as an indirect measure of intestinal integrity (Albers *et al*., 2005). No differences in serum LPS concentrations that would have been attributable to diet or phenotype were found at either time point. It was surprising there were no differences associated with HE feeding in the DIO rats, considering associations of high fat diets and elevated LPS concentrations in other studies (Erridge *et al.,* 2007; Ghanim *et al.,* 2009). A gut leakiness test was performed by project collaborators at Health Canada; rats were gavaged with a sugar probe and temporarily housed into metabolic cages for urine collection. Analysis of the gut leakiness test results are pending, and could reveal information about the integrity of the IEC barrier that may not have been apparent after analysis of circulating LPS concentrations.

After the preliminary HE feeding trial, no differences were found in serum cytokine or CRP concentrations. At the end of the trial, no differences due to diet were found in concentrations of circulating chemokines or regulatory cytokines; however, differences were apparent between the two phenotypes within specific diets. On the HE FOS diet, DIO rats had higher CINC-1 concentrations than DR rats, and had higher CINC-2αβ and CINC-3 concentrations on the HE RS diet. On the HE oat bran diet however, DR rats had significantly higher serum CINC-3 concentrations than DIO rats. On the control diet, active TGF-β1 concentrations were higher in DR rats than in DIO rats, but this difference was not apparent on any of the test diets. Overall, these differences in CINC-3 and active TGF-β1 were the only instances where DR rats had higher circulating cytokine concentrations than DIO rats.

CRP is a marker of inflammatory disorders; the protein interacts with the complement system by binding to complement C1q to activate the classical complement pathway, and is functionally analogous to IgG, although it is not antigen specific (Pagana and Pagana, 2002). Rats on the HE RS diet had lower serum CRP concentrations than rats on control, HE control and HE wheat bran diets, however it was not suspected that any of the rats were in a state of inflammatory disorder since concentrations of proinflammatory cytokines and chemokines did not reflect this. DIO rats had higher concentrations of sICAM-1 than DR rats on the HE control diet. The serum represented one means of measuring immune activity at the systemic level, however it did not appear that diet or phenotype consistently influenced circulating cytokine or chemokine concentrations. Spleen and liver tissue may have been more appropriate tissues to measure the impact of HE diets on systemic immune activity since blood concentrations only represent the net outcome of cytokine production from all tissues in the body (Albers *et al.*, 2005). Consequently, these systemic tissues were examined.

Histological scoring of tissue collected from section of the small and large bowel did not reveal any pathological differences in intestinal mucosa after the 2-week preliminary HE feeding or at the end of the trial. This was a surprising finding since RS and FOS are rapidly fermented in the lower gut and rapidly decrease the pH of the lumen due to increased SCFA production, which could potentially lead to epithelial injury (Ten Bruggencate *et al*., 2003). It was expected that RS and FOS would have different histological scorings from control, wheat bran or oat bran diets to due how quickly and completely RS and FOS are fermented in the caecum and proximal colon (Cummings *et al*., 1996; Singh and Singh, 2010), and due to the changes in gross morphology evident in

the caecum of these rats. However, this was not the case. In a study investigating the effects of FOS and lactulose-supplemented diets in Wistar rats, histological analysis of the intestinal mucosa did not reveal mucosal inflammation in rats fed FOS diets before they were infected orally with *Salmonella enteritidis* (Bovee-Oudenhoven *et al.*, 2003). After infection however, the rats consuming the FOS diet had more severe mucosal inflammation compared to rats on a control diet (Bovee-Oudenhoven *et al.*, 2003). This work suggests that even though differences were not apparent with the HE FOS diet alone, differences might be evident if there was an infectious or pro-inflammatory challenge. IEL counts from the duodenum and jejunum of DR and DIO rats did not differ between phenotypes or diets after the preliminary trial or at the end of the trial. The IELs represent movement of immune cells from the MLN to the lumen for surveillance and are influenced by the microbiota (Hooper and Macpherson, 2010). Abnormally high IEL counts, greater than 40 IELs per 100 epithelial cells, would be indicative of inflammation, and are seen in inflammatory bowel disease and coeliac disease (Dickson *et al.*, 2006). However, the rats in this trial were healthy and these histopathology measures indicated that severe inflammation was not induced with the HE and HE DFM-supplemented diets.

In the analysis of ileal tissue after the preliminary trial, it was found that DR rats tended to have higher total TGF-β concentrations than DIO rats. By the end of the trial, no differences were apparent in the ileum for any of the proinflammatory or regulatory cytokines measured. This was not a surprising finding since the selected DFMs used in this study were not fermented in the small intestine, but further along the GI tract at either the caecum, proximal or distal colon. A study which involved mice on high fat diets found that the expression of glucose and lipid metabolism-related genes had been altered in the small

intestine, providing an adaptation to the metabolic changes induced by the high fat diet (Clara *et al*., 2017). It is possible that the rats in this trial may have metabolically adapted to the HE DFM supplemented diets over the 11-12 week period, however sampling after DFM supplementation was limited to the trial end point. It would be interesting to determine whether there was early evidence of proinflammatory immune activation followed by regulation, perhaps 2-3 weeks into the trial.

In the analysis of caecal cytokines after the preliminary trial, it was found that DIO rats tended to have higher total TGF-β concentrations than DR rats. By the end of the trial, rats on the HE wheat bran, HE RS and HE FOS diets had lower concentrations of CINC-2αβ than rats on the HE control diet. Rats on the HE RS starch diet had the lowest caecal IL-6 concentrations, which was interesting since RS is quickly fermented in the caecum. Since the caeca of rats on the HE RS diet were visibly wrinkled and shriveled, and the caeca of rats on the HE FOS diet were notably bloated and distended, it was expected that these caeca would have had altered immune activity in comparison to the other diets. However, there were no differences in the cytokine profiles that would be in keeping with alterations in immune activity in the caeca of HE RS and HE FOS fed rats.

In the analysis of proximal colon tissue after the preliminary trial, concentrations of IL-10 and TNF-α were higher in DIO rats than in DR rats, and IL-17F concentrations tended to be higher in the DIO rats. In the distal colon, DIO rats had a tendency toward higher IL-17F, IL-22 and total TGF-β concentrations compared to the DR rats. These initial findings suggested involvement of mucosal IL-17F/IL-22 activity in the DIO phenotype apparent after only 2 weeks of a HE diet. IL-17F is produced by T_H17 cells which are involved in the clearance of pathogens (Korn *et al*., 2009), and IL-22 is involved in mucosal

epithelial repair mediated through activation of STAT3 in IEC (Mizoguchi, 2012; Neufert *et al*., 2010), although the roles of IL-17 and IL-22 in the context of HE diets and metabolism remains to be explored. By the end of the trial, rats on the HE oat bran and HE FOS diets had lower IL-6 concentrations than rats on the control diet in the proximal colon. DIO rats on the HE FOS diet had lower IL-17F concentrations than rats on the control diet, and DIO rats on the HE RS diet had higher active and total TGF-β1 concentrations than DR rats in the proximal colon. In the distal colon, IL-10 concentrations were higher in DR rats on the HE control diet than DIO rats, and DR rats had higher IL-22 concentrations than DIO rats on the HE RS diet. Also, DIO rats had higher total TGF-β concentrations than DR rats on the control diet. In the assessment of immune activity within gut tissues, it was difficult to associate an overall pattern with diet. The patterns found in the preliminary trial due to phenotype dissipated by the end of the trial. It did not appear that HE DFM-supplemented diets had major or long-term effects on immune activity in the GI tissues after 11-12 weeks of consumption.

After 2 weeks on a HE diet, DIO rats had higher liver CINC-1 concentrations than DR rats. By the end of the trial, rats on the HE oat bran, HE RS and HE control had higher CINC-2αβ concentrations in the liver than rats on the control diet. Rats of the DIO phenotype on the HE RS diet had lower liver CRP concentrations than rats on the control diet. Rats on the HE oat bran diet had higher liver IL-1β concentrations than rats on the control diet. A rather large panel of cytokines was measured in the liver and no other differences were found. In the analysis of splenic tissue after the preliminary trial, DIO rats had higher TNF-α concentrations than DR rats. At the end of the trial, DR rats had higher CINC-3 concentrations in the spleen than DIO rats. A diet effect was observed for GM-

CSF, as rats on HE wheat bran had lower splenic GM-CSF concentrations than rats on the HE oat bran diet, and DIO rats on the HE wheat bran diet had lower GM-CSF concentrations than DR rats. As in the liver analysis, a very large panel of cytokines was measured in the spleen, but only a few minor differences were found between phenotypes within specific diets. At the systemic level, it does not appear that the HE DFM-supplement diets induced major changes in immune activity.

In the analysis of the MLN after the preliminary trial, it was found that DR rats had higher concentrations of sICAM-1 than DIO rats. By the end of the trial, this effect on sICAM-1 had dissipated in the MLN, however there were several differences found between phenotypes on specific diets. CINC-2αβ concentrations were higher in DIO rats than in DR rats on the control diet, and DIO rats had lower concentrations of CINC-3 than DR rats on the HE control diet. There was an overall phenotype effect on CINC-3; DIO rats had lower concentrations than DR rats. On the HE FOS diet, DR rats had higher IFN-γ concentrations than DIO rats, and on the HE RS and control diets, DR rats had higher IL-1β and IL-2 concentrations in the MLN than DIO rats. DIO rats had higher MLN IL-10 concentrations on the control diet than did DR rats, while DIO rats on the HE wheat bran diet had lower IL-10 concentrations than on the control diet. After 2 weeks on the HE diet, no differences were apparent in the ileal PP between phenotypes. By the end of the trial, differences due to phenotype and to diet were apparent in CINC-2αβ concentrations of the ileal PP; DIO rats had higher concentrations than DR rats, and rats on the HE oat bran diet had higher concentrations than rats on the HE control or HE FOS diets. These small changes in the CINC-2αβ concentrations may represent increased neutrophil activity in the DIO rats and those which consumed the HE oat bran diet, as monocytes and macrophages

are key sources of this cytokine. At the local immune tissue level, the most pronounced differences were that CINC-3 concentrations were lower in the MLN and CINC-2αβ concentrations were higher in the ileal PP of DIO rats. Small differences between phenotypes within diet groups were apparent, although it did not appear that any specific diet produced a long-lasting effect on cytokine or chemokine production in the MLN and ileal PP. Lymph fluids from the ileum, caecum and proximal colon drain into the MLN (Mowat and Agace, 2014), making it a suitable tissue to examine immune activity from these regions. Lymph fluid from the distal colon drains into the caudal lymph node (Mowat and Agace, 2014), and this tissue may have been more representative of some immune activity influenced by the DFM which are fermented further along the proximal colon and in the distal colon.

No differences were found in the analysis of epididymal fat cytokine production after the preliminary trial. At the end of the trial, DIO rats had higher TNF-α concentrations than DR rats in the epididymal fat, suggesting potential for higher proinflammatory activity. Since no other differences were apparent between phenotype or diet in concentrations of IL-1β, IL-6 and IL-10, it was not possible to determine if there were be fluctuations in the activity of M1 and M2 macrophages in the adipose tissue. A rise in IL-10 production on a particular diet or in one phenotype over the other may have indicated increased M2 macrophage activity, since heightened IL-10 expression is a marker of M2 macrophage activity (Mantovani *et al*., 2002). Although an appropriate ELISA format for IL-12 was not available at the time of analysis, increased concentrations of IL-12 in the adipose tissue may have indicated increased M1 macrophage activity (Mantovani *et al*., 2004). Increased M1 macrophage polarization has been shown in obese models and it

exacerbates inflammation to a point where it may influence metabolism and insulin resistance (Lumeng *et al.*, 2007).

Leptin was the only adipokine measured after the preliminary analysis. Higher leptin concentrations were found in DIO rats in all tissues measured, with significantly higher concentrations than DR rats in the distal colon. Leptin concentrations tended to be higher in the serum and mesenteric fat, and no differences were found in epididymal fat. At the end of the trial, adiponectin, leptin, glucagon and insulin were measured in serum. Rats on the HE FOS diet had higher glucagon concentrations that rats on the HE oat bran diet. DIO rats had higher serum insulin and leptin concentrations than DR rats overall, and this result was expected due to the difference in body mass between the phenotypes. In the distal colon, rats on the HE oat bran diet had higher leptin concentrations than rats on the HE RS and control diets. No differences were found in adiponectin concentrations in serum or epididymal fat. Adipokines, specifically leptin, are produced in proportion to body fat mass (Matarese and La Cava, 2004) and are involved in immunomodulation by adipocytes. Leptin influences TLR expression and responsiveness in pre-adipocytes and mature adipocytes (Batra *et al.*, 2007). Elevated concentrations of serum and tissue leptin were quantified in the DIO rats compared to the DR, and this finding, combined with the observed occasional increases in proinflammatory cytokine concentrations in systemic and local immune tissues, suggested that DIO rats would be predicted to have heightened sensitivity to TLR stimulation in the whole blood TLR agonist assay. However, the results of the *ex vivo* TLR assay did not suggest that DIO rats were more sensitive than DR rats to TLR stimulation through TLR2, TLR4 or TLR5.

Resistin is another adipokine which would have been interesting to measure, however Luminex or ELISA rat formats were not readily available during the time of this study. Increased resistin concentrations are capable of influencing inflammation and the expression of adhesion molecules, including ICAM-1 (Verma *et al.*, 2003). Resistin has also been shown to upregulate proinflammatory cytokine production by PMBCs *in vitro* (Bokarewa *et al.,* 2005), and correlations have been found between plasma resistin concentrations and IL-6, CRP and ICAM-1 concentrations in non-diabetic women (Zhang *et al*., 2010). DIO rats from the current trial had elevated concentrations of sICAM-1 on the HE control diet compared to DR rats, however it has not been established if resistin has involvement in sICAM-1 concentrations in obesity.

Composition of the gut bacterial community has been linked to obesity in the literature (Ley, 2010; Yoshimoto *et al.*, 2013). Most research investigating this topic has been in mice (Ley *et al*., 2005; Vijay-Kumar *et al*., 2010), however there are inconsistencies with human research (Duncan *et al*., 2008; Finucane *et al*., 2014; Walters *et al.*, 2014). Gut community analysis of the faecal and caecal microbial communities by research collaborators at Health Canada and AAFC will provide further insight into the role of the gut microbiota and effects of DFM in the context of HE diets and their impact on obesity and metabolism-associated immune parameters in DIO and DR rats.

This study has examined how HE DFM-supplemented diets influence mucosal, systemic and obesity-associated biomarkers under homeostatic immune conditions. Although two distinct phenotypes of DR and DIO were investigated, the obese rats were disease-free and represented a resting condition. Much of the focus for this study was on baseline and innate measures of immune activity. The rats were not immunized since there

was interest in determining how DFM impact the immune system in the absence of an adaptive immune challenge. Since several differences were observed between diets in lymphocyte populations at the systemic and local immune tissue levels, it could be useful to investigate the response to immunization. A strong T cell dependent antigen such as KLH (Harris and Markl, 1999) should be used in determining if DFM influence responsiveness to immunization during HE diet exposure.

The observed impact of a HE diet on the immune system at the systemic as well as the mucosal level after only two weeks indicates the extent to which a HE diet can rapidly influence immune parameters, even in the absence of additional immune challenges such as infection or defined sources of inflammation. DIO rats had elevated leptin concentrations in serum and tissue, and elevated concentrations of regulatory and proinflammatory cytokine activity in the distal and proximal colon, liver and spleen in comparison to the DR rats. Addition of DFM with distinct physical and chemical characteristics and wide range of fermentability to the HE diet had varied impacts on cytokine and adipokine profiles associated with diet-induced obesity. Many of the observed effects of the HE diet between phenotypes dissipated after DFM supplementation. The phenotypic differences by the end of the trial were that DIO rats had higher concentrations of CINC-3 in the MLN, higher concentrations of CINC-2αβ in the ileal PP, and higher serum insulin and leptin concentrations than DR rats. The most frequently observed influences on immune activity according to diet involved the HE oat bran and HE FOS diets. Although rats on the HE oat bran diet consumed more food and weighed more than rats on other diets in the trial, this group of rats displayed lower IL-6 concentrations in the proximal colon and had higher concentrations of CINC-2αβ and IL-1β in the liver, higher

CINC-2αβ concentrations in the ileal PP and higher leptin concentrations in the distal colon relative to rats on the control diet. HE FOS-fed rats displayed decreased splenic lymphocyte population percentages in comparison to rats on the control diet and decreased lymphocyte and DC population percentages in the MLN in comparison to rats on other HE DFM diets. The effects of DFM-supplemented HE diets on gut microbial communities and the network of interactions with the immune system and host metabolism remain to be explored, as do the ramifications for the immune changes observed in the context of a defined immune challenge.

Table 3-1. Structural and fermentation properties of DFM added to HE diets used in the obese rat trial.

Fibre	Solubility	Structural Properties	Fermentation Location	References
Wheat Bran	Insoluble	Arabinoxylan; branched chain polymer of β1-4 linked D-xylopyranosyl units	Slowly and incompletely fermented in the distal colon	Chen *et al*. 2011; Izydorcyzck and Biliaderis, 1993
Oat Bran	Soluble	β-glucan; β1-4 linked glucose units separated by β1-3 linked glucose every 2-3 units	Slowly fermented in the proximal colon	Andersson *et al*. 2010; Drzikova *et al*. 2005
Resistant Starch Type II	Soluble	Amylose; polymer of α1-4 linked glucosidic chains	Rapidly and completely fermented in the caecum	Cummings *et al*. 1996; Tharanathan, 2002
Fructo-oligosaccharide	Soluble	Composed of short-length chains of 2-8 fructose units with a terminal glucose, linked by β2-1 glycosidic bonds	Rapidly and completely fermented in the proximal colon	Roberfroid, 2005; Singh and Singh, 2010

Table 3-2. Cytokines and chemokines measured to determine the impact of DFM-supplemented HE diets on rat immune parameters.

Cytokine or Chemokine	Cellular Source	Cellular Target & Role	References
CINC-1 /CXCL1	Monocytes, macrophages and epithelial cells	Neutrophil activation	(Shibata *et al*., 2000)
CINC-2αβ /CXCL3	Monocytes, macrophages and epithelial cells	Neutrophil activation, first chemokine produced in the presence of LPS	(Takano and Nakagawa, 2001)
CINC-3 /CXCL2	Monocytes, macrophages and epithelial cells (previously named MIP-2)	Neutrophil activation, most potent of CINCs but produced in smaller quantities	(Shibata *et al*., 1995; Shibata *et al*., 2000)
GM-CSF	Monocytes, T-lymphocytes, fibroblasts and endothelial cells	Stimulates development of neutrophils, dendritic cells and macrophages, promotes eosinophil proliferation and development	(Caux *et al.*, 1992)
IFN-γ	Activated NK cells, $CD4^+$ and $CD8^+$ T lymphocytes	Impact on macrophage activation, MHC class I and II expression, antigen processing and presentation ability; T_H1 signature cytokine.	(Schroder *et al*., 2004)
IL-1β	Monocytes, tissue macrophages and dendritic cells	Proinflammatory cytokine, promotes IL-2 release, B-cell maturation and proliferation	(Dinarello, 1989)
IL-2	T lymphocytes	Promotes signaling required for T-cell proliferation	(Popmihajlov *et al*., 2012)
IL-4	Mast cells, T lymphocytes and bone marrow stromal cells	Regulates the differentiation of naïve $CD4^+$ T cells into helper T_H2 cells; T_H2 signature cytokine	(Paul, 1991)
IL-6	T lymphocytes, monocytes, macrophages, fibroblasts and endothelial cells	Proinflammatory cytokine and also considered an adipokine, regulates immune responses and stimulates antibody production	(Mauer *et al*., 2015)

This table is continued on the next page.

Table 3-2 *continued*.

Cytokine or Chemokine	Cellular Source	Cellular Target & Role	References
IL-10	Monocytes, macrophages (M2), T lymphocytes, B cells and keratinocytes	Regulatory, inhibits T_H1 cell activity, prevents macrophage cytokine release; T_{reg} signature cytokine.	(Borish, 1998)
IL-17F	T_H17 cells	Stimulates proliferation and activation of T cells, stimulates epithelial cells to secrete chemokines	(Korn *et al.*, 2009)
IL-22	T lymphocytes	Regulatory mucosal cytokine, inhibits IL-4 production by T_H2 cells, protective in preserving the epithelial barrier	(Zheng *et al.*, 2008)
sICAM-1 /sCD54	Endothelial and smooth muscle cells; ICAM-1 is membrane bound (provides adhesion between endothelial cells and leukocytes after injury); sICAM-1 is released and circulating	Competes with bound ICAM-1 and stimulates leukocyte cytokine production	(Witkowska, 2005)
TGF-β1	Produced by almost every cell type	Immunoregulatory, regulates cell proliferation, growth and differentiation, involved in class switching to IgA	(Annes *et al.*, 2003)
TNF-α	T_H1 cells, adipocytes, B cells, activated monocytes and macrophages	Proinflammatory, cytotoxic, activates macrophages and induces nitric oxide production	(Bradley, 2008)

Table 3-3. AIN-93G modified diets* with high fat and wheat bran, oat bran, RS or FOS.

Product #	**D14062202**	**D14062203**	**D14062204**	**D14062205**	**D14062206**	**D14062207**
Diet Name	*Control (AIN-93G Modified)*	*High-Energy Control (AIN-93G Modified)*	*HE Wheat Bran*	*HE Oat Bran*	*HE Resistant Starch*	*HE FOS*
Ingredient	**g**	**g**	**g**	**g**	**g**	**g**
Casein	200	190	169.2	152.7	190	190
L-Cystine	3	3	3	3	3	3
Corn Starch	387.5	173.3	158.4	110.3	155.3	172.6
Maltodextrin 10	122	68.3	53.4	5.3	50.3	67.6
Sucrose	100	283.3	268.4	220.3	265.3	282.6
Cellulose, BW200	70	70	3.3	40	40	40
Corn Starch, High Amylose	0	0	0	0	83.9	0
Wheat Bran	0	0	133.3	0	0	0
Oat Bran	0	0	0	260.9	0	0
Oligofructose	0	0	0	0	0	32.2
Soybean Oil	70	120.3	120.3	120.3	120.3	120.3
Milk Fat, Anhydrous	0	44.2	44.2	44.2	44.2	44.2
tBHQ	0.014	0.014	0.014	0.014	0.014	0.014
Mineral Mix S10022G	35	35	35	35	35	35
Potassium Phosphate, Monobasic	0	0	0	0	0	0
Vitamin Mix V10037	10	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
FD&C Red Dye #5	0	0	0	0	0.05	0.025
FD&C Yellow Dye #40	0	0.05	0	0.025	0	0
FD&C Blue Dye #1	0	0	0.05	0.025	0	0.025
Total	**1000.014**	**999.964**	**1001.0644**	**1004.564**	**999.864**	**1000.064**
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**
g						
Protein	179.0	170.2	172.7	174.7	170.2	170.2
Carbohydrate (digestible)	609.5	524.9	508.8	495.0	520.6	522.8
Fat	70.0	164.5	170.1	183.0	164.5	164.5
Fiber	70.0	70.0	70.0	70.0	70.0	72.2
Resistant Starch	0.0	0.0	0.0	0.0	30.0	0.0
Potassium	3.6	3.6	3.6	3.6	3.6	3.6
Phosphorus	3.0	2.9	2.7	2.6	2.9	2.9
g%						
Protein	17.9	17.0	17.3	17.4	17.0	17.0
Carbohydrate (digestible)	60.9	52.5	50.8	49.3	52.1	52.3
Fat	7.0	16.5	17.0	18.2	16.5	16.4
Fiber	7.0	7.0	7.0	7.0	7.0	7.2
Resistant Starch	7.0	7.0	7.0	7.0	7.0	7.2
kcal						
Protein	716	681	691	699	681	681
Carbohydrate	2438	2100	2035	1980	2082	2091
Fat	630	1481	1531	1647	1481	1481
Total	3784	4261	4257	4326	4244	4253
kcal%						
Protein	**19**	**16**	**16**	**16**	**16**	**16**
Carbohydrate	**64**	**49**	**48**	**46**	**49**	**49**
Fat	**17**	**35**	**36**	**38**	**35**	**35**

* These formulations were created by Dr. Stephen PJ Brooks (Bureau of Nutritional Sciences, Health Canada) and this table was reproduced with permission.

Table 3-4. Anti-rat antibodies used in flow cytometry for analysis of cell populations in spleen, MLN and ileal PP.

Marker	Description	Clone	Conjugate*	Manufacturer
Control	Isotype – IgG1	F8-11-13	AF700	AbD Serotec
CD3	T lymphocytes	IF4	APC	BD Biosciences
CD3**	T lymphocytes	G4.18	PerCP-eFluor	eBioscience
CD4	T helper cells	OX-35	PE-Cy7	BD Biosciences
CD4***	T helper cells	OX-35	V450	BD Biosciences
CD8a	Cytotoxic T cells	OX-8	V450	BD Biosciences
CD25	T cell IL-2R α chain	OX-39	FITC	BD Biosciences
CD45	Leukocytes	OX-1	APC-Cy7	BD Biosciences
CD45RA	B cells	OX-33	PE-Cy5	BD Biosciences
CD62L	T cell subsets, effector/memory	11RL1	PE	BD Biosciences
CD68	Pan macrophage	ED1	AF700	AbD Serotec
CD103	Dendritic cells	OX-62	AF647	BioLegend
CD161a	NK cells	10/78	FITC	BD Biosciences
CD163	M2 macrophage	ED2	AF647	AbD Serotec
CD197	M1 macrophage	Y59	PE-Cy7	abcam
Foxp3	Treg	FJK-16s	PE	eBioscience
HIS36	Pan macrophage	HIS36	PE	BD Biosciences
γδ-TCR	γδ-T cells	V65	FITC	eBioscience
Viability dye	Labels dead cells	-----	eFluor 506	eBioscience

* Conjugates: AF647: Alexa Fluor 647, APC: allophycocyanin, FITC: fluorescein isothiocyanate, MHC II: Major histocompatibility complex class molecule II, PE: phycoerythrin, PerCP: peridinin–chlorophyll–protein complex.
** Alternate fluorochrome-conjugated anti-CD3 antibody used to differentiate populations in panel 2.
*** Alternate fluorochrome-conjugated anti-CD4 antibody used to differentiate populations in panel 3.
Manufacturers: BD Biosciences (San Jose, CA, USA); BioLegend (San Diego, CA, USA); AbD Serotec (Oxford, UK); abcam (Cambridge UK); eBioscience (San Diego, CA, USA).

Table 3.5. Preliminary trial TLR stimulating concentrations used in *ex vivo* whole blood assay.

Receptor	Agonist	Concentrations Tested
TLR2	P3C	10 µg/mL and 20 µg/mL
TLR4	LPS	5 µg/mL and 10 µg/mL
TLR5	Flagellin	100 ng/mL and 200 ng/mL
TLR9	*E. coli* ssDNA	2.5 µg/mL and 5 µg/mL
TLR9	CpG ODN 2006	5 µg/mL and 10 µg/mL
Control	RPMI-1640 medium	*n/a*

Table 3-6. Hematological measures from the preliminary trial.

Measure*	Phenotype	Value	2-tailed *P* value
WBC 10^9/L	DR	7.1 ± 0.7	0.748
	DIO	7.4 ± 0.7	
RBC 10^9/L	DR	6.50 ± 0.10	0.124
	DIO	6.31 ± 0.06	
Hgb g/L	DR	134 ± 1.07	0.683
	DIO	133 ± 1.89	
Hct L/L	DR	0.386 ± 0.004	0.674
	DIO	0.383 ± 0.006	
MCV fL	DR	59 ± 0.67	0.192
	DIO	61 ± 0.79	
MCH pg	DR	20.6 ± 0.25	0.171
	DIO	21.1 ± 0.23	
MCHC g/L	DR	347 ± 0.88	0.863
	DIO	347 ± 0.73	
RDW %	DR	12.1 ± 0.14	0.032
	DIO	12.5 ± 0.14	
PLT 10^9/L	DR	822 ± 24.68	0.028
	DIO	899 ± 20.94	
MPV fL	DR	6.2 ± 0.13	0.013
	DIO	6.7 ± 0.10	
NE %	DR	9.0 ± 0.92	0.203
	DIO	10.6 ± 0.76	
LY %	DR	87.2 ± 1.02	0.134
	DIO	84.8 ± 1.13	
MO %	DR	2.4 ± 0.23	0.147
	DIO	3.2 ± 0.46	
EO %	DR	1.0 ± 0.14	0.760
	DIO	1.1 ± 0.36	
BA %	DR	0.3 ± 0.06	0.899
	DIO	0.3 ± 0.05	

* WBC, White blood cell count; RBC, Red blood cell count; Hgb, Hemoglobin; Hct, Hematocrit; MCV, Mean corpuscular volume, MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration, RDW, Red cell distribution width; PLT, platelet count; MPV, mean platelet volume; NE, neutrophil count, LY, lymphocyte count; MO, monocyte count; EO, eosinophil count; BA, basophil count. Concentrations or percentages are presented as mean ± SEM, $n = 10$. Statistical analyses were performed using unpaired *t*-tests.

Table 3-7. Serum cytokine/chemokine and biochemistry measures from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
CINC-1 pg/mL	DR	132.4 ± 14.3	0.376
	DIO	115.8 ± 11.4	
CINC-2αβ pg/mL	DR	30.7 ± 3.1	0.668
	DIO	29.0 ± 2.2	
CINC-3 pg/mL	DR	14.0 ± 0.6	0.811
	DIO	13.8 ± 0.5	
Active TGF-β pg/mL	DR	419.3 ± 22.3	0.141
	DIO	380.0 ± 11.8	
Total TGF-β ng/mL	DR	14.6 ± 1.1	0.777
	DIO	15.0 ± 1.2	
sICAM-1/sCD54 pg/mL	DR	6412.0 ± 475.0	0.355
	DIO	5893.5 ± 270.1	
CRP pg/mL	DR	277.6 ± 18.6	0.516
	DIO	296.3 ± 21.4	
LPS (EU/mL)	DR	0.443 ± 0.063	0.456
	DIO	0.548 ± 0.122	
BUN mmol/L	DR	4.41 ± 0.32	0.640
	DIO	4.64 ± 0.36	
Glucose mmol/L	DR	7.70 ± 0.34	0.460
	DIO	8.09 ± 0.39	

* Concentrations are presented as mean ± SEM, $n = 10$. Statistical analyses were performed using unpaired *t*-tests.

Table 3-8. Cytokine/chemokine concentrations measured from whole blood cultures after TLR stimulation in the preliminary trial.

		Measure* (pg/mL)					
Agonist	**Phenotype**	**CINC-1**	**IFN-γ**	**IL-1β**	**IL-6**	**IL-10**	**TNF-α**
P3C 10 µg/mL	DR unstim[3]	4.4 ± 4.0	0.0 ± 0.0	59.1 ± 145.8	100.4 ± 53.1	48.8 ± 48.8	37.9 ± 37.9
	DR stim[3]	4.8 ± 4.8	21.5 ± 21.5	145.8 ± 42.7	89.5 ± 52.1	82.1 ± 69.6	31.2 ± 21.2
	DIO unstim[2]	5.4 ± 5.4	20.8 ± 20.8	61.5 ± 61.5	149.0 ± 98.0	34.8 ± 6.4	32.7 ± 11.7
	DIO stim[2]	2.4 ± 2.4	0.0 ± 0.0	587.5 ± 506.6	120.0 ± 105.1	61.1 ± 12.0	44.4 ± 11.4
P3C 20 µg/mL	DR unstim[2]	6.7 ± 6.7	64.4 ± 64.4	41.7 ± 41.7	402.7 ± 387.8	48.8 ± 48.8	37.9 ± 37.9
	DR stim[2]	6.7 ± 6.7	0.0 ± 0.0	47.3 ± 47.3	185.0 ± 98.2	82.1 ± 69.6	31.2 ± 31.2
	DIO unstim[3]	5.1 ± 5.1	0.0 ± 0.0	24.8 ± 24.8	104.8 ± 36.3	34.8 ± 6.4	32.7 ± 11.7
	DIO stim[3]	7.1 ± 7.1	0.0 ± 0.0	82.8 ± 46.4	92.9 ± 51.4	61.1 ± 12.0	44.4 ± 11.4
LPS 5 µg/mL	DR unstim[3]	4.4 ± 4.0	0.0 ± 0.0	59.1 ± 31.6	100.5 ± 53.1	50.2 ± 15.7	31.3 ± 24.4
	DR stim[3]	10.4 ± 8.2	0.0 ± 0.0	453.2 ± 95.9	112.5 ± 33.3	80.8 ± 10.0	174.6 ± 19.6
	DIO unstim[2]	5.4 ± 5.4	20.8 ± 20.8	61.4 ± 61.4	149.0 ± 98.0	58.8 ± 32.5	9.4 ± 9.4
	DIO stim[2]	42.5 ± 42.5	0.0 ± 0.0	414.0 ± 64.1	119.8 ± 83.1	67.4 ± 30.3	88.5 ± 9.2
LPS 10 µg/mL	DR unstim[2]	6.7 ± 6.7	64.4 ± 64.4	41.7 ± 41.7	402.7 ± 387.8	48.8 ± 48.8	37.9 ± 37.9
	DR stim[2]	10.3 ± 10.3	0.0 ± 0.0	168.3 ± 71.3	138.2 ± 123.4	81.4 ± 81.4	104.7 ± 54.2
	DIO unstim[3]	5.1 ± 5.1	0.0 ± 0.0	24.8 ± 24.8	104.8 ± 36.3	34.8 ± 6.4	32.7 ± 11.7
	DIO stim[3]	11.9 ± 6.1	0.0 ± 0.0	510.1 ± 171.2	80.4 ± 46.6	86.1 ± 20.3	203.3 ± 68.3
Flagellin 100 ng/mL	DR unstim[2]	2.4 ± 2.4	0.0 ± 0.0	111.9 ± 33.9	257.9 ± 10.9	110.7 ± 25.7	13.1 ± 13.1
	DR stim[2]	3.0 ± 3.0	0.0 ± 0.0	172.0 ± 14.6	276.0 ± 21.7	123.4 ± 10.8	50.1 ± 1.8
	DIO unstim[3]	5.4 ± 5.4	0.0 ± 0.0	69.9 ± 36.1	113.4 ± 59.8	44.0 ± 28.6	17.1 ± 8.8
	DIO stim[3]	5.8 ± 5.8	5.8 ± 5.8	234.8 ± 93.9	127.2 ± 63.6	52.5 ± 35.1	30.2 ± 1.8
Flagellin 200 ng/mL	DR unstim[3]	71.9 ± 71.9	0.0 ± 0.0	86.2 ± 44.3	117.2 ± 58.9	51.2 ± 28.6	27.1 ± 7.2
	DR stim[3]	50.1 ± 50.1	0.0 ± 0.0	117.1 ± 60.8	148.9 ± 53.6	34.1 ± 2.0	42.4 ± 15.7
	DIO unstim[2]	0.0 ± 0.0	0.0 ± 0.0	53.0 ± 53.0	101.2 ± 71.8	18.4 ± 12.5	44.9 ± 18.7
	DIO stim[2]	0.0 ± 0.0	0.0 ± 0.0	170.3 ± 57.4	108.8 ± 108.8	41.5 ± 41.5	0.0 ± 0.0

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Table 3-8 *continued.*

		Measure* (pg/mL)					
Agonist	**Phenotype**	**CINC-1**	**IFN-γ**	**IL-1β**	**IL-6**	**IL-10**	**TNF-α**
***E. coli* ssDNA** 2.5 µg/mL	DR unstim[2]	110.3 ± 105.5	0.0 ± 0.0	146.5 ± 0.6	186.6 ± 82.2	79.4 ± 57.0	19.4 ± 6.8
	DR stim[2]	77.9 ± 73.7	0.0 ± 0.0	138.3 ± 15.3	197.4 ± 93.0	79.1 ± 50.7	45.2 ± 30.7
	DIO unstim[1]	0.0	0.0	0.0	0.0	0.0	29.4
	DIO stim[1]	0.0	0.0	0.0	0.0	12.5	0.0
***E. coli* ssDNA** 5 µg/mL	DR unstim	-----	-----	-----	-----	-----	-----
	DR stim	-----	-----	-----	-----	-----	-----
	DIO unstim[2]	8.1 ± 8.1	0.0 ± 0.0	60.4 ± 60.4	83.3 ± 53.9	20.1 ± 14.3	42.7 ± 20.9
	DIO stim[2]	5.1 ± 5.1	0.0 ± 0.0	22.1 ± 5.6	100.8 ± 28.3	39.5 ± 5.1	10.4 ± 7.6
CpG ODN 2006 5 µg/mL	DR unstim[1]	0.0	0.0	78.0	247.0	85.1	0.0
	DR stim[1]	0.0	0.0	72.5	268.8	97.7	65.7
	DIO unstim[1]	0.0	0.0	89.1	202.9	97.7	0.0
	DIO stim[1]	0.0	0.0	123.0	225.1	110.5	0.0
CpG ODN 2006 10 µg/mL	DR unstim[2]	0.0 ± 0.0	0.0 ± 0.0	55.8 ± 55.8	123.6 ± 101.5	65.6 ± 42.8	34.3 ± 0.5
	DR stim[2]	0.0 ± 0.0	0.0 ± 0.0	72.9 ± 72.9	220.7 ± 98.4	121.2 ± 15.2	61.3 ± 12.9
	DIO unstim[1]	0.0	0.0	105.9	173.0	31.0	26.2
	DIO stim[1]	0.0	0.0	128.6	276.0	80.9	44.8

* Concentrations are presented as mean ± SEM. CINC-2αβ concentrations was also measured; however, this chemokine was below the level of quantification in every sample.

[1] $n = 1$

[2] $n = 2$

[3] $n = 3$

Table 3-9. Histological analysis of IEL counts in small bowel sections collected from the preliminary trial.

Small Bowel Section	Phenotype	IEL count*	2-tailed *P* value
Duodenum	DR	2.53 ± 1.56	0.449
	DIO	4.49 ± 1.90	
Jejunum**	DR	0.00	*n/a*
	DIO	0.00	

* IELs were enumerated per 100 villous enterocytes for 5 villi per section in the small bowel. Counts are presented as mean ± SEM, $n = 5$. Statistical analyses were performed using unpaired *t*-tests.
** There were no visible IELs in the jejunum of any DR or DIO rat examined.

Table 3-10. Ileal cytokine concentrations measured in rats from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
IL-1β ng/mL	DR	11.7 ± 1.9	0.327
	DIO	14.8 ± 2.4	
IL-6 pg/mL	DR[1]	1199.7 ± 396.0	0.093
	DIO[2]	498.1 ± 97.4	
IL-22 pg/mL	DR[3]	88.5 ± 29.7	0.582
	DIO[3]	134.2 ± 70.3	
TNF-α pg/mL	DR	17.9 ± 2.0	0.429
	DIO	26.6 ± 10.6	
Active TGF-β1 pg/mL	DR[2]	635.9 ± 65.7	0.942
	DIO[4]	647.6 ± 134.2	
Total TGF-β1 pg/mL	DR[4]	6324.9 ± 735.4	0.051
	DIO[4]	4339.9 ± 543.2	

* Concentrations are presented as mean ± SEM, $n = 10$ (unless otherwise specified). Statistical analyses were performed using unpaired *t*-tests.

[1] $n = 5$ for IL-6 in DR rats.

[2] $n = 6$ for IL-6 in DIO rats and active TGF-β in DR rats.

[3] $n = 3$ for IL-22 in DR and DIO rats.

[4] $n = 7$ for active TGF-β in DIO rats, and total TGF-β in both DR and DIO rats.

Table 3-11. Caecal cytokine/chemokine concentrations measured in rats from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
CINC-1 pg/mL	DR	292.3 ± 12.8	0.308
	DIO	264.8 ± 13.3	
CINC-2αβ pg/mL	DR	426.1 ± 56.6	0.261
	DIO	335.6 ± 53.7	
IL-6 pg/mL	DR	2182.6 ± 84.4	0.192
	DIO	1972.0 ± 130.4	
IL-10 pg/mL	DR	1048.2 ± 73.2	0.679
	DIO	1097.0 ± 90.0	
IL-22 pg/mL	DR[1]	126.0 ± 29.7	0.482
	DIO[1]	160.2 ± 32.6	
TNF-α pg/mL	DR	63.8 ± 7.7	0.500
	DIO	92.8 ± 41.4	
Active TGF-β1 pg/mL	DR	1092.2 ± 21.4	0.183
	DIO	1176.9 ± 57.2	
Total TGF-β1 pg/mL	DR	2986.3 ± 148.5	0.061
	DIO	3445.3 ± 175.4	

* Concentrations are presented as mean ± SEM, $n = 10$ (unless otherwise specified). Statistical analyses were performed using unpaired *t*-tests.

[1] $n = 3$ for IL-22 in DR and DIO rats.

Table 3-12. Proximal colon cytokine concentrations measured in rats from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
IL-6 pg/mL	DR	4736.4 ± 299.8	0.230
	DIO	5350.6 ± 393.2	
IL-10 pg/mL	DR	4155.1 ± 136.6	0.032
	DIO	4644.6 ± 160.2	
IL-17F pg/mL	DR	505.8 ± 38.6	0.070
	DIO	609.0 ± 37.0	
TNF-α pg/mL	DR	1481.0 ± 85.3	0.016
	DIO	1827.2 ± 97.7	
Active TGF-β1 pg/mL	DR	527.7 ± 59.8	0.280
	DIO	597.1 ± 17.3	
Total TGF-β1 pg/mL	DR	1494.9 ± 137.7	0.096
	DIO	1799.0 ± 104.8	

* Concentrations are presented as mean ± SEM, $n = 10$. Statistical analyses were performed using unpaired *t*-tests.

Table 3-13. Distal colon cytokine concentrations measured in rats from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
IL-6 pg/mL	DR	3920.9 ± 358.4	0.152
	DIO	4503.5 ± 152.1	
IL-10 pg/mL	DR	2638.9 ± 142.1	0.368
	DIO	2817.9 ± 131.5	
IL-17F pg/mL	DR	395.1 ± 34.3	0.061
	DIO	501.3 ± 40.4	
IL-22 pg/mL	DR[1]	67.4 ± 13.9	0.059
	DIO[1]	132.9 ± 20.8	
TNF-α pg/mL	DR	1026.1 ± 56.1	0.110
	DIO	1148.2 ± 46.3	
Active TGF-β1 pg/mL	DR	591.4 ± 34.4	0.107
	DIO	675.2 ± 35.5	
Total TGF-β1 pg/mL	DR	1522.0 ± 129.8	0.072
	DIO	1914.7 ± 158.7	

* Concentrations are presented as mean ± SEM, $n = 10$ (unless otherwise specified). Statistical analyses were performed using unpaired *t*-tests.

[1] $n = 3$ for IL-22 in both DR and DIO rats.

Table 3-14. Liver cytokine/chemokine concentrations measured in rats from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
CINC-1 pg/mL	DR	2679.4 ± 164.8	0.017
	DIO	3387.9 ± 211.3	
CINC-2αβ ng/mL	DR	12.9 ± 1.3	0.815
	DIO	13.3 ± 0.8	
CINC-3 pg/mL	DR	3845.7 ± 491.9	0.149
	DIO	4809.8 ± 409.3	
IL-1β ng/mL	DR	64.8 ± 6.2	0.098
	DIO	82.7 ± 8.1	
IL-4 pg/mL	DR	7283.7 ± 626.8	0.339
	DIO	8070.9 ± 500.0	
IL-6 ng/mL	DR	61.8 ± 4.8	0.109
	DIO	74.6 ± 5.9	
IL-10 ng/mL	DR	30.4 ± 2.4	0.185
	DIO	35.3 ± 2.6	
TNF-α ng/mL	DR	23.2 ± 2.1	0.163
	DIO	27.7 ± 2.3	
Active TGF-β1 pg/mL	DR	4811.0 ± 298.7	0.902
	DIO	4864.4 ± 311.1	
Total TGF-β1 pg/mL	DR	4663.0 ± 321.5	0.696
	DIO	4830.1 ± 272.0	

* Concentrations are presented as mean ± SEM, $n = 10$. Statistical analyses were performed using unpaired *t*-tests.

Table 3-15. Spleen cytokine/chemokine concentrations measured in rats from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
CINC-1 pg/mL	DR	1572.2 ± 57.2	0.972
	DIO	1575.6 ± 77.8	
CINC-2αβ pg/mL	DR	1722.2 ± 83.8	0.769
	DIO	1755.0 ± 71.5	
CINC-3 pg/mL	DR	239.7 ± 25.8	0.391
	DIO	263.9 ± 9.3	
IL-1β ng/mL	DR	81.6 ± 6.0	0.295
	DIO	90.9 ± 6.1	
IL-6 pg/mL	DR	170.4 ± 53.8	0.244
	DIO	273.6 ± 66.5	
IL-10 pg/mL	DR	53.3 ± 0.0	0.331
	DIO	55.6 ± 2.2	
TNF-α pg/mL	DR	141.5 ± 28.6	0.049
	DIO	215.0 ± 19.8	

* Concentrations are presented as mean ± SEM, $n = 10$. Statistical analyses were performed using unpaired *t*-tests.

Table 3-16. MLN cytokine concentrations measured in rats from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
IL-1β ng/mL	DR	51.8 ± 15.9	0.705
	DIO	44.9 ± 8.4	
IL-6 pg/mL	DR	1662.0 ± 480.2	0.260
	DIO	1089.7 ± 105.1	
IL-10 pg/mL	DR	505.7 ± 155.8	0.738
	DIO	568.6 ± 99.5	
IL-22 pg/mL	DR[1]	168.7 ± 86.3	0.835
	DIO[1]	143.9 ± 70.3	
sICAM-1/sCD54 ng/mL	DR	73.1 ± 14.8	0.018
	DIO	32.5 ± 5.1	

* Concentrations are presented as mean ± SEM, $n = 10$ (unless otherwise specified). Statistical analyses were performed using unpaired *t*-tests.

[1] $n = 3$ for IL-22 in both DR and DIO rats.

Table 3-17. Ileal PP cytokine/chemokine concentrations measured in rats from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
CINC-3** pg/mL	DR	BLQ	*n/a*
	DIO	BLQ	
IFN-γ pg/mL	DR	224.0 ± 58.5	0.601
	DIO	187.2 ± 37.0	
IL-4 pg/mL	DR	226.4 ± 27.1	0.940
	DIO	223.2 ± 31.2	
IL-6 pg/mL	DR	674.8 ± 206.8	0.957
	DIO	704.0 ± 488.2	
IL-10 pg/mL	DR	721.7 ± 77.4	0.480
	DIO	814.1 ± 101.9	
IL-17F pg/mL	DR	501.5 ± 41.0	0.952
	DIO	504.4 ± 23.8	
TNF-α pg/mL	DR	364.8 ± 63.4	0.205
	DIO	514.5 ± 94.4	

* Concentrations are presented as mean ± SEM, $n = 10$. Statistical analyses were performed using unpaired *t*-tests.

** CINC-3 was below the level of quantification in all PP analyzed.

Table 3-18. Mesenteric fat cytokine/chemokine concentrations measured in rats from the preliminary trial.

Measure	Phenotype	Concentration*	2-tailed *P* value
CINC-1 pg/mL	DR	418.6 ± 70.3	0.169
	DIO	309.2 ± 30.0	
CINC-2αβ pg/mL	DR	699.6 ± 106.9	0.053
	DIO	459.6 ± 45.1	
IL-6 pg/mL	DR	5869.2 ± 1131.5	0.111
	DIO	3497.4 ± 849.0	
IL-10 pg/mL	DR	5542.4 ± 1157.4	0.154
	DIO	3491.5 ± 748.1	
TNF-α pg/mL	DR	1615.1 ± 369.9	0.092
	DIO	896.0 ± 161.6	

* Concentrations are presented as mean ± SEM, $n = 10$. Statistical analyses were performed using unpaired *t*-tests.

Table 3-19. Epididymal fat cytokine/chemokine concentrations measured in rats from the preliminary trial.

Measures	Phenotype	Concentrations*	2-tailed *P* value
CINC-1 pg/mL	DR	1280.7 ± 121.2	0.944
	DIO	1296.3 ± 184.6	
CINC-2αβ pg/mL	DR	3861.4 ± 352.1	0.690
	DIO	4089.7 ± 439.8	
CINC-3 pg/mL	DR	586.9 ± 139.3	0.712
	DIO	665.8 ± 157.4	
IL-6 ng/mL	DR	13.1 ± 1.4	0.712
	DIO	13.8 ± 1.3	
IL-10 ng/mL	DR	13.3 ± 1.8	0.566
	DIO	11.9 ± 1.6	
TNF-α pg/mL	DR	4591.2 ± 533.4	0.562
	DIO	5095.9 ± 651.4	

* Concentrations are presented as mean ± SEM, $n = 10$. Statistical analyses were performed using unpaired *t*-tests.

Table 3-20a. Hematological measures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
WBC 10^9/L	DR	4.4 ± 0.6	4.0 ± 0.2	4.8 ± 0.4	4.5 ± 0.3$^{\alpha}$	4.4 ± 0.4	5.1 ± 0.6	0.008	0.233	0.368
	DIO	4.4 ± 0.3	4.9 ± 0.3	4.9 ± 0.3	5.9 ± 0.4$^{\beta}$	5.6 ± 0.6	4.9 ± 0.4			
RBC 10^9/L	DR	8.23 ± 0.10$^{\alpha}$	8.25 ± 0.12	8.41 ± 0.09	8.26 ± 0.09	8.30 ± 0.11	8.58 ± 0.13$^{\beta}$	0.316	0.017	0.737
	DIO	8.05 ± 0.13$^{\alpha}$	8.20 ± 0.17	8.37 ± 0.12	8.40 ± 0.13	8.06 ± 0.14	8.51 ± 0.15$^{\beta}$			
Hgb g/L	DR	146 ± 1.85	146 ± 1.94	149 ± 2.62	146 ± 1.47	148 ± 2.22	150 ± 1.83	0.708	0.638	0.586
	DIO	146 ± 2.50	148 ± 2.04	149 ± 1.67	151 ± 2.65	147 ± 2.21	147 ± 1.44			
Hct L/L	DR	0.447 ± 0.006	0.447 ± 0.007	0.456 ± 0.008	0.439 ± 0.011$^{\alpha}$	0.451 ± 0.007	0.457 ± 0.006	0.605	0.739	0.400
	DIO	0.448 ± 0.007	0.451 ± 0.006	0.456 ± 0.005	0.459 ± 0.008$^{\beta}$	0.444 ± 0.007	0.452 ± 0.004			
MCV fL	DR	54 ± 0.83	54 ± 1.08	54 ± 0.79	54 ± 0.53	54 ± 0.54	53 ± 0.64	0.168	0.358	0.938
	DIO	56 ± 0.31	55 ± 0.86	54 ± 0.72	55 ± 1.16	56 ± 0.75	53 ± 1.10			
MCH pg	DR	17.8 ± 0.28	17.8 ± 0.34	17.7 ± 0.25	17.7 ± 0.19	17.8 ± 0.22	17.4 ± 0.22	0.142	0.275	0.982
	DIO	18.1 ± 0.18	18.1 ± 0.28	17.9 ± 0.29	18.0 ± 0.35	18.2 ± 0.30	17.4 ± 0.35			
MCHC g/L	DR	326.5 ± 1.06	327.3 ± 1.02	326.9 ± 0.75	326.4 ± 1.01	328.2 ± 1.59	327.1 ± 1.25	0.784	0.491	0.844
	DIO	325.2 ± 1.92	328.7 ± 1.10	327.2 ± 1.33	328.1 ± 1.48	328.4 ± 1.82	326.1 ± 1.35			

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Table 3-20a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
RDW %	DR	11.4 ± 0.24	11.4 ± 0.24	11.6 ± 0.32	11.8 ± 0.22	11.3 ± 0.11	11.7 ± 0.19	0.091	0.456	0.652
	DIO	10.8 ± 0.09	11.4 ± 0.23	11.7 ± 0.16	11.4 ± 0.34	11.3 ± 0.23	10.9 ± 0.73			
PLT 10^9/L	DR	672 ± 29.12	720 ± 56.34	668 ± 20.08	691 ± 26.71	603 ± 50.74	644 ± 31.20	0.758	0.138	0.681
	DIO	631 ± 26.96	704 ± 44.38	675 ± 19.96	681 ± 33.50	624 ± 40.61	723 ± 37.53			
MPV fL	DR	6.2 ± 0.10	6.6 ± 0.21	6.2 ± 0.09	6.1 ± 0.08	6.3 ± 0.08	6.4 ± 0.07	0.924	0.127	0.469
	DIO	6.4 ± 0.09	6.3 ± 0.14	6.2 ± 0.09	6.3 ± 0.11	6.1 ± 0.10	6.4 ± 0.15			
NE %	DR	5.3 ± 0.58$^{\alpha}$	6.7 ± 0.52	5.8 ± 0.55	7.1 ± 0.58	7.2 ± 0.91	6.2 ± 0.14	0.327	0.413	0.306
	DIO	7.1 ± 0.69$^{\beta}$	7.1 ± 0.80	6.5 ± 0.72	6.9 ± 0.30	5.8 ± 0.40	7.1 ± 0.93			
LY %	DR	91.0 ± 0.86	88.5 ± 0.56	89.7 ± 0.71	87.3 ± 0.99	88.5 ± 1.23	89.0 ± 0.29	0.067	0.195	0.423
	DIO	88.3 ± 1.01	87.5 ± 1.16	87.8 ± 1.46	87.4 ± 0.51	89.4 ± 0.63	87.7 ± 0.98			
MO %	DR	3.0 ± 0.29$^{a\alpha}$	3.9 ± 0.34$^{ab\beta}$	3.8 ± 0.25$^{ab\beta}$	4.3 ± 0.20$^{b\beta}$	3.6 ± 0.30^{ab}	4.0 ± 0.18$^{ab\beta}$	0.022	<0.001	0.995
	DIO	3.5 ± 0.50$^{a\alpha}$	4.7 ± 0.51$^{ab\beta}$	4.7 ± 0.54$^{ab\beta}$	5.0 ± 0.44$^{b\beta}$	4.1 ± 0.31^{ab}	4.4 ± 0.37$^{ab\beta}$			

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Table 3-20a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
EO	DR	0.4 ± 0.06	0.5 ± 0.08	0.3 ± 0.05	1.0 ± 0.55	0.4 ± 0.10	0.4 ± 0.05	0.981	0.831	0.331
%	DIO	0.7 ± 0.21	0.4 ± 0.09	0.7 ± 0.43	0.4 ± 0.04	0.4 ± 0.09	0.5 ± 0.13			
BA	DR	0.3 ± 0.05	0.3 ± 0.04	0.3 ± 0.03	0.3 ± 0.05	0.3 ± 0.03	0.4 ± 0.01	0.644	0.185	0.429
%	DIO	0.4 ± 0.05	0.3 ± 0.05	0.4 ± 0.05	0.4 ± 0.03	0.4 ± 0.04	0.4 ± 0.03			

* WBC, White blood cell count; RBC, Red blood cell count; Hgb, Hemoglobin; Hct, Hematocrit; MCV, Mean corpuscular volume, MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration, RDW, Red cell distribution width; PLT, platelet count; MPV, mean platelet volume; NE, neutrophil count, LY, lymphocyte count; MO, monocyte count; EO, eosinophil count; BA, basophil count. Concentrations or percentages are presented as mean ± SEM, $n = 10$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significance differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-20b. Hematological measures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure	Phenotype	Value*
WBC 10^9/L	DR	4.5 ± 0.2
	DIO	5.1 ± 0.2
RBC 10^9/L	DR	8.34 ± 0.05
	DIO	8.26 ± 0.06
Hgb g/L	DR	147 ± 0.81
	DIO	148 ± 0.86
Hct L/L	DR	0.449 ± 0.003
	DIO	0.452 ± 0.003
MCV fL	DR	54 ± 0.30
	DIO	55 ± 0.35
MCH pg	DR	17.7 ± 0.10
	DIO	17.9 ± 0.12
MCHC g/L	DR	327.1 ± 0.45
	DIO	327.3 ± 0.62
RDW %	DR	11.5 ± 0.09
	DIO	11.2 ± 0.15
PLT 10^9/L	DR	666 ± 15.73
	DIO	674 ± 14.47
MPV fL	DR	6.3 ± 0.05
	DIO	6.3 ± 0.05
NE %	DR	6.4 ± 0.25
	DIO	6.7 ± 0.27
LY %	DR	89.0 ± 0.36
	DIO	88.0 ± 0.41
MO %	DR	3.8 ± 0.12
	DIO	4.4 ± 0.19
EO %	DR	0.5 ± 0.10
	DIO	0.5 ± 0.08
BA %	DR	0.3 ± 0.02
	DIO	0.4 ± 0.02

* Concentrations or percentages are presented as mean ± SEM, n = 60.

Table 3-21a. Total immune cell population percentages within the spleen of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Cell Marker*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CD3$^+$	DR[1]	5.23 ± 1.12$^{\alpha}$	5.71 ± 1.39$^{\alpha\beta}$	5.68 ± 0.42$^{\alpha\beta}$	5.90 ± 0.43$^{\alpha\beta}$	6.23 ± 1.29$^{\alpha\beta}$	7.18 ± 1.00$^{\beta}$	0.435	0.019	0.488
	DIO[1]	6.26 ± 1.88$^{a\alpha}$	7.00 ± 1.25$^{ab\alpha\beta}$	6.48 ± 0.81$^{ab\alpha\beta}$	3.60 ± 0.52$^{ab\alpha\beta}$	6.14 ± 1.17$^{b\alpha\beta}$	4.70 ± 1.32$^{ab\beta}$			
CD3$^+$ CD62L$^+$	DR[1]	0.63 ± 0.59$^{a\alpha\gamma\delta}$	1.02 ± 0.59$^{ab\alpha\gamma\delta}$	1.65 ± 0.55$^{ab\alpha\gamma\delta}$	1.87 ± 0.66$^{ab\beta\gamma\delta}$	1.69 ± 0.70$^{b\beta\delta}$	1.37 ± 0.61$^{b\beta}$	0.632	<0.001	0.729
	DIO[1]	1.64 ± 1.17$^{a\alpha\gamma\delta}$	1.21 ± 0.69$^{a\alpha\gamma\delta}$	1.55 ± 0.63$^{abc\alpha\gamma\delta}$	0.82 ± 0.34$^{abc\beta\gamma\delta}$	1.94 ± 0.86$^{bc\beta\delta}$	1.01 ± 0.77$^{bc\beta}$			
CD3$^+$ CD4$^+$	DR[1]	2.76 ± 0.47	3.14 ± 0.82	3.23 ± 0.31	3.23 ± 0.21	3.77 ± 0.78	4.27 ± 0.58	0.339	0.063	0.444
	DIO[1]	3.26 ± 0.96	3.96 ± 0.88	3.55 ± 0.53	1.92 ± 0.28	3.52 ± 0.55	2.88 ± 0.71			
CD3$^+$ CD8a$^+$	DR[1]	2.13 ± 0.63$^{\alpha}$	2.27 ± 0.53$^{\alpha\beta}$	2.13 ± 0.23$^{\alpha\beta}$	2.26 ± 0.38$^{\alpha\beta}$	2.18 ± 0.55$^{\beta}$	2.50 ± 0.41$^{\beta}$	0.779	0.005	0.656
	DIO[1]	2.63 ± 0.85$^{a\alpha}$	2.66 ± 0.38$^{ab\alpha\beta}$	2.62 ± 0.34$^{ab\alpha\beta}$	1.51 ± 0.26$^{ab\alpha\beta}$	2.28 ± 0.71$^{ab\beta}$	1.59 ± 0.55$^{b\beta}$			
CD3$^+$ CD45$^+$	DR[1]	11.97 ± 1.92$^{\alpha}$	11.74 ± 2.71$^{\alpha\beta}$	12.76 ± 1.80$^{\alpha\beta}$	12.63 ± 0.80$^{\alpha\beta}$	13.08 ± 2.49$^{\beta}$	15.88 ± 1.79$^{\beta}$	0.371	<0.001	0.437
	DIO[1]	14.07 ± 3.49$^{a\alpha}$	15.80 ± 1.89$^{ab\alpha\beta}$	13.25 ± 1.53$^{ab\alpha\beta}$	8.18 ± 1.22$^{ab\alpha\beta}$	13.00 ± 2.15$^{b\beta}$	9.97 ± 3.22$^{b\beta}$			

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Table 3-21a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Cell Marker*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CD3⁻ CD45RA⁺	DR[1]	3.19 ± 0.28$^{\alpha}$	2.28 ± 0.70$^{\alpha\beta}$	3.09 ± 0.60$^{\alpha\beta}$	3.46 ± 0.21$^{\alpha\beta}$	2.98 ± 0.41$^{\alpha\beta}$	3.39 ± 0.32$^{\beta}$	0.851	0.015	0.645
	DIO[1]	4.08 ± 0.92$^{\alpha}$	4.16 ± 0.47$^{\alpha\beta}$	2.99 ± 0.25$^{\alpha\beta}$	1.98 ± 0.29$^{\alpha\beta}$	2.93 ± 0.50$^{\alpha\beta}$	3.09 ± 1.01$^{\beta}$			
CD3⁻ CD161a⁺	DR[1]	0.76 ± 0.04$^{\alpha\gamma}$	0.80 ± 0.14$^{\alpha\beta\gamma}$	0.61 ± 0.05$^{\alpha\beta\gamma}$	0.66 ± 0.05$^{\alpha\gamma}$	0.80 ± 0.12$^{\alpha\beta\gamma}$	1.12 ± 0.16$^{\beta}$	0.350	0.006	0.218
	DIO[1]	0.87 ± 0.27$^{a\alpha\gamma}$	1.06 ± 0.23$^{ab\alpha\beta\gamma}$	0.74 ± 0.11$^{ab\alpha\beta\gamma}$	0.53 ± 0.13$^{a\alpha\gamma}$	0.84 ± 0.08$^{ab\alpha\beta\gamma}$	0.47 ± 0.16$^{b\beta}$			
CD3⁻CD4⁻ CD161a⁺	DR[1]	0.66 ± 0.04	0.69 ± 0.12	0.48 ± 0.06	0.57 ± 0.07	0.65 ± 0.09	0.94 ± 0.15	0.254	0.045	0.163
	DIO[1]	0.74 ± 0.21^{a}	0.86 ± 0.17^{ab}	0.59 ± 0.06^{ab}	0.46 ± 0.12^{ab}	0.69 ± 0.06^{ab}	0.39 ± 0.12^{b}			
CD3⁻ CD103⁺	DR	0.14 ± 0.02	0.10 ± 0.02	0.11 ± 0.02	0.10 ± 0.01	0.17 ± 0.04	0.12 ± 0.03	0.159	0.548	0.583
	DIO	0.10 ± 0.02	0.14 ± 0.04	0.10 ± 0.02	0.08 ± 0.02	0.12 ± 0.04	0.09 ± 0.02			
CD3⁻ His36⁺	DR	0.35 ± 0.09	0.35 ± 0.11	0.34 ± 0.06	0.27 ± 0.03	0.49 ± 0.10	0.52 ± 0.09$^{\alpha}$	0.973	0.687	0.089
	DIO	0.31 ± 0.06	0.66 ± 0.22	0.55 ± 0.26	0.44 ± 0.19	0.40 ± 0.08	0.20 ± 0.10$^{\beta}$			
CD3⁺ γδTCR⁺	DR	0.24 ± 0.04	0.17 ± 0.05	0.19 ± 0.03	0.17 ± 0.02	0.28 ± 0.06	0.20 ± 0.04	0.911	0.083	0.300
	DIO	0.20 ± 0.04	0.21 ± 0.04	0.27 ± 0.04	0.18 ± 0.03	0.25 ± 0.03	0.13 ± 0.03			

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Table 3-21a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Cell Marker*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
$CD3^+$ $CD4^+$ $Foxp3^+$ $CD25^{+}$ **	DR	1.89 ± 0.31	1.97 ± 0.22	2.02 ± 0.21	1.88 ± 0.14	2.31 ± 0.33	2.02 ± 0.16	0.796	0.367	0.863
	DIO	1.72 ± 0.28	1.71 ± 0.24	2.21 ± 0.36	1.82 ± 0.18	2.12 ± 0.26	2.28 ± 0.19			

* Data are shown as the mean percentage of viable cells ± SEM, $n = 5$ (unless otherwise specified). Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

** $Foxp3^+$ ISO control has been removed from total $CD4^+Foxp3^+CD25^+$ T_{reg} cell percentage.

[1] For antibody panel 1, $n = 4$ for DR rats on control, HE control, HE wheat bran and HE oat bran only diets, and $n = 4$ for DIO rats on control and HE control diets.

Table 3-21b. Total immune cell population percentages within the spleen of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Cell Marker*	Phenotype	Cell Percentage
CD3$^+$	DR[1]	6.04 ± 0.40
	DIO[1]	5.63 ± 0.49
CD3$^+$ **CD62L$^+$**	DR[1]	1.38 ± 0.24
	DIO[1]	1.36 ± 0.29
CD3$^+$ **CD4$^+$**	DR[1]	3.45 ± 0.24
	DIO[1]	3.15 ± 0.27
CD3$^+$ **CD8a$^+$**	DR[1]	2.25 ± 0.18
	DIO[1]	2.19 ± 0.22
CD3$^+$ **CD45$^+$**	DR[1]	13.12 ± 0.80
	DIO[1]	12.22 ± 0.99
CD3$^-$ **CD45RA$^+$**	DR[1]	3.07 ± 0.18
	DIO[1]	3.14 ± 0.27
CD3$^-$ **CD161a$^+$**	DR[1]	0.80 ± 0.05
	DIO[1]	0.74 ± 0.07
CD3$^-$CD4$^-$ **CD161a$^+$**	DR[1]	0.68 ± 0.05
	DIO[1]	0.61 ± 0.06
CD3$^-$ **CD103$^+$**	DR	0.12 ± 0.01
	DIO	0.11 ± 0.01
CD3$^-$ **His36$^+$**	DR	0.39 ± 0.04
	DIO	0.43 ± 0.07
CD3$^+$ **γδTCR$^+$**	DR	0.21 ± 0.02
	DIO	0.21 ± 0.01
CD3$^+$CD4$^+$Foxp3$^+$ **CD25$^+$**	DR	2.01 ± 0.09
	DIO	1.98 ± 0.11

* Percentages are presented as mean ± SEM, *n* = 30 (unless otherwise specified)

[1] *n* = 26 for DR rats and *n* = 28 for DIO rats in antibody panel 1.

Table 3-22a. Total immune cell population percentages within the MLN of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Cell Marker*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CD3$^+$	DR[1]	67.55 ± 1.56	72.65 ± 2.70	69.25 ± 2.10	67.48 ± 4.32	64.30 ± 4.11	64.84 ± 3.38	0.196	0.650	0.320
	DIO[1]	59.15 ± 3.09	64.63 ± 4.64	67.52 ± 3.80	60.84 ± 5.40	70.82 ± 3.72	65.60 ± 3.78			
CD3$^+$ CD62L$^+$	DR[1]	3.23 ± 2.86	10.94 ± 6.39	17.59 ± 6.48	18.87 ± 6.81	13.70 ± 5.65	11.77 ± 4.62	0.973	0.555	0.950
	DIO[1]	9.41 ± 5.40	10.82 ± 6.17	14.99 ± 6.04	13.27 ± 5.43	16.20 ± 6.76	10.70 ± 6.90			
CD3$^+$ CD4$^+$	DR[1]	40.65 ± 2.01$^{\alpha}$	44.05 ± 2.13	42.20 ± 2.18	36.60 ± 4.45	39.58 ± 5.13	40.62 ± 2.08	0.135	0.327	0.445
	DIO[1]	29.30 ± 4.45$^{\beta}$	38.08 ± 4.20	39.38 ± 3.56	35.18 ± 3.99	43.20 ± 3.93	39.52 ± 2.07			
CD3$^+$ CD8a$^+$	DR[1]	25.05 ± 1.01	27.13 ± 1.95	25.30 ± 2.80	29.18 ± 1.41	22.90 ± 2.48	22.46 ± 1.81	0.982	0.811	0.526
	DIO[1]	26.58 ± 0.73	24.78 ± 1.63	26.54 ± 2.63	23.84 ± 1.95	25.92 ± 3.91	24.86 ± 3.39			
CD3$^+$ CD45$^+$	DR[1]	83.85 ± 2.64	86.18 ± 2.58	86.65 ± 1.57	88.70 ± 2.43	81.50 ± 3.22	81.66 ± 2.49	0.206	0.345	0.475
	DIO[1]	76.35 ± 2.72	84.80 ± 3.74	85.18 ± 2.34	81.90 ± 3.51	85.54 ± 4.62	80.66 ± 3.49			

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Table 3-22a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Cell Marker*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CD3⁻ CD45RA⁺	DR[1]	12.43 ± 1.47$^{\gamma\delta}$	10.26 ± 1.28$^{\alpha\gamma\delta}$	14.75 ± 1.88$^{\gamma\delta}$	17.25 ± 2.46$^{\gamma}$	13.80 ± 2.09$^{\gamma\delta}$	12.64 ± 1.75$^{\delta}$	0.727	0.045	0.254
	DIO[1]	13.48 ± 1.40$^{\gamma\delta}$	16.05 ± 2.51$^{\beta\gamma\delta}$	13.58 ± 1.12$^{\gamma\delta}$	17.28 ± 1.42$^{\gamma}$	11.57 ± 1.67$^{\gamma\delta}$	11.27 ± 0.91$^{\delta}$			
CD3⁻ CD161a⁺	DR[1]	0.58 ± 0.31	0.34 ± 0.08	0.40 ± 0.13	0.54 ± 0.05	0.60 ± 0.17	0.89 ± 0.29	0.893	0.851	0.320
	DIO[1]	0.45 ± 0.13	1.01 ± 0.46	0.49 ± 0.16	0.52 ± 0.14	0.68 ± 0.21	0.31 ± 0.06			
CD3⁻CD4⁻ CD161a⁺	DR[1]	0.24 ± 0.07	0.17 ± 0.02	0.21 ± 0.07	0.27 ± 0.07	0.20 ± 0.02	0.32 ± 0.13	0.845	0.895	0.759
	DIO[1]	0.19 ± 0.06	0.30 ± 0.09	0.16 ± 0.03	0.21 ± 0.05	0.32 ± 0.11	0.18 ± 0.04			
CD3⁻ CD103⁺	DR	0.22 ± 0.02	0.20 ± 0.02	0.15 ± 0.01$^{\alpha}$	0.25 ± 0.03	0.31 ± 0.06	0.28 ± 0.05$^{\gamma}$	0.708	0.580	0.002
	DIO	0.22 ± 0.04^{ab}	0.25 ± 0.05^{ab}	0.34 ± 0.05$^{a\beta}$	0.31 ± 0.05^{ab}	0.20 ± 0.04^{ab}	0.15 ± 0.03$^{b\delta}$			
CD3⁻ His36⁺	DR	0.08 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.12 ± 0.03$^{\alpha}$	0.09 ± 0.03	0.178	0.514	0.137
	DIO	0.04 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.10 ± 0.02	0.05 ± 0.01$^{\beta}$	0.07 ± 0.01			
CD3⁺ γδTCR⁺	DR	0.64 ± 0.14	0.70 ± 0.08$^{\alpha}$	0.56 ± 0.07	0.71 ± 0.09	0.67 ± 0.10	0.48 ± 0.02	0.360	0.243	0.127
	DIO	0.55 ± 0.06^{ab}	0.41 ± 0.03$^{a\beta}$	0.66 ± 0.10^{ab}	0.56 ± 0.06^{ab}	0.76 ± 0.09^{b}	0.56 ± 0.06^{ab}			

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Table 3-22a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Cell Marker*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CD3$^+$ CD4$^+$ Foxp3$^+$ CD25^{+**}	DR	2.60 ± 0.25$^{ab\alpha\beta}$	2.55 ± 0.30$^{ab\alpha\beta}$	2.37 ± 0.30$^{ab\alpha\beta}$	2.20 ± 0.41$^{a\alpha}$	3.32 ± 0.35$^{b\beta}$	2.59 ± 0.10$^{ab\alpha\beta}$	0.133	0.043	0.700
	DIO	2.42 ± 0.27$^{\alpha\beta}$	2.11 ± 0.11$^{\alpha\beta}$	2.45 ± 0.21$^{\alpha\beta}$	2.15 ± 0.08$^{\alpha}$	2.67 ± 0.24$^{\beta}$	2.51 ± 0.12$^{\alpha\beta}$			

* Data are shown as the mean percentage of viable cells ± SEM, $n = 5$ (unless otherwise specified). Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

** $Foxp3^+$ ISO control has been removed from total $CD4^+Foxp3^+CD25^+$ T_{reg} cell percentage.

[1] For antibody panel 1, $n = 4$ for DR rats on control, HE control, HE wheat bran and HE oat bran diets, and $n = 4$ for DIO rats on control and HE control diets.

Table 3-22b. Total immune cell population percentages within the MLN of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Cell Marker*	Phenotype	Cell Percentage
CD3$^+$	DR[1]	67.44 ± 1.34
	DIO[1]	64.96 ± 1.72
CD3$^+$ CD62L$^+$	DR[1]	12.69 ± 2.27
	DIO[1]	12.74 ± 2.34
CD3$^+$ CD4$^+$	DR[1]	40.58 ± 1.32
	DIO[1]	37.71 ± 1.59
CD3$^+$ CD8a$^+$	DR[1]	25.13 ± 0.88
	DIO[1]	25.35 ± 1.04
CD3$^+$ CD45$^+$	DR[1]	84.51 ± 1.10
	DIO[1]	82.54 ± 1.43
CD3$^-$ CD45RA$^+$	DR[1]	13.50 ± 0.81
	DIO[1]	13.81 ± 0.70
CD3$^-$ CD161a$^+$	DR[1]	0.57 ± 0.08
	DIO[1]	0.57 ± 0.09
CD3$^-$CD4$^-$ CD161a$^+$	DR[1]	0.24 ± 0.03
	DIO[1]	0.23 ± 0.03
CD3$^-$ CD103$^+$	DR	0.24 ± 0.02
	DIO	0.25 ± 0.02
CD3$^-$ His36$^+$	DR	0.08 ± 0.01
	DIO	0.07 ± 0.01
CD3$^+$ γδTCR$^+$	DR	0.63 ± 0.04
	DIO	0.58 ± 0.03
CD3$^+$CD4$^+$Foxp3$^+$ CD25$^+$	DR	2.60 ± 0.13
	DIO	2.39 ± 0.08

* Percentages are presented as mean ± SEM, n = 30 (unless otherwise specified).

[1] n = 26 for panel 1 DR rats and n = 28 for DIO rats in antibody panel 1.

Table 3-23a. Total immune cell population percentages within the ileal PP of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Cell Marker*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CD3$^+$	DR[1]	7.95 ± 2.75$^{\alpha}$	4.09 ± 2.26	8.17 ± 5.11	4.27 ± 1.87	1.82 ± 0.36	2.36 ± 1.10	0.587	0.074	0.117
	DIO[1]	1.75 ± 0.61$^{\beta}$	5.83 ± 1.09	4.04 ± 0.33	4.28 ± 1.29	4.32 ± 1.42	1.65 ± 0.46			
CD3$^+$ CD62L$^+$	DR[1]	0.50 ± 0.31	0.40 ± 0.20	1.67 ± 1.22	0.71 ± 0.54	0.29 ± 0.14	0.53 ± 0.23	0.760	0.624	0.784
	DIO[1]	0.36 ± 0.22	0.46 ± 0.28	0.68 ± 0.29	0.72 ± 0.36	0.69 ± 0.28	0.25 ± 0.14			
CD3$^+$ CD4$^+$	DR[1]	5.19 ± 2.22$^{\alpha}$	2.70 ± 1.66	4.84 ± 2.96	2.63 ± 1.24	1.14 ± 0.19	1.53 ± 0.75	0.601	0.118	0.154
	DIO[1]	1.01 ± 0.39$^{\beta}$	3.76 ± 0.90	2.68 ± 0.26	2.42 ± 0.75	2.68 ± 0.88	1.09 ± 0.34			
CD3$^+$ CD8a$^+$	DR[1]	2.17 ± 0.60$^{a\alpha}$	1.11 ± 0.51^{ab}	2.78 ± 2.09^{ab}	1.20 ± 0.44^{ab}	0.50 ± 0.15^{b}	0.56 ± 0.27^{b}	0.651	0.021	0.069
	DIO[1]	0.51 ± 0.16$^{\beta}$	1.51 ± 0.28	0.94 ± 0.13	1.27 ± 0.36	0.85 ± 0.25	0.42 ± 0.10			
CD3$^+$ CD45$^+$	DR[1]	21.88 ± 7.61$^{\alpha\beta}$	14.51 ± 6.28$^{\alpha\beta}$	15.99 ± 4.36$^{\alpha}$	19.81 ± 10.47$^{\alpha\beta}$	5.21 ± 1.33$^{\alpha\beta}$	9.20 ± 4.02$^{\beta}$	0.675	0.022	0.196
	DIO[1]	7.00 ± 1.68$^{\alpha\beta}$	23.63 ± 2.30$^{\alpha\beta}$	20.74 ± 5.24$^{\alpha}$	16.28 ± 3.87$^{\alpha\beta}$	15.75 ± 5.03$^{\alpha\beta}$	5.36 ± 2.53$^{\beta}$			

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Table 3-23a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Cell Marker*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CD3⁻ CD45RA⁺	DR[1]	12.43 ± 4.65$^{a\alpha\beta}$	8.90 ± 3.54$^{ab\alpha}$	7.22 ± 1.24$^{ab\alpha\beta}$	14.31 ± 8.30$^{a\alpha\beta}$	2.76 ± 0.95$^{b\alpha\beta}$	6.16 ± 2.82$^{ab\beta}$	0.408	0.039	0.203
	DIO[1]	4.66 ± 1.52$^{ac\alpha\beta}$	15.50 ± 1.80$^{b\alpha}$	14.72 ± 4.37$^{ab\alpha\beta}$	10.80 ± 2.44$^{ab\alpha\beta}$	10.68 ± 3.76$^{abc\alpha\beta}$	3.40 ± 1.98$^{c\beta}$			
CD3⁻ CD161a⁺	DR[1]	0.12 ± 0.07	0.08 ± 0.05$^{\alpha}$	0.09 ± 0.05	0.10 ± 0.04	0.10 ± 0.04	0.11 ± 0.06	0.381	0.249	0.104
	DIO[1]	0.09 ± 0.04^{ab}	0.29 ± 0.07$^{a\beta}$	0.08 ± 0.01^{ab}	0.07 ± 0.02^{ab}	0.22 ± 0.11^{ab}	0.03 ± 0.02^{b}			
CD3⁻CD4⁻ CD161a⁺	DR[1]	0.09 ± 0.04	0.04 ± 0.02$^{\alpha}$	0.09 ± 0.05	0.06 ± 0.01	0.06 ± 0.02$^{\alpha}$	0.06 ± 0.03	0.246	0.185	0.040
	DIO[1]	0.06 ± 0.02^{ab}	0.21 ± 0.05$^{a\beta}$	0.05 ± 0.01^{ab}	0.05 ± 0.02^{ab}	0.17 ± 0.10$^{ab\beta}$	0.02 ± 0.01^{b}			
CD3⁻ CD103⁺	DR[2]	0.11 ± 0.04	0.07 ± 0.03	0.17 ± 0.11	0.14 ± 0.05	0.04 ± 0.04$^{\alpha}$	0.08 ± 0.05	0.340	0.381	0.272
	DIO[2]	0.18 ± 0.11	0.19 ± 0.07	0.11 ± 0.02	0.09 ± 0.02	0.18 ± 0.07$^{\beta}$	0.03 ± 0.01			
CD3⁻ His36⁺	DR[2]	0.05 ± 0.02	0.04 ± 0.01	0.08 ± 0.05	0.11 ± 0.07	0.28 ± 0.18$^{\alpha}$	0.06 ± 0.02	0.806	0.810	0.207
	DIO[2]	0.08 ± 0.05	0.17 ± 0.15	0.15 ± 0.08	0.07 ± 0.06	0.03 ± 0.01$^{\beta}$	0.06 ± 0.03			
CD3⁺ γδTCR⁺	DR[2]	0.06 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	0.05 ± 0.01	0.03 ± 0.02	0.03 ± 0.01	0.501	0.482	0.714
	DIO[2]	0.06 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.07 ± 0.03	0.02 ± 0.02			

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Table 3-23a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Cell Marker*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
$CD3^+$	DR[3]	0.62 ± 0.16	0.77 ± 0.13	0.84 ± 0.20	0.53 ± 0.16	0.96 ± 0.32	0.72 ± 0.23	0.435	0.419	0.737
$CD4^+$ $Foxp3^+$ $CD25^{+**}$	DIO[3]	0.63 ± 0.15	0.52 ± 0.18	0.79 ± 0.19	0.61 ± 0.08	1.62 ± 0.73	0.87 ± 0.25			

* Data are shown as the mean percentage of viable cells ± SEM, $n = 5$ (unless otherwise specified). Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

** $Foxp3^+$ ISO control has been removed from total $CD4^+Foxp3^+CD25^+$ T_{reg} cell percentage.

[1] For antibody panel 1, $n = 4$ for DR rats on control, HE control, HE wheat bran and HE oat bran diets, and $n = 4$ for DIO rats on control and HE control diets.

[2] For antibody panel 2, $n = 4$ for DR rats on control, HE control and HE FOS diets, and $n = 3$ for DIO rats on the HE control diet.

[3] For antibody panel 3, $n = 4$ for DR and DIO rats on the HE control diet.

Table 3-23b. Total immune cell population percentages within the ileal PP of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Cell Marker*	Phenotype	Cell Percentage
CD3$^+$	DR[1]	4.57 ± 1.03
	DIO[1]	3.64 ± 0.46
CD3$^+$ CD62L$^+$	DR[1]	0.66 ± 0.21
	DIO[1]	0.54 ± 0.11
CD3$^+$ CD4$^+$	DR[1]	2.87 ± 0.67
	DIO[1]	2.26 ± 0.30
CD3$^+$ CD8a$^+$	DR[1]	1.32 ± 0.36
	DIO[1]	0.91 ± 0.11
CD3$^+$ CD45$^+$	DR[1]	13.88 ± 2.47
	DIO[1]	14.75 ± 1.91
CD3$^-$ CD45RA$^+$	DR[1]	8.31 ± 1.68
	DIO[1]	9.95 ± 1.41
CD3$^-$ CD161a$^+$	DR[1]	0.10 ± 0.02
	DIO[1]	0.13 ± 0.03
CD3$^-$CD4$^-$ CD161a$^+$	DR[1]	0.06 ± 0.01
	DIO[1]	0.09 ± 0.02
CD3$^-$ CD103$^+$	DR[2]	0.10 ± 0.02
	DIO[2]	0.13 ± 0.03
CD3$^-$ His36$^+$	DR[2]	0.11 ± 0.04
	DIO[2]	0.09 ± 0.02
CD3$^+$ γδTCR$^+$	DR[2]	0.04 ± 0.01
	DIO[2]	0.05 ± 0.01
CD3$^+$CD4$^+$Foxp3$^+$ CD25$^+$	DR[3]	0.74 ± 0.08
	DIO[3]	0.85 ± 0.15

* Percentages are presented as mean ± SEM.

[1] *n* = 26 for DR rats and *n* = 28 for DIO rats in antibody panel 1.

[2] *n* = 27 for DR rats and *n* = 28 for DIO rats in antibody panel 2.

[3] *n* = 29 for both DR and DIO rats in antibody panel 3.

Table 3-24a. CINC-1 concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		CINC-1 pg/mL[*]								
		Control	High Energy Diets					Statistics - *P* value		
TLR Agonist[**]		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
Control	DR	59.3 ± 22.2	74.3 ± 41.2	65.5 ± 35.0	22.1 ± 7.8	27.5 ± 14.5	14.8 ± 6.5$^{\alpha}$	0.852	0.873	0.061
	DIO	30.5 ± 12.6	33.1 ± 13.7	8.5 ± 4.1	87.6 ± 63.9	25.5 ± 7.2	96.2 ± 28.8$^{\beta}$			
TLR2 P3C	DR	43.2 ± 23.9	103.4 ± 53.2	32.0 ± 11.7	13.0 ± 5.0	17.1 ± 7.7	10.3 ± 5.9	0.516	0.619	0.123
	DIO	25.6 ± 13.5	30.7 ± 15.5	7.6 ± 4.6	104.1 ± 93.6	20.3 ± 9.8	114.1 ± 50.3			
TLR4 LPS	DR	70.2 ± 29.8	92.3 ± 49.4	105.8 ± 62.0$^{\alpha}$	38.0 ± 17.6	43.5 ± 28.5	33.7 ± 13.9	0.201	0.866	0.145
	DIO	59.5 ± 26.4	21.2 ± 14.9	8.3 ± 5.5$^{\beta}$	33.1 ± 10.1	32.2 ± 15.3	94.9 ± 31.1			
TLR5 Flagellin	DR	49.1 ± 20.6	43.6 ± 31.6	70.9 ± 49.1$^{\alpha}$	30.0 ± 6.3	30.6 ± 18.1	10.4 ± 8.9$^{\gamma}$	0.664	0.485	0.037
	DIO	47.7 ± 25.0	36.6 ± 15.6	7.8 ± 4.6$^{\beta}$	133.8 ± 107.5	24.1 ± 8.8	78.2 ± 31.5$^{\delta}$			

[*] CINC-1 concentrations (pg/mL) are presented as mean ± SEM. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

[**] $n = 10$ for unstimulated controls and $n = 6$-7 for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-24b. CINC-1 concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

TLR Agonist*	Phenotype	CINC-1 (pg/mL)
Control (Unstimulated)	DR	43.9 ± 10.2
	DIO	47.2 ± 12.6
TLR2 P3C	DR	36.5 ± 11.3
	DIO	49.9 ± 18.5
TLR4 LPS	DR	65.2 ± 14.9
	DIO	42.3 ± 8.9
TLR5 Flagellin	DR	38.3 ± 10.5
	DIO	52.9 ± 17.7

* In the DR phenotype, $n = 60$ for control, $n = 39$ for TLR2, $n = 39$ for TLR4 and $n = 41$ for TLR5 stimulated blood cultures. In the DIO phenotype, $n = 59$ for control and $n = 40$ for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-25a. IL-1β concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		IL-1β pg/mL*								
		Control	High Energy Diets					Statistics - *P* value		
TLR Agonist**		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
Control	DR	8.0 ± 6.3	11.4 ± 9.8	7.2 ± 5.6	28.0 ± 15.5$^{\alpha}$	5.0 ± 2.4	1.7 ± 0.0	0.285	0.628	0.149
	DIO	2.7 ± 1.0	1.7 ± 0.0	1.7 ± 0.0	3.1 ± 1.4$^{\beta}$	15.5 ± 12.2	10.2 ± 6.2			
TLR2 P3C	DR	4.5 ± 2.9^{a}	9.2 ± 7.5^{a}	12.0 ± 5.7^{a}	42.5 ± 24.0$^{b\alpha}$	2.3 ± 0.6^{a}	1.7 ± 0.0^{a}	0.439	0.156	0.040
	DIO	14.5 ± 12.1	2.2 ± 0.5	15.8 ± 8.5	7.0 ± 3.5$^{\beta}$	49.9 ± 48.3	11.9 ± 10.2			
TLR4 LPS	DR	77.4 ± 31.5	93.7 ± 33.1	111.5 ± 41.6	131.4 ± 33.3	91.5 ± 23.3	89.5 ± 30.6	0.929	0.729	0.559
	DIO	79.1 ± 34.8	72.5 ± 35.3	49.6 ± 17.0	162.3 ± 51.2	55.2 ± 29.4	87.7 ± 32.4			
TLR5 Flagellin	DR	22.8 ± 14.4	13.3 ± 4.7	67.3 ± 62.4	17.7 ± 10.5	6.2 ± 4.1	2.5 ± 0.8	0.634	0.818	0.463
	DIO	15.0 ± 13.4	3.5 ± 1.2	7.1 ± 5.5	22.3 ± 12.3	25.4 ± 23.6	20.4 ± 9.5			

* IL-1β concentrations (pg/mL) are presented as mean ± SEM. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

** n = 10 for unstimulated controls and n = 6-7 for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-25b. IL-1β concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

TLR Agonist*	Phenotype	IL-1β (pg/mL)
Control (Unstimulated)	DR	10.2 ± 3.4
	DIO	5.8 ± 2.3
TLR2 P3C	DR	11.5 ± 4.3
	DIO	17.0 ± 8.8
TLR4 LPS	DR	98.5 ± 12.8
	DIO	85.4 ± 14.7
TLR5 Flagellin	DR	21.8 ± 11.1
	DIO	15.5 ± 5.1

* In the DR phenotype, *n* = 60 for control, *n* = 39 for TLR2, *n* = 39 for TLR4 and *n* = 41 for TLR5 stimulated blood cultures. In the DIO phenotype, *n* = 59 for control and *n* = 40 for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-26a. IL-6 concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		IL-6 pg/mL[*]								
		Control	High Energy Diets					Statistics - *P* value		
TLR Agonist[**]		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
Control	DR	17.3 ± 13.0	22.2 ± 10.1	2.6 ± 0.0	18.4 ± 9.9	31.2 ± 14.1	22.6 ± 13.3	0.921	0.377	0.768
	DIO	13.0 ± 8.5	11.5 ± 6.3	21.4 ± 10.7	7.0 ± 3.1	36.8 ± 16.6	20.7 ± 16.1			
TLR2 P3C	DR	25.9 ± 15.4$^{\alpha}$	12.1 ± 6.1	2.6 ± 0.0	24.8 ± 12.0	14.1 ± 5.3	33.1 ± 27.4	0.256	0.331	0.320
	DIO	10.8 ± 6.0$^{\beta}$	15.0 ± 8.1	22.1 ± 12.9	2.6 ± 0.0	57.7 ± 28.8	2.6 ± 0.0			
TLR4 LPS	DR	9.8 ± 6.1	35.0 ± 17.1$^{\alpha}$	5.3 ± 2.7	13.5 ± 10.9	25.3 ± 22.6	2.6 ± 0.0	0.888	0.404	0.134
	DIO	5.0 ± 2.4	2.6 ± 0.0$^{\beta}$	37.2 ± 17.3	4.0 ± 1.4	26.2 ± 13.2	10.9 ± 8.3			
TLR5 Flagellin	DR	22.5 ± 14.6	15.5 ± 12.9	26.9 ± 24.3	27.5 ± 14.5	15.7 ± 9.6	8.8 ± 5.5	0.491	0.244	0.312
	DIO	2.6 ± 0.0	14.1 ± 8.4	17.5 ± 14.5	2.8 ± 0.2	39.4 ± 21.5	4.9 ± 2.3			

[*] IL-6 concentrations (pg/mL) are presented as mean ± SEM. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

[**] $n = 10$ for unstimulated controls and $n = 6$-7 for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-26b. IL-6 concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

TLR Agonist*	Phenotype	IL-6 (pg/mL)
Control (Unstimulated)	DR	19.1 ± 4.5
	DIO	18.5 ± 4.6
TLR2 P3C	DR	18.9 ± 6.0
	DIO	18.8 ± 7.1
TLR4 LPS	DR	15.6 ± 5.2
	DIO	14.3 ± 4.2
TLR5 Flagellin	DR	19.6 ± 5.7
	DIO	14.1 ± 4.9

* In the DR phenotype, $n = 60$ for control, $n = 39$ for TLR2, $n = 39$ for TLR4 and $n = 41$ for TLR5 stimulated blood cultures. In the DIO phenotype, $n = 59$ for control and $n = 40$ for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-27a. IL-10 concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

		IL-10 pg/mL[*]								
		Control	High Energy Diets					Statistics - *P* value		
TLR Agonist[**]		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
Control	DR	1.4 ± 0.0	3.4 ± 1.9	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	0.800	0.588	0.233
	DIO	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	2.5 ± 1.1	1.4 ± 0.0	3.0 ± 1.0			
TLR2 P3C	DR	1.4 ± 0.0	6.9 ± 4.0	1.4 ± 0.0	8.4 ± 4.4	2.0 ± 0.6	1.4 ± 0.0	0.159	0.864	0.644
	DIO	1.4 ± 0.0	4.7 ± 3.3	10.9 ± 8.8	7.2 ± 5.8	18.4 ± 17.0	12.2 ± 8.7			
TLR4 LPS	DR	$8.1 \pm 4.9^{\alpha}$	$13.9 \pm 8.4^{\alpha\beta}$	$3.1 \pm 1.0^{\alpha\beta}$	$15.3 \pm 9.3^{\alpha}$	$46.0 \pm 23.5^{\beta}$	$1.4 \pm 0.0^{\alpha}$	0.987	0.019	0.841
	DIO	$1.4 \pm 0.0^{\alpha}$	$19.1 \pm 17.2^{\alpha\beta}$	$11.7 \pm 6.6^{\alpha}$	$10.9 \pm 7.0^{\alpha\beta}$	$32.4 \pm 17.0^{\beta}$	$11.5 \pm 5.4^{\alpha}$			
TLR5 Flagellin	DR	3.9 ± 2.5	1.4 ± 0.0	1.4 ± 0.0	3.7 ± 2.2	1.43± 0.0	5.0 ± 3.5	0.183	0.363	0.780
	DIO	1.4 ± 0.0	5.8 ± 3.6	1.4 ± 0.0	8.6 ± 7.2	4.1 ± 2.7	13.0 ± 8.9			

[*] IL-10 concentrations (pg/mL) are presented as mean ± SEM. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

[**] $n = 10$ for unstimulated controls and $n = 6$-7 for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-27b. IL-10 concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

TLR Agonist*	Phenotype	IL-10 (pg/mL)
Control (Unstimulated)	DR	1.8 ± 0.3
	DIO	1.9 ± 0.3
TLR2 P3C	DR	3.6 ± 1.0
	DIO	9.0 ± 3.6
TLR4 LPS	DR	14.5 ± 4.7
	DIO	14.0 ± 4.1
TLR5 Flagellin	DR	2.8 ± 0.8
	DIO	5.8 ± 2.0

* In the DR phenotype, *n* = 60 for control, *n* = 39 for TLR2, *n* = 39 for TLR4 and *n* = 41 for TLR5 stimulated blood cultures. In the DIO phenotype, *n* = 59 for control and *n* = 40 for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-28a. TNF-α concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		TNF-α pg/mL*								
		Control	High Energy Diets					Statistics - *P* value		
TLR Agonist**		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
Control	DR	1.2 ± 0.4	0.8 ± 0.0	1.6 ± 0.8	0.8 ± 0.0	1.1 ± 0.3	0.8 ± 0.0$^{\alpha}$	0.205	0.172	0.143
	DIO	18.2 ± 16.6^{ac}	1.5 ± 0.7^{ac}	0.8 ± 0.0^{abc}	2.5 ± 1.7^{ac}	2.8 ± 2.0^{abc}	12.3 ± 7.2$^{b\beta}$			
TLR2 P3C	DR	2.1 ± 1.3	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	5.6 ± 4.7	0.8 ± 0.0	0.235	0.535	0.955
	DIO	0.8 ± 0.0	2.1 ± 1.3	5.6 ± 4.8	4.7 ± 3.9	8.1 ± 7.3	2.9 ± 1.4			
TLR4 LPS	DR	28.9 ± 10.2	2.6 ± 1.8$^{\alpha}$	8.4 ± 4.8	16.8 ± 12.7	4.5 ± 2.6$^{\alpha}$	13.0 ± 6.8	0.467	0.240	0.026
	DIO	10.9 ± 6.0^{ab}	29.6 ± 13.7$^{ab\beta}$	44.5 ± 39.5^{a}	5.8 ± 3.5^{ab}	39.6 ± 17.8$^{b\beta}$	20.6 ± 7.8^{b}			
TLR5 Flagellin	DR	1.2 ± 0.4	0.8 ± 0.0	2.73 ± 1.9	0.8 ± 0.0	1.6 ± 0.8	0.8 ± 0.0	0.149	0.729	0.498
	DIO	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	6.8 ± 6.0	5.1 ± 2.9	6.9 ± 6.1			

* TNF-α concentrations (pg/mL) are presented as mean ± SEM. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

** $n = 10$ for unstimulated controls and $n = 6$-7 for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-28b. TNF-α concentrations measured from TLR agonist-stimulated whole blood cultures of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

TLR Agonist*	Phenotype	TNF-α (pg/mL)
Control (Unstimulated)	DR	1.1 ± 0.2
	DIO	6.2 ± 2.8
TLR2 P3C	DR	1.8 ± 0.8
	DIO	4.0 ± 1.6
TLR4 LPS	DR	12.6 ± 3.4
	DIO	24.7 ± 7.7
TLR5 Flagellin	DR	1.3 ± 0.4
	DIO	3.5 ± 1.5

* In the DR phenotype, *n* = 60 for control, *n* = 39 for TLR2, *n* = 39 for TLR4 and *n* = 41 for TLR5 stimulated blood cultures. In the DIO phenotype, *n* = 59 for control and *n* = 40 for TLR2, TLR4 and TLR5 stimulated blood cultures.

Table 3-29a. Serum cytokines/chemokines, CRP, LPS, BUN and glucose concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CINC-1 pg/mL	DR	187.0 ± 25.2	238.7 ± 37.8	213.3 ± 29.8	159.5 ± 22.2	156.0 ± 25.7	154.3 ± 23.8$^{\alpha}$	0.123	0.132	0.121
	DIO	198.3 ± 34.9	222.6 ± 16.9	176.0 ± 22.4	209.4 ± 28.8	152.4 ± 17.4	249.2 ± 24.6$^{\beta}$			
CINC-2αβ pg/mL	DR	20.6 ± 0.6	21.1 ± 1.0	19.9 ± 0.9	20.6 ± 0.6	19.8 ± 1.0$^{\alpha}$	19.9 ± 0.7	0.668	0.937	0.148
	DIO	19.7 ± 0.7	19.5 ± 0.5	20.3 ± 0.9	20.1 ± 0.5	22.2 ± 1.4$^{\beta}$	21.4 ± 0.8			
CINC-3 pg/mL	DR	13.9 ± 0.5	14.6 ± 1.1	13.3 ± 0.6	16.2 ± 1.6$^{\alpha}$	12.9 ± 0.4$^{\alpha}$	13.5 ± 0.7	0.646	0.676	0.036
	DIO	13.5 ± 0.6	14.0 ± 1.1	15.3 ± 1.5	13.5 ± 0.7$^{\beta}$	16.2 ± 1.2$^{\beta}$	13.4 ± 0.4			
sICAM-1/sCD54 ng/mL	DR	9.9 ± 0.6	8.7 ± 0.57$^{\alpha}$	10.4 ± 0.6	10.3 ± 0.8	10.6 ± 0.6	11.0 ± 0.9	0.068	0.311	0.044
	DIO	9.8 ± 0.8	11.9 ± 0.7$^{\beta}$	10.9 ± 0.7	11.0 ± 0.7	9.5 ± 0.3	11.8 ± 0.6			
Active TGF-β1 pg/mL	DR	711.2 ± 73.6$^{\alpha}$	611.5 ± 28.2	652.9 ± 46.2	641.5 ± 34.6	566.4 ± 61.6	612.1 ± 42.4	0.318	0.801	0.523
	DIO	573.3 ± 77.5$^{\beta}$	619.4 ± 22.1	595.5 ± 39.0	617.5 ± 22.4	583.5 ± 27.0	639.8 ± 56.8			
Total TGF-β1 ng/mL	DR	20.3 ± 1.5	20.7 ± 2.7	19.1 ± 1.5	20.2 ± 1.5	17.1 ± 1.9	20.5 ± 1.6	0.480	0.695	0.900
	DIO	21.2 ± 1.9	19.3 ± 2.0	20.9 ± 2.4	21.1 ± 1.7	19.7 ± 1.5	19.8 ± 1.6			

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Table 3-29a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure[*]		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CRP µg/mL	DR	439.3 ± 60.9^{abα}	515.7 ± 85.6^{aα}	410.4 ± 37.2^{abα}	381.6 ± 39.7^{abαβ}	341.5 ± 49.2^{bβ}	346.4 ± 31.0^{abαβ}	0.731	0.043	0.737
	DIO	479.0 ± 34.6^{aα}	411.9 ± 47.3^{abα}	441.0 ± 51.1^{abα}	368.8 ± 47.5^{abαβ}	333.5 ± 35.7^{bβ}	406.4 ± 41.5^{abαβ}			
LPS EU/mL	DR	0.472 ± 0.046	0.520 ± 0.055	0.564 ± 0.059	0.490 ± 0.041	0.449 ± 0.035	0.505 ± 0.033	0.766	0.299	0.670
	DIO	0.459 ± 0.031	0.522 ± 0.043	0.499 ± 0.041	0.646 ± 0.096	0.461 ± 0.049	0.489 ± 0.046			
BUN mmol/L	DR	7.4 ± 0.1^{aα}	7.1 ± 0.4^{abβ}	6.6 ± 0.2^{abβ}	6.6 ± 0.2^{abβ}	6.5 ± 0.1^{bβ}	8.4 ± 0.2^{abβ}	0.887	<0.001	0.389
	DIO	7.8 ± 0.2^{aα}	6.6 ± 0.3^{bβ}	6.9 ± 0.2^{abβ}	6.5 ± 0.1^{bβ}	6.6 ± 0.2^{bβ}	6.4 ± 0.2^{bβ}			
Glucose mmol/L	DR	8.9 ± 0.3	8.7 ± 0.3	8.8 ± 0.3	8.6 ± 0.3	8.7 ± 0.3	6.7 ± 0.4	0.821	0.262	0.637
	DIO	8.8 ± 0.2	8.9 ± 0.5	9.1 ± 0.2	9.1 ± 0.3	8.2 ± 0.2	8.4 ± 0.3			

[*] Concentrations are presented as mean ± SEM, $n = 10$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-29b. Serum cytokine/chemokine, CRP, LPS, BUN and glucose concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure[*]	**Phenotype**	**Concentration**
CINC-1 pg/mL	DR	184.9 ± 11.7
	DIO	201.3 ± 10.6
CINC-2αβ pg/mL	DR	20.3 ± 0.3
	DIO	20.5 ± 0.4
CINC-3 pg/mL	DR	14.1 ± 0.4
	DIO	14.3 ± 0.4
sICAM-1/sCD54 ng/mL	DR	10.1 ± 0.3
	DIO	10.8 ± 0.3
Active TGF-β1 pg/mL	DR	632.6 ± 20.5
	DIO	604.8 ± 18.0
Total TGF-β1 ng/mL	DR	19.7 ± 0.7
	DIO	20.3 ± 0.7
CRP µg/mL	DR	405.8 ± 22.4
	DIO	406.8 ± 18.0
LPS EU/mL	DR	0.500 ± 0.019
	DIO	0.513 ± 0.023
BUN mmol/L	DR	6.8 ± 0.1
	DIO	6.8 ± 0.1
Glucose mmol/L	DR	8.7 ± 0.1
	DIO	8.7 ± 0.1

[*] *n* = 60 for DR and DIO rats.

Table 3-30. Histological analysis of IEL counts in small bowel sections collected from DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

	Control	High Energy Diets					1-way ANOVA
Section*	**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	***P* value**
Duodenum	8.93 ± 1.19	7.33 ± 0.07	7.87 ± 1.57	7.20 ± 1.20	7.00 ± 1.42	8.73 ± 0.59	0.753
Jejunum	6.93 ± 3.73	4.93 ± 2.53	12.13 ± 0.79	2.67 ± 2.67	3.47 ± 3.47	13.83 ± 3.28	0.086

* IELs were enumerated per 100 villous enterocytes for 5 villi per section in the small bowel. Counts are presented as mean ± SEM, $n = 5$. Statistical analyses were performed using one-way ANOVA.

Table 3-31a. Ileal tissue cytokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure[*]		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
IL-1β ng/g	DR	10.6 ± 2.5	9.7 ± 2.5	9.1 ± 2.3	11.1 ± 2.8	7.4 ± 1.5	8.3 ± 2.2	0.624	0.577	0.822
	DIO	9.1 ± 3.2	5.2 ± 2.2	8.4 ± 2.3	11.4 ± 2.5	7.6 ± 2.1	10.4 ± 2.0			
IL-6 pg/g	DR	1586.7 ± 171.7	1382.7 ± 246.1	1296.8 ± 286.8	1852.7 ± 280.2	1069.8 ± 278.0	1161.7 ± 250.8	0.323	0.235	0.788
	DIO	1579.6 ± 236.0	1148.8 ± 267.2	1358.4 ± 335.5	1230.1 ± 257.5	998.6 ± 255.0	1149.1 ± 185.7			
IL-10 pg/g	DR	477.8 ± 86.1	457.9 ± 106.5	391.7 ± 85.0	526.9 ± 67.3	243.3 ± 49.7	451.3 ± 85.2	0.232	0.219	0.509
	DIO	465.5 ± 85.0	295.0 ± 57.7	372.0 ± 55.3	406.2 ± 83.5	354.4 ± 64.6	338.8 ± 66.7			
IL-17F pg/g	DR	495.3 ± 92.8	502.1 ± 111.2	452.0 ± 94.4	452.0 ± 85.1	449.4 ± 57.6	470.2 ± 103.0	0.421	0.833	0.689
	DIO	455.5 ± 65.9	333.8 ± 67.0	351.5 ± 60.6	554.6 ± 92.0	432.5 ± 81.2	461.7 ± 57.7			
Active TGF-β1 ng/g	DR	1.8 ± 0.7	1.9 ± 0.6	1.3 ± 0.2	1.3 ± 0.2	2.8 ± 0.5	1.8 ± 0.3	0.204	0.163	0.721
	DIO	1.6 ± 0.2	1.7 ± 0.4	2.3 ± 0.5	2.0 ± 0.5	2.9 ± 0.7	2.7 ± 0.7			
Total TGF-β1 ng/g	DR	9.0 ± 0.5	8.5 ± 1.2	8.4 ± 0.8	9.8 ± 0.6	10.3 ± 0.9	9.3 ± 1.1	0.087	0.420	0.913
	DIO	10.0 ± 1.1	8.8 ± 1.4	9.8 ± 0.7	9.9 ± 0.7	10.7 ± 1.0	10.7 ± 0.9			

[*] Concentrations are presented as mean ± SEM, $n = 10$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. A Tukey's multiple comparison test did not detect any differences between phenotype or diet in this dataset.

Table 3-31b. Ileal tissue cytokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure*	Phenotype	Concentration
Il-1β ng/g	DR	9.4 ± 0.9
	DIO	8.7 ± 1.0
IL-6 pg/g	DR	1391.7 ± 105.4
	DIO	1244.1 ± 104.3
IL-10 pg/g	DR	424.8 ± 34.1
	DIO	372.0 ± 28.1
IL-17F pg/g	DR	470.2 ± 36.1
	DIO	431.6 ± 29.6
Active TGF-β1 ng/mL	DR	1.8 ± 0.2
	DIO	2.2 ± 0.2
Total TGF-β1 ng/mL	DR	9.2 ± 0.4
	DIO	10.1 ± 0.4
Protein Quantification by Bradford Assay mg/g	DR	49.8 ± 1.8
	DIO	49.5 ± 1.8

* n = 60 for DR and DIO rats.

Table 3-32a. Caecal tissue cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure[*]		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CINC-1 pg/g	DR	198.2 ± 29.2	250.5 ± 36.8	222.6 ± 32.5	205.4 ± 30.3	164.5 ± 21.3	191.9 ± 30.2	0.880	0.604	0.834
	DIO	183.8 ± 26.9	203.1 ± 25.7	233.2 ± 33.8	198.1 ± 36.4	203.4 ± 34.4	195.1 ± 22.0			
CINC-2αβ pg/g	DR	305.4 ± 28.0^{acd}	411.4 ± 88.3^{acd}	225.7 ± 58.6^{b}	363.9 ± 60.0^{abcd}	155.4 ± 31.3^{b}	210.2 ± 47.2^{abd}	0.597	0.014	0.638
	DIO	303.7 ± 44.1	246.0 ± 35.2	216.5 ± 39.6	266.5 ± 61.1	210.0 ± 36.9	172.8 ± 36.5			
CINC-3 pg/g	DR	7.4 ± 1.7	26.6 ± 12.3	29.9 ± 15.1	37.3 ± 13.0	6.2 ± 1.3	5.1 ± 0.2	0.296	0.111	0.372
	DIO	13.8 ± 5.6	18.9 ± 9.9	12.4 ± 7.5	15.4 ± 10.5	6.0 ± 1.1	15.3 ± 6.9			
GM-CSF pg/g	DR	95.5 ± 32.0	102.6 ± 35.2	64.3 ± 25.1	73.2 ± 23.4	67.2 ± 28.3	102.6 ± 30.3	0.775	0.617	0.357
	DIO	27.3 ± 7.9	80.3 ± 32.5	52.2 ± 24.4	126.2 ± 40.4	105.0 ± 33.2	85.2 ± 29.1			
IL-6 pg/g	DR	1028.51± 422.0^{abα}	624.6 ± 151.4^{aα}	722.0 ± 229.7^{aαβ}	831.0 ± 124.0^{abαβ}	510.4 ± 100.3^{bβ}	622.3 ± 118.9^{aα}	0.291	0.003	0.523
	DIO	404.2 ± 50.5^{α}	502.4 ± 158.7^{α}	633.5 ± 171.4^{αβ}	627.7 ± 182.4^{αβ}	528.2 ± 119.7^{β}	596.2 ± 94.2^{α}			
IL-10 pg/g	DR	286.3 ± 70.2	358.3 ± 128.6	251.2 ± 67.7	277.8 ± 61.8	141.7 ± 35.4	299.6 ± 73.8	0.467	0.813	0.704
	DIO	263.3 ± 62.2	222.4 ± 68.7	290.4 ± 74.3	194.7 ± 50.6	222.4 ± 59.7	244.9 ± 60.3			

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Table 3-32a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
IL-17F pg/g	DR	156.6 ± 70.8	210.4 ± 87.1	215.0 ± 84.5	203.0 ± 82.2	125.9± 48.4	201.2 ± 72.2	0.752	0.436	0.680
	DIO	227.4 ± 80.8	236.9 ± 96.4	318.8 ± 111.4	58.6 ± 16.9	138.0 ± 39.1	216.3 ± 77.3			
IL-22 pg/g	DR	110.8 ± 30.5	125.4 ± 29.8	174.7 ± 52.2	149.4 ± 38.5	209.0 ± 58.0	221.6 ± 87.6	0.431	0.359	0.459
	DIO	159.1 ± 28.8	187.9 ± 39.5	154.6 ± 35.3	141.9 ± 42.9	234.2 ± 57.6	145.0 ± 29.1			
Active TGF-β1 pg/g	DR	998.6 ± 85.3	726.6 ± 71.7	767.6 ± 79.0	917.2 ± 113.4	873.7 ± 84.7	992.5 ± 53.3	0.713	0.212	0.799
	DIO	863.0 ± 90.2	825.2 ± 122.2	878.1 ± 119.8	941.1 ± 78.6	900.5 ± 76.8	985.5 ± 99.7			
Total TGF-β1 pg/g	DR	5798.7 ± 293.1	5511.7 ± 310.5	5969.3 ± 188.8	6230.7 ± 410.8	5623.6 ± 480.6	6135.4 ± 536.1	0.047	0.379	0.813
	DIO	6271.4 ± 295.1	6109.1 ± 453.6	7128.7 ± 551.8	6283.9 ± 369.9	5924.5 ± 424.1	6381.4 ± 410.4			

* Concentrations are presented as mean ± SEM, $n = 10$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-32b. Caecal tissue cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure*	Phenotype	Concentration
CINC-1 pg/g	DR	205.5 ± 12.4
	DIO	202.8 ± 12.7
CINC-2αβ pg/g	DR	278.7 ± 24.8
	DIO	235.9 ± 17.7
CINC-3 pg/g	DR	18.8 ± 4.1
	DIO	13.5 ± 3.0
GM-CSF pg/g	DR	84.2 ± 11.6
	DIO	79.4 ± 12.4
IL-6 pg/g	DR	723.1 ± 89.1
	DIO	548.7 ± 54.8
IL-10 pg/g	DR	269.1 ± 31.7
	DIO	239.7 ± 25.0
IL-17F pg/g	DR	185.4 ± 29.7
	DIO	199.3 ± 32.1
IL-22 pg/g	DR	165.2 ± 21.5
	DIO	170.5 ± 16.20
Active TGF-β1 pg/mL	DR	879.4 ± 35.2
	DIO	898.9 ± 39.5
Total TGF-β1 pg/mL	DR	5878.3 ± 155.4
	DIO	6349.8 ± 173.0
Protein Quantification by Bradford Assay mg/g	DR	37.0 ± 1.2
	DIO	38.2 ± 1.0

* n = 60 for DR and DIO rats.

Table 3-33a. Proximal colon cytokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure*		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
IL-6 pg/g	DR	1192.9 ± 154.0$^{\alpha}$	1610.8 ± 153.3	1526.4 ± 171.0	1702.1 ± 154.0	1243.9 ± 168.2	1317.9 ± 162.8	0.612	0.060	0.016
	DIO	1974.1 ± 192.3$^{a\beta}$	1569.4 ± 236.0^{ab}	1761.9 ± 166.2^{ab}	1241.4 ± 168.2^{b}	1294.4 ± 225.4^{ab}	1067.2 ± 164.8^{b}			
IL-10 pg/g	DR	1096.6 ± 176.0	1318.5 ± 164.1	1175.3 ± 134.3	1430.5 ± 168.7	1002.0 ± 193.6	1347.2 ± 179.1	0.879	0.793	0.182
	DIO	1428.2 ± 172.6	1156.1 ± 189.0	1406.7 ± 152.4	1171.4 ± 205.7	1188.8 ± 192.0	927.4 ± 145.0			
IL-17F pg/g	DR	350.2 ± 67.4	375.7 ± 53.3	336.0 ± 48.1	413.0 ± 49.6	275.1 ± 48.9	343.0 ± 57.9	0.737	0.110	0.255
	DIO	459.5 ± 45.1^{a}	337.5 ± 64.0^{ab}	370.5 ± 53.3^{ab}	355.2 ± 59.6^{ab}	309.2 ± 56.0^{ab}	197.3 ± 45.5^{b}			
TNF-α pg/g	DR	122.4 ± 20.5	207.0 ± 64.0	192.2 ± 43.4	180.5 ± 27.4	149.4 ± 41.3	156.6 ± 36.6	0.764	0.458	0.422
	DIO	229.5 ± 55.5	189.6 ± 51.7	223.8 ± 45.7	121.2 ± 27.8	113.8 ± 22.8	173.9 ± 41.7			
Active TGF-β1 pg/g	DR	978.0 ± 64.3	929.8 ± 52.1	868.9 ± 76.9	972.0 ± 57.9	787.6 ± 89.7$^{\alpha}$	1001.1 ± 57.9	0.694	0.883	0.312
	DIO	928.9 ± 39.7	906.1 ± 80.2	956.0 ± 78.8	911.5 ± 61.9	993.2 ± 70.2$^{\beta}$	937.4 ± 91.1			

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Table 3-33a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure[*]		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
Total TGF-β1 pg/g	DR	4178.9 ± 282.2	4200.6 ± 270.9	4666.8 ± 526.6	4591.0 ± 384.5	3893.3 ± 241.4$^{\alpha}$	4375.6 ± 334.1	0.433	0.823	0.357
	DIO	4146.1 ± 317.3	4740.3 ± 539.2	4863.0 ± 382.0	4039.3 ± 201.0	5199.1 ± 522.4$^{\beta}$	4323.6 ± 462.8			

[*] Concentrations are presented as mean ± SEM, $n = 10$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-33b. Proximal colon cytokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure	Phenotype	Concentration
IL-6 pg/g	DR	1432.3 ± 67.5
	DIO	1484.7 ± 86.2
IL-10 pg/g	DR	1228.4 ± 69.3
	DIO	1213.1 ± 72.7
IL-17F pg/g	DR	348.8 ± 22.0
	DIO	338.2 ± 23.6
TNF-α pg/g	DR	168.0 ± 16.5
	DIO	175.3 ± 17.6
Active TGF-β1 pg/mL	DR	922.9 ± 28.1
	DIO	938.9 ± 28.5
Total TGF-β1 pg/mL	DR	4317.7 ± 142.1
	DIO	4551.9 ± 173.2
Protein Quantification by Bradford Assay mg/g	DR	50.1 ± 1.6
	DIO	49.0 ± 1.2

[*] *n* = 60 for DR and DIO rats.

Table 3-34a. Distal colon tissue cytokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
IL-6 pg/g	DR	1841.7± 309.9	2427.0 ± 343.7	2039.4 ± 276.0	2069.9 ± 268.6	2082.5 ± 329.4	1612.6 ± 381.5	0.778	0.963	0.445
	DIO	2513.6 ± 436.0	1664.5 ± 381.2	1980.6 ± 440.3	2187.1 ± 303.7	1997.1 ± 316.7	2084.7 ± 484.7			
IL-10 pg/g	DR	564.8 ± 167.6	1060.2 ± 298.2$^{\alpha}$	587.3 ± 154.1	768.9 ± 193.6	519.1 ± 112.5	720.8 ± 183.7	0.954	0.972	0.108
	DIO	726.3 ± 222.8	496.3 ± 162.1$^{\beta}$	804.5 ± 159.2	496.6 ± 154.9	962.4 ± 179.8	697.0 ± 213.3			
IL-17F pg/g	DR	366.0 ± 75.0	519.7 ± 85.6	476.8 ± 86.2	506.1 ± 80.3	354.9 ± 58.6	439.3 ± 79.2	0.552	0.305	0.227
	DIO	549.7 ± 72.1	474.1 ± 91.3	602.0 ± 70.1	331.6 ± 62.7	385.7 ± 62.2	475.5 ± 74.4			
IL-22 pg/g	DR	126.7 ± 10.1	126.0 ± 10.8	158.2 ± 15.6	139.4 ± 16.4	247.6 ± 47.2$^{\alpha}$	194.0 ± 36.6	0.924	0.155	0.192
	DIO	157.1 ± 10.7	163.6 ± 22.0	145.0 ± 23.6	156.9 ± 36.3	156.8 ± 27.2$^{\beta}$	239.8 ± 64.7			
TNF-α pg/g	DR	188.6 ± 49.8	231.5 ± 46.6	281.9 ± 42.2	212.8 ± 19.9	159.1 ± 40.9	220.4 ± 71.8	0.903	0.444	0.341
	DIO	271.5 ± 58.6	162.1 ± 29.2	206.7 ± 24.7	216.2 ± 73.9	196.3 ± 53.3	197.8 ± 39.8			

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Table 3-34a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
Active TGF-β1 pg/g	DR	1354.6 ± 51.1	1450.6 ± 82.9	1613.1 ± 73.0	1547.2 ± 47.4	1512.3 ± 76.9	1500.6 ± 54.6	0.361	0.435	0.173
	DIO	1538.8 ± 86.8	1437.0 ± 89.1	1453.5 ± 48.0	1553.3 ± 102.8	1340.3 ± 58.2	1419.9 ± 90.4			
Total TGF-β1 pg/g	DR	3065.1 ± 276.1$^{\alpha}$	3240.4 ± 273.8	4129.4 ± 252.6	3850.2 ± 260.8	3689.1 ± 209.8	3632.9 ± 291.3	0.753	0.096	0.089
	DIO	3993.1 ± 281.7$^{\beta}$	3572.5 ± 326.1	4172.9 ± 359.5	3302.1 ± 330.7	3142.5 ± 297.7	3732.9 ± 176.6			

[*] Concentrations are presented as mean ± SEM, $n = 10$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-34b. Distal colon tissue cytokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure	Phenotype	Concentration
IL-6 pg/g	DR	2012.2 ± 129.3
	DIO	2071.3 ± 159.4
IL-10 pg/g	DR	703.5 ± 79.2
	DIO	697.2 ± 75.0
IL-17F pg/g	DR	443.8 ± 31.6
	DIO	469.8 ± 30.8
IL-22 pg/g	DR	165.3 ± 11.9
	DIO	169.9 ± 14.3
TNF-α pg/g	DR	215.7 ± 19.3
	DIO	208.5 ± 19.9
Active TGF-β1 pg/mL	DR	1496.4 ± 27.7
	DIO	1457.1 ± 33.2
Total TGF-β1 pg/mL	DR	3601.2 ± 112.4
	DIO	3652.7 ± 126.7
Protein Quantification by Bradford Assay mg/g	DR	40.0 ± 1.0
	DIO	41.0 ± 1.0

* n = 60 for DR and DIO rats.

Table 3-35a. Liver cytokine/chemokine and CRP concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CINC-1 ng/g	DR	5.8 ± 0.5	10.0 ± 1.4	7.4 ± 0.8	9.8 ± 1.3	8.3 ± 0.9	7.2 ± 0.4	0.423	0.081	0.835
	DIO	7.6 ± 0.7	8.8 ± 1.3	8.2 ± 1.1	9.7 ± 2.2	9.3 ± 1.4	8.2 ± 0.5			
CINC-2αβ ng/g	DR	18.7 ± 1.2$^{a\alpha\gamma}$	25.8 ± 2.6$^{b\beta}$	21.6 ± 1.3$^{ab\alpha\beta}$	26.7 ± 2.1$^{b\beta}$	23.7 ± 1.9$^{b\beta}$	21.6 ± 1.3$^{ab\alpha\beta}$	0.569	0.005	0.221
	DIO	21.8 ± 1.2$^{\alpha\delta}$	22.8 ± 0.8$^{\beta}$	22.4 ± 1.3$^{\alpha\beta}$	25.4 ± 3.8$^{\beta}$	25.8 ± 2.5$^{\beta}$	21.1 ± 0.9$^{\alpha\beta}$			
CINC-3 ng/g	DR	7.9 ± 1.4	9.8 ± 1.9	7.0 ± 1.4	9.6 ± 2.0	8.1 ± 1.6	10.8 ± 0.8	0.563	0.415	0.440
	DIO	6.5 ± 1.5	9.5 ± 1.7	9.5 ± 1.3	10.5 ± 2.3	11.7 ± 1.3	8.6 ± 1.4			
CRP µg/g	DR	65.8 ± 5.1$^{\alpha}$	70.2 ± 7.7	64.8 ± 5.6	63.0 ± 6.7	57.2 ± 4.4	66.9 ± 2.8	0.409	0.140	0.488
	DIO	84.9 ± 13.6$^{a\beta}$	65.2 ± 4.4^{ab}	69.6 ± 4.7^{ab}	65.4 ± 3.2^{ab}	56.8 ± 5.6^{b}	64.4 ± 6.2^{ab}			
IFN-γ ng/g	DR	31.8 ± 4.5	44.2 ± 5.8	36.1 ± 5.2	39.9 ± 6.8	39.3 ± 4.2	48.1 ± 8.0	0.502	0.250	0.635
	DIO	33.2 ± 8.1	34.7 ± 5.0	38.1 ± 5.6	42.5 ± 4.5	42.7 ± 3.4	33.8 ± 4.1			
IL-1β ng/g	DR	120.2 ± 13.6$^{\alpha}$	155.3 ± 15.1$^{\alpha\beta}$	134.6 ± 15.3$^{\alpha\beta}$	155.7 ± 16.1$^{\beta}$	149.8 ± 11.9$^{\alpha\beta}$	141.5 ± 16.9$^{\alpha\beta}$	0.502	0.046	0.724
	DIO	122.4 ± 15.7$^{\alpha}$	134.8 ± 11.0$^{\alpha\beta}$	128.3 ± 12.5$^{\alpha\beta}$	174.1 ± 17.4$^{\beta}$	134.2 ± 7.9$^{\alpha\beta}$	124.1 ± 8.7$^{\alpha\beta}$			
IL-4 ng/g	DR	52.1 ± 7.3	66.9 ± 7.7	59.6 ± 8.9	68.8 ± 11.2	62.2 ± 5.8	69.8 ± 13.6	0.605	0.375	0.861
	DIO	55.40 ± 11.0	56.1 ± 7.8	62.9 ± 9.6	69.2 ± 6.4	66.6 ± 7.8	51.4 ± 7.4			

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Table 3-35a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
IL-6 ng/g	DR	154.9 ± 21.9	190.8 ± 22.5	188.4 ± 29.9	20.0 ± 34.0	180.3 ± 19.6	194.5 ± 40.7	0.681	0.334	0.918
	DIO	173.2 ± 33.2	175.0 ± 27.3	193.6 ± 34.3	196.4 ± 18.1	180.4 ± 23.7	147.4 ± 20.3			
IL-10 ng/g	DR	34.1 ± 3.3	39.5 ± 2.8	41.6 ± 2.9	41.5 ± 4.0	41.7 ± 2.9	41.1 ± 4.1	0.328	0.172	0.975
	DIO	34.6 ± 4.4	36.4 ± 2.7	41.2 ± 3.4	42.3 ± 4.5	39.3 ± 3.8	35.9 ± 2.9			
TNF-α pg/g	DR	6251.8 ± 540.9	7444.0 ± 660.9	7165.0 ± 523.9	7871.8 ± 781.7	6520.8 ± 4278.0	7453.9 ± 441.2	0.911	0.250	0.498
	DIO	6485.5 ± 683.4	7039.8 ± 492.0	7524.8 ± 359.8	7713.2 ± 686.2	7740.5 ± 760.3	6427.2 ± 290.8			
Active TGF-β1 pg/g	DR	2696.4 ± 141.5	3068.3 ± 290.3	2808.5 ± 223.7	2821.8 ± 165.8	2835.0 ± 205.8	2604.5 ± 225.6	0.215	0.443	0.845
	DIO	2329.5 ± 109.3	2730.7 ± 176.2	2629.2 ± 201.6	2916.3 ± 206.4	2742.9 ± 274.1	2639.5 ± 184.6			
Total TGF-β1 pg/g	DR	3242.2 ± 94.3	3311.3 ± 254.8	3219.2 ± 221.8	3354.1 ± 227.0	3322.5 ± 215.3	3130.8 ± 161.1	0.383	0.562	0.848
	DIO	3114.6 ± 412.0	2921.3 ± 82.0	3217.8 ± 287.0	3560.1 ± 244.2	3122.4 ± 106.5	2951.6 ± 180.4			

[*] Concentrations are presented as mean ± SEM, $n = 10$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-35b. Liver cytokine/chemokine and CRP concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure	Phenotype	Concentration
CINC-1 ng/g	DR	8.1 ± 0.4
	DIO	8.6 ± 0.5
CINC-2αβ ng/g	DR	23.0 ± 0.8
	DIO	23.2 ± 0.8
CINC-3 ng/g	DR	8.9 ± 0.6
	DIO	9.4 ± 0.7
CRP µg/g	DR	64.6 ± 2.2
	DIO	67.7 ± 3.0
IFN-γ ng/g	DR	39.9 ± 2.4
	DIO	37.5 ± 2.1
IL-1β ng/g	DR	142.9 ± 6.1
	DIO	136.3 ± 5.4
IL-4 ng/g	DR	63.2 ± 3.8
	DIO	60.3 ± 3.4
IL-6 pg/g	DR	184.8 ± 11.5
	DIO	177.7 ± 10.7
IL-10 pg/g	DR	39.9 ± 1.4
	DIO	38.3 ± 1.5
TNF-α pg/g	DR	7117.9 ± 236.7
	DIO	7155.2 ± 234.9
Active TGF-β1 ng/mL	DR	2805.8 ± 85.7
	DIO	2664.7 ± 80.7
Total TGF-β1 ng/mL	DR	3262.0 ± 79.8
	DIO	3148.0 ± 99.8
Protein Quantification by Bradford Assay mg/g	DR	113.9 ± 2.7
	DIO	108.7 ± 2.8

* n = 60 for DR and DIO rats.

Table 3-36a. Spleen cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CINC-1 pg/g	DR	1461.1 ± 183.1	1481.4 ± 201.3	1221.0 ± 85.8	1523.2 ± 106.2	1565.6 ± 152.2	1412.9 ± 73.0	0.645	0.499	0.564
	DIO	1311.3 ± 104.2	1500.8 ± 101.7	1341.1 ± 61.2	1527.6 ± 130.1	1260.9 ± 104.1	1418.9 ± 119.3			
CINC-2αβ pg/g	DR	1482.9 ± 140.1	1243.0 ± 129.0	1382.1 ± 129.9	1570.7 ± 131.1	1404.0 ± 167.0	1555.6 ± 125.6	0.833	0.953	0.381
	DIO	1509.5 ± 189.6	1591.2 ± 126.2	1522.3 ± 148.3	1371.0 ± 87.0	1340.1 ± 142.0	1405.8 ± 124.9			
CINC-3 pg/g	DR	293.3 ± 43.5	383.8 ± 41.4$^{\alpha}$	271.4 ± 25.6	368.9 ± 46.2	265.2 ± 31.8	255.4 ± 24.7	0.181	0.458	0.311
	DIO	299.8 ± 35.4	277.2 ± 58.9$^{\beta}$	242.2 ± 22.4	292.2 ± 27.9	289.5 ± 45.5	280.7 ± 33.3			
GM-CSF pg/g	DR	126.7 ± 44.5$^{\alpha\beta}$	134.8 ± 28.4$^{\alpha\beta}$	102.2 ± 25.4$^{\alpha}$	121.5 ± 19.8$^{\beta}$	144.8 ± 24.3$^{\alpha\beta}$	80.4 ± 22.3$^{\alpha\beta}$	0.479	0.017	0.371
	DIO	131.0 ± 20.9$^{ab\alpha\beta}$	76.7 ± 20.7$^{ab\alpha\beta}$	48.7 ± 13.0$^{a\alpha}$	178.8 ± 45.0$^{b\beta}$	132.9 ± 31.7$^{ab\alpha\beta}$	82.0 ± 25.4$^{ab\alpha\beta}$			
IFN-γ pg/g	DR	546.7 ± 124.5	452.7 ± 93.0	555.0 ± 115.5	566.5 ± 144.1	406.2 ± 97.2	661.1 ± 124.2	0.643	0.688	0.552
	DIO	608.0 ± 116.8	604.2 ± 117.8	738.2 ± 121.4	488.5 ± 106.2	489.4 ± 136.0	450.3 ± 110.6			
IL-1β ng/g	DR	60.1 ± 3.3	57.9 ± 4.2	66.0 ± 5.8	62.7 ± 5.2	66.8 ± 3.9	61.8 ± 5.6	0.504	0.705	0.598
	DIO	69.9 ± 6.8	68.8 ± 8.7	66.9 ± 7.7	64.4 ± 6.9	65.4 ± 5.4	53.6 ± 5.0			

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Table 3-36a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
IL-4 pg/g	DR	84.4 ± 36.8	169.7 ± 62.2	171.1 ± 47.0	185.1 ± 59.6	197.7 ± 56.9	117.1 ± 55.8	0.516	0.669	0.729
	DIO	152.6 ± 59.5	74.9 ± 37.9	115.8 ± 35.7	141.6 ± 60.5	192.9 ± 65.6	126.0 ± 55.8			
IL-6 pg/g	DR	1304.4 ± 614.7	1181.5 ± 288.9	895.9 ± 304.2	1082.1 ± 373.3	1333.0 ± 313.9	1121.6 ± 263.1	0.381	0.125	0.260
	DIO	1074.0 ± 250.2	726.5 ± 298.7	782.6 ± 420.8	2300.7 ± 870.7	2494.4 ± 708.6	944.8 ± 311.4			
IL-10 pg/g	DR	578.7 ± 76.8	652.7 ± 83.3	577.4 ± 86.9	650.1 ± 88.5	574.4 ± 90.0	549.7 ± 80.9	0.447	0.798	0.838
	DIO	643.0 ± 81.9	544.9 ± 71.2	461.0 ± 53.6	593.4 ± 78.6	563.2 ± 81.4	568.9 ± 65.9			
TNF-α pg/g	DR	236.2 ± 73.0	361.3 ± 64.6	312.5 ± 62.6	289.1 ± 74.1	300.4 ± 45.6	212.0 ± 51.0	0.254	0.868	0.209
	DIO	320.0 ± 69.0	207.1 ± 58.2	158.7 ± 34.0	293.9 ± 39.7	244.9 ± 52.6	253.8 ± 63.9			
Active TGF-β1 ng/g	DR	8.4 ± 1.4	6.9 ± 0.8	6.4 ± 0.8	7.5 ± 0.9	7.0 ± 1.0	7.4 ± 0.8	0.971	0.721	0.973
	DIO	8.5 ± 1.3	7.4 ± 0.9	6.7 ± 0.6	7.0 ± 1.0	7.0 ± 0.8	6.6 ± 0.5			
Total TGF-β1 ng/g	DR	86.3 ± 10.6	78.4 ± 10.4	91.0 ± 9.6	92.4 ± 10.6	78.4 ± 8.9	81.0 ± 11.6	0.351	0.269	0.456
	DIO	96.0 ± 8.4	94.8 ± 10.9	111.5 ± 9.7	74.4 ± 7.3	76.48 ± 10.0	87.3 ± 12.5			

[*] Concentrations are presented as mean ± SEM, $n = 10$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-36b. Spleen cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure	Phenotype	Concentration
CINC-1 pg/g	DR	1444.2 ± 57.3
	DIO	1393.4 ± 43.2
CINC-2αβ pg/g	DR	1439.7 ± 55.8
	DIO	1456.6 ± 55.8
CINC-3 pg/g	DR	306.3 ± 15.7
	DIO	280.3 ± 15.5
GM-CSF pg/g	DR	118.4 ± 11.5
	DIO	108.4 ± 12.3
IFN-γ ng/g	DR	531.4 ± 47.2
	DIO	563.1 ± 48.0
IL-1β ng/g	DR	62.6 ± 1.9
	DIO	64.8 ± 2.8
IL-4 ng/g	DR	154.2 ± 21.6
	DIO	134.0 ± 21.5
IL-6 pg/g	DR	1153.1 ± 149.2
	DIO	1387.2 ± 227.6
IL-10 pg/g	DR	597.2 ± 33.4
	DIO	562.4 ± 29.4
TNF-α pg/g	DR	285.3 ± 25.3
	DIO	246.4 ± 22.3
Active TGF-β1 ng/mL	DR	7.3 ± 0.4
	DIO	7.2 ± 0.4
Total TGF-β1 ng/mL	DR	84.6 ± 4.1
	DIO	90.1 ± 4.2
Protein Quantification by Bradford Assay mg/g	DR	52.6 ± 1.5
	DIO	56.2 ± 1.6

* n = 60 for DR and DIO rats.

Table 3-37a. MLN cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CINC-2αβ pg/g	DR	26.9 ± 7.4$^{\alpha}$	33.1 ± 5.7	24.6 ± 4.8	37.2 ± 6.8	40.9 ± 5.3	35.8 ± 4.4	0.233	0.760	0.064
	DIO	46.2 ± 7.6$^{\beta}$	38.2 ± 6.0	36.6 ± 3.0	29.4 ± 5.2	29.4 ± 4.1	41.6 ± 3.2			
CINC-3 pg/g	DR	62.7 ± 10.3	74.9 ± 11.2$^{\alpha}$	141.9 ± 86.8	43.7 ± 13.2	51.0 ± 11.5	45.0 ± 8.7	0.005	0.597	0.257
	DIO	50.3 ± 17.9	31.1 ± 1.4$^{\beta}$	45.1 ± 8.7	43.0 ± 11.9	42.8 ± 7.7	32.7 ± 2.3			
GM-CSF pg/g	DR	28.0 ± 6.0	27.3 ± 5.1	26.3 ± 3.6	29.0 ± 4.9	23.8 ± 3.9	29.3 ± 5.6	0.401	0.685	0.887
	DIO	32.6 ± 8.3	25.1 ± 4.2	25.9 ± 6.3	23.2 ± 2.8	19.4 ± 0.6	23.0 ± 3.6			
sICAM-1 /sCD54 ng/g	DR	32.5 ± 4.9	23.6 ± 4.0	32.1 ± 7.0	26.0 ± 6.2	14.3 ± 3.4	21.2 ± 5.4	0.621	0.099	0.582
	DIO	23.9 ± 4.3	27.2 ± 6.9	27.3 ± 4.8	25.8 ± 4.7	21.8 ± 3.2	15.2 ± 3.2			
IFN-γ pg/g	DR	1092.6 ± 299.9	1623.5 ± 418.4	849.5 ± 273.1	2063.0 ± 308.9	1325.1 ± 311.8	1205.6 ± 236.8$^{\alpha}$	0.112	0.215	0.218
	DIO	2023.5 ± 760.5	964.3 ± 288.3	1134.6 ± 632.4	930.4 ± 266.0	1177.1 ± 231.1	532.6 ± 169.5$^{\beta}$			
IL-1β pg/g	DR	1576.8 ± 344.1	1858.9 ± 429.8	1556.5 ± 316.4	1732.1 ± 309.7	1965.2 ± 214.5$^{\alpha}$	1783.7 ± 212.5	0.318	0.602	0.072
	DIO	2321.0 ± 194.6^{a}	1621.8 ± 286.2^{ab}	1693.5 ± 138.6^{ab}	1329.5 ± 190.4^{ab}	1052.7 ± 193.5$^{b\beta}$	1523.5 ± 221.8^{ab}			
IL-2 pg/g	DR	173.6 ± 44.6	151.4 ± 21.4	149.0 ± 24.2	155.7 ± 11.8	180.5 ± 31.5	208.0 ± 36.7	0.601	0.193	0.285
	DIO	246.5 ± 21.9^{a}	176.9 ± 28.8^{ab}	178.9 ± 23.3^{ab}	156.6 ± 18.8^{ab}	117.7 ± 19.1^{b}	193.9 ± 41.7^{ab}			

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Table 3-37a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
IL-4 pg/g	DR	31.7 ± 8.8	47.7 ± 10.5	31.8 ± 9.1	33.3 ± 10.0	48.1 ± 3.4	33.0 ± 10.1	0.487	0.771	0.273
	DIO	48.5 ± 9.7	28.6 ± 11.0	27.6 ± 7.8	38.7 ± 8.2	29.5 ± 6.2	30.8 ± 9.6			
IL-6 pg/g	DR	1796.1 ± 386.7	4714.4 ± 2542.0	1905.2 ± 378.0	1508.2 ± 425.3	2137.3 ± 252.0	2710.3 ± 323.5	0.664	0.398	0.224
	DIO	2345.4 ± 477.5	1454.1 ± 376.1	1748.6 ± 81.7	1554.6 ± 197.9	1248.5 ± 131.6	2006.0 ± 270.9			
IL-10 pg/g	DR	26.3 ± 4.0$^{\alpha}$	34.2 ± 8.2	20.6 ± 1.7	32.0 ± 6.9	27.7 ± 3.0	23.5 ± 2.8	0.518	0.144	0.231
	DIO	50.7 ± 10.8$^{a\beta}$	27.9 ± 6.6^{ab}	22.3 ± 2.8^{b}	28.6 ± 2.4^{ab}	22.5 ± 3.0^{ab}	28.6 ± 5.3^{ab}			
TNF-α pg/g	DR	137.7 ± 41.2	165.9 ± 41.7	199.8 ± 40.2	160.4 ± 41.1	103.9 ± 34.2	143.9 ± 53.4	0.092	0.508	0.529
	DIO	172.1 ± 71.8	140.7 ± 50.8	184.3 ± 45.2	90.5 ± 35.5	125.1 ± 43.1	111.8 ± 39.3			

[*] Concentrations are presented as mean ± SEM, $n = 5$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-37b. MLN cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure*	Phenotype	Concentration
CINC-2αβ pg/g	DR	33.1 ± 2.4
	DIO	36.9 ± 2.2
CINC-3 pg/g	DR	69.9 ± 15.1
	DIO	40.8 ± 3.9
GM-CSF pg/g	DR	27.3 ± 1.9
	DIO	24.9 ± 2.0
sICAM-1/sCD54 withng/g	DR	24.9 ± 2.3
	DIO	23.5 ± 1.9
IFN-γ ng/g	DR	1359.9 ± 137.2
	DIO	1127.1 ± 187.0
IL-1β ng/g	DR	1745.6 ± 119.6
	DIO	1590.3 ± 106.0
IL-2 pg/g	DR	169.7 ± 11.9
	DIO	178.4± 12.3
IL-4 ng/g	DR	37.6 ± 3.6
	DIO	33.9 ± 3.6
IL-6 pg/g	DR	2461.9 ± 450.5
	DIO	1726.2 ± 127.3
IL-10 pg/g	DR	27.4 ± 2.1
	DIO	30.1 ± 2.8
TNF-α pg/g	DR	151.9 ± 16.7
	DIO	137.4 ± 19.2
Protein Quantification by Bradford Assay** mg/g	DR	NC
	DIO	NC

* *n* = 30 for DR and DIO rats.

** Not completed (NC), as there was not enough tissue to perform the Bradford assay.

Table 3-38a. Ileal PP cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CINC-2αβ pg/g	DR	$23.0 \pm 3.1^{\alpha\beta}$	$16.0 \pm 2.5^{\alpha}$	$15.8 \pm 1.8^{\alpha\beta}$	$29.8 \pm 11.0^{\beta}$	$21.8 \pm 4.0^{\alpha\beta}$	$15.2 \pm 4.0^{\alpha}$	0.038	0.007	0.672
	DIO	$22.2 \pm 5.6^{ab\alpha\beta}$	$18.7 \pm 3.4^{a\alpha}$	$24.5 \pm 4.0^{ab\alpha\beta}$	$42.4 \pm 9.7^{b\beta}$	$26.5 \pm 2.0^{ab\alpha\beta}$	$18.2 \pm 1.9^{ab\alpha}$			
CINC-3 pg/g	DR	68.0 ± 47.0	32.3 ± 7.9	24.9 ± 2.3	32.9 ± 7.0	25.1 ± 1.3	24.0 ± 1.7	0.601	0.214	0.172
	DIO	19.8 ± 4.9	23.0 ± 3.3	22.9 ± 0.9	148.4 ± 94.3	26.5 ± 1.3	22.4 ± 2.5			
GM-CSF pg/g	DR	20.4 ± 3.9	16.9 ± 4.6	16.0 ± 1.6	19.3 ± 4.4	19.7 ± 4.9	22.9 ± 9.8	0.492	0.921	0.956
	DIO	21.3 ± 5.7	23.1 ± 6.8	18.9 ± 5.2	25.5 ± 5.5	17.3 ± 3.5	22.3 ± 5.9			
sICAM-1 /sCD54 ng/g	DR	29.3 ± 7.2	24.4 ± 5.4	24.0 ± 5.0	28.7 ± 3.7	20.2 ± 2.4	18.6 ± 2.5	0.976	0.889	0.681
	DIO	29.0 ± 8.4	21.0 ± 4.2	21.8 ± 5.6	26.5 ± 5.0	23.9 ± 3.4	32.4 ± 8.5			
IFN-γ pg/g	DR	321.5 ± 113.1	3016.7 ± 2744.1	227.4 ± 72.8	6589.0 ± 270.6	205.3 ± 23.3	236.5 ± 68.7	0.459	0.088	0.988
	DIO	1086.2 ± 815.1	602.4 ± 275.4	207.8 ± 29.4	2884.9 ± 2037.4	198.7 ± 28.8	401.3 ± 215.0			
IL-1β pg/g	DR	600.2 ± 112.6	497.0 ± 263.2	609.6 ± 197.6	1207.2 ± 266.6	727.9 ± 294.0	611.0 ± 327.7	0.929	0.545	0.417
	DIO	905.4 ± 290.1	848.0 ± 386.8	717.5 ± 206.1	484.3 ± 33.3	390.6 ± 94.5	480.3 ± 68.7			

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Table 3-38a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
IL-2 pg/g	DR	84.3 ± 23.5	72.7 ± 32.2	69.8 ± 27.8	66.2 ± 18.2	74.7 ± 28.4	65.7 ± 28.7	0.390	0.928	0.886
	DIO	87.6 ± 24.2	92.9 ± 32.0	70.5 ± 32.3	124.0 ± 39.0	67.9 ± 24.0	75.2 ± 20.8			
IL-4 pg/g	DR	13.4 ± 1.6	12.4 ± 2.0	13.1 ± 2.5	15.9 ± 2.4	13.9 ± 2.6	13.8 ± 2.1	0.400	0.565	0.753
	DIO	25.9 ± 15.6	11.3 ± 2.8	12.8 ± 2.0	24.1 ± 10.7	12.0 ± 1.3	13.5 ± 2.6			
IL-6 pg/g	DR	418.8 ± 95.1	425.8 ± 107.2	515.1 ± 155.4	876.2 ± 273.0	618.2 ± 200.9	468.6 ± 145.1	0.676	0.105	0.105
	DIO	373.2 ± 125.7	623.3 ± 202.2	453.6 ± 114.1	859.8 ± 195.9	407.1 ± 49.8	462.0 ± 203.2			
IL-10 pg/g	DR	12.4 ± 1.8	13.4 ± 1.1	12.5 ± 1.7	16.6 ± 1.8	18.8 ± 4.1	17.2 ± 2.9	0.151	0.496	0.446
	DIO	12.8 ± 2.1	10.7 ± 2.1	15.7 ± 1.9	13.4 ± 3.0	12.9 ± 2.8	13.7 ± 0.8			
TNF-α pg/g	DR	108.2 ± 4.7	131.7 ± 30.7	117.1 ± 17.4	126.6 ± 23.5	135.9 ± 31.4	139.2 ± 40.5	0.307	0.110	0.579
	DIO	148.6 ± 26.5	142.8 ± 43.9	127.5 ± 24.1	152.5 ± 27.0	127.4 ± 22.2	161.6 ± 36.4			

[*] Concentrations are presented as mean ± SEM, $n = 5$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-38b. Ileal PP cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure*	Phenotype	Concentration
CINC-2αβ pg/g	DR	20.3 ± 2.2
	DIO	25.4 ± 2.4
CINC-3 pg/g	DR	34.5 ± 7.9
	DIO	43.9 ± 16.8
GM-CSF pg/g	DR	19.2 ± 2.1
	DIO	21.4 ± 2.1
sICAM-1/sCD54 ng/g	DR	24.2 ± 1.9
	DIO	25.8 ± 2.4
IFN-γ ng/g	DR	777.7 ± 459.0
	DIO	896.9 ± 379.4
IL-1β ng/g	DR	708.8 ± 103.6
	DIO	637.7 ± 89.6
IL-2 pg/g	DR	72.2 ± 10.0
	DIO	86.3 ± 11.5
IL-4 ng/g	DR	13.8 ± 0.9
	DIO	16. ± 3.2
IL-6 pg/g	DR	553.8 ± 70.9
	DIO	529.8 ± 66.7
IL-10 pg/g	DR	15.2 ± 1.0
	DIO	13.2 ± 0.9
TNF-α pg/g	DR	126.4 ± 10.3
	DIO	143.4 ± 11.7
Protein Quantification by Bradford Assay* mg/g	DR	NC
	DIO	NC

* n = 30 for DR and DIO rats.
** Not completed (NC), as there was not enough tissue to perform the Bradford assay.

Table 3-39a. Epididymal fat cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
CINC-1 pg/g	DR	164.7 ± 35.0	164.3 ± 21.5	201.5 ± 52.4	221.3 ± 52.1	215.1 ± 45.8	105.6 ± 31.9	0.113	0.403	0.202
	DIO	222.6 ± 62.1	131.7 ± 35.1	68.0 ± 20.8	133.3 ± 20.4	164.4 ± 45.2	171.7 ± 56.8			
CINC-2 pg/g	DR	762.7 ± 130.5	661.4 ± 124.8	866.4 ± 154.2$^{\alpha}$	645.7 ± 150.5	840.3 ± 127.4	589.2 ± 139.2	0.260	0.449	0.194
	DIO	949.0 ± 206.9^{a}	590.0 ± 154.3	346.5 ± 131.6$^{c\beta}$	573.0 ± 88.7	644.5 ± 119.7	697.8 ± 167.7			
CINC-3 pg/g	DR	255.7 ± 63.6	277.9 ± 52.8	287.9 ± 77.8	289.4 ± 80.5	366.8 ± 87.5	227.9 ± 75.3	0.274	0.554	0.434
	DIO	345.1 ± 98.6	177.5 ± 42.5	91.8 ± 31.5	237.5 ± 43.4	227.7 ± 67.4	287.7 ± 89.3			
IL-1β pg/g	DR	570.5 ± 93.4	665.3 ± 127.4	697.2 ± 100.0	740.7 ± 141.7	733. ± 96.2	582.8 ± 126.2	0.461	0.476	0.578
	DIO	574.9 ± 137.5	575.9 ± 150.7	723.4 ± 155.9	646.5 ± 138.0	902.4 ± 125.2	897.3 ± 135.3			
IL-6 pg/g	DR	726.9 ± 167.3	673.6 ± 79.7	981.6 ± 226.9	969.2 ± 231.0	962.7 ± 212.8	554.2 ± 81.6	0.434	0.888	0.150
	DIO	1009.4 ± 203.6	781.2 ± 143.2	559.6 ± 117.5	666.5 ± 99.2	608.7 ± 92.1	771.8 ± 271.8			
IL-10 pg/g	DR	632.6 ± 120.9	650.3 ± 120.7	679.3 ± 85.7	837.1 ± 182.0	978.3 ± 149.9	613.8 ± 131.5	0.385	0.612	0.399
	DIO	791.5 ± 72.7	655.7 ± 110.8	522.4 ± 95.8	699.0 ± 123.0	655.8 ± 148.6	645.4 ± 159.7			

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Table 3-39a *continued*.

		Control	High Energy Diets					Statistics - *P* value		
Measure		**AIN-93G**	**AIN-93G**	**Wheat Bran**	**Oat Bran**	**Resistant Starch II**	**FOS**	**Phenotype**	**Diet**	**Phenotype × Diet**
TNF-α pg/g	DR	19.5 ± 0.0$^{\alpha}$	19.5 ± 0.0	19.5 ± 0.0	19.5 ± 0.0	19.5 ± 0.0	29.8 ± 10.4	0.141	0.418	0.363
	DIO	40.2 ± 14.5$^{\beta}$	19.5 ± 0.0	34.9 ± 13.2	19.5 ± 0.0	21.5 ± 2.1	25.8 ± 6.4			
Active TGF-β1 pg/g	DR	1176.7 ± 72.0^{a}	1884.2 ± 295.7$^{b\alpha}$	1095.0 ± 97.9$^{a\beta}$	1308.6 ± 86.4	1330.0 ± 114.6$^{\beta}$	1397.9 ± 151.0$^{\beta\gamma}$	0.292	0.012	0.133
	DIO	1374.8 ± 162.5	1549.7 ± 133.0$^{\alpha}$	1390.8 ± 163.4$^{\beta}$	1230.3 ± 106.1	1101.1 ± 97.7$^{\beta}$	1034.2 ± 141.9$^{\beta\delta}$			
Total TGF-β1 pg/g	DR	2312.8 ± 198.5	2806.3 ± 380.8$^{\alpha}$	2030.6 ± 340.4$^{\beta}$	2052.8 ± 178.9$^{\beta}$	2300.531 ± 239.5	2634.6 ± 279.0	0.262	0.026	0.830
	DIO	2505.2 ± 146.0	2578.2 ± 245.8$^{\alpha}$	1966.0 ± 179.0$^{\beta}$	1911.4 ± 185.5$^{\beta}$	2090.9 ± 187.2	2158.5 ± 165.0			

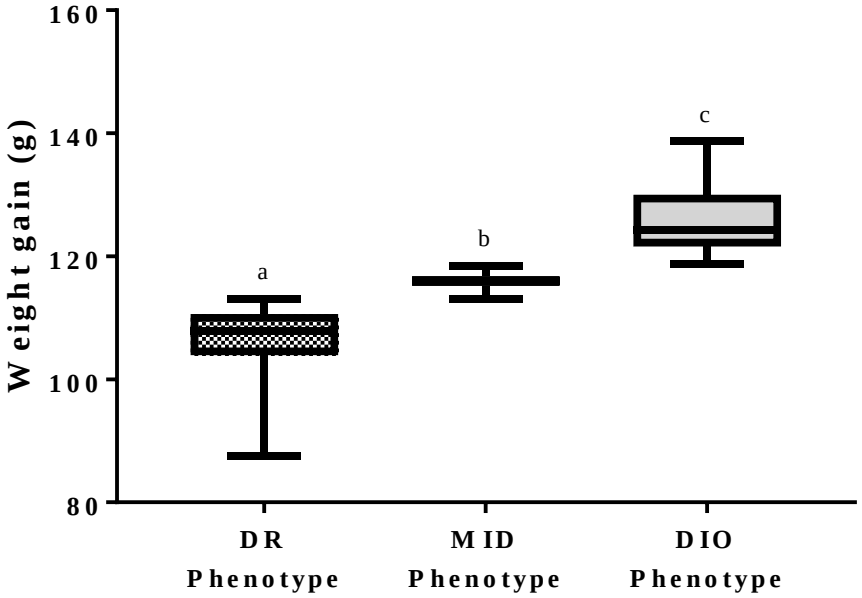
[*] Concentrations are presented as mean ± SEM, $n = 10$. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. Superscripted letters denote significant differences between diets within a phenotype, and symbols denote significant differences between overall diet or overall phenotype as determined by Tukey's multiple comparison test.

Table 3-39b. Epididymal fat cytokine/chemokine concentrations of DR and DIO rats after consuming AIN-93G modified diets for 11-12 weeks, separated by phenotype.

Measure*	Phenotype	Concentration
CINC-1 pg/g	DR	178.7 ± 17.0
	DIO	148.6 ± 18.0
CINC-2 pg/g	DR	727.6 ± 55.6
	DIO	633.5 ± 62.9
CINC-3 pg/g	DR	284.2 ± 29.3
	DIO	227.9 ± 28.2
IL-1β pg/g	DR	665.0 ± 46.0
	DIO	720.1 ± 57.8
IL-6 pg/g	DR	811.4 ± 73.1
	DIO	732.8 ± 68.1
IL-10 pg/g	DR	731.9 ± 55.5
	DIO	661.6 ± 48.8
TNF-α pg/g	DR	21.2 ± 1.7
	DIO	26.9 ± 3.5
Active TGF-β1 pg/mL	DR	1365.4 ± 69.2
	DIO	1280.2 ± 58.1
Total TGF-β1 pg/mL	DR	2356.3 ± 115.1
	DIO	2201.7 ± 80.3
Protein Quantification by Bradford Assay mg/g	DR	7.0 ± 0.5
	DIO	7.1 ± 0.5

* n = 60 for DR and DIO rats.

a)



b)

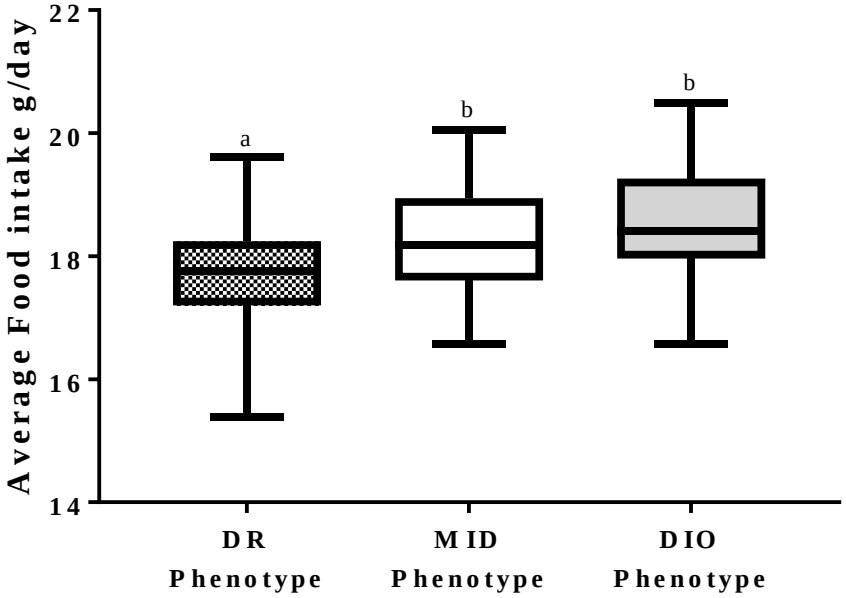


Figure 3-1. Rat weight gain and food consumption during the two-week preliminary trial on the HE control diet. Significance ($P < 0.05$) for phenotype was determined by one-way ANOVA. **a**. Weight gained; rats in the upper 40% weight gain were considered DIO ($n = 70$), rats in the lower 40% weight gain were considered DR ($n = 70$), and rats in the middle were labelled as MID ($n = 36$). Phenotypes were significantly different from each other ($P < 0.001$) and bars with differing letters represent differences between phenotypes. **b**. Average amount of food consumed per day.

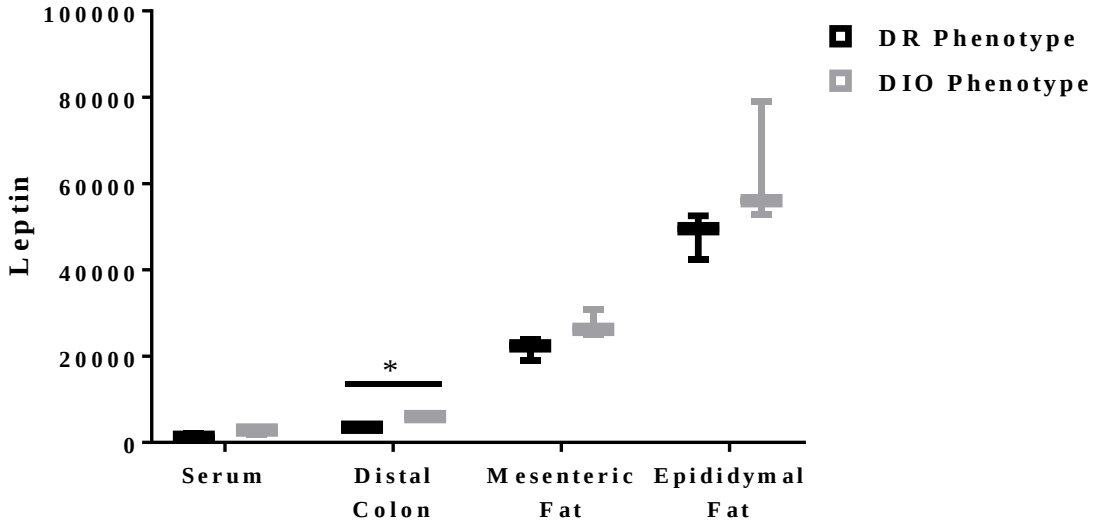
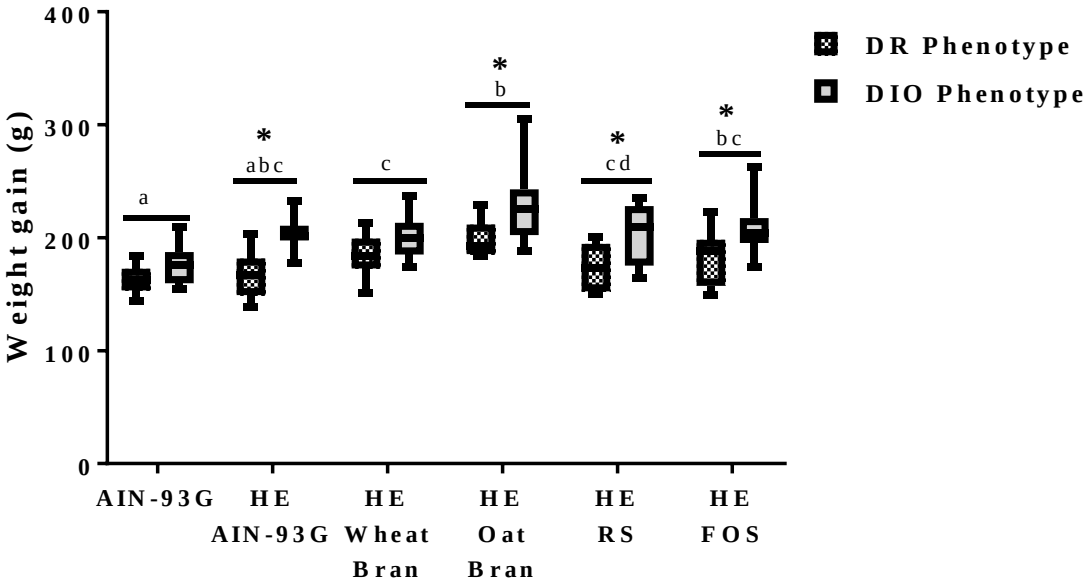


Figure 3-2. Serum leptin (pg/mL) and tissue leptin (pg/g) concentrations measured in rats from the preliminary trial. Statistical analyses were performed using unpaired *t*-tests (n = 3). * denotes a significant difference between DR and DIO rats in the distal colon (P < 0.001).

a)



b)

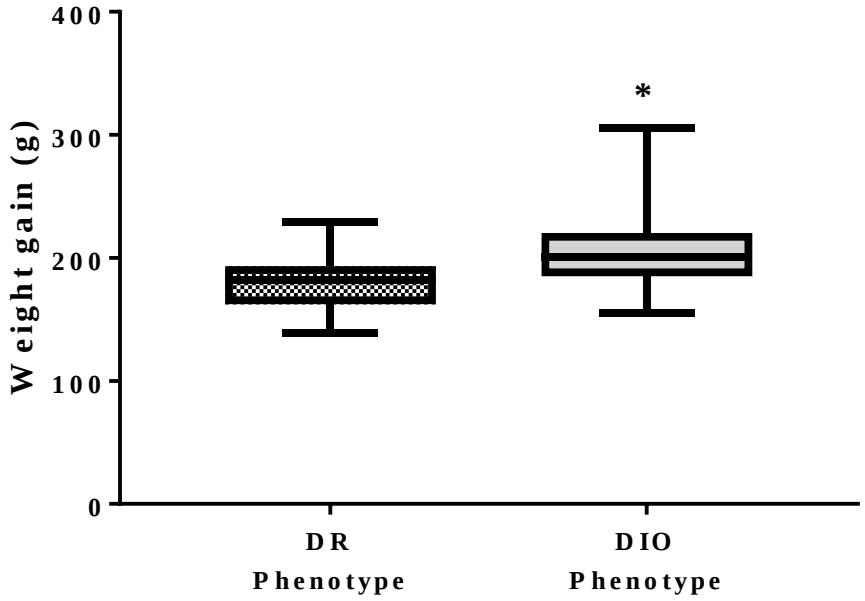
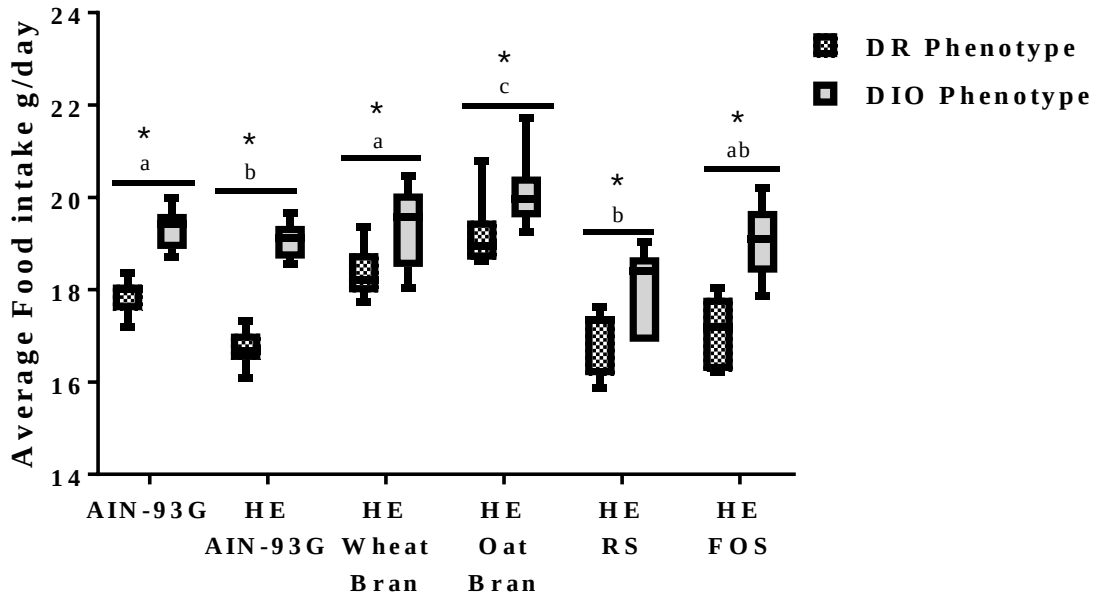


Figure 3-3. Weight gained after rats consumed AIN-93G modified diets for 11-12 weeks. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. **a**. Weight gain separated by diet ($n = 10$), * denotes a significant difference between DR and DIO rats on a specific diet, and bars with differing letters represent significant differences between diets overall as determined by Tukey's multiple comparison test. **b**. Weight gain separated by phenotype only ($n = 60$). DIO rats had higher weight gain than DR rats ($P < 0.001$).

a)



b)

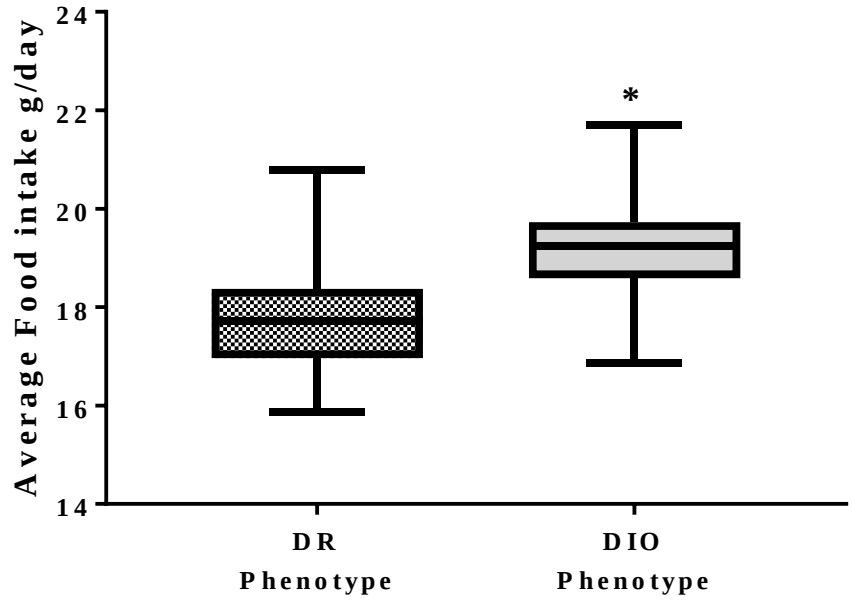
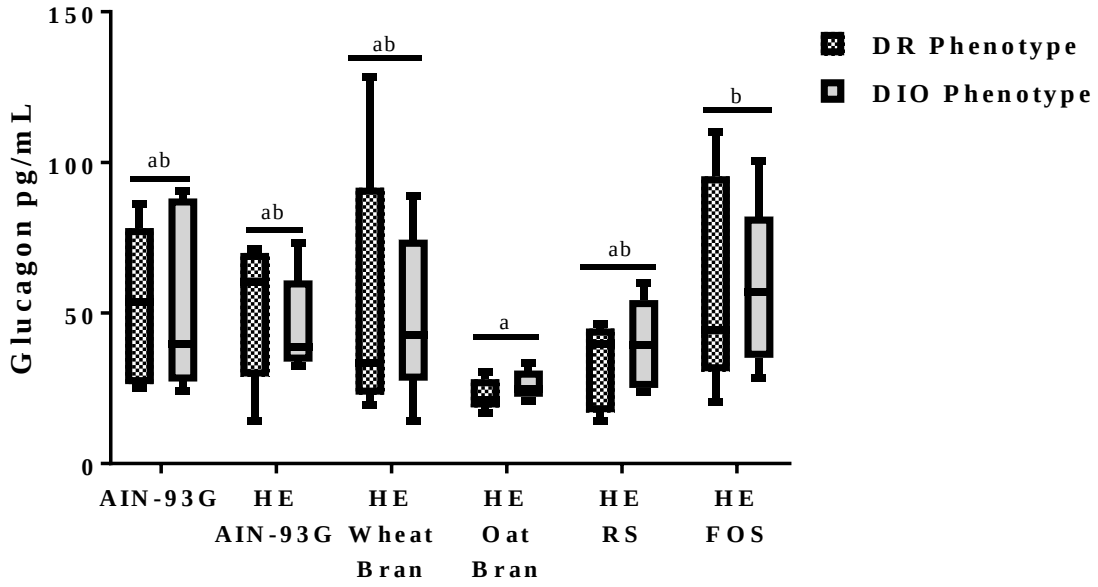


Figure 3-4. Average daily food consumption of AIN-93G modified diets over 11-12 weeks. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. **a**. Food consumption separated by diet group ($n = 10$), * denotes a significant difference between phenotypes on specific diets, and bars with differing letters represent significant differences between diets overall as determined by Tukey's multiple comparison test. **b**. Food consumption separated by phenotype only ($n = 60$). DIO rats consumed more food than DR rats ($P < 0.001$).

a)



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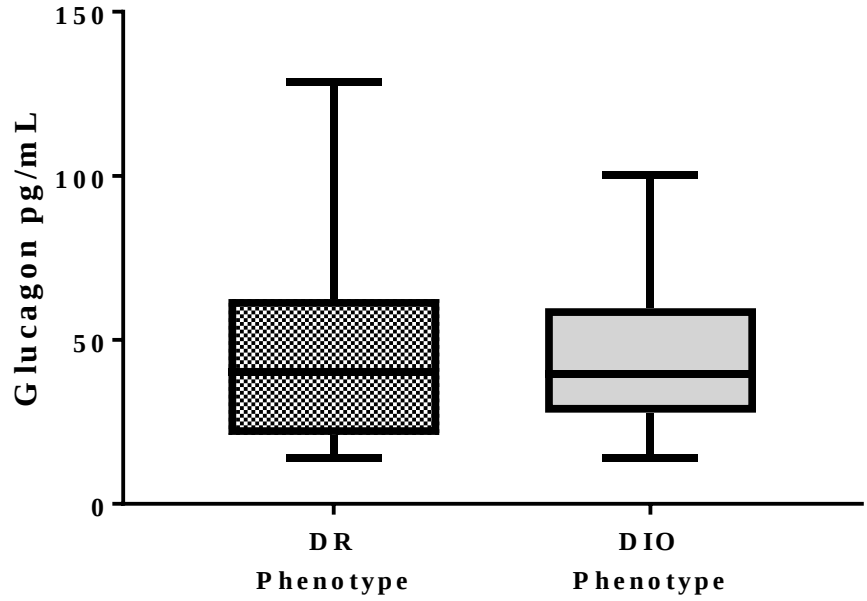
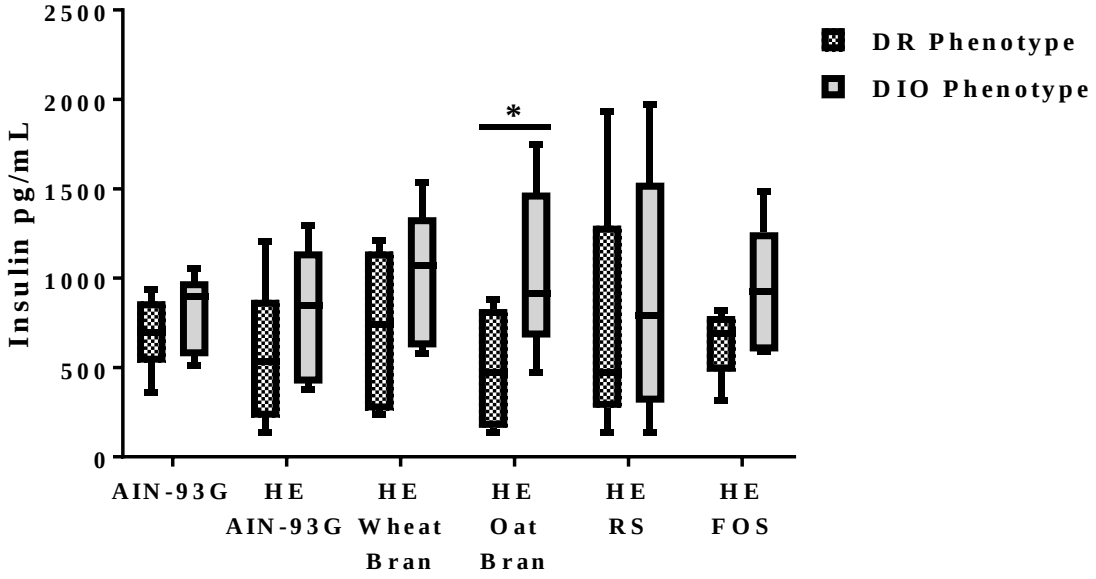


Figure 3-5. Serum glucagon concentrations measured in rats after consuming AIN-93G modified diets for 11-12 weeks. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. **a**. Serum glucagon concentrations separated by diet group ($n = 5$), Bars with differing letters represent significant differences between diets overall as determined by Tukey's multiple comparison test. **b**. Serum glucagon concentrations separated by phenotype ($n = 30$).

a)



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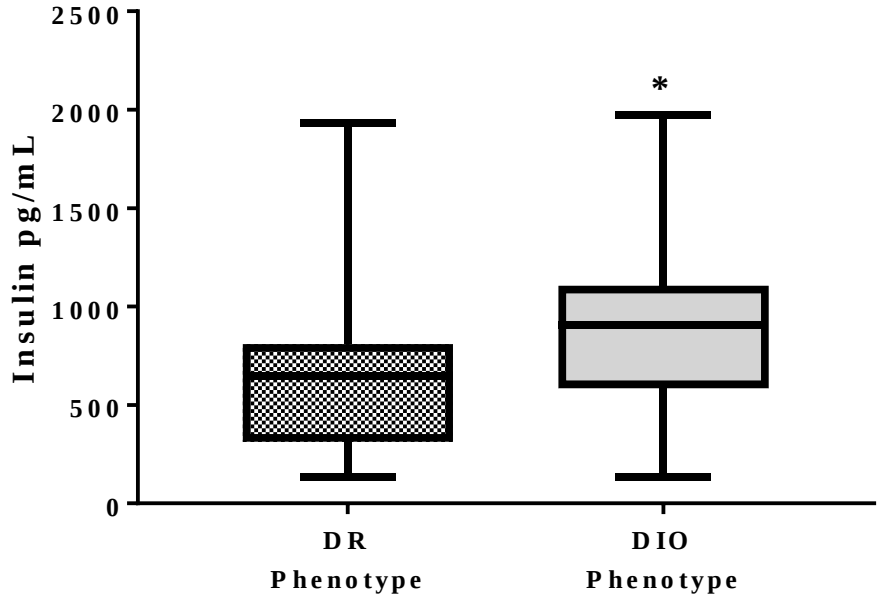
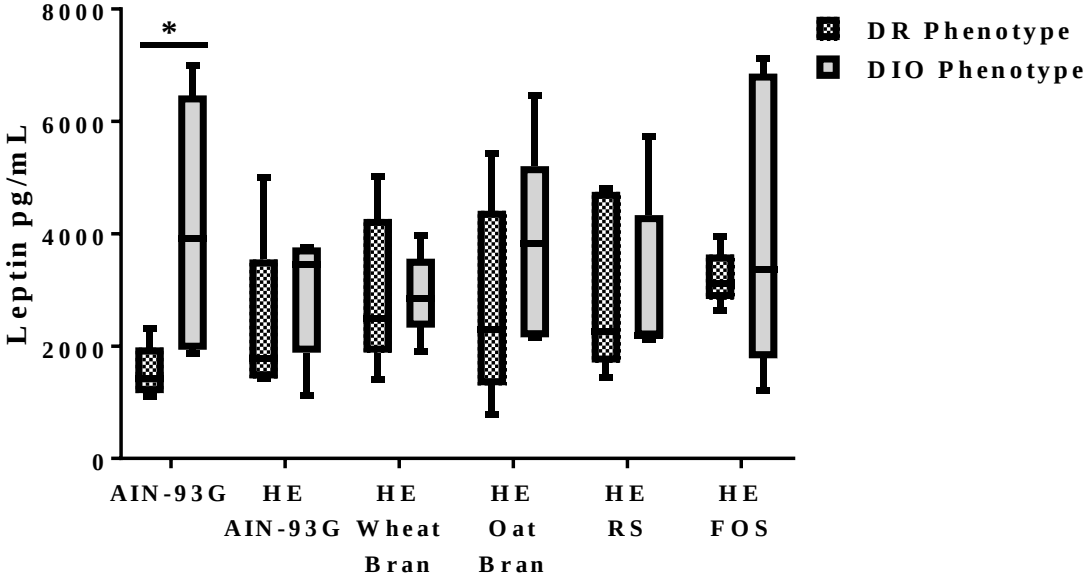


Figure 3-6. Serum insulin concentrations measured in rats after consuming AIN-93G modified diets for 11-12 weeks. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. **a**. Insulin concentrations separated by diet group ($n = 5$), * denotes a significant difference between DR and DIO rats on the HE oat bran diet ($P = 0.032$). **b**. Insulin concentrations separated by phenotype ($n = 30$). DIO rats displayed higher serum insulin concentrations than DR rats ($P = 0.013$).

a)



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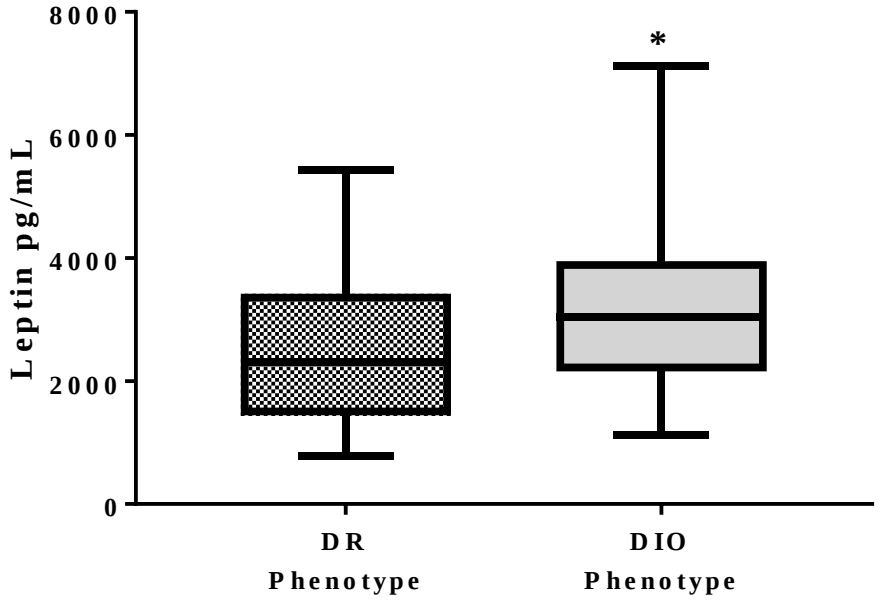
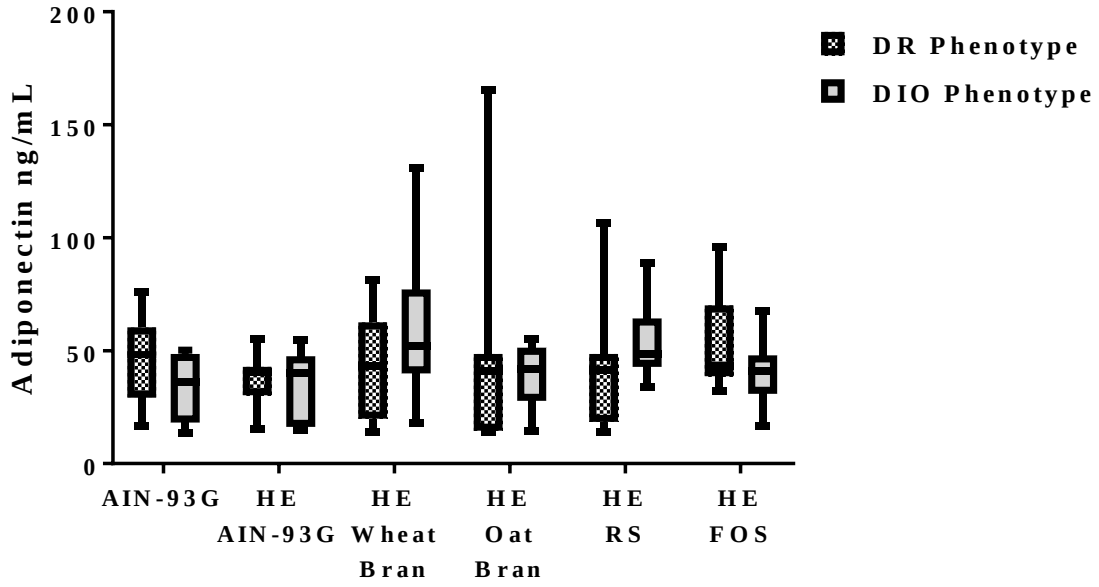


Figure 3-7. Serum leptin concentrations measured in rats after consuming AIN-93G modified diets for 11-12 weeks. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. **a**. Leptin concentration separated by diet group ($n = 5$), * denotes a significant difference between DR and DIO rats on the control diet ($P = 0.004$). **b**. Leptin concentrations separated by phenotype ($n = 30$), DIO rats displayed higher serum leptin concentrations than DR rats ($P = 0.034$).

a)



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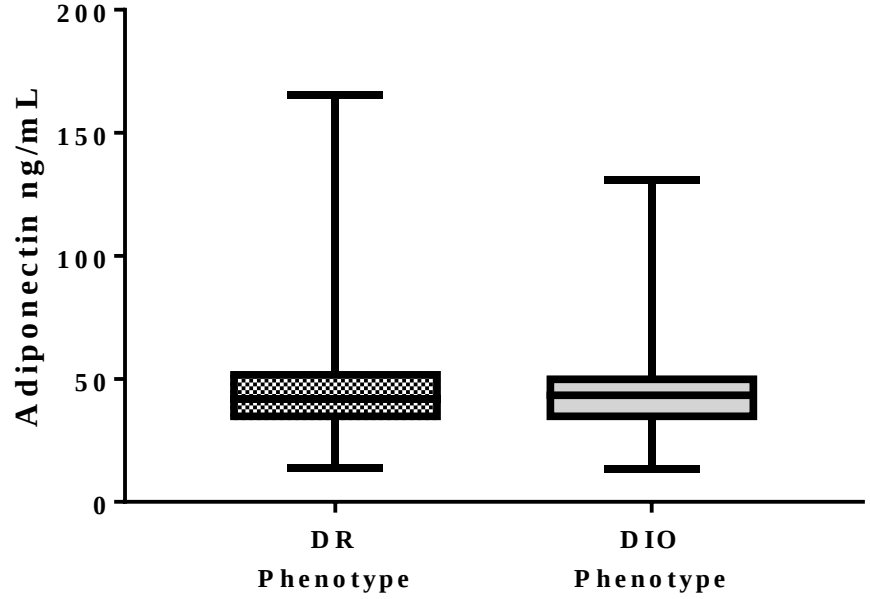
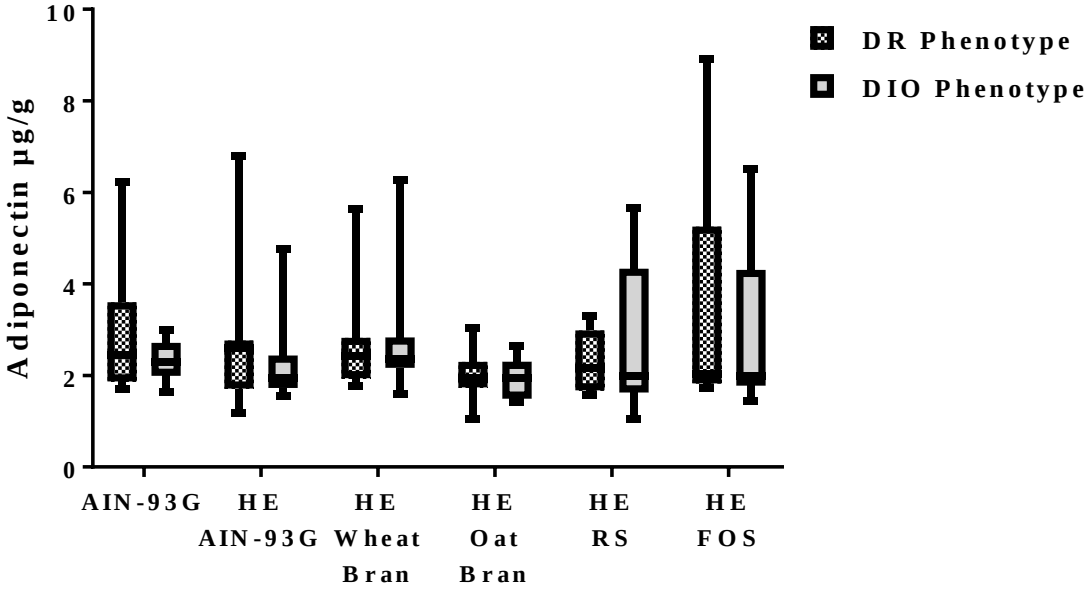


Figure 3-8. Serum adiponectin concentrations measured in rats after consuming AIN-93G modified diets for 11-12 weeks. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. **a**. Adiponectin concentrations separated by diet group ($n = 10$). **b**. Adiponectin concentrations separated by phenotype only ($n = 60$).

a)



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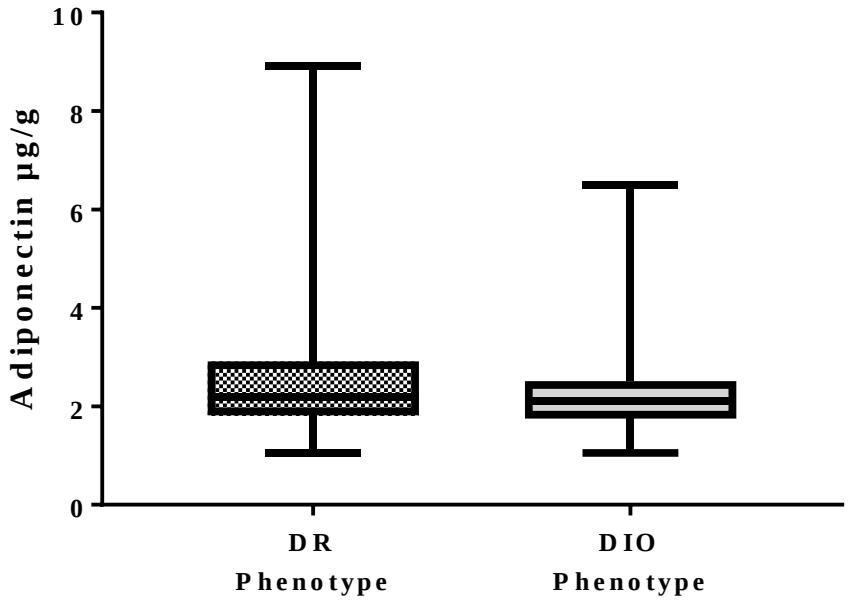
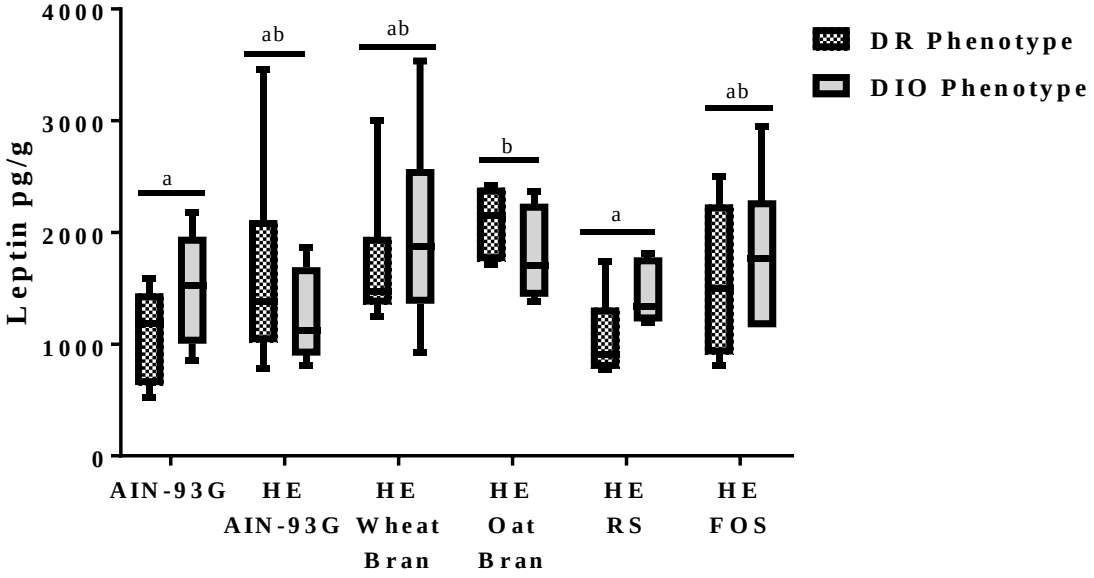


Figure 3-9. Epididymal fat adiponectin concentrations measured in rats after consuming AIN-93G modified diets for 11-12 weeks. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. **a**. Adiponectin concentrations separated by diet group (n = 10). **b**. Adiponectin concentrations separated by phenotype (n = 60).

a)



b)

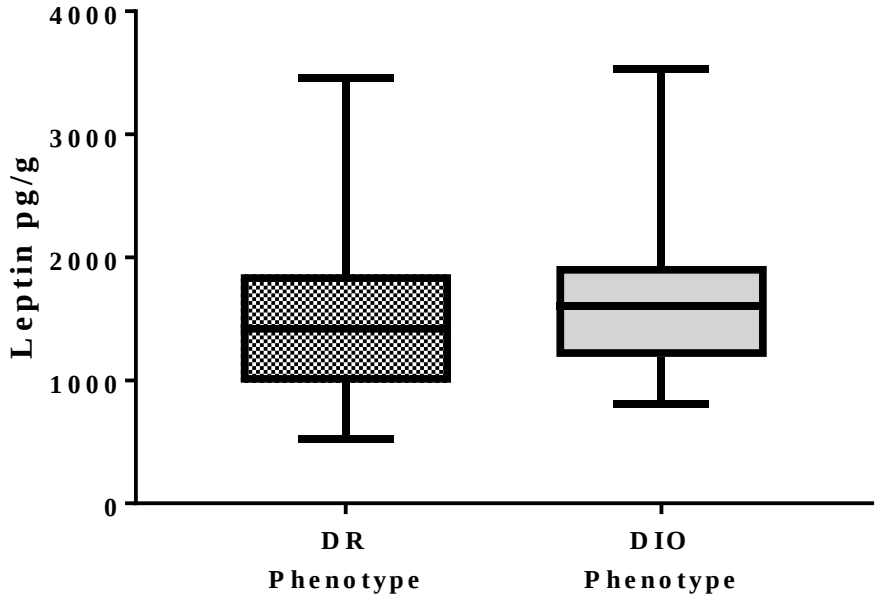


Figure 3-10. Distal colon leptin concentrations measured in rats after consuming AIN-93G modified diets for 11-12 weeks. Significance ($P < 0.05$) for phenotype, diet, and interaction between phenotype × diet was determined by two-way ANOVA. **a**. Leptin concentrations separated by diet group ($n = 6$). Bars with differing letters represent significant differences between diets overall as determined by Tukey's multiple comparison test. **b**. Leptin concentrations separated by phenotype only ($n = 36$).

Conclusions

In summation of these three distinct studies, the main goal of this research project was to understand the impact of diet on immunity in realistic settings. Human subjects, bovine and rat models were used to investigate the influence of dietary interventions on the immune system. Although these were diverse models, the findings demonstrated that dietary components do influence immune activity to an extent.

In the first study, Holstein dairy cattle were utilized as model to investigate how altering the feeding regime with omega-3 fatty acid supplementation and altering the environment between separate feed management systems affects the immune system. Holstein cattle are an outbred population, they are considered to be genetically diverse, and are a breed of cattle that are ranched in Canada using both traditional and industrial farming practices. The results of this study have shown that supplementation with omega-3 fatty acids in dairy cattle, especially in the form of microalgae, improved responses to immunization with KLH. However, the strongest influence on the immune response was the feed management system, which suggests cattle respond to immunization more efficiently in a pasture grazing environment than when confined in barn tie stalls. It is possible that cattle freely grazing pasture may have encountered different PPR ligands such as bacterial or fungal components which could have bolstered the immune response, or that cattle confined in the barn may have been exposed to stressors which contributed to suppressed immunization responses.

In future bovine studies investigating immunization responses during alterations to feed management systems or dietary supplements, it will be important to consider sampling time points, the types of samples that will be collected, and the use of a vaccine relevant to a cattle population. Small volume blood draws to sample antibody production should be

collected more frequently (every one to two days) so that the kinetics in the time to respond to immunization may be studied. It is unknown in the current trial if dietary omega-3 supplements or feed management systems affected the rate of Ig production based on the chosen sampling points, and this information would be useful to determine the impact on timing of a maximal response. To further investigate mucosal responses to immunization, other mucosal secretions besides milk should be collected for Ig analysis, such as nasal or salivary secretions or faecal samples. It will also be meaningful to determine if feed management systems and omega-3 dietary supplements influence immunization responses in cattle to infectious agents. Immunization with the bovine vaccine J-VAC® (Merial Canada Inc., Baie d'Urfé, Québec, Canada), which confers protection against *E. coli* mastitis and endotoxemia caused by *E. coli* and *S. typhimurium*, could be a relevant strategy to study antibody production in this context.

Human subjects were utilized in the second study to investigate how supplementing regular daily diets of healthy adults with β2-1 fructans influences the immune system. In this experiment, there were no controls on environmental factors, and the subjects would have been genetically diverse. β2-1 fructans have been widely claimed as beneficial to the health of those who consume them. Results of this human clinical trial suggested that immune activity had been impacted at a systemic level in healthy people, while consuming ordinary diets supplemented with β2-1fructans. These findings of increased serum IL-4, GM-CSF and LPS concentrations, decreased serum IL-10 concentrations, increased circulating percentages of $CD282^{+}/TLR2^{+}$ mDC and increased *ex vivo* responsiveness to TLR2 ligand stimulation do not necessarily reflect a benefit to the immune system in healthy individuals. For example, increases in IL-4 could offer potential enhancement in

response to immunization, however decreases in regulatory cytokine IL-10 could be detrimental while fighting an infection. Alterations in immune activity are context dependent, and although some disease-states may benefit from alterations in circulating cytokines and immune cell populations, others may not. Other researchers involved in the trial investigated gut community structure and determined that β2-1 fructan supplementation increased faecal Bifidobacteria, however this change was not related to any of the measured immunological parameters.

Human diet intervention studies are very useful; however, they do have limitations. Participant compliance is extremely important for the validity of the results. In this trial, participant compliance rates were high, as the placebo phase compliance was 97.6 % and the β2-1 fructan phase compliance was 99.9 %. However, compliance rates were determined based on the amount of returned unopened supplement sachets. In a study conducted by Lomax *et al*. (2012), compliance rates were calculated on the total amount of returned supplement sachets, including both consumed and unconsumed. This may be a more effective way to measure compliance and to deter dishonesty. It may also be useful to control for BMI, as the average BMI in the current trial was 24.2 kg/m^2, and several participants would have been considered to be over-weight. It could be important to ask participants about social habits during pre-screenings, as cigarette smoking has been shown to influence TLR2 and TLR4 expression on monocytes (Versteeg *et al*., 2009), and binge drinking has been shown to increase serum and bacterial DNA levels in healthy subjects (Bala *et al*., 2014). Future studies could include a routine flu vaccination strategy to examine impacts of dietary interventions on response to vaccination, as a previous study investigating β2-1 fructan supplementation found alterations in responsiveness (Lomax *et*

al., 2015). Additionally, it may be important to examine male and female subjects separately, as this trial demonstrated that healthy subjects did differ between immune cell markers and cytokines when separated by sex.

For the final study, a rat model was used to investigate the impact of supplementing a HE diet with DFM on immune activity and obesity-related biomarkers. Sprague Dawley rats, an outbred and genetically diverse strain with a history of experimental use in medical and metabolic studies, were utilized and selected for obese or non-obese phenotypes based on their genetic propensity to put on weight in a controlled environmental setting. After the development of an obese state in rats over a two-week period on a HE diet, interventions with DFM were not able to ameliorate obesity, and the obese and non-obese phenotypes remained distinct in not only their body weight, but also in their relative levels of systemic and local inflammation. The DFM-supplemented HE diets were not capable of influencing immune activity to counter the deleterious effect of the high fat diet.

Several results of this obese-rat study were surprising, including the fact that DFM did not appear to decrease inflammation in DIO rats and the histological analysis of gut tissue did not reveal differences between diets in DIO rats. Other studies in mouse models have previously demonstrated that high-fiber diets and SCFA production have decreased allergic inflammation in the lung (Trompette *et al*., 2014), suppressed allergic airway disease through enhancing the function of T_{reg} cells (Thorburn *et al*., 2015), and enhanced oral tolerance while contributing to protection from food allergy (Tan *et al*., 2016). It was anticipated that the increase in SCFA production through fermentation of the selected DFM used in this current trial would have led to dampening down of inflammatory mediators in the obese rats, and that there would be distinct differences in proinflammatory biomarkers,

and the extent to which they were affected by DFM, between obese and non-obese rats. The ceca of rats on the highly fermented diets, HE RS and HE FOS, were distinguishable from ceca collected from rats on other diets due to their shriveled, stretched or distended appearance. However, histological analysis using haemotoxylin and eosin staining of gut tissue above and below anatomical location of the caecum did not reveal differences in tissue level inflammation between any of the HE diets compared the control. Perhaps in future HE DFM supplemented diet studies in rat models, alternate staining with agents such as methyl green or methylene blue (Lee's Stain) could be used to examine differences during histological analysis of tissues, or immunohistochemistry approaches specific for lymphocyte subsets (Ward *et al*., 2006) or macrophage M1 and M2 markers could be implemented to interrogate effects of diet at the tissue level.

It was also unexpected that there were not more pronounced differences in obesity-associated biomarkers between DR and DIO rats. Based on results collected from the human β2-1fructan trial, it was predicted that the rats on the HE FOS diet would have had higher levels of inflammation in comparison to the control diets. Healthy Sprague Dawley rats have been previously shown to adapt to high fat and high fructose diets after a three-month intervention period (Stark *et al*., 2000). These rats did not develop the classical signs of insulin resistance or impaired glucose tolerance during the dietary intervention period (Stark *et al*., 2000), an effect that was also noted in the current study. To combat this resistance to metabolic dysfunction, the Wistar rat breed could be used as model since the effects caused by a HE diet appear to be more pronounced and these rats will display worsened features of metabolic syndrome (Marques *et al*., 2016), thought to be associated with differences in gut bacterial community structure between the two breeds. However,

using Wistar rats would not allow for the direct comparison between obese-resistant and obese animals, and Sprague Dawley rats have been repeatedly used as an effective model to investigate two distinct phenotypes while on the same diet (Levin *et al*., 1997, Aziz *et al*., 2009, Barbier de la Serre *et al*., 2010).

The most sensitive measures of the influence of HE diet and the DIO phenotype on immune activity in this rat model were CINC-2αβ and TGF-β1, as this proinflammatory chemokine and regulatory cytokine were most frequently associated with differences between diet groups or phenotypes. It was unfortunate that the M1 and M2 macrophage immunofluorescent cell markers utilized in this study were not suitable with our flow cytometry application. Although efforts were made to examine cytokine profiles of M1 and M2 macrophages, distinctive alterations were not found between phenotype or diet groups. It would have been very interesting to determine if the DFM supplemented in the HE diet influenced the M1/M2 macrophage phenotype in DIO rats. It is possible rat-specific flow cytometry antibodies for M1 and M2 macrophages may have increased in availability since this rat trial took place, increasing the likelihood for successful flow cytometry measurements. An alternate approach would be to use real-time polymerase chain reaction (qPCR) to investigate the relative levels of gene expression associated with these macrophage phenotypes (Martinez and Gordon, 2014); this would be an interesting avenue to explore in future studies.

To further interrogate a possible mechanism for differences between phenotypes in the onset of obesity and links with inflammation, other variables could be measured. Animal gene knockout models may be beneficial in determining molecular mechanisms of obesity development, as studies in mice have shown that animals lacking TLR4 have

protection from obesity-associated inflammation and insulin resistance (Jia *et al*. 2014). The reason DIO rats gained more weight throughout this current trial was due to their increased daily food consumption. It is possible that differences in metabolism may have been linked to adipokine production, as DIO rats produced higher concentrations of leptin than DR rats throughout the trial. The peptide hormone ghrelin is involved in controlling hunger and could be influenced by leptin, and it would be interesting to measure potential fluctuations of this hormone in DR and DIO rats. In this study, I was unable to determine whether inflammation proceeded obesity since there were no measures to assess the level of inflammation before the introduction of the HE diet in the preliminary phase of the trial. For future studies, it will be important to perform a small volume blood draw following the acclimatization period and prior to starting dietary interventions to assess whether inflammation precedes obesity, occurs alongside obesity, or if it is an outcome caused by obesity in this rat model.

In conclusion, this research investigated the impact of diet on immunity in realistic settings and determined that immune activity is affected by dietary interventions of omega-3 fatty acids, β2-1 fructans, and HE diets supplemented with various DFM. However, the outcomes of the impact of diet on immune activity may differ in intensity due to environmental factors, exercise and lifestyle, health status, genetic background or obesity, reflecting the complex interactions involved.

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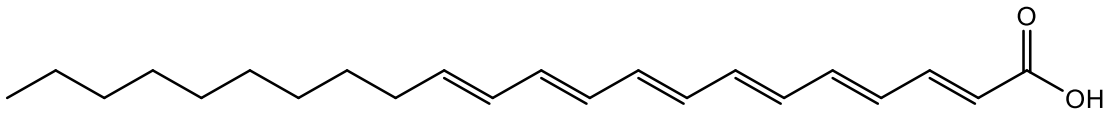
Appendix A

a)



b)

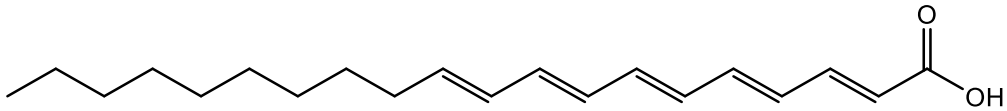


Figure A1. Diagrams of basic chemical structures of omega-3 fatty acids in the microalgae and fish oil supplements of AIF Trial #7. **a.** Docosahexaenoic Acid (DHA) 22:6(n-3). **b**. Eicosapentaenoic Acid (EPA) 20:5(n-3). These structures were drawn using ChemDraw Professional 15.1 (PerkinElmer Informatics, Waltham, MA, USA).

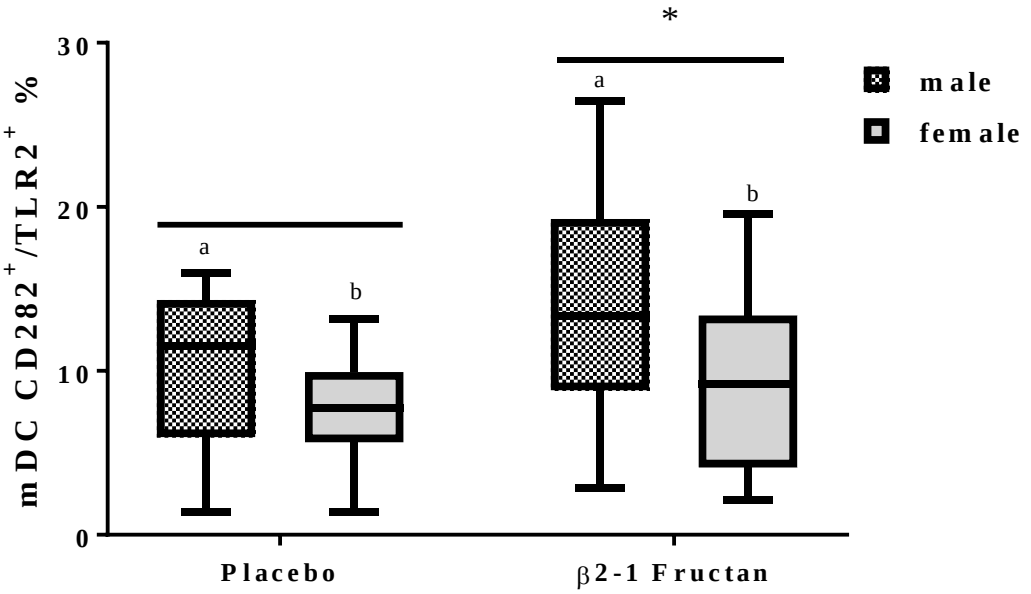
Appendix B

Table B1. Demographic data of individual participants at study baseline.

ID	Gender	Age	Height (m)	Weight (kg)	BMI (kg/m^2)	Sys BP[1]	Dia BP
001	M	26	1.790	73.4	22.91	135	75
002	M	28	1.846	90.3	26.50	135	76
003	F	27	1.496	50.4	22.52	107	70
004	F	29	1.699	59.4	20.58	115	79
005	F	25	1.670	69.5	24.92	112	84
006	M	34	1.830	79.0	23.59	131	83
007	M	28	1.830	71.7	21.41	123	82
008	F	27	1.635	52.0	19.45	104	69
009	F	20	1.603	52.7	20.51	112	68
010	F	23	1.617	70.2	26.85	115	68
011	F	25	1.634	57.9	21.69	94	65
012	M	25	1.770	73.4	23.43	135	79
013	F	32	1.765	72.0	23.11	132	89
015	M	27	1.780	78.4	24.74	120	63
016	M	36	1.718	73.3	24.83	139	87
018	M	35	1.657	80.0	29.14	130	65
022	F	26	1.595	57.5	22.60	121	87
023	F	22	1.622	67.2	25.54	116	82
024	M	24	1.860	91.3	26.39	152	94
025	M	25	1.770	75.5	24.10	136	76
026	F	24	1.695	66.6	23.18	116	68
027	F	30	1.630	77.0	28.98	111	82
028	M	29	1.680	82.8	29.34	119	86
029	F	42	1.515	58.0	25.27	122	93
030	F	24	1.600	57.8	22.58	110	78
031	M	38	1.840	96.7	28.56	122	76
032	F	34	1.655	62.4	22.78	108	81
033	M	23	1.660	66.2	24.02	127	72
034	F	25	1.652	81.0	29.68	114	79
035	F	30	1.665	50.0	18.04	120	80
Median		27	1.668	71.0	23.8	120	79
Mean		28.1	1.693	69.8	24.2	121	78
95% CI		26.3 – 29.9	1.66 – 17.3	65.4 – 74.2	23.2 – 25.3	116.7 – 125.	74.9 – 80.9

[1] Sys BP, systolic blood pressure; Dia BP, diastolic blood pressure.

a)



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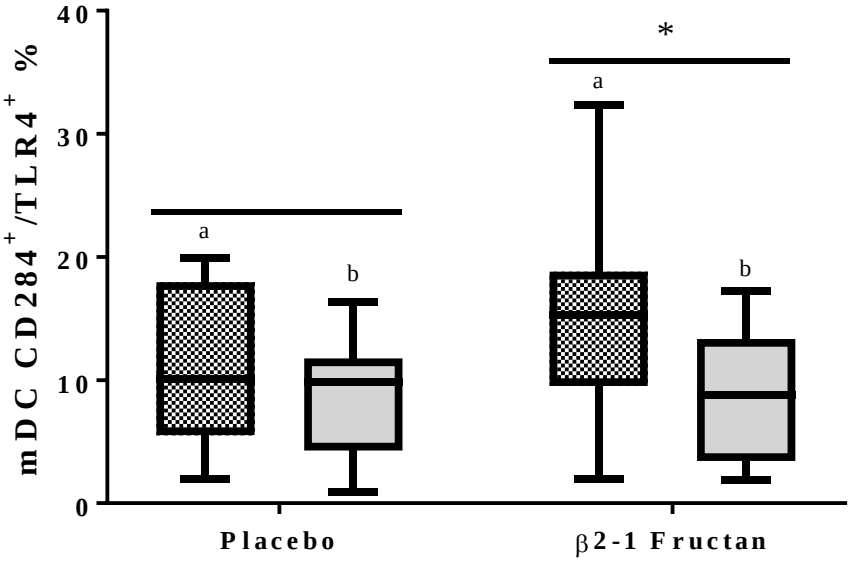

Figure B1. Peripheral mDC CD282$^+$/TLR2$^+$ and CD284$^+$/TLR4$^+$ percentages on day 28 of placebo and β2-1 fructan phases, separated by sex. Significance ($P < 0.05$) for gender and phase were determined by rANOVA ($n = 13$ for males, $n = 17$ for females). **a**. Peripheral mDC CD282$^+$/TLR2$^+$ percentages; * denotes a significant difference between phases ($P = 0.016$), and bars with differing letters represent significant differences between sex as determined by Tukey's HSD ($P = 0.025$). **b**. Peripheral mDC CD284$^+$/TLR4$^+$ percentages; phase was significantly different ($P < 0.05$) as was sex ($P = 0.003$).

Table B2. Impact of supplement on serum LPS, LBP and cytokine/chemokine concentrations in males and females.

Cytokine	Sex	Placebo d-0	Placebo d-28	β2-1 Fructan d-0	β2-1 Fructan d-28
LPS (EU/mL)	male[1]	0.32 ± 0.08	0.43 ± 0.12	0.35 ± 0.07	0.38 ± 0.07[3]
	female[2]	0.67 ± 0.14	0.61 ± 0.12	0.67 ± 0.11	0.80 ± 0.16
LBP (μg/mL)	male	11.1 ± 1.2	14.8 ± 2.9	11.7 ± 1.6	10.9 ± 1.1
	female	14.2 ± 2.0	15.4 ± 2.2	15.5 ± 2.3	16.7 ± 2.2
sCD-40L (ng/mL)	male	14.2 ± 10.8	14.7 ± 11.2	13.6 ± 10.9	12.1 ± 9.1
	female	0.8 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.4 ± 0.1
G-CSF (pg/mL)	male	11.0 ± 6.4	11.6 ± 4.6	3.4 ± 3.1	4.6 ± 3.3
	female	12.4 ± 5.7	16.5 ± 5.4	2.4 ± 1.5	6.0 ± 3.6
GM-CSF (pg/mL)	male	500.0 ± 392.4	532.6 ± 401.4	495.8 ± 396.8	549.6 ± 403.9
	female	12.1 ± 6.1	13.1 ± 6.0	9.8 ± 4.5	6.6 ± 3.8
IFN-γ (pg/mL)	male	BLQ	0.4 ± 0.4	24.6 ± 16.9	93.1 ± 43.7
	female	BLQ	4.4 ± 3.7	7.9 ± 5.5	46.1 ± 23.1
IL-1β (pg/mL)	male	370.6 ± 234.3	363.5 ± 235.4	366.7 ± 235.8	384.5 ± 256.7
	female	6.7 ± 3.1	8.4 ± 3.0	11.0 ± 5.9	5.2 ± 3.2
IL-1Ra (pg/mL)	male	166.8 ± 89.2	177.6 ± 113.3	135.5 ± 94.6	1300.3 ± 935.0
	female	15.7 ± 7.3	53.2 ± 25.7	21.6 ± 7.6	17.7 ± 9.6
IL-4 (pg/mL)	male	27.7 ± 14.1	25.3 ± 14.0	22.5 ± 12.2	29.3 ± 16.1
	female	1.7 ± 0.9	1.1 ± 0.6	1.1 ± 0.7	2.3 ± 0.7
IL-6 (pg/mL)	male	209.7 ± 128.8	177.6 ± 108.6	103.1 ± 70.1	186.9 ± 113.9
	female	4.3 ± 1.7	3.0 ± 1.4	3.5 ± 1.5	3.4 ± 1.8
IL-8 (pg/mL)	male	30.5 ± 10.8	41.8 ± 18.3	39.7 ± 16.4	59.7 ± 19.3
	female	9.1 ± 1.0	8.9 ± 1.8	16.8 ± 7.3	7.9 ± 1.4
IL-10 (pg/mL)	male	140.7 ± 80.2	156.8 ± 94.2	98.4 ± 62.9	82.6 ± 66.4
	female	18.6 ± 10.1	20.9 ± 9.7	16.2 ± 7.0	9.5 ± 3.6
IL-12p70 (pg/mL)	male	475.0 ± 303.6	442.6 ± 314.7	341.5 ± 270.2	460.5 ± 369.5
	female	17.4 ± 12.5	14.1 ± 8.4	10.5 ± 6.74	12.6 ± 7.7
IP-10 (pg/mL)	male	818.3 ± 593.8	1031.4 ± 632.9	996.7 ± 589.0	966.3 ± 619.4
	female	56.7 ± 8.5	71.5 ± 10.8	86.5 ± 15.4	61.8 ± 10.1
TNF-α (pg/mL)	male	114.8 ± 87.4	260.8 ± 194.2	223.8 ± 190.4	270.1 ± 231.4
	female	20.5 ± 17.6	17.6 ± 11.7	17.1 ± 13.0	11.3 ± 7.7

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Table B2 *continued*.

Cytokine	Sex	Placebo d-0	Placebo d-28	β2-1 Fructan d-0	β2-1 Fructan d-28
Active TGF-β1 (pg/mL)	male	3.8 ± 2.5	5.3 ± 2.51	5.8 ± 2.6	9.7 ± 2.7
	female	1.5 ± 1.1	1.9 ± 1.5	0.9 ± 0.8	2.2 ± 1.6
Total TGF-β1 (pg/mL)	male	4183.6 ± 462.4	4177.8 ± 745.7	4078.0 ± 685.9	3755.6 ± 498.1
	female	3908.7 ± 446.5	3445.7 ± 339.6	3925.9 ± 436.1	3700.0 ± 55.8

[1] n = 13 for male subjects.
[2] n = 17 for female subjects.
[3] LPS concentrations were significantly different between sexes 28 (rANOVA P = 0.001).

Table B3. Concentrations of serum and faecal Ig in males and females at baseline and endpoint of placebo and β2-1 fructan phases.

Sample	Class	Sex	Placebo		β2-1 Fructan		rANOVA
			d-0	d-28	d-0	d-28	*P* Value[3]
Serum[1] (ng/mL)	**IgA**	male	731.3 ± 386.8	1805.7 ± 357.5	1721.8 ± 405.6	928.3 ± 128.9	0.271
		female	673.3 ± 117.7	1249.9 ± 305.0	1136.2 ± 268.0	898.9 ± 227.0	
	IgM	male	354.1 ± 44.7	852.1 ± 287.4	649.3 ± 160.1	411.8 ± 63.8	0.565
		female	441.9 ± 46.9	797.7 ± 117.2	874.1 ± 183.7	542.3 ± 106.6	
	IgG$_1$	male	2675.7 ± 226.3	7114.5 ± 1933.0	5408.8 ± 1335.1	2952.8 ± 346.1	0.975
		female	2801.4 ± 348.7	7258.99 ± 1706.4	4949.6 ± 1003.8	2821.9 ± 279.3	
	IgG$_2$	male	1731.6 ± 211.6	29 228.7 ± 12 518.2	12 485.6 ± 4883.7	1631.8 ± 246.5	0.247
		female	1596.5 ± 169.3	14 282.3 ± 3464.0	7497.7 ± 1813.5	15 168.0 ± 265.1	
	IgG$_3$	male	9609.98 ± 2122.8	27 806.5 ± 5904.8	25 331.6 ± 348.5	28 999.4 ± 5807.2	0.428
		female	11 096.2 ± 177.0	28 468.7 ± 4740.8	31 182.4 ± 3617.6	31 754.72 ± 4058.1	
	IgG$_4$	male	1064.5 ± 177.0	3375.6 ± 738.3	2619.6 ± 549.2	2880.6 ± 568.2	0.027
		female	380.9 ± 132.1	1307.6 ± 496.5	1115.1 ± 436.2	1587.7 ± 623.9	
Faecal[2] (ng/g)	**IgA**	male	109 093.5 ± 23 318.2	108 350.95 ± 26 502.9	106 134.8 ± 33 951.3	100 341.8 ± 24 954.4	0.913
		female	83 687.5 ± 25 248.7	79 830.5 ± 17 378.9	68 284.0 ± 9685.0	77 554.2 ± 10 000.5	
	IgG	male	9006.8 ± 5520.0	12 898.94 ± 4084.3	25 319.1 ± 7705.6	25 897.9 ± 10 289.7	0.232
		female	3573.854 ± 1329.1	13 782.0 ± 4283.3	20 386.5 ± 12 699.2	5093.3 ± 1516.0	
	IgM	male	1961.7 ± 806.3	1227.0 ± 535.7	852.9 ± 295.3	4360.1 ± 2173.6	0.259
		female	3307.6 ± 2662.7	682.5 ± 213.0	578.1 ± 140.2	913.3 ± 250.5	

[1] n = 13 for males and n = 17 for females.
[2] n = 13 for males, n = 17 for females (except for placebo day 0, n = 16).
[3] rANOVA with phase and gender as variables, followed by LSD test when significant.

Table B4. *Ex vivo* cytokine production in unstimulated and TLR agonist-stimulated whole blood cultures from males and females at the endpoints of placebo and β2-1 fructan phases.

Cytokine	Sex	Unstimulated Placebo d-28	Unstimulated β2-1 Fructan d-28	LPS Placebo d-28	LPS β2-1 Fructan d-28	P3C Placebo d-28	P3C β2-1 Fructan d-28
IFN-α (pg/mL)	male[1]	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	female[2]	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
IL-1β (ng/mL)	male	BLQ	0.1 ± 0.1	7.8 ± 0.8	8.2 ± 0.8	0.3 ± 0.1	0.4 ± 0.1
	female	BLQ	0.01 ± 0.01	8.5 ± 1.8	10.0 ± 2.7	0.4 ± 0.1	0.5 ± 0.2
IL-6 (ng/mL)	male	0.1 ± 0.1	0.7 ± 0.6	39.4 ± 1.9	39.7 ± 2.3	8.5 ± 1.0	12.2 ± 3.3
	female	0.1 ± 0.1	106.5 ± 0.1	30.6 ± 1.6	34.2 ± 2.3	8.5 ± 1.2	10.9 ± 1.7
IL-8 (ng/mL)	male	6.0 ± 2.6	28.9 ± 17.3	65.1 ± 3.7	72.3 ± 3.9	60.4 ± 4.2	60.8 ± 7.8
	female	4.7 ± 1.2	9.3 ± 5.1	64.6 ± 2.0	73.0 ± 3.0	54.1 ± 3.9	60.5 ± 5.0
IL-10 (pg/mL)	male	BLQ	48.8 ± 49.8	2352.3 ± 200.1	2119.4 ± 245.0	33.9 ± 13.0	78.9 ± 34.0[3]
	female	BLQ	BLQ	1807.4 ± 195.3	1910.2 ± 278.3	BLQ	28.7 ± 10.9
IL-12p70 (pg/mL)	male	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	female	BLQ	BLQ	BLQ	BLQ	BLQ	6.1 ± 5.4
TNF-α (ng/mL)	male	BLQ	0.3 ± 0.2	3.2 ± 0.3	4.0 ± 0.6	0.3 ± 0.0	0.2 ± 0.1
	female	BLQ	0.01 ± 0.01	2.2 ± 0.5	2.3 ± 0.3	0.1 ± 0.0	0.2 ± 0.0

[1] n = 13 for unstimulated and LPS stimulated samples, and n = 11 for P3C stimulated samples in males.
[2] n = 17 for unstimulated and LPS stimulated samples, and n = 13 for P3C stimulated samples in females.
[3] IL-10 production was significantly different between males and females over all time points (rANOVA with LSD test, P = 0.022).

Appendix C

Table C1. Diet formulation and nutritional profile of the test diet, AIN-93G.

Ingredients %	
Corn Starch	39.7
Casein	20.0
Maltodextrin	13.2
Sucrose	10.0
Soybean Oil	7.0
Powdered Cellulose	5.0
AIN-93G Mineral Mix	3.5
AIN-93G Vitamin Mix	1.0
L-Lysine	0.3
Choline bitartrate	0.25
t-Butylhydroquinone	0.0014
Energy (kcal/g)	**3.89**
From:	Kcal
Protein	0.731 (18.8 %)
Fat	0.637 (16.4 %)
Carbohydrates	2.528 (65.1 %)

Nutritional Profile	
Protein %	**18.3**
Arginine	0.70
Histidine	0.52
Isoleucine	0.96
Leucine	1.73
Lysine	1.45
Methionine	0.52
Cysteine	0.37
Phenylalanine	0.96
Tyrosine	1.01
Threonine	0.77
Tryptophan	0.22
Valine	1.14
Alanine	0.55
Aspartic Acid	1.29
Glutamic Acid	4.08
Glycine	0.39
Proline	2.36
Serine	1.10
Taurine	0.00
Fat %	**7.1**
Cholesterol ppm	0.00
Linoleic acid %	3.58
Linolenic acid %	0.55
Arachidonic acid, %	0.00
n-3 fatty acids, %	0.55
Total saturated fatty acids, %	1.05
Total monounsaturated fatty acids, %	1.54
Polyunsaturated fatty acids, %	3.78
Fibre %	**5.0**
Carbohydrates %	**63.2**

Minerals	
Calcium %	0.51
Phosphorus %	0.32
Potassium %	0.36
Magnesium %	0.05
Sodium %	0.13
Chloride %	0.22
Fluorine ppm	1.0
Iron ppm	40
Zinc ppm	35
Manganese ppm	11
Copper ppm	6.0
Cobalt ppm	0.0
Iodine ppm	0.21
Chromium ppm	1.0
Molybdenum ppm	0.14
Selenium ppm	0.24
Vitamins	
Vitamin A IU/g	4.0
Vitamin D3 IU/g	1.0
Vitamin E IU/g	81.6
Vitamin K ppm	0.75
Thiamin hydrochloride ppm	6.1
Riboflavin ppm	6.7
Niacin ppm	30
Pantothenic acid ppm	16
Folic acid ppm	2.1
Pyridoxine ppm	5.8
Biotin ppm	0.2
Vitamin B12 µg/kg	29
Choline chloride ppm	1250
Ascorbic acid ppm	0.0