

INVESTIGATING THE ROLE OF INULIN FOR ENHANCING IMMUNE FUNCTION IN ZINC DEFICIENT RATS

By Natasha Ryz

A thesis submitted to the Department of Human Nutritional Sciences in partial
fulfillment of the requirements for the degree of
Master of Science

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FACULTY OF GRADUATE STUDIES

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**INVESTIGATING THE ROLE OF INULIN FOR ENHANCING IMMUNE FUNCTION
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**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of
Manitoba in partial fulfillment of the requirement of the degree**

MASTER OF SCIENCE

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ABSTRACT

Zinc deficient (ZD) states are characterized by suppressed immune function. Prebiotic fibers have a beneficial effect on immune responses. Thus, it was hypothesized that prebiotics may indirectly protect against immunosuppression due to ZD.

The objectives of this research were to determine if the prebiotic inulin i) alters the proportion of immune cells and ii) modulates *ex vivo* cytokine secretion of immune cells from the spleen, mesenteric lymph nodes (MLN) and Peyer's patches (PP), and iii) alters systemic immunoglobulin (Ig) secretion in ZD rats. Weanling female Sprague Dawley rats were fed a control diet (5% cellulose) supplemented with 5% cellulose (CEL) or with 5% long chain inulin (PRE) for 4 weeks. The rats continued to receive the CEL or PRE diet *ad libitum* (ZN) or in restricted amounts (DR), or deficient in zinc (ZD) for another 4 weeks.

PRE reduced feed intake by 7% but did not affect final body weight. Furthermore, PRE had 19% less adipose tissue, while lean muscle mass was conserved. PRE had a higher proportion of T-helper cells in the spleen, dendritic cells in the PP and greater secretion of the cytokines interleukin-2 (IL-2), IL-10 and interferon- γ from both spleen and PP cells. Conversely, PRE had a lower proportion of cytotoxic T cells in both spleen and PP and B cells in the MLN. The only immune marker that was specifically hindered by ZD was serum IgG2a, while DR had a higher proportion of total T cells and cytotoxic T cells in the PP. The femur zinc concentration of DR-PRE was higher than DR-CEL; thus, it appears that inulin helps conserve femur zinc levels in DR animals.

PRE elevated the proportion of dendritic cells, T helper cells and the cytokines involved with the initiation of immune responses, while reducing the proportion of effector cells. Overall, long-chain inulin was not protective against immune dysfunction caused by DR or ZD in young adult female rats.

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LIST OF ABBREVIATIONS

AIDS = Acquired Immunodeficiency Syndrome

ANOVA = Analysis of Variance

APC = Antigen Presenting Cell

BASE = Baseline

Bb12 = *Bifidobacterium lactis* Bb12

BP = sonicated Bifidus componenets from *Bifidobacterium pseudocatenulatum*
7041

BSA = Bovine Serum Albumin

CD4⁺ = T-helper cell

CD8⁺ = Cytotoxic T-cell

CDF = Cation Diffusion Facilitator

CEL = Cellulose

ConA = Concanavalin A

CTL = Control

BB = Inoculated by mouth with *Bacteriodes vulgatus* and *Bifidobacterium longum*

DEXA = Dual Energy X-ray Absorptiometry

GLP = Glucagon-like peptide

DP = Degree of Polymerization

DR = Diet Restricted

DTT = Dithiothreitol

EDTA = Ethylenediaminetetraacetic acid

ELISA = Enzyme Linked Immunosorbent Assay

F = Fructose

FITC = Fluorescein Isothiocyanate

FOS = Fructooligosaccharides

G = Glucose

GALT = Gut Associated Lymphoid Tissue

GF = Germ Free

HF = High Fat Diet

HB = Inoculated by mouth with bacteria obtained from human feces

HIV = Human Immunodeficiency Virus

IBD = Inflammatory Bowel Disease

ICI = Inflammatory Cell Infiltration

IEL = Intraepithelial

IFN- γ = Interferon-gamma

Ig = Immunoglobulin

IL = Interleukin

IN = Inulin

LAB = Lactic Acid Bacteria

LGG = *Lactobacillus rhamnosus GG*

LI = Labeling Index

LPS = Lipopolysaccharide

MLN = Mesenteric Lymph Nodes

MZD = Marginal Zinc Deficiency

OF = Oligofructose

PBS = Phosphate Buffered Saline

PE = Phycoerythrin

PHA = Phytohemagglutinin

PMSF = Phenylmethyl Sulfonylfluoride

PP = Peyer's Patches

PRE = Prebiotics

PRO = Probiotics

RPM = Revolutions per Minute

SE = Standard Error

SCFA = Short Chain Fatty Acids

slgA = Secretory Immunoglobulin A

SYN = Synbiotics

TCR = T-Cell Receptor

Th = T-helper lymphocytes

TG = Transgenic

TGF = Transforming Growth Factor

TNF = Tumor Necrosis Factor

ZD = Zinc Deficient

ZIP = Zrt and Irt-like Proteins

I. LITERATURE REVIEW

INTRODUCTION

Zinc is a mineral known to play a central role in the immune system. Zinc deficiency has been shown to damage the epithelial lining of the gastrointestinal tract and the cells associated with it, thus weakening the innate defense of the body. The acquired immune response is also affected; cytotoxic T-cell activity is suppressed, B-cell antibody production is disturbed and cytokine secretion is altered by zinc deficiency (Shankar & Prasad, 1998).

Prebiotic fibers are nondigestible food products that selectively stimulate the growth and activity of health promoting bacteria already present in the colon (Kolida et al, 2002). In animal models, prebiotics have been shown to increase the proportion of lymphocytes and increase cytokine secretion in the Peyer's patch (Roller et al, 2004; Manhart et al, 2003; Hosono et al, 2003). Thus, prebiotics may attenuate the damage caused by zinc deficiency.

To date, no research has explored the effects of prebiotic fibers on immune function using a zinc deficient model. The purpose of this thesis is to investigate if the prebiotic inulin can enhance immune function in a zinc deficient rat model. This literature review will discuss research on the separate effects of zinc and prebiotic fibers on immune function and the intestinal tract.

THE IMMUNE SYSTEM

The immune system defends the body against invading pathogens and altered self-cells, which are foreign to the body. Immune responses can be classified as either innate or acquired. Innate immunity is a nonspecific host defense and functions as a barrier to any microorganism or virus. Barriers include skin and mucous membranes, chemical mediators, endocytic and phagocytic cells and inflammatory mechanisms (Goldsby et al, 2003).

Acquired immunity is a specific host defense, which is mediated by B and T lymphocytes. This type of defense is very diverse, exhibits immunological memory and can recognize self from non-self. The basic steps of the acquired immune response are antigen processing and presentation, activation of T helper cells ($CD4^+$), which can then activate cell-mediated immunity and/or humoral immunity. Cell-mediated immunity involves cytotoxic T cells ($CD8^+$) and acts mainly on altered self-cells, cancer cells or virally infected cells. Humoral immunity refers to immune responses that involve antibodies produced by B-cells (Goldsby et al, 2003).

Dendritic cells play an important link between the innate and acquired immune systems. The major function of dendritic cells is to process and present antigen to T cells in a stimulatory or tolerogenic fashion (Smits et al, 2005). Activated T helper cells (Th) are initially naïve Th0 cells, which can differentiate into Th1, Th2 or Th3 cells depending on the type of antigen and cytokines it encounters. Viruses and bacteria activate macrophages, dendritic cells and other antigen presenting cells (APC) to release the cytokine interleukin-12 (IL-12),

which influences naïve T cells to become Th1 cells (Kidd, 2003). The presence of IL-4, released by mast cells and eosinophils in response to allergens and parasites favors the Th2 pathway (Kidd, 2003). Dietary antigens and commensal bacterial are taken up by dendritic cells and influence naïve T cells to become Th3 cells (Smits et al, 2005).

Th1 cells are hypothesized to drive cell-mediated immunity, by synthesizing the cytokines IL-2, interferon-gamma (IFN- γ) and tumor necrosis factor beta (TNF- β), which activates natural killer cells, macrophages and CD8⁺ T cells. Thus, the Th1 subset is responsible for destroying altered self-cells, attacking intracellular pathogens, and inducing antibody-class switching to immunoglobulin (Ig) G2a, which supports phagocytosis and fixation of complement. The Th1 pathway is associated with the promotion of inflammation and delayed type hypersensitivity. IFN- γ produced by Th1 cells inhibits the generation of Th2 cells (Benjamini et al, 2000).

Th2 cells are essential for humoral immunity and they can synthesize the cytokines IL-4, IL-5, IL-10 and IL-13. These cytokines are involved in the growth, development and proliferation of B cells. IL-4 also promotes antibody-class switching to IgG1, which does not activate complement. The Th2 subset supports allergic reactions and are believed to provide protection against extracellular pathogens such as multicellular parasites. IL-4 and IL-10 can inhibit the generation of Th1 cells (Goldsby et al, 2003).

Th3 cells are regulatory T cells that secrete IL-10 and transforming growth factor beta (TGF- β); cytokines which suppress the activation of Th1 and Th2 cells

and induce B cells to switch from IgM to an IgA isotype (Allez & Mayer, 2004). TGF- β is important for the regulation of intestinal inflammation and tolerance to normal intestinal bacteria.

Th1/Th2/Th3 balance is a theory of immune regulation, in which a healthy immune system is able to utilize pathways as required. In certain disease conditions, it is believed that this balance is altered; for instance, an overactive Th1 response has been linked to autoimmune disease, while an overactive Th2 response may lead to allergies (Kidd et al, 2003). Furthermore, an inappropriate response against dietary antigens or commensal bacteria can lead to inflammatory disorders such as food allergies and Crohn's disease, respectively (Makala et al, 2004).

THE GUT ASSOCIATED LYMPHOID TISSUE

The gut associated lymphoid tissue (GALT) provides the body with specialized host defense. GALT is composed of aggregated lymphoid tissue, which includes Peyer's patches (PP) and solitary lymphoid follicles, and non-aggregated tissue, including intraepithelial (IEL) lymphocytes and lymphoid cells found in the lamina propria (Schley & Field, 2002). PPs are found in the mucosa and submucosa of the small intestine; these follicles are rich in $CD4^+$ and $CD8^+$ T-cells, B-cells, macrophages and dendritic cells. Overlying the patches and interspersed along the mucous membrane are specialized M cells, which can endocytose and transport antigens to the underlying follicles. In the PPs, antigens are processed and presented to activate B and T cells. The activated B cells differentiate into plasma cells and produce IgA (Goldsby et al, 2003). Solitary lymphoid follicles are present mainly in the colon and rectum. These follicles are thought to function like PPs (Schley & Field, 2002).

Activated immune cells leave the PPs and populate the lamina propria and IEL regions of the intestine. The lamina propria is rich in $CD4^+$ T cells and IgA secreting plasma cells. IEL lymphocytes are located in the interstitial spaces of the mucosal epithelium and are rich in $CD8^+$ T-cells (Schley & Field, 2002).

Mesenteric lymph nodes (MLN) are not situated within the intestinal mucosa, but are still considered as part of the GALT. When $CD4^+$ and $CD8^+$ cells mature in the PPs, they leave via the MLN to enter systemic circulation, and then return via the MLN to the lamina propria regions of the gut. Thus, MLN are rich in $CD4^+$ and $CD8^+$ cells (Schley & Field, 2002).

ZINC AND THE BODY

Zinc is an essential mineral that is found in almost every cell in the body. In biologic systems, zinc is almost universally found in the divalent state (Zn^{2+}). Rich food sources of zinc include meat, eggs and seafood. Whole grains and plant proteins are relatively good sources of zinc, but contain phytic acid, which can bind zinc and decrease its absorption (King & Keen, 1999). Other factors can influence the absorption of zinc; enhancers include citric acid, sulfur containing amino acids and histidine, whereas inhibitors include oxalate, polyphenols and nutrients with divalent cations, such as calcium (Ca^{2+}) and iron (Fe^{2+}) (Groff & Gropper, 2000).

During digestion, zinc is released from dietary protein by gastric acid and enzymes in the stomach and small intestine, respectively (Groff & Gropper, 2000). Zinc is absorbed predominantly in the duodenum and jejunum; "zinc is incorporated into the intestinal epithelium from its apical side and transferred through an unknown intracellular pathway to the basolateral side, from where zinc is released into portal circulation" (Kambe et al, 2004, p. 49).

Mammalian zinc transporters are found within two gene families: the ZnT/CDF (cation diffusion facilitator) proteins and the ZIP (Zrt and Irt-like proteins) family (Liuzzi & Cousins, 2004). ZnT4 is highly expressed in rat small intestine, especially in villus cells, which suggests that this transporter may play a role in zinc absorption (reviewed by Liuzzi & Cousins, 2004). hZIP4 mRNA is detected mostly in the mature enterocytes of the intestinal villus, and hZIP4

protein resides on the apical surface of the enterocytes, thus hZIP4 may also be involved in zinc absorption (reviewed by Kambe et al, 2004). Cragg et al (2002) identified HZTL1, a human ZnT-like transporter, expressed at the apical membrane of the enterocyte. The researchers found that hZTL1 can mediate Zn^{2+} uptake across the plasma membrane. Movement of zinc through the enterocyte is not well understood. It is believed that zinc binds to metallothionein, a sulphur-rich metal binding protein, or other intracellular proteins such as cysteine-rich intestinal proteins and is transported to the basolateral membrane (Semrad, 1999). Immunolocalization studies in growing rats have shown that ZnT1 is expressed at the basolateral membrane and thus may be involved in mediating zinc transport from enterocyte to blood (reviewed by Liuzzi and Cousins, 2004).

Most circulating zinc is bound to albumin and α 2-macroglobulin. Protein-bound zinc is transported via the portal circulation to the liver, where it is taken up and released, and then distributed to other tissues. Zinc is found in all body organs, especially the liver, kidney, muscle, skin and bones (Groff and Gropper, 2000).

Zinc has many diverse functions and is essential for growth and development. As a component of metalloenzymes, zinc can have a catalytic, structural or regulatory role. Zinc plays a part in gene expression, cell growth and replication, bone formation, skin integrity, and also affects behavior and learning (Groff & Gropper, 2000). Zinc is necessary for the normal function of the immune system, and dysfunction is observed, even in mild zinc deficiency (Ibs & Rink, 2003).

Zinc Deficiency

The first symptom of zinc deficiency in experimental animals is a decrease in food intake, leading to a reduction in growth. If zinc deficiency is prolonged, other symptoms include dermatitis, impaired growth and altered immune function (King & Keen, 1999).

In humans, reduced plasma zinc concentrations are common in patients with cancer, atherosclerosis, human immunodeficiency virus (HIV), acquired immunodeficiency virus (AIDS), liver diseases and inflammatory bowel disease (King & Keen, 1999). Severe zinc deficiency has been observed in humans with acrodermatitis enteropathica, an autosomal recessive disease characterized by zinc malabsorption. It should be noted that severe zinc deficiency is rare, whereas mild to moderate zinc deficiency is common, especially in children, women and the elderly (Walker & Black, 2004). Overall, it is estimated that 21% of the world's population is at risk of inadequate zinc supply (Wuehler et al, 2005).

Clinical manifestations of zinc deficiency in humans include growth retardation, delayed sexual maturation, hair loss, eye and skin lesions, behavioral disturbances, impaired appetite and taste, immunodeficiency and delayed wound healing (King & Keen, 1999). For the purpose of this thesis, the literature review will focus on zinc and immune function.

ZINC AND IMMUNE FUNCTION

Zinc and Innate Immunity

Anatomic barriers include skin and mucous membranes, which are effective in preventing the entry of most pathogens (Goldsby et al, 2003). Zinc deficiency damages the epithelium, resulting in skin lesions and damage to the gastrointestinal and pulmonary tract (Shankar & Prasad, 1998). Villous atrophy, edema, mucosal ulceration, necrosis and inflammation have all been observed in the small intestine of zinc deficient rat models (Elmes & Jones, 1980; Mengheri et al, 1999).

Goblet cells are found in the epithelia lining the digestive tract. These cells secrete mucinogen, which is mucin in its hydrated form and a component of mucous. Mucous functions as a lubricant and forms a barrier to prevent pathogens from binding to the epithelium (Deplancke & Gaskins, 2001). Klf4 is a zinc-finger transcription factor found in the epithelia of the gastrointestinal tract. Katz et al (2002) have shown that in mice, Klf4 is required for the differentiation of goblet cells in the colon.

Physiological barriers, such as temperature, pH and soluble factors, are another component of the innate immune system. Paneth cells, which are found in the small intestinal crypts of Lieberkuhn, have granules that store and release antimicrobial peptides such as α -defensins and lysozyme into the crypt lumen (Kelly et al, 2004). Patients with acrodermatitis enteropathica have Paneth cells with small abnormal granules (Lombeck et al, 1974) or in some cases, the secretory granules are empty (Bohane et al, 1977).

Phagocytosis and endocytosis, which degrade extracellular molecules and cells, constitute important defense mechanisms. In phagocytosis, entire microorganisms and particles are enveloped and digested with the help of lysosomes. Phagocytic cells include neutrophils, blood monocytes and tissue macrophages. In zinc deficiency, the cells of the innate immune system, including natural killer cells and the phagocytic cells are impaired (reviewed by Shankar & Prasad, 1998; Rink & Gabriel, 2001; Ibs & Rink, 2003).

During the inflammatory response, there is an influx of phagocytic cells from the blood stream to the damaged tissue (Goldsby et al, 2003). The chemotaxis of neutrophils, which is the step where cells move through the tissue to the infected site, is decreased with zinc deficiency (Rink & Gabriel, 2001).

Zinc and Acquired Immunity

The numbers of CD8⁺ CD73⁺ T cells, which are precursors to cytotoxic T cells, are found to decrease during zinc deficiency (Prasad, 2000). The cytotoxic activity of T cells is also suppressed during zinc deficiency (Shankar & Prasad, 1998). Zinc is an essential cofactor for the hormone thymulin, which regulates the differentiation of immature T cells in the thymus and the function of mature T cells in the periphery. Thymulin induces proliferation of CD8⁺ T-cells in combination with IL-2 (Rink & Gabriel, 2001). Thymulin activity, in both animals and humans, is dependent on plasma zinc, thus zinc deficiency reduces thymulin activity (Dardenne, 2002).

During zinc deficiency, B-cells and their precursors are reduced in number, whereas mature B-cells are more resistant to change (Ibs & Rink, 2003).

B-cell antibody production is disturbed during zinc depletion and zinc deficient patients show a reduced response to vaccination (reviewed by Shankar & Prasad, 1998; Rink & Gabriel, 2001; Ibs & Rink, 2003).

Zinc and Cytokines

The effect of zinc on cytokine balance has been investigated in both rodent and human subjects. The majority of work has focused on IL-2 production. Tanaka et al (1990) examined the effect of zinc ions on T-cells isolated from human peripheral blood mononuclear cells. The researchers found that zinc enhanced the proliferation of T-cells in response to IL-2 and production of IL-2 in vitro. Zinc also induced the expression of high-affinity receptors for IL-2 on lymphocytes. These results indicate that zinc may be required for IL-2 mediated T-cell activation.

Bao et al (2003) examined the effect of zinc deficiency on IL-2 and IFN- γ in HUT-78 (Th0) and D1.1 (Th1) cell lines. Cells were separated into two groups: one set of cells was incubated in zinc-deficient medium and the other set of cells was incubated in zinc-sufficient medium for 4 days. The cells were stimulated with mitogens and supernatant fractions were measured. Northern blot analysis was used to determine the amount of IL-2 and IFN- γ mRNAs in cells exposed to different concentrations of zinc. Zinc deficiency decreased the levels of IL-2 and IFN- γ mRNAs in HUT-78 and D1.1 cells after mitogen stimulation compared with the zinc sufficient cells. This suggests that zinc may mediate the gene expression of IL-2 and IFN- γ in Th0 and Th1 cells.

One of the first human studies to look at the effect of zinc on cytokine status was done by Prasad et al (1988). The researchers measured IL-2 activity in human volunteers (n=2) in whom a specific and mild deficiency of zinc was induced by dietary means. There was an indication that IL-2 activity was lower in the human volunteers during the zinc depletion phase, which was corrected after repletion with zinc. However, the results were not significant due to the small sample size.

Healthy human subjects were studied to determine if a mild zinc deficiency would lead to an imbalance of cytokines. The subjects were placed on a semi-purified diet that contained ~3 mg of zinc/day and phytic acid to reduce the bioavailability of zinc. At the end of the zinc-restricted phase (~20 weeks), subjects were supplemented with 25-50 mg elemental zinc for 12 wk. Mitogen-stimulated IFN- γ production was significantly decreased during zinc restriction, compared to baseline and zinc repletion values. The decrease in IL-2 production during zinc restriction was borderline significant ($p=0.07$). No significant changes were seen in the production of IL-4, IL-6 and IL-10 at baseline or at the end of zinc restriction or at the end of zinc repletion (Beck et al, 1997). In another study, cytokine production was assessed in three groups of mildly zinc-deficient subjects; head and neck cancer patients, healthy volunteers who were found to be mildly zinc deficient and normal, healthy volunteers with induced zinc deficiency. The production of IL-2 was decreased significantly in zinc-deficient subjects in both cancer and healthy volunteer groups. The production of IL-4, IL-5 and IL-6 were not affected by zinc status (Prasad et al, 1997). Thus, it can be

suggested that mild zinc deficiency is associated with an imbalance between Th1 (IL-2, IFN- γ) and Th2 (IL-4, IL-5, IL-6, IL-10) cytokines.

Most studies have found that zinc deficiency impairs Th1 responses and has no effect on Th2 cytokines. However, in a *Heligomosomoides polygyrus* infected mouse model, researchers have shown that zinc deficiency interferes with the ability of T cells to produce IL-4 and IL-5 (reviewed by Scott & Koski, 2000).

PREBIOTIC FIBERS

Prebiotics are nondigestible food products that selectively stimulate the growth and activity of health promoting bacteria, such as Bifidobacteria and Lactobacillus, already present in the colon. To be considered a prebiotic, a food ingredient must: (1) pass through the upper gastrointestinal tract undigested and unabsorbed, (2) be fermented by a select number of beneficial bacterial in the colon, and (3) alter the composition of the colonic microflora to benefit the host (Kolida et al, 2002). Prebiotics function complementary to, and possibly synergistically with probiotics, which are live microorganisms that are beneficial to the host (Reid et al, 2003).

Unlike most starches, prebiotic fibers can escape digestion in the small intestine and arrive intact in the colon. Humans do not have the appropriate enzymes to cleave the sugar units of prebiotic fibers. However, bacteria in the large intestine can completely degrade the fibers to yield lactate, short chain fatty acids, H₂ and CO₂ (Bornet et al, 2002).

Inulin and oligofructose are considered prebiotics and are found naturally in many plants, including wheat, onions, garlic, asparagus, artichokes, chicory and bananas (Niness, 1999). The chicory plant (*Cichorium intybus*) is nearly exclusively processed for commercial inulin and oligofructose (Coussement, 1999). Chicory is a biennial plant, with roots that look like small oblong-shaped beets. Inulin is produced by hot water extraction; the chicory roots are washed, diced and extracted by diffusion in hot water. The semi-refined syrup is then purified using physical separation techniques followed by evaporation and spray

drying to yield a product of over 99% purity (Carabin & Flamm, 1999). Inulin is a polydisperse β (2-1) fructan, which is made up of both GF_n and F_m compounds, where n or m represents the number of fructose units (F) linked to each other, with one terminal glucose (G) (Carabin & Flamm, 1999). The chain lengths of these fructans range from 2 to 60 units, with an average degree of polymerization (DP) of ~ 10 (Niness, 1999).

Inulin can be further broken down using an endo-inulase enzyme to yield oligofructose. Oligofructose is similar in structure compared to inulin, but contains shorter chain lengths ranging from 2 to 10 units, with an average DP of 4. Oligofructose can also be synthesized from sucrose by transfructosylation, a process that enzymatically adds one, two or three additional fructose units to the sucrose molecule (Niness, 1999). Oligofructose synthesized from sucrose is generally referred to as fructooligosaccharides (FOS). These synthesized materials have a GF_n type structure with a chain length of 2 to 4 units and an average DP of 3.5 (Carabin & Flamm, 1999).

Inulin and oligofructose are legally classified as food or food ingredients in all countries in which they are used. They are well accepted for food use without limitations (Kolida et al, 2002). inulin-type fructans “do not result in mortality, morbidity, target organ toxicity, reproductive or developmental toxicity or carcinogenicity” (Carabin & Flamm, 1999, p.268). However, signs of gastrointestinal intolerance can be seen with intakes above 20-30 g/day (Carabin & Flamm, 1999).

PREBIOTICS AND IMMUNE FUNCTION

Prebiotics and Innate Immunity

One of the major functions of the intestinal mucosa is its barrier activity, which prevents pathogenic molecules from entering the systemic circulation. The effectiveness of the barrier depends on “the physical integrity of the mucosa and the reactivity of dynamic defensive factors, such as mucosal blood flow, mucosal secretions and epithelial cell function” (Bourlioux et al, 2003, p. 679).

The mucosa is composed of three sections, called the epithelium, the lamina propria and the muscularis mucosa. The epithelium of the small intestine contains absorptive enterocytes, enteroendocrine cells, goblet cells and Paneth cells. The epithelium of the large intestine is similar to that of the small intestine, except it contains more goblet cells and does not contain Paneth cells (Klein et al, 1999).

The epithelial lining of the small intestine is structured to maximize surface area. Large folds of mucosa protrude into the lumen of the small intestine. The mucosal surface is also covered with villi; long fingerlike projections lined with enterocytes. Furthermore, the plasma membranes of enterocytes that make up the villi are lined with microvilli. The microvilli have a surface coat, which forms the brush border of the enterocytes (Groff & Gropper, 2000).

In between the villi, there are numerous invaginations that form intestinal glands called crypts of Lieberkuhn. The crypts are lined with absorptive enterocytes, goblet cells, Paneth cells, enteroendocrine cells and regenerative cells (Gartner & Hiatt, 1997). Regenerative cells are stem cells “that extensively

proliferate to repopulate the epithelium of the crypts, mucosal surface and villi” (Gartner & Hiatt, 1997, p. 327). Goblet cells, Paneth cells, enteroendocrine cells and absorptive enterocytes are all derived from regenerative cells located near the base of each intestinal crypt. The new cells migrate upward and out of the crypts toward the tips of the villi. Intestinal cell turnover is rapid; every 3-5 days the old cells are sloughed off and excreted in the feces (Groff & Gropper, 2000).

The lamina propria lies between the epithelium and the muscularis mucosa. The main part of the lamina propria forms the core of the villi and consists of connective tissue and small blood and lymphatic vessels. The rest of the lamina propria is “compressed into thin sheets of highly vascularized connective tissue by the numerous ... crypts of Lieberkuhn” (Gartner & Hiatt, 1997, p. 327). The muscularis mucosa is a thin layer of muscle fibers, which forms the lower boundary of the mucosa (Groff & Gropper, 2000).

In a pig model, Tsukahara et al (2003) looked at the influence of dietary FOS on the histological variables of the large intestinal mucosa. Six male piglets received either a control diet or the control diet supplemented with 10% FOS for 10 days. Once terminated, the large intestine was removed, sections were treated and stained. The mucosa in the hindgut of FOS-fed pigs appeared thicker than those in the controls. The crypt depth was significantly larger and the crypt density was significantly smaller in the FOS-fed pigs than in the controls. The number of epithelial, mitotic and mucin containing cells was higher in piglets fed FOS than in the controls.

Kleesen et al (2003) investigated the effects of oligofructose (OF) and long chain inulin (IN) on the mucosal morphometry (height of villi, depth of crypts, number of goblet cells) and the thickness of the epithelial mucus layer in the distal jejunum and the distal colon of rats. Male 5-week-old germ-free Wistar rats were randomly divided into six groups. Two groups were inoculated by mouth with bacteria obtained from human faeces from a healthy male subject (HB rats). Two other groups were inoculated first with *Bacteriodes vulgatus* and then with *Bifidobacterium longum* (BB rats). The final two groups were kept germ free (GF rats). Ten days after inoculation, one group each of HB, BB and GF rats was fed either a control diet (8% fiber derived from cereal products) or control diet supplemented with 5% OF and IN/kg (OF and IN were mixed 1:1) for 28 days. Once terminated, the small intestine and colon were removed and sections were treated and stained. Villus height and crypt depth were significantly higher in HB-rats consuming OF and IN than in rats consuming the control diet or in GF rats. OF and IN fed rats had a higher number of goblet cells in the jejunal and colonic mucosa of both BB and HB groups, whereas a thicker epithelial mucus layer was only observed in HB rats. In GF rats, OF and IN had no effect on the thickness of the epithelial mucus layer or the number of goblet cells. The results suggest that the changes in mucosal morphometry and thickness of epithelial mucus layer are caused indirectly by the fermentation of OF and IN by intestinal bacteria.

Other researchers have looked at the effect of dietary fibers on mucosal morphology. In mice fed graded levels of cellulose, there was a gradual increase

in villus length as cellulose content increased (Komai et al, 1980). Dirks et al (1987) found that in rats fed different sources of fiber, cellulose and pectin increased jejunal (but not ileal) villus and crypt heights, whereas hemicellulose increased ileal (but not jejunal) mucosal height. Cellulose and pectin also increased crypt heights in the cecum and proximal colon. In another study, researchers fed rats high fiber diets and found that the main effect of fiber was an increase in the number of crypts per circumference and also the number of branched crypts in the proximal colon. Fiber also had a direct effect on goblet cells in the small intestine and colon (McCullough et al. 1998). In hamsters fed oat bran, rye bran or soybean hull, researchers found that all high fiber diets, compared to controls, resulted in significant increases of goblet cells in the small intestine (Lundin et al, 1993). In growing pigs fed topinambur powder (Jerusalem artichoke), Breves et al (2001) found an increase in the number of ileal goblet cells and in the thickness of the colonic mucosa.

Cells of the innate immune system may also be affected by prebiotic fibers. Researchers examined the effect of inulin and oligofructose on natural killer cell activity and macrophage phagocytosis in a mouse model. For six weeks, mice were fed a diet with 10% cellulose or 10% inulin or 10% oligofructose. The researchers found that natural killer cell activity of splenocytes was enhanced in mice fed the oligofructose or inulin diets compared to the controls. Peritoneal macrophages from mice fed the oligofructose or inulin diets had greater phagocytic capabilities compared to those fed the control diet (Kelly-Quagliana et al, 2003). However, in rats fed inulin enriched with oligofructose for

4 weeks, the researchers found that the phagocytic capacity of neutrophils and monocytes isolated from the blood, MLN, PP and spleen were not affected by the prebiotic (Roller et al, 2004).

Prebiotics and Acquired Immunity

The effects of prebiotic fibers on B and T lymphocyte populations in the GALT, spleen and blood have been studied in rodent models. Manhart et al (2003) looked at the influence of FOS on PP lymphocyte numbers in healthy and endotoxemic mice. Six to eight -week old mice were fed a control diet, or control diet plus 10% FOS for 16 days. To induce endotoxemia, mice were injected with lipopolysaccharide (LPS) on day 15. Peyer's patches were removed from mice and lymphocyte populations were determined by flow cytometry. The FOS diet increased the total cell yield in healthy and endotoxemic mice. Similarly, B-lymphocytes were also increased. However, the T lymphocytes were only increased in LPS-challenged mice fed the FOS diet. In FOS-fed endotoxemic mice, there was an increase in the CD4:CD8 ratio. The researchers concluded that FOS exerts positive effects on intestinal immune status by increasing the number of lymphocytes in PP in healthy and endotoxemic mice.

Roller et al (2004) hypothesized that probiotics (PRO) and prebiotics (PRE), alone or in combination (SYN) would enhance T-cell functions in the GALT. Male Fischer rats, aged 14 to 15 weeks, were assigned to one of four groups for 4 weeks. The control group was fed a high fat (HF) diet based on the AIN76 diet, modified to contain a high level of fat (231 g corn oil/kg) and low fiber (20 g cellulose/kg) to reflect a typical Western diet. The carbohydrate sources of

the diet were sucrose (361 g/kg) and maltodextrins (100 g/kg). The PRE group was fed a HF diet with the maltodextrins replaced by 100 g/kg of inulin enriched with oligofructose. The PRO group was fed a HF diet supplemented with *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium lactis* Bb12 (Bb12). The SYN group was fed the PRE-group diet supplemented with LGG and Bb12. T-lymphocytes from the blood, spleen and MLN were investigated by immunofluorescence. No significant differences in CD4⁺ and CD8⁺ T-lymphocytes were observed among the different dietary groups in any of the tissues studied. Thus prebiotics had no effect on T-cell populations in the blood, spleen or MLN.

The researchers also measured secretory IgA (sIgA) in the ileum and cecum of rats. sIgA serves an important protective function because it can bind to bacterial and viral surface antigens, thus preventing them from attaching to and penetrating the epithelial surface (Goldsby et al, 2003 c). The PRE treatment enhanced the sIgA concentration in the cecum, and the SYN treatment increased it in the ileum. The PRO alone had no effect on sIgA levels. In the cecum there is a large quantity of endogenous microorganisms, thus PRE supported the growth of these bacteria, which also stimulated sIgA production. However, in rats treated with SYN, the PRE may have been metabolized in the ileum and therefore could not further support bacterial growth in the cecum (Roller et al, 2004).

Nakamura et al (2004) studied the effects of FOS on the mucosal IgA response in the intestines of infant mice. Timed pregnant mice were fed a non-

purified diet *ad libitum*. Two days following birth, each dam and her pups were placed together in their own cage. The dams were fed either a control diet or a control diet supplemented with 5% FOS. Pups were weaned at 21 days of age and fed the same diets until analysis. Pups were terminated at 23, 30, 38 or 44 days of age. The total IgA levels in the intestinal tissue extracts were higher in the FOS diet group compared with the control group in the jejunum, ileum and colon. The IgA content of faeces and the ileal secretions from the FOS group were both significantly higher than the control diet group. The IgA response of PP cells in the FOS diet was also significantly higher than the control group. These findings suggest that dietary FOS increases the intestinal IgA response in the small intestine as well as the colon of infant mice.

Hosono et al (2003) investigated the influences of orally administered FOS on intestinal mucosal immunity. Female 6-week-old BALB/c mice were fed one of 3 experimental diets for 4 or 6 weeks: control, control + 2.5 % FOS or control + 7.5 % FOS. In the control diet, sucrose replaced the FOS weight. PP cells from mice given the experimental diet were co-cultured with different doses of sonicated Bifidus components from *Bifidobacterium pseudocatenulatum* 7041 (BP). The culture supernatants were collected after 7 days and measured for total IgA. The total IgA production from both FOS-fed groups showed upregulated responses *in vitro*. Twenty-four hour fecal samples were collected, processed and assayed for total IgA. Total IgA was higher in the group given 2.5% FOS than in the control group at the second week. The mice fed the 7.5% FOS had diarrhea during the initial week of the experimental diet and their fecal

IgA was not different from that of the controls. The results indicate that IgA secretion is increased in the intestinal mucosa of mice in response to FOS administration.

As for the systemic immune response, total IgG1 and IgG2a in the sera was measured for comparison with the mucosal immune system. The IgG1 subclass reflects a Th2 immune response and IgG2a reflects a Th1 immune response. A high level of total IgG1 was observed in control mice after the fifth week and a low level of IgG1 was found in the FOS-fed mice. There was no significant difference in the total IgG2a between groups. Thus FOS supplementation can suppress serum IgG1 level, which reflects a Th2 type response. Although mucosal IgA production was increased at 2 weeks, systemic immune responses (IgG1 and IgG2a production) were affected at 6 weeks by FOS administration. This indicates that FOS first change mucosal immune responses for protective immunity and then affect the systemic immune response.

Prebiotics and Cytokines

Hosono et al (2003) also assayed CD4⁺ cultured cells for IL-5, IL-6, IL-10 and IFN- γ production. CD4⁺ T cells derived from PP had increased secretions of IL-10 and IFN- γ in response to orally administered FOS in a dose-dependent manner. IL-4 was not detected and secretion of IL-5 and IL-6 was maintained at a high level. In CD4⁺ T cells derived from spleen, the IFN- γ secretion was similar to PP, however lower amounts of IL-5 and IL-6 were observed. Thus, CD4⁺ T

cells derived from PP from mice that consumed FOS can induce both Th1 and Th2 cytokine responses when stimulated by the mitogen BP.

Roller et al (2004) fed rats inulin enriched with oligofructose for 4 weeks. The dietary treatment did not modulate cytokine production in the spleen or MLN, but significantly stimulated IL-10 production by PP cells, compared to the control group. The production of IFN- γ and IL-10 in the PP was also correlated ($r = 0.90$, $P < 0.0001$). The strong correlation between the levels of production of the two cytokines suggests that the PRE treatment simultaneously activated different T-lymphocyte subpopulations.

Mechanism of Action

The exact mechanism by which prebiotics affect immune function is still unknown. However, a few hypotheses have been proposed and will be discussed.

Short Chain Fatty Acids

Bacteria in the large intestine can degrade prebiotic fibers to yield short chain fatty acids (SCFA), which include butyrate, acetate and propionate (Bourlioux et al, 2003). Most studies have focused on the effects of oligofructose on SCFA formation. In three separate studies, rats fed oligofructose showed a significant increase in butyrate concentrations (Campbell et al, 1997; Roland et al, 1995; Poulsen & Molck, 2002). Levrat et al (1999) found a significant dose-dependent increase in the total amount of caecal SCFA when rats received inulin at varying levels. However, in humans fed oligofructose or inulin, little effect has been seen on fecal concentration or of molar proportions of SCFA (Gibson et al,

1995; Alles et al, 1996; Kleessen et al, 1997; Clausen et al, 1998; Brighenti et al, 1999; Kruse et al, 1999). This may be due to the fact that studies in rats focus on SCFA patterns in the caecum, whereas studies in humans focus on the SCFA found in feces, and not at the actual site of fermentation (Nyman, 2002).

SCFA help maintain the mucosal barrier of the intestinal tract through a number of mechanisms. The pK value of SCFA is approximately 4.8 (Wrong, 1995), thus these compounds decrease the pH of the large intestine and prevent certain pathogens from growing. The acidic environment can also lead to "the dissociation of alkaline compounds with toxic or carcinogenic potential and so inhibits their absorption" (Bird et al, 2000. p. 28). SCFA have been shown to increase the mucus layer of the intestine. Finnie et al (1995) looked at the effect of sodium butyrate on colonic mucus glycoprotein (mucin) synthesis using tissue from colonic resection samples. The researchers demonstrated that butyrate increases the mucin synthesis by normal colonic epithelium *in vitro* and in a dose dependent manner. In a rat model, SCFA have been shown to stimulate mucus release *in vivo* (Sakata & Setoyama, 1995; Shimotoyodome et al, 2000). SCFA also stimulate colonic blood flow, which supports the epithelial cell mass by increasing the delivery of oxygen and nutrients (Sakata, 1997). Furthermore, butyrate is the prime energy substrate for intestinal cells and can enhance the growth of normal cells and inhibit malignant ones (reviewed by Bird et al, 2000).

In vitro, SCFA exert inhibitory effects on neutrophil chemotaxis (Botta et al, 1985), degranulation (Eftimiadi et al, 1987; Rotstein et al, 1989), phagocytosis (Eftiadi et al, 1990) and phagocytic killing (Eftiadi et al, 1990; Tonetti et al,

1991). Millard et al (2002) studied the *in vitro* effects of butyric acid on differentiation, maturation and function of macrophages and dendritic cells generated from human monocytes. The researchers found that butyrate altered the monocyte-derived macrophage differentiation and reduced the phagocytic capacity of treated cells. Butyrate also altered the terminal maturation of dendritic cells and consequently, decreased their ability to prime alloreactive naïve T cells. Similarly, Saemann et al (2002) found that butyrate was able to prevent LPS-induced maturation of dendritic cells resulting in a reduced capacity to stimulate T-cells.

Butyrate has been shown to inhibit T cell activation in response to mitogens (Novogrodsky et al, 1980), alloantigens (Bohmig et al, 1995) and soluble antigens (Kyner et al, 1976). Butyric acid has also been shown to inhibit B-cell function. Eftimiadi et al (1995) investigated the *in vitro* proliferation response of B-cells, in the presence of butyric acid, to a T-cell independent mitogen. B-cells were purified from human tonsil tissues removed at surgery. A dose-dependent inhibition of the mitogenic response and immunoglobulin production in B-cells was observed.

Studies have indicated that SCFA have an effect on cytokine secretion. In a rat model, MLN were removed and lymphocytes were cultured with concanavalin A (ConA) and butyrate, acetate and/or propionate. Butyrate inhibited IL-2 production, while acetate and propionate had no effect. Butyrate also inhibited IFN- γ production, while acetate and butyrate increased the cytokine. When used in combination, acetate and propionate were able to

decrease the inhibitory effect of butyrate on IL-2 and prevent the inhibitory effect of butyrate on IFN- γ . Acetate and propionate increased IL-10 production, whereas butyrate had no effect. Thus butyrate significantly inhibits Th1-type responses, while in some cases acetate and propionate oppose the effects of butyrate (Cavaglieri et al, 2003). Saemann et al (2000) investigated the interaction of bacterial products on human monocytes in the presence of butyrate. When cells were exposed to bacterial products, they produced IL-12 and tumor necrosis factor alpha (TNF- α). However, when butyrate was present during bacterial encounters, the Th1 cytokines IL-12 and TNF- α were suppressed, while the Th2 cytokine IL-10 was increased. In healthy human subjects, Nancey et al (2002) studied the effects of butyrate on the stimulated release of cytokines *in vitro*. Butyrate induced a significant decrease in both type 1 (IL-12, IFN- γ) and type 2 (IL-5, IL-10, IL-13) cytokines, whereas IL-6 was not altered. In summary, this data indicates that butyrate has an anti-inflammatory effect on the immune system.

Beneficial Bacteria

Prebiotic fibers stimulate the proliferation of health promoting bacteria such as *Bifidobacterium* and to a lesser extent *Lactobacillus* (Kolida et al, 2002). Kaplan et al (2000) screened a selection of twenty-eight lactic acid bacteria (LAB) and bifidobacteria for their ability to ferment inulin and oligofructose on agar. Twelve of sixteen *Lactobacillus* and seven of eight *Bifidobacterium* strains tested were able to ferment the substrates. In a continuous culture study, Sghir et al (1998) demonstrated that inulin and oligofructose were selectively

fermented by both bifidobacteria and lactobacilli. However, Menne et al (2000) found that feeding human volunteers oligofructose for up to 5 weeks significantly increased the number of bifidobacteria in the feces, while no change in lactobacilli was observed. Numerous studies have shown that inulin can significantly increase most strains of bifidobacteria in humans (Wang & Gibson, 1993; Gibson et al, 1995; Kleessen et al, 1997; Kruse et al, 1999). Bifidobacteria are gram-positive anaerobic bacteria, which represent a major part of the normal human intestinal flora (Carabin & Flamm, 1999). Bifidobacteria produce relatively high amounts of β -fructosidase, which can degrade the β -(1,2) glycosidic links present in inulin (Bornet et al, 2002).

Probiotic bacteria can affect immune function, thus it is hypothesized that prebiotic fibers may modulate immune function indirectly through the beneficial bacteria already in the gut. In human subjects, researchers have shown that dietary consumption of *Bifidobacterium lactis* and/or *Lactobacillus rhamnosus* led to significant increases in the phagocytic capacity of polymorphonuclear cells (Arunachalam et al, 2000; Chiang et al, 2000; Gill et al, 2001a, Gill et al, 2001b; Sheih et al, 2001) and the tumoricidal activity of natural killer cells (Chiang et al, 2000; Gill et al, 2001a, Gill et al, 2001b; Sheih et al, 2001).

The majority of studies looking at the effects of probiotic bacteria on acquired immune function have used animal models. Perdigon et al (1999) fed mice different strains of LAB and found that *Lactobacillus casei* and *Lactobacillus plantarum* were able to increase CD4⁺ T cells in the lamina propria of the small intestine. Pestka et al (2001) found that in mice fed yogurt supplemented with

Bifidobacterium and *Lactobacillus acidophilus*, the percentage of CD⁺ T-cells was significantly increased in the spleen, however no effect was observed in the PP lymphocytes. Studies in mice have shown that probiotic bacteria can increase the number of IgA secreting plasma cells in the GALT (Perdigon et al, 1999; Bibas Bonet et al, 1999; Gauffin Cano et al, 2002; Park et al, 2002).

Researchers have also looked at the effect of probiotic bacteria on cytokine secretion. Bifidobacteria has been shown to “induce cytokine secretion in a strain and species specific manner” (He et al, 2002, p. 784). For instance, to determine if different strains of bifidobacteria could alter macrophage cytokine production, a murine macrophage cell line J774.1 was cultured in the presence of 27 strains of bifidobacteria. Adult type bifidobacteria such as *B. adolescentis* and *B. longum* increased IL-6 and IL-12 secretion more than the infant-type bifidobacteria, *B. bifidum*, *B. breve* and *B. infantile*. All bifidobacteria strains, except *B. adolescentis* stimulated IL-10 production from macrophage cells (He et al, 2002).

Peyer’s patches, collected from mice, were stimulation by *B. infantis* or *B. bifidum* and cytokine levels were measured. Neither strain of bifidobacteria stimulated an increase in IL-6 or IFN- γ (Griffiths et al, 2004). In a rat model fed *B. lactis* (HN019) there was no difference in the IL-4 or IFN- γ produced from spleen cells (Gill et al, 2000).

Vinderola et al (2004) fed mice either *B. bifidum* or *B. animalis* for 5 days, then the small and large intestine were removed and processed for histological preparation. The number of IL-6⁺ and IL-10⁺ cells in the small and large intestine

was determined by immunostaining. Mice fed *B. bifidum* or *B. animalis* showed a significant decrease in the number of IL-6⁺ cells in the small intestine.

Interestingly, mice fed *B. animalis* also had increased IL-6⁺ cells in the large intestine. Animals fed *B. animalis* had increased IL-10⁺ cells in the small and large intestine, while mice fed *B. bifidum* only had increased IL-10⁺ cells in the large intestine. The results indicate that probiotic bacteria not only induce cytokine secretion in a strain specific manner, but may also affect cytokine secretion depending on the location of the bacteria.

PREBIOTICS/PROBIOTICS AND ZINC

Researchers have investigated the effect of prebiotic fibers on zinc metabolism in animal and human subjects. Delzenne et al (1995) found a significant increase in zinc absorption when rats were fed inulin or oligofructose. Raschka & Daniel (2005) found that femur zinc was increased by 8% in male adolescent Sprague Dawley rats when fed Synergy1 (inulin and oligofructose mixture) for 15 days. Coudray et al (2006) also investigated the effect of inulin intake on zinc status. Male Wistar rats of 4 different ages (2, 5, 10, and 20 months) were assigned to a control group or a group administered 3.75% inulin in their diet for 4 days followed by 7.5% inulin for 26 days. Plasma and tibia zinc concentrations were not affected by inulin intake, however the researchers found a positive correlation between Zn absorption and cecal amounts of acetate, propionate, and butyrate (SCFAs), which may confirm the involvement of inulin-fermentation products in intestinal Zn absorption.

In healthy adult men (n=9), Coudray et al (1997) investigated the effect of inulin on absorption and balance of zinc by using a chemical balance technique in a crossover design with 28-day treatment periods. The researchers found that inulin had little effect on the absorption of zinc and did not significantly alter urinary excretion of zinc. However, a trend to increase zinc balance was observed. In ileostomy subjects (n=10), Ellegard et al (1997) studied the effects of inulin and oligofructose on excretion of zinc. It was determined that the excretion of zinc was unaffected by inulin and oligofructose. Thus to date,

studies have shown that prebiotic fibers do not affect zinc metabolism in human subjects.

To my knowledge, no studies have looked at the effects of prebiotic fibers in zinc deficiency. However, Mengheri et al (1999) looked at the effect of the probiotic *Bifidobacterium animalis* on intestinal damage using zinc deficient rats as a model for intestinal alterations. Zinc deficient rats were fed diets containing 1 mg Zn/kg for 20 (ZD₂₀) or 40 (ZD₄₀) days to induce intestinal damage. Subgroups of these rats, ZD₂₀ + B and ZD₄₀ + B, received *B. animalis* daily for the last 10 days of the experiment. Zinc deficiency caused ulcerations, edema, inflammatory cell infiltration (ICI) and dilation of blood vessels in the duodenum, jejunum and ileum. However, after *B. animalis* administration, the morphologic alterations induced by zinc deficiency did not develop or were notably less.

APPLICATIONS

Zinc deficiency and energy malnutrition lead to compromised immunity with increased susceptibility to disease. These conditions may also accompany chronic diseases such as AIDS, and chronic gastrointestinal disorders, such as the inflammatory bowel diseases.

Patients with HIV and/or AIDS exhibit clinical symptoms similar to those associated with zinc deficiency, including immune deficiency, gastrointestinal malfunction with diarrhea, villus atrophy and epithelial lesions (Baum et al, 2003). The amount of zinc supplementation is important because deficiency, as well as excessive intakes have been linked with faster disease progression and mortality (Tang et al, 1993; Tang et al, 1996; Baum et al, 2003). The association between zinc intake and increased rate of disease progression may be due to zinc as a component of the HIV-1 virus. The nucleocapsid protein of HIV-1 has two zinc fingers (Baba et al, 2003) and both zinc fingers are required for HIV-1 replication (Guo et al, 2000). Zinc fingers are DNA binding motifs that contain cysteine and histidine residues that bind zinc to create a finger-like loop. Zinc fingers are also involved in protein-to-protein interactions (Matthews & Sunde, 2002). Perhaps excessive zinc intake stimulates HIV-1.

Alternative therapies such as prebiotic fibers may be beneficial for people with HIV/AIDS who are zinc deficient, but cannot take zinc supplements. To my knowledge, the effect of prebiotic fibers on HIV or AIDS has not been investigated. Wolf et al (1998) looked at the safety and tolerance of the probiotic *Lactobacillus reuteri* in a population infected with HIV. Overall, the researchers

determined that *L. reuteri* may be fed to HIV-positive individuals without any safety concerns.

The inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis are chronic inflammatory disorders of the intestinal tract. Ojuawo & Keith (2002) found that children with Crohn's disease had a lower serum zinc level compared to controls. It was also demonstrated that adolescents with Crohn's disease had low zinc absorption and inappropriately high endogenous fecal zinc losses, compared to controls (Griffin et al 2004).

The role of intestinal bacteria in the pathogenesis of IBD is well-recognized, and has been demonstrated in the HLA-B27 transgenic (TG) rat. This model develops severe, immune-mediated colitis in a specific pathogen-free (SPF) environment, however germ-free TG rats, as well as non-TG rats colonized with SPF bacteria remain disease-free (Hoentjen et al, 2005). Hoentjen et al (2005) have shown that feeding prebiotics to SPF TG rats significantly reduced colitis and had immunomodulatory effects; there was a decrease in IFN- γ , while TGF- β , which is important for the regulation of intestinal inflammation, was increased. These beneficial effects were associated with the growth of intestinal lactobacilli and bifidobacteria, intestinal bacteria shown to prevent colitis in animal models and in patients with IBD (Sartor, 2005).

In disease conditions such as HIV/AIDS and IBD, zinc deficiency is common and can exacerbate symptoms. There may be advantages of consuming prebiotic fibers to ameliorate the intestinal damage caused by zinc deficiency, however more research is required.

II. STUDY RATIONALE

It is well documented that zinc deficiency impairs immune function. However, research looking at the effect of zinc on cytokine balance is limited. Most studies have been done *in vitro*, which may not be reliable because cells typically undergo changes when removed from the body. Most human studies looking at the effect of zinc on cytokine balance use small sample sizes, which reduces the statistical power of the research.

Some researchers have failed to include controls for the reduced food intake that typically occurs during zinc deficiency. Zinc deficiency commonly occurs with energy malnutrition, which on its own has immunosuppressive effects. Thus changes in cytokine secretion may have more to do with energy deficiency than zinc deficiency. Most researchers have not indicated the nutrient status of the population studied. It is important to determine if there are other nutrient deficiencies, because cytokine balance may be influenced by other nutrients. For instance, selenium has been shown to enhance IL-2 production, thus a deficiency may impair IL-2 secretion (Kidd, 2003). It is also difficult to assess the effect of a certain nutrient on cytokine balance in human subjects, because many factors can influence Th1/Th2 maturation, including antigen dose, nature of the antigen, the diversity and relative intensity of these interactions and the cytokine receptors available on the naïve cell (Kidd, 2003).

Despite these limitations, researchers have demonstrated that zinc deficiency alters cytokine levels in the body, especially IL-2 (Kaplan et al, 1988; Prasad et al, 1988; Prasad et al, 1997). It can also be noted that the zinc

deficient studies have only measured cytokine production in peripheral blood mononuclear cells or splenocytes. To my knowledge, no studies have looked at the effect of zinc deficiency on Peyer's patch lymphocytes.

There is evidence to show that dietary fibers beneficially alter the intestinal morphology, and studies show that prebiotic fibers also have a beneficial effect. Prebiotic fibers have been shown to increase goblet cells, epithelial cells and mitotic cells in the intestine. Few studies have focused on prebiotics and acquired immune function, and fewer have researched how prebiotics alter cytokine production. To my knowledge, no studies have looked at how IL-2 production is affected by prebiotics. Few studies have looked specifically at the effect of inulin on immune function; most research has used oligofructose, fructooligosaccharides or some combination. It is assumed that prebiotics will indirectly affect immune function through the modulation of intestinal flora and production of short chain fatty acids. However, it is still unknown exactly how probiotic bacteria and short chain fatty acids affect the immune system. The research that has looked at probiotic bacteria and cytokine production is often contradictory. Thus there are many unanswered questions.

No studies have looked at the immune effects of prebiotic fibers using a zinc deficient model. Mengheri et al (1999) found that when the probiotic *Bifidobacteria animalis* was fed to zinc deficient rats, there was a significant reduction in intestinal damage. However, the prebiotic inulin is not highly fermented by *B. animalis* (Wang, 1993). Thus the results obtained by Mengheri et al (1999) may not apply to the prebiotic inulin.

It is hypothesized that the prebiotic inulin may improve immune function in a zinc deficient rat model. If we can understand how inulin and zinc deficiency interact in an otherwise healthy model, it may help us understand what would happen in a disease situation. The objectives of this research were to determine if inulin (i) alters the proportion of immune cells in the spleen, MLN and PP, (ii) modulates *ex vivo* cytokine secretion of immune cells in the spleen, MLN and PP, and (iii) alters systemic immunoglobulin secretion in zinc deficient rats.

IV. PRELIMINARY STUDIES

PRELIMINARY STUDY 1:

“ZINC DEFICIENCY IN YOUNG ADULT RATS”

STUDY RATIONALE

In the Main Study, weanling rats will be fed inulin for up to 6 weeks before consuming a zinc deficient diet. The length of time required to induce zinc deficiency in young adult rats is presently unknown. Thus, a preliminary study was required to determine the conditions and time frame for developing zinc deficiency in young adult rats.

MATERIALS AND METHODS

Animals and Diets

Twenty-four 9-week-old male Sprague Dawley rats (Charles River Laboratories, St. Constant, Canada) were acclimatized for 5 days and randomly assigned to the baseline group (BASE; n=4) or fed a severe ZD diet (<1 mg Zn/Kg diet) for 3, 6 or 9 weeks (3ZD, n=4; 6ZD, n=4; 9ZD, n=4, respectively), a marginally ZD diet (5 mg Zn/Kg diet; 9MZD, n=4) or a control diet (30 mg Zn/Kg diet; 9CTL, n=4) for 9 weeks (Figure 1). Rats were provided with diet and Millipore water *ad libitum*, available in glass dishes and plastic bottles with stainless steel sipper tubes, respectively. The diet formulations, based on the AIN-93G diet (Reeves et al, 1997), are provided in Table 1. The protein source

casein was replaced with spray-dried egg white (Harland Teklad) because casein is a significant source of zinc. Spray-dried egg white contains avidin, which binds biotin, thus the biotin content of the diet was increased. Rats were housed individually in hanging wire mesh cages, and were maintained in an environment of controlled temperature (21-23°C), humidity (55%), and light cycle (14:10 hr light:dark). Body weights were determined weekly, and feed intake was determined daily. Clean lab coats and disposable gloves were always worn when handling rats to minimize zinc exposure.

Figure 1: Experimental Outline for Preliminary Study 1

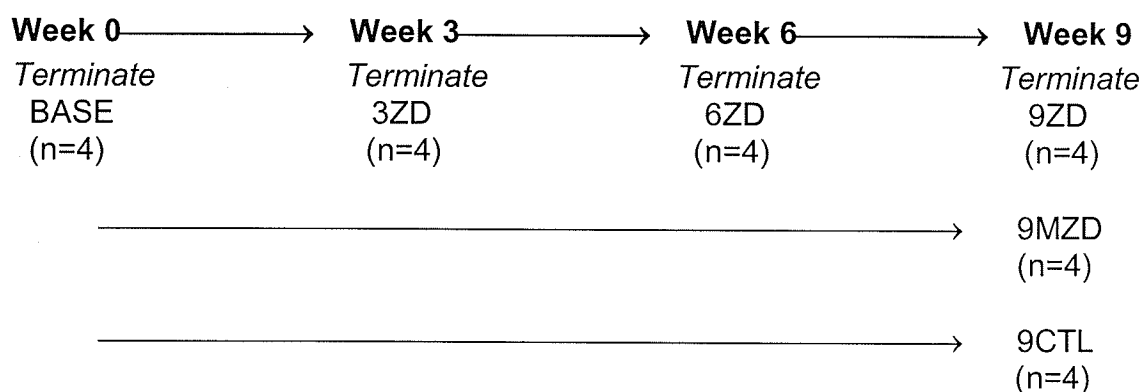


Table 1: Diet Formulations for Preliminary Study 1¹

Ingredient (g)	Severe ZD 1 mg Zn/kg diet	Marginal ZD 5 mg Zn/kg diet	Control 30 mg Zn/kg diet
Dextrose (cerelose)	604.58	603.27	594.60
Egg white	212.5	212.5	212.5
Cellulose	50	50	50
Mineral mix (AIN-93 MX, Zn free)	35	35	35
Potassium phosphate	5.4	5.4	5.4
Vitamin mix (AIN-93-VX)	10	10	10
Choline	2.5	2.5	2.5
Biotin mix ³	10	10	10
Zinc premix ⁴	0	1.33	10
Soybean oil	70	70	70

¹The above diet formulations were used to make 1 kg of each diet. All ingredients from Harlan Teklad (Madison, WI) except dextrose (Moonshiners, Winnipeg, MB) and potassium phosphate (Fisher Scientific, Mississauga, ON).

²Actual amount of zinc in diet as determined by atomic absorption was 2.42 (severe ZD), 5.34 (Marginal ZD) and 27.2 (Control) mg Zn/kg diet.

³Biotin mix was 200 mg biotin/kg cerelose

⁴Zinc premix was 5.775 g Zn carbonate/kg cerelose

Tissue Collection

Rats were terminated at baseline (n=4), week 3 (n=4), week 6 (n=4) and week 9 (n=12) (Figure 1) by CO₂ asphyxiation and decapitation. Trunk blood was collected, centrifuged at 2400 RPM (revolutions per minute) at 4°C for 10 minutes to obtain serum and stored at -80°C until analysis.

Mineral Analysis

Whole femurs and diet samples were wet-ashed using trace element grade nitric acid as previously described (Clegg et al, 1981). After obtaining wet weights, whole femurs and bovine liver reference (standard reference material 1577b, U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) were placed on labeled aluminum foil and dried for ~68 hours in an 85°C drying oven. Dry weights were recorded, and then dried femurs, diet samples and quality controls were placed into individual labeled glass test tubes that had previously been acid washed 3x with 30% nitric acid and rinsed 3x with Millipore water. Trace element grade nitric acid (Fisher Scientific, XXX, ON) was added to femur, diet and quality control samples as required: Femur (~0.3 - 0.4 g dry) = 3-4 mL acid; Diets (0.5 g dry) = 4 mL; Bovine Liver Reference = (0.2 – 0.5 g dry) = 3-4 mL. An equal amount of nitric acid was added to 2-3 glass tubes to serve as blanks. Samples were covered with an acid washed glass marble and kept at 85°C in a dry bath until completely dissolved. Once cooled, samples were diluted with Millipore water to a final volume of 50 mL. Serum zinc samples were not treated with acid digestion, and samples were appropriately diluted with Millipore water. Zinc concentrations were determined

by atomic absorption spectroscopy using a Spectra AA-30 Spectrophotometer (Varian Canada, Georgetown, ON).

Statistical Analysis

Data was analyzed by one-way analysis of variance (ANOVA) using the general linear models procedure (SAS software release 8.2, SAS Institute).

Body weight and feed efficiency were analyzed by one-way ANOVA with repeated measures. When necessary, data was normalized by log transformation for statistical analysis, but nontransformed means are reported. Significant differences between means were determined using Duncan's multiple range test. Differences were considered significant at $p < 0.05$.

RESULTS

Feed Intake

The total feed intake of 9ZD was 14% less at week 1, 37-39% less at week 2, 30-33% less at week 3, 38% less at week 6 and 41% less at week 9 compared to 9MZD and 9CTL at the respective time points (Figure 2).

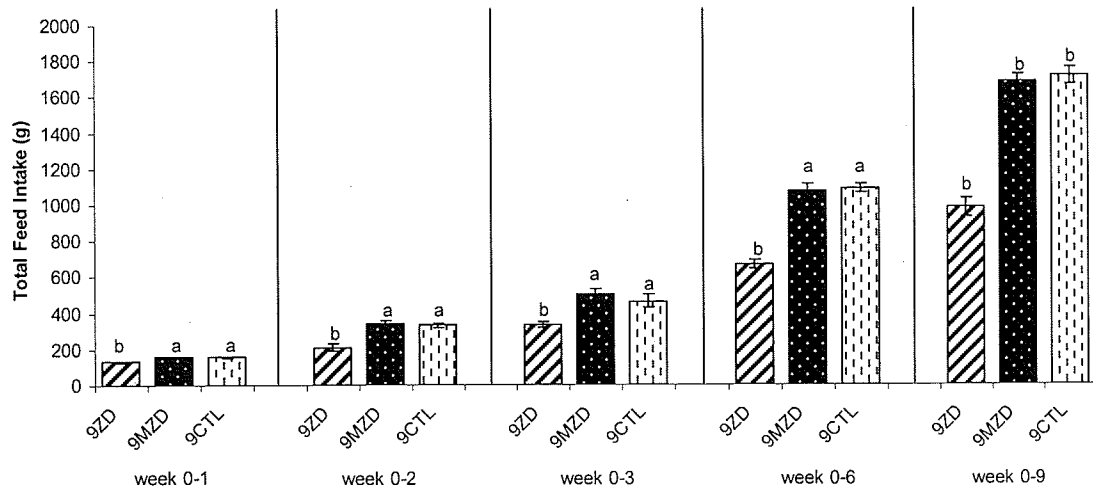


Figure 2: Total Feed Intake

Values are means $\pm$ SE, $n = 4$ per group. Data for each time period were analyzed by one-way ANOVA and different letters indicate significant differences within a time period. 9ZD = 9 week zinc deficient; 9 MZD = 9 week marginally zinc deficient; 9CTL = 9 week control.

Body Weight

Over Time: The 9ZD group weighed less than 9MZD and 9CTL groups, ranging from 7-10% less at week 1, 17-21% less at week 3, 36% less at week 6 and up to 43% less at week 9. Body weight of 9MZD and 9CTL was not different throughout the study.

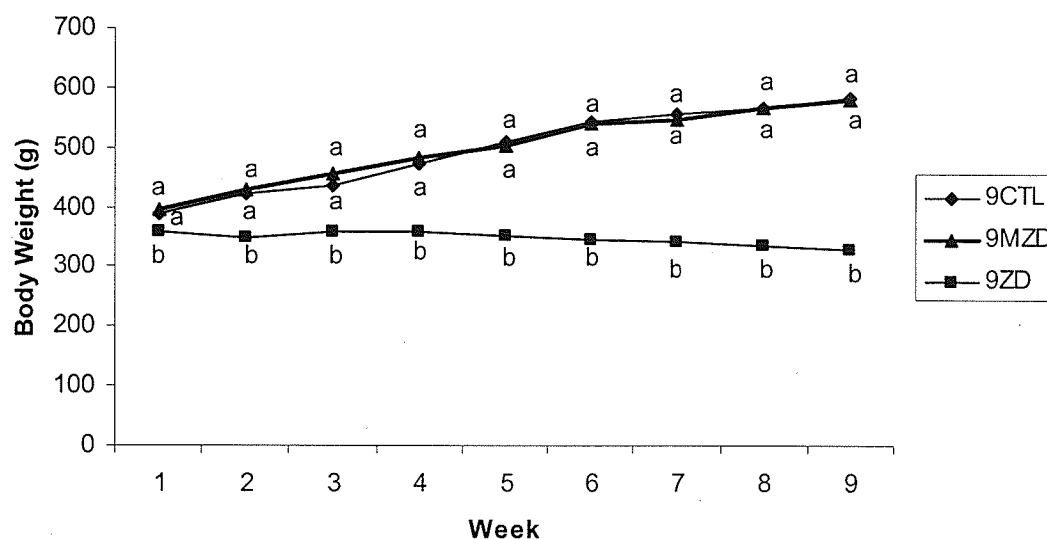


Figure 3: Body Weight Over Time

Values are means $\pm$ SE, $n = 4$ per group. Data were analyzed by one-way ANOVA with repeated measures. Statistical differences among means ($p \leq 0.05$) are indicated by lowercase letters. 9CTL = 9 week control; 9 MZD = 9 week marginally zinc deficient; 9ZD = 9 week zinc deficient.

End Point: There were no differences in termination body weights among BASE, 3ZD, 6ZD or 9ZD. 9ZD weighed 43% less than both 9MZD and 9CTL (Figure 4).

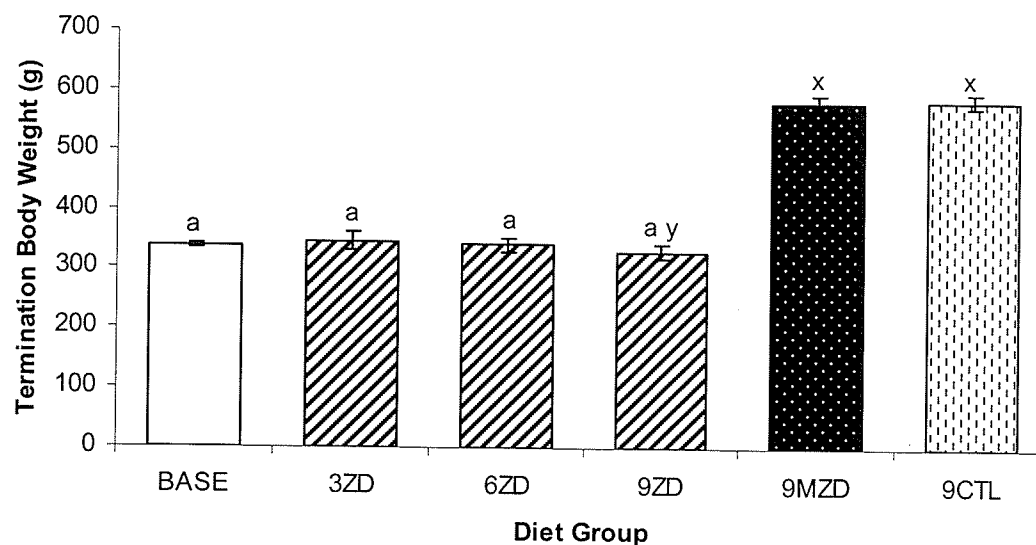


Figure 4: Termination Body Weight

Values are means $\pm$ SE, $n = 4$ per group. Statistical differences ($P < 0.05$) are indicated by symbols: ^{a,b} indicate statistical differences among BASE, 3ZD, 6ZD, and 9ZD; ^{x,y} indicate statistical differences among 9ZD, 9MZD, and 9CTL. BASE = baseline; 3ZD = 3 week zinc deficient; 6ZD = 6 week zinc deficient; 9ZD = 9 week zinc deficient; 9 MZD = 9 week marginally zinc deficient; 9CTL = 9 week control.

Feed Efficiency

At weeks 1, 2, 6, 7 and 8, ZD animals had a lower feed efficiency compared to both CTL and MZD. There was no difference among any groups at week 3, 4 or 9. At week 5, CTL was higher than MZD, and MZD was higher than ZD (Figure 5).

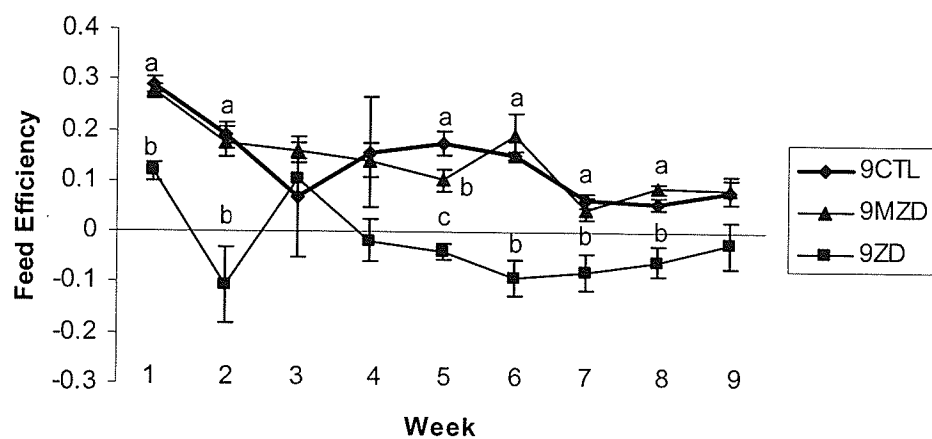


Figure 5: Feed Efficiency

Statistical analysis and abbreviations as described in Figure 2. Feed efficiency was calculated as g of weight gain per week/ g of feed intake per week. Food spillage was not taken into account until week 3, and may explain variable response from week 1 to 3.

Serum Zinc

Over time: At week 3, serum zinc of 3ZD decreased by 76% compared to BASE.

There were no differences in serum zinc among 3ZD, 6ZD or 9ZD (Figure 6).

End point: At week 9, serum zinc of 9CTL was 56% higher compared to 9MZD and 88% higher compared to 9ZD (Figure 6).

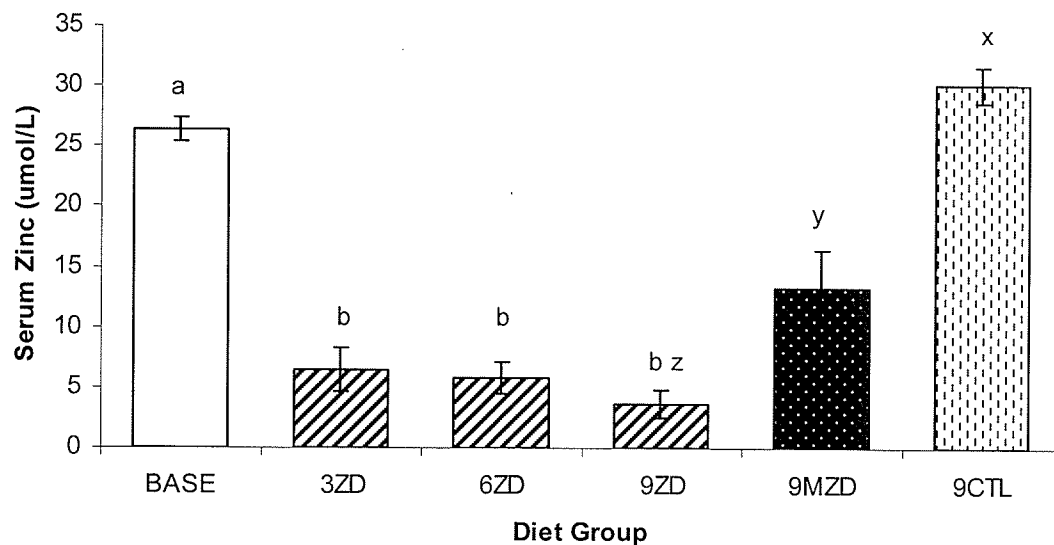


Figure 6: Serum Zinc

Statistical analysis and abbreviations as described in Figure 4.

Femur Zinc

Over time: Femur zinc decreased by 30% at week 3, 40% at week 6, and 44% at week 9, compared to BASE (Figure 7).

End point: At week 9, femur zinc of 9CTL was 56% higher compared to both 9MZD and 9ZD (Figure 7).

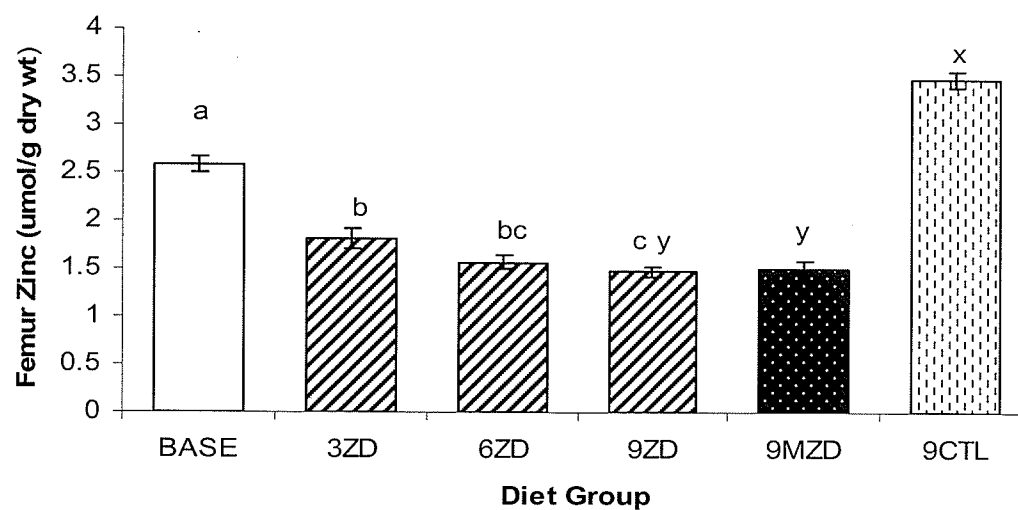


Figure 7: Femur Zinc

Statistical analysis and abbreviations as described in Figure 4.

DISCUSSION AND CONCLUSIONS

Feed Intake and Growth

In young-adult male rats, consumption of a ZD diet produced a 14% decrease in feed intake within 1 week, while a MZD diet had no effect on feed intake (Figure 2). Throughout the 9 week study, the MZD and CTL animals continued to gain weight, while the body weight of ZD animals seemed to remain at ~350 g (Figure 3). At termination, there was no difference in body weight among BASE, 3ZD, 6ZD or 9ZD animals (Figure 4), which demonstrates that young adult rats fed ZD diet do not lose weight over this time frame, rather, they stop growing. As expected, ZD animals had a lower feed efficiency compared to MZD and CTL (Figure 5).

In a similar study, 5-week-old male Sprague Dawley rats were fed a ZD, MZD (5 mg Zn/kg diet) or control diet for 6 weeks. Throughout the study, rats fed ZD diets consumed significantly less feed than rats fed either the MZD or the control diets. There was no difference in growth for the first week, but by week 2 the ZD animals remained at 220 g, while MZD and CTL animals continued to grow at the same rate. The feed efficiency of ZD animals was also significantly lower than both MZD and CTL (Scrimgeour et al, 2007).

Zinc Status

Over Time

Serum zinc was $\sim 5 \mu\text{mol/L}$ in ZD animals terminated at week 3, 6 and 9 compared to $26 \mu\text{mol/L}$ in BASE animals (Figure 6). It appears that serum zinc dropped after 3 weeks of consuming a 1 mg Zn/kg diet, but then remained steady for up to 9 weeks.

It should be noted that serum zinc may not be an accurate indicator of zinc status, since several factors, including time of day, dietary intakes, growth, infection, and stress can affect serum zinc levels (Panemangalore & Bebe, 1997). For instance, Wilkins et al (1972) found that plasma zinc concentrations in rats fell from 18.35 to $10.71 \mu\text{mol/L}$ after 1 day on a ZD diet. After 5 days the plasma zinc concentration was $\sim 7.65 \mu\text{mol/L}$. Similarly, Pallauf & Kirchgessner (1972) found that serum zinc levels in rats fell by more than 30% within 2 days of consuming a ZD diet, and addition of zinc to the diet restored serum zinc levels to normal. Thus serum/plasma zinc seems to be an indicator of short term zinc status, since it falls rapidly when no dietary source of zinc is available, and is quickly restored when zinc is re-introduced into the diet.

Bone zinc can be considered an indicator of long term zinc status. Femur zinc concentration dropped by 30% after 3 weeks of consuming a 1 mg Zn/kg diet, and then dropped another 13% by week 6, and another 6% by week 9 (Figure 7). Windisch (2003) fed 12-week-old female Sprague-Dawley rats a zinc deficient diet (1 mg Zn/kg diet) for up to 29 days, and skeletal zinc was measured on day 1, 2, 4, 7, 11, 16, 22 and 29. Within 2 days, the skeleton lost 6% zinc, but

remained constant for the next ~3 weeks. By day 29, the skeletal zinc concentration was 20% below normal levels. Zhou et al (1993) suggest the existence of two zinc pools in the skeleton: "one pool is a rapidly turning-over pool from which zinc can be readily removed when needed, and the other is a slowly turning-over pool or sink from which zinc cannot be utilized without bone loss" (Zhou et al, 1993, p. 1386).

End Point

After 9 weeks of consuming a marginally zinc deficient diet, serum zinc was 13 $\mu\text{mol/L}$, compared to 30 $\mu\text{mol/L}$ in control animals (Figure 6), and femur zinc was 1.51 $\mu\text{mol/g}$ dry wt, compared to 3.48 $\mu\text{mol/g}$ dry wt in control animals (Figure 7). This demonstrates that a marginal zinc deficiency can occur in young adult rats after consuming a 5 mg Zn/kg diet for 9 weeks.

Interestingly, at 9 weeks, there was no difference in femur zinc of 9MZD and 9ZD, but the serum zinc of 9MZD was 56% higher than 9ZD. A potential explanation is that with a marginal zinc deficiency, the zinc that is consumed is being used for growth instead of being stored in the bone.

Conclusions

- In young adult male rats fed a MZD diet for 9 weeks, feed intake and growth were not affected, however, serum and femur zinc were both reduced by 56%, compared to control.
- In young adult male rats fed a severely ZD diet for up to 9 weeks, feed intake and growth were decreased within 1 week. By week 3 serum and femur zinc were reduced by 76% and 30%, respectively, compared to control.
- Both serum and femur zinc can be significantly lowered in young-adult male rats by consuming a 1 mg Zn/kg diet zinc diet for 3 weeks or a 5 mg Zn/kg diet for 9 weeks. However, due to time constraints, a severe ZD will be used for the Main Study.
- It should be noted that for the Main Study, female rats will be used instead of males. In collaboration with Dr. Hope Weiler (McGill University), rat carcasses will be analyzed using dual energy x-ray absorptiometry to determine how prebiotics affect bone in a zinc deficient rat model. Females were chosen because according to the Third National Health and Nutrition Examination Survey (1988-94), only 39% of female adolescents have a zinc intake at or above 77% of the 1989 RDA, compared with 62% of adolescent males (Briefel et al, 2000).

PRELIMINARY STUDY 2:

THE EFFECT OF INULIN ON BODY WEIGHT AND FEED

INTAKE IN RATS

STUDY RATIONALE

In the Main Study, 3-week old Sprague-Dawley rats will be fed inulin for 4 weeks. Previous studies have shown that inulin may alter body weight in rats, however, the studies are conflicting, and show either an increase in body weight (Juskiewicz & Zdunczyk, 2004), or a decrease in body weight (Roberfroid et al, 2002; Verghese et al, 2002), while others show no change (Fontaine et al, 1996; Reddy et al, 1997; Hughes & Rowland, 2001; Kelly-Quagliana et al, 2003). Thus, a preliminary study was required to determine if inulin alters feed intake and weight gain in healthy rats. This will help determine if a pair-fed group is necessary for the Main Study.

MATERIALS AND METHODS

Animals and Diets

Twelve 3-week-old male Sprague Dawley rats (Charles River Laboratories, St. Constant, Canada) were acclimatized for 5 days, then fed a diet containing 10% cellulose (n=6) or 10% long chain inulin (Beneo HP, gift from Orafit Group, Tienen, Belgium; n=6) for 6 weeks. The diet formulations (Table 2) are based on the AIN-93G diet (Reeves et al, 1997) and were modified as described under Preliminary Study 1. Rats were provided with diet and distilled

water *ad libitum*, available in glass dishes and plastic bottles with stainless steel sipper tubes, respectively. Rats were housed individually in hanging wire mesh cages, and were maintained in an environment of controlled temperature (21-23°C), humidity (55%), and light cycle (14:10 hr light:dark). Body weights were determined weekly, and feed intake was determined daily. Rats were terminated by CO₂ asphyxiation and decapitation.

Table 2: Diet Formulations for Preliminary Study 2¹

Ingredient (g)	Inulin	Control
Dextrose (cerelose)	544.6	544.6
Egg white	212.5	212.5
Cellulose	0	100
Inulin	100	0
Mineral mix (AIN-93 MX, Zn free)	35	35
Potassium phosphate	5.4	5.4
Vitamin mix (AIN-93-VX)	10	10
Choline	2.5	2.5
Biotin mix ²	10	10
Zinc premix ³	10	10
Soybean oil	70	70

¹The above diet formulations were used to make 1 kg of each diet. All ingredients from Harlan Teklad (Madison, WI) except dextrose (Moonshiners, Winnipeg, MB) and potassium phosphate (Fisher Scientific, Mississauga, ON).

²Biotin mix was 200 mg/kg cerelose

³Zinc premix was 5.775 g Zn carbonate/kg cerelose

Statistical Analysis

Data was analyzed by one-way ANOVA using the general linear models procedure (SAS software release 8.2, SAS Institute). Feed intake, body weight and feed efficiency were analyzed by one-way ANOVA with repeated measures. When necessary, data was normalized by log transformation for statistical analysis, but nontransformed means are reported. Significant differences between means were determined using Duncan's multiple range test. Differences were considered significant at $p < 0.05$.

RESULTS

Stool Characteristics

Inulin fed rats suffered from bloating and diarrhea for first 2 weeks of experiment. After 2 weeks, inulin fed rats had formed stool, but it was still looser and darker compared to cellulose fed rats.

Weekly Feed Intake

The feed intake of cellulose-fed rats was higher than inulin-fed rats at weeks 1, 2, 3, 5 and 6. There was no difference at week 4 (Figure 8).

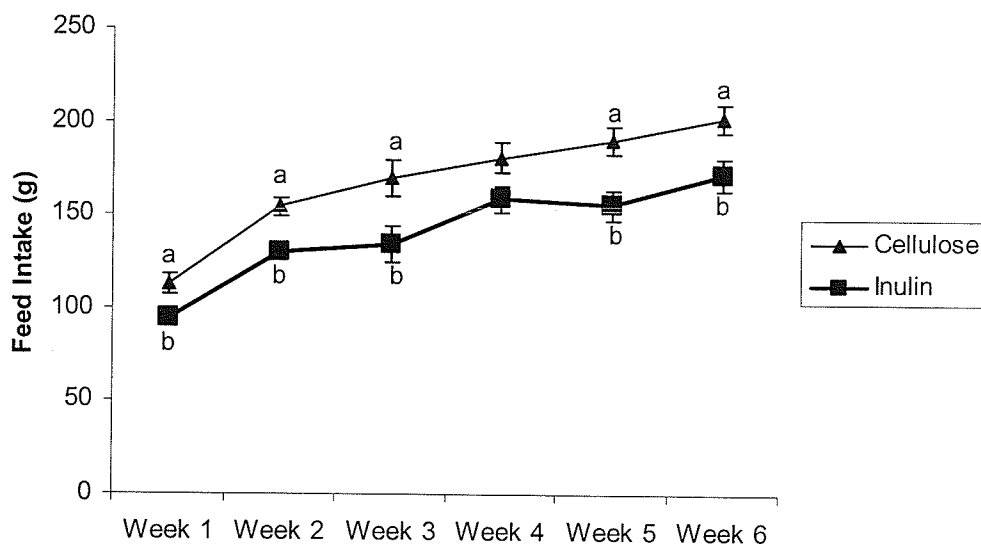


Figure 8: Weekly Feed Intake

Values are means $\pm$ SE, $n = 6$ per group. Data were analyzed by one-way ANOVA with repeated measures. Statistical differences between means ($p \leq 0.05$) at the same time point are indicated by lowercase letters.

Total Feed Intake

After 6 weeks, the total feed intake of cellulose-fed rats was 16% higher compared to inulin-fed rats (Figure 9).

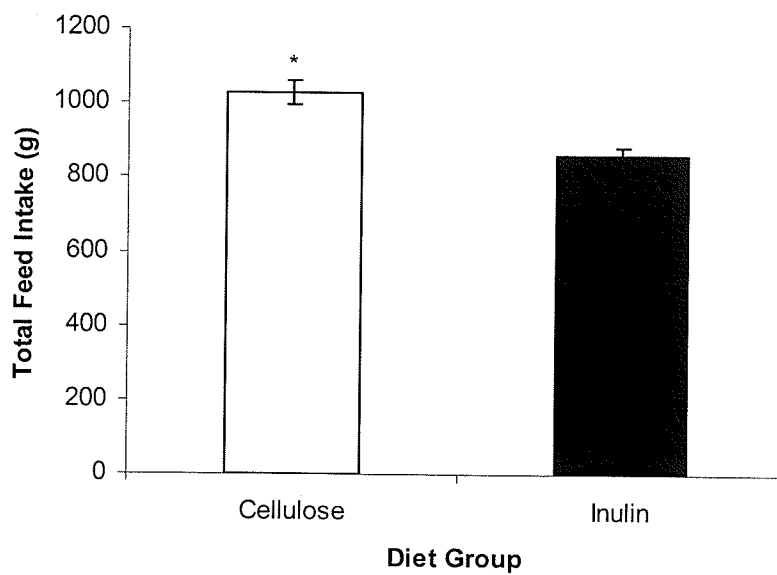


Figure 9: Total Feed Intake

Values are means $\pm$ SE, $n = 6$ per group. Data were analyzed by one-way ANOVA and statistical differences between means ($p \leq 0.05$) are indicated by an asterisk.

Body Weight

There was no difference in body weight between cellulose- and inulin-fed rats at any time point (Figure 10).

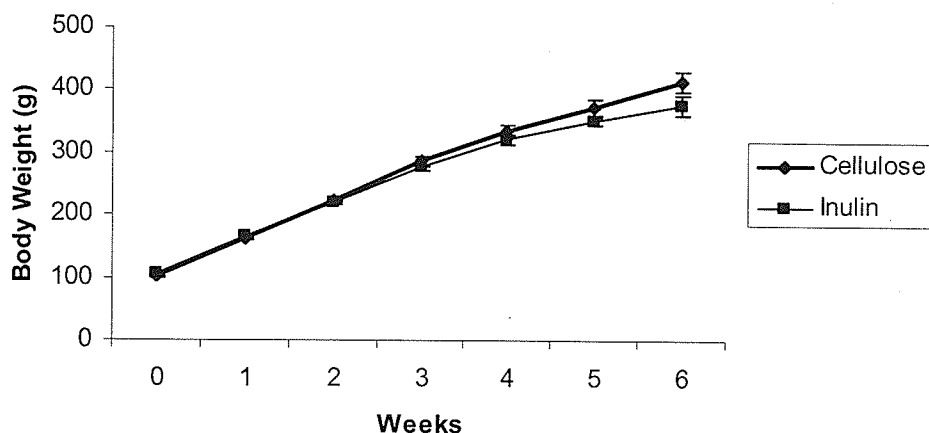


Figure 10: Body Weight

Statistical analysis and abbreviations as described in Figure 8.

Feed Efficiency

There was no difference in feed efficiency between cellulose- and inulin-fed rats at any time point (Figure 11).

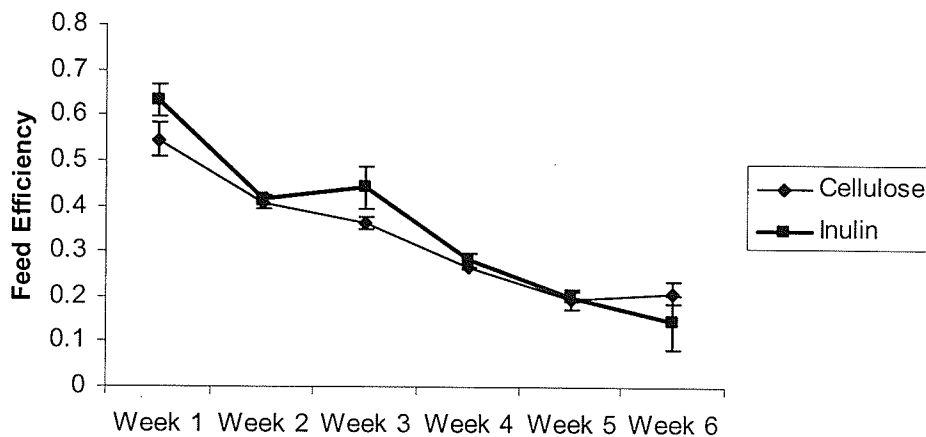


Figure 11: Feed Efficiency

Statistical analysis and abbreviations as described in Figure 8.

DISCUSSION AND CONCLUSIONS

Stool Characteristics

In weanling rats, 10% inulin induced bloating and diarrhea for the first 2 weeks of the experiment. Prebiotic fibers cause an osmotic effect in the intestinal lumen, which increases the water flow rate and can lead to diarrhea if the capacity of the colon to absorb water and electrolytes is exceeded. Diarrhea also occurs when prebiotics are consumed in high amounts and are not completely fermented. Fermentation in the colon leads to the production of SCFAs and gas, which can lead to bloating, yet helps prevent diarrhea by reducing the osmotic effect (Marteau & Seksik, 2004). Thus, intolerance symptoms may be the result of the osmotic effect and/or fermentation.

The inulin diet may have also induced diarrhea because there was no source of insoluble fiber. Insoluble fibers, such as cellulose, have a direct bulking effect and adsorb water, thus increasing fecal volume and stool weight (Roberfroid, 2005). Insoluble fibers that are not fermented, or only poorly fermented are the most effective stool bulking agents. For instance, cellulose has a bulking index (g increase in stools per g of intake) of 3.4, while inulin-type fructans have a bulking index of 1.7 (Roberfroid, 2005). However, even when a source of insoluble fiber is added, 10% inulin may still be too high. Verghese et al (2002) found that feeding rats a mix of 5% cellulose and 10% long-chain inulin (Orafti, Raftilin, average DP ~40) induced diarrhea after 2 weeks of feeding, but the animals recovered by ~4 weeks. Similarly, Hosono et al (2003) found that mice fed a mix diet containing 5% cellulose and 7.5% FOS had diarrhea during

the initial week of the experiment, while a mix diet with 5% cellulose and 2.5% FOS was well tolerated and still showed beneficial effects.

It can be concluded that 10% inulin is too high for a short term study because adaptation to a high dose does not have a chance to occur. To give animals a chance to adapt they could start with a lower amount of inulin and gradually increase the dose, however this would require a longer acclimatization period. Since other researchers have shown immune effects with lower doses of prebiotics, the experimental diet will contain 5% inulin for the Main Study.

A source of insoluble fiber must also be included in the experimental diet. To determine if the immune effects are due to prebiotic fibers, or simply a high fiber diet, both control and experimental diets in the Main Study will contain a total of 10% fiber. Thus, the control diet will contain 10% cellulose, while the experimental diet will contain a mix of 5% cellulose with 5% long-chain inulin.

Feed Intake, Body Weight and Feed Efficiency

Cellulose fed animals consumed 16% more diet than inulin fed animals (Figure 9). However there was no difference in body weight or feed efficiency (Figure 10, 11), thus a pair-fed group is not required for the Main Study. These findings will be discussed further with the Main Study discussion.

IV. MAIN STUDY

MATERIALS AND METHODS

Animals and Diets

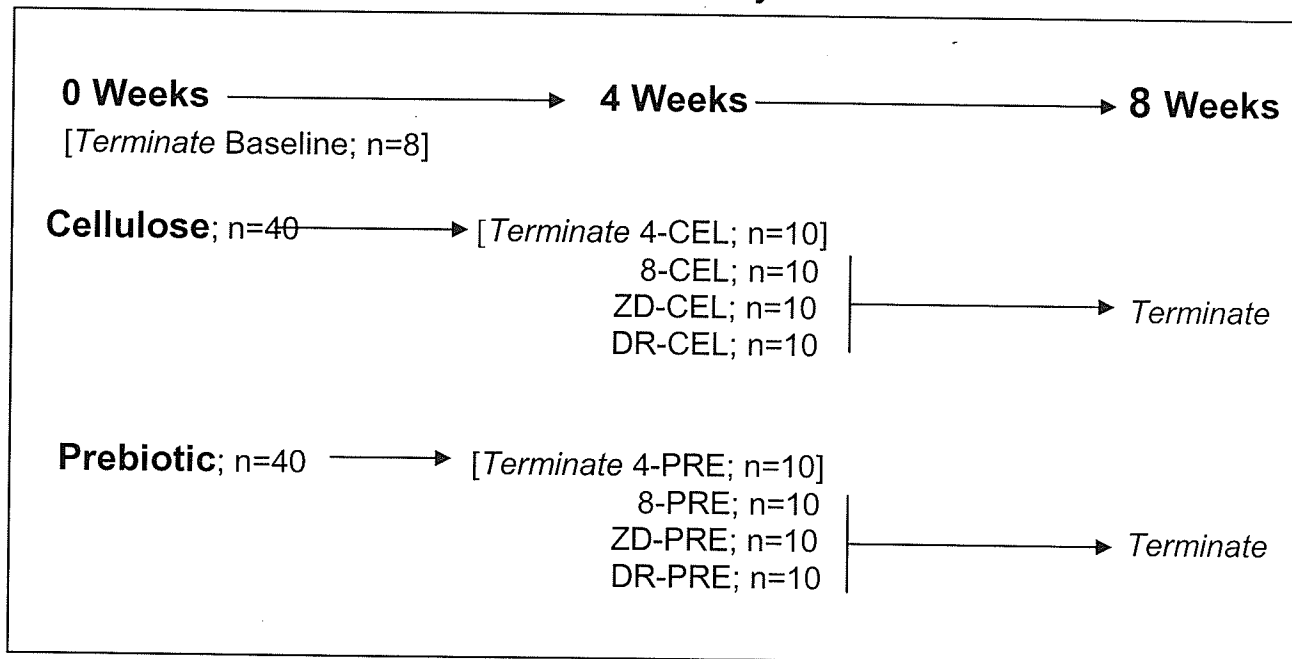
Eighty-eight 3-week-old female Sprague Dawley rats (Charles River Laboratories, St. Constant, Canada) were acclimatized for 7 days and randomly assigned to be terminated at baseline (BASE; n=8), or were fed a control diet (30 mg Zn/kg diet, 5% cellulose) supplemented with either cellulose (CEL, 5% cellulose; n=40) or inulin (PRE, 5% inulin from Beneo HP, gift from Orafit Group, Tienen, Belgium; n=40) for 4 weeks. At the end of 4 weeks, ten rats were terminated from each group (4-CEL; n=10 and 4-PRE; n=10), and ten rats remained in their original groups for another 4 weeks (8-CEL; n=10 and 8-PRE; n=10). The remaining rats were fed a zinc deficient diet (1 mg Zn/kg diet) supplemented with cellulose or inulin (ZD-CEL; n=10 and ZD-PRE; n=10) or fed the zinc normal cellulose or inulin diets in restricted amounts (DR-CEL; n=10 and DR-PRE; n=10) for 4 weeks (Figure 12). A consequence of zinc deficiency is a reduction in feed intake and growth, thus a diet-restricted group is required to differentiate the effects of malnutrition verses zinc deficiency *per se*.

The diet formulations (Table 4) are based on the AIN-93G diet (Reeves et al, 1997) and were modified as described under preliminary study 1. Tert-butylhydroquinone was added as an antioxidant to prevent deterioration of the diet. Rats were provided with diet and Millipore water ad libitum, available in glass dishes and plastic bottles with stainless steel sipper tubes, respectively.

The rats were maintained in an environment of controlled temperature (21-23°C), humidity (55%), and light cycle (14:10 hr light:dark). Body weights were determined weekly, and feed intake was determined daily. Clean lab coats and disposable gloves were always worn when handling rats to minimize zinc exposure.

In collaboration with Dr. J. Meddings (University of Calgary), animals were tested for intestinal permeability (results will not be reported in this thesis). Five days prior to termination, animals were fasted (no food or water) for 4 hours, then gavaged with a 2 ml solution containing 1 g sucrose, 120 mg lactulose, 80 mg mannitol and 60 mg sucralose. Animals were placed in individual metabolic cages for 2 hours with no food or water, and then allowed free access to water for the next 22 hours. During this time, urine was collected into plastic tubes containing 10% thymol as an antibacterial agent and 100 µl of mineral oil to prevent evaporation.

Figure 12: Experiment Outline for Main Study



Abbreviations: 4-CEL= Cellulose for 4 weeks; 8-CEL = Cellulose for 8 weeks; ZD-CEL = Zinc Deficient Cellulose; DR-CEL = Diet Restricted Cellulose; 4-PRE = Prebiotic for 4 weeks; 8-PRE = Prebiotic for 8 weeks; ZD-PRE = Zinc Deficient Prebiotic; DR-PRE = Diet Restricted Prebiotic

Table 3. Diet Formulations For Main Study¹

Ingredient (g)	CEL	PRE	ZD-CEL	ZD-PRE
Cornstarch	312.59	312.59	322.59	322.59
Maltodextrin	132	132	132	132
Sucrose	100	100	100	100
Egg white	212.5	212.5	212.5	212.5
Biotin mix ³	10	10	10	10
Mineral mix (AIN-93 MX, Zn free)	35	35	35	35
Zinc premix ⁴	10	10	0	0
Vitamin mix (AIN-93-VX)	10	10	10	10
Potassium Phosphate	5.4	5.4	5.4	5.4
Choline	2.5	2.5	2.5	2.5
Tert-butylhydroquinone	0.014	0.014	0.014	0.014
Cellulose	100	50	100	50
Inulin	0	50	0	50
Soybean oil	70	70	70	70

¹The above diet formulations were used to make 1 kg of each diet. All ingredients from Harlan Teklad (Madison, WI) except potassium phosphate (Fisher Scientific, Mississauga, ON).

²Actual amount of zinc in diet as determined by atomic absorption was 35 (CEL), 34 (PRE), 1.2 (ZD-CEL) and 1.1 (ZD-PRE) mg Zn/kg diet.

³Biotin mix was 200 mg biotin/kg cerelose

⁴Zinc premix was 5.775 g Zn carbonate/kg cerelose

Tissue Collection

Rats were terminated by CO₂ asphyxiation and decapitation. Trunk blood was collected, centrifuged at 2400 RPM at 4°C for 10 minutes to obtain serum and stored at -80°C until analysis. To remove tissues aseptically, 70% ethanol was poured on animal prior to opening the abdominal cavity, and sterile scissors and tweezers were used. The spleens were removed and placed in 50 ml sterile polypropylene centrifuge tubes with 15 ml of sterile KRH buffer (130 mM NaCl, 10 mM HEPES, 5.2 mM KCl, 1.4 mM CaCl₂, 1.0 mM NaH₂PO₄, 1.4 mM MgSO₄ with 0.5% bovine serum albumin (BSA) and 1% antibiotic/antimycotic, pH 7.4) and stored on ice. The gut was removed and the MLN were extracted with sterile tweezers, and placed in 50 ml sterile polypropylene centrifuge tubes with 15 ml of sterile KRH buffer and stored on ice. The small intestine was flushed with 30 ml of Hank's buffer (136 mM NaCl, 5.36 mM KCL, 0.44 mM KH₂PO₄, 0.30 mM Na₂HPO₄ · 7H₂O, 5.55 mM glucose, 2.01 mM Dithiothreitol (DTT), 1.56 mM Ethylenediaminetetraacetic acid (EDTA) with 1% antibiotic/antimycotic, pH 7.4) to remove fecal material, and then weighed and measured. PP were cut out of the small intestine with sterile scissors and placed in 15 ml sterile polypropylene centrifuge tubes with 10 ml sterile Hank's buffer and stored on ice. Parametrical fat pads were removed and weighed.

Preparation of Lymphocytes

The following procedures were obtained from Field et al, 1999 and Stillie et al, 2005.

From Spleen

Each spleen was pushed through nylon mesh (soaked in 70% ethanol and air dried immediately prior to use), using the barrel of a 10 ml sterile syringe, into a Petri dish containing 5 ml of KRH buffer. Remaining matter was rinsed off screen into Petri dish with ~ 5 ml KRH buffer using a 30 ml syringe with an 18 gauge needle. Solution was sucked up with 10 ml sterile syringe and put into 50 ml sterile polypropylene centrifuge tube (total volume of ~ 12 ml). The samples were centrifuged at 1000 RPM for 10 minutes at 4°C to pellet the cells. The supernatant was discarded and 10 ml ACK lysis buffer (155 mM NH_4Cl , 0.1 mM EDTA and 10 mM KHCO_3 , pH 7.2) was added to remove excess red blood cells. Samples were vortexed, left on ice for five minutes, then 20 ml KRH buffer was added to stop reaction. Samples were centrifuged at 1000 RPM for 10 minutes at 4°C to pellet the cells and supernatant was discarded. The pellet was resuspended in cell culture medium (RPMI-1640, 10 mmol/L HEPES, 10 mmol/L sodium bicarbonate, 1 mmol sodiumpyruvate, 0.1 mmol/L non-essential amino acids, 50 $\mu\text{mol/L}$ 2-mercaptoethanol, 1% antibiotic/antimycotic and 5% fetal bovine serum, pH 7.2). Cells were counted using a hemacytometer and trypan blue.

From Mesenteric Lymph Nodes

Mesenteric lymph nodes were removed from liquid and pushed through nylon mesh (soaked in 70% ethanol and air dried immediately prior to use), using the barrel of a 10 ml sterile syringe, into a Petri dish containing 5 ml of KRH buffer. Remaining matter was rinsed off screen into Petri dish with ~ 5 ml KRH buffer using a 30 ml syringe with an 18 gauge needle. The solution was sucked up with 10 ml sterile syringe and put into 15 ml sterile polypropylene centrifuge tube (total volume of ~ 12 ml). The samples were centrifuged at 1000 RPM for 10 minutes at 4°C to pellet the cells. The supernatant was discarded and the samples were resuspended in 10 ml of cell culture medium. Cells were counted using a hemacytometer and trypan blue.

From Peyer's Patches

Tubes containing PP were vortexed (Vortex-Genie, Scientific Industries INC, Model K-550-G) at highest speed for 4 minutes to remove mucus and epithelial cells, and then stored on ice for 30 minutes. Liquid was removed with a sterile transfer pipette (discarded) and 10 ml of fresh Hank's buffer was added and PP were vortexed at highest speed for another 2 minutes. PP were removed from liquid and pushed through nylon mesh (soaked in 70% ethanol and air dried), using the barrel of a 10 ml sterile syringe, into a Petri dish containing ~ 5 ml of Hank's buffer. Remaining matter was rinsed off screen into Petri dish with ~ 5 ml Hank's buffer using a 30 ml syringe with an 18 gauge needle. Solution was sucked up with 10 ml sterile syringe and put into 15 ml sterile polypropylene centrifuge tube (total volume of ~ 12 ml).

The samples were centrifuged at 1000 RPM for 10 minutes at 4°C to pellet the cells. The supernatant was discarded and the samples were resuspended in 10 ml of cell culture medium and filtered with a 40 micron cell strainer (VWR International) to remove excess debris. Cells were counted using a hemacytometer and trypan blue.

Immunofluorescence Staining

The expression of the cell surface markers TCR $\alpha\beta$, TCR $\gamma\delta$, CD4, CD8, CD45RA and OX62 on the immune cells of the spleen, MLN and PP were investigated by immunofluorescence. The majority of T cells in circulation and lymphoid organs express the $\alpha\beta$ T-cell receptor (TCR), commonly with the co-receptor of either CD4 (specific for T-helper cells) or CD8 (specific for cytotoxic T-cells) (Dyugovskaya et al, 2003). A less frequent population of T cells express the $\gamma\delta$ TCR. Although the majority of mouse and approximately half of human peripheral $\gamma\delta$ T-cells express neither CD4 nor CD8, rat $\gamma\delta$ T-cells in lymph nodes and spleen are almost exclusively (>90%) of the CD4⁻CD8⁺ phenotype (Kuhnlein et al, 1994). CD45 is found on most B-lymphocytes, including developing B cells in the bone marrow and peripheral B cells, but not plasma cells (BD Pharmingen, 2003). OX62 is expressed by rat dendritic cells and by $\gamma\delta$ T-cells (Serotec, 2006).

Prepared cells were split into two 5 ml polystyrene tubes, adjusted to a density of 1×10^6 cells/ml and centrifuged at 1000 RPM for 6 minutes. Supernatant was discarded and pellet was resuspended in 100 μ l stain buffer (phosphate buffered saline (PBS) containing 0.2% BSA and 0.09% sodium azide,

pH 7.2). In tube 1, cells were incubated with R-phycoerythrin (R-PE)-conjugated mouse anti-rat $\alpha\beta$ T-cell receptor monoclonal antibody (5 μ l/test; BD biosciences), fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat $\gamma\delta$ T-cell receptor monoclonal antibody (2 μ l/test; BD biosciences), allophycocyanin (APC)-conjugated mouse anti-rat CD4 monoclonal antibody (5 μ l/test; BD biosciences) and peridinin chlorophyll-a protein (PerCP)-conjugated mouse anti-rat CD8a monoclonal antibody (5 μ l/test; BD biosciences) for 30 minutes in the dark at 4°C. In tube 2, cells were incubated with PE-Cy5-conjugated mouse anti-rat CD45RA monoclonal antibody (5 μ l/test; BD biosciences), fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat $\gamma\delta$ T-cell receptor monoclonal antibody (2 μ l/test; BD biosciences) and mouse anti-rat OX62: RPE (10 μ l/test; Serotec) for 30 minutes in the dark at 4°C. After washing with stain buffer, cells were fixed with 1% (v/v) paraformaldehyde. The following morning, cells were strained (in the dark) into 5 ml polystyrene round-bottom tubes with cell-strainer caps (BD Falcon, Fisher Scientific). Samples were then placed on ice and transported in a dark container for ~30 minutes to Health Sciences Centre and then analyzed with a BD FACSCalibur cytometer (BD Biosciences, San Jose, California) with dual lasers (blue argon 488 nm and He-Ne red diode 635 nm). Cells were analyzed using the BD FACStation data management system software provided with the instrument.

Mitogen Stimulation

Prepared cells from the spleen, MLN and PP were stimulated with ConA (from *Canavalia ensiformis*; MP Biomedicals; #150710), a potent mitogen used to stimulate cell proliferation in T-cells (Alexander & Brutlag, 2005).

Prepared cell suspensions (1 ml) were transferred into 5 ml polystyrene sterile culture tubes and 100 μ l of 2.5 μ g/ml ConA or cell culture media (for unstimulated cells) was added and incubated for 48 hours at 37°C with 5% CO₂. After incubation, samples were spun at 1500 RPM for 10 minutes to pellet cells. Supernatant was removed and stored at -80°C until analysis.

Cytokines

The supernatant fractions of ConA stimulated cells was analyzed for the following cytokines using a rat LINCOplex kit (Linco Research Inc, St. Charles, Missouri): IL-2 and IFN- γ , which represent Th1 cells and IL-10, which represents Th2/Th3 cells. The LINCOplex kit uses the Luminex system (version 1.7 of the xMAP technology operating system). Systems using xMAP technology “perform discrete bioassays on the surface of color-coded beads known as microspheres, which are then read in a compact analyzer. Using multiple lasers and high-speed digital-signal processors, the analyzer reads multiplex assay results by reporting multiple colors on each individual microsphere particle” (Luminex Corporation, 2004). This technology enables multiplexing of bioassays, reducing time, labor and costs over traditional methods.

The following procedure has been previously described (Linco Research, 2004). All buffers and solutions were provided with the LINCOplex kit unless

otherwise noted. The 96-well filtration plates were coated with 200 μ l of Assay buffer, incubated for 10 minutes at room temperature, and liquid was removed by vacuum. Standards, controls and undiluted samples (25 μ l/well) were added, followed by 25 μ l of mixed beads. To prepare mixed beads, 0.15 ml from each of the three bead sets (anti-rtIL-2, anti-rtIL10 and anti-rtIFN- γ) was added to the mixing bottle, along with 2.55 ml of Assay buffer. The plates were sealed, covered with aluminum foil and incubated with gentle agitation on a plate shaker overnight (18-20 hours) at 2-8°C. Fluid was removed by vacuum filtration and plates were washed 2 times with 200 μ l/well of Wash Buffer. Detection Antibody Cocktail (25 μ l/well) was added and plates were sealed, covered and incubated for 2 hours with agitation on a plate shaker at room temperature. Streptavidin-Phycoerythrin (25 μ l/well) was added and the plates were sealed, covered and incubated for 30 minutes with agitation on a plate shaker at room temperature. All contents were removed by vacuum and the plates were washed 2 times with 200 μ l/well of Wash Buffer. Sheath Fluid (Luminex Corporation, Austin, Texas) was added (100 μ l/well) and plates were covered with aluminum foil and mixed on a plate shaker for 5 minutes. Prepared plates were run on Luminex (Lincoplex 200, Luminex Corporation, Austin, Texas) and data was evaluated using GraphPad Prism, Version 2 (GraphPad Software Inc, San Diego, CA).

Immunoglobulins

The following procedure was adapted from the ELISA protocol by BD Biosciences, 2007.

A sandwich enzyme linked immunosorbent assay (ELISA) method was used to measure the amount of Th1 (IgG2a) and Th2 (IgG1) type immunoglobulins in the serum. Maxisorb 96-well microtiter plates (NUNC, Roskilde, Denmark) were coated with 100 μ L of appropriate coating antibody [mouse anti-rat IgG1 monoclonal antibody (BD Pharmingen; diluted 0.20 μ g/ml in PBS: 136.89 mM NaCl, 8.17 mM Na₂HPO₄, 1.46 mM KH₂PO₄, 2.68 mM KCl, pH 7.2) or mouse anti-rat IgG2a monoclonal antibody (BD Pharmingen; diluted 0.50 μ g/ml in PBS)] and incubated for 1 hour at room temperature on shaker (200 rpm). After incubating with 200 μ L of blocking buffer (PBS containing 1% w/v BSA) for 30 minutes and washing 1x (PBS, containing 0.05% v/v Tween 20), 100 μ L of blanks (blocking buffer) standards (rat reference serum diluted over a range of 1000 ng/ml to ~2 ng/ml; Bethyl Laboratories, Montgomery, Texas) and samples (diluted 40 000x with blocking buffer) were added and incubated for 1 hour at room temperature on shaker. After washing 3x, 100 μ L of appropriate detection antibody [biotin-conjugated mouse anti-rat IgG1 monoclonal antibody (BD Biosciences; diluted 0.20 μ g/ml in blocking buffer) or biotin-conjugated mouse anti-rat IgG2a monoclonal antibody (BD Biosciences; diluted 0.50 μ g/ml in blocking buffer)] were added and incubated for 1 hour at room temperature on shaker. Plates were washed 6x and avidin-horseradish peroxidase conjugate (BD Biosciences) diluted 1:1000 in blocking buffer was added and incubated for

30 minutes at room temperature on shaker. Plates were washed 6x, and 100 μ L TMB substrate (mixed equal parts of reagent A and B; BD Biosciences) was added, incubated for 20 minutes at room temperature on shaker, followed by addition of 50 μ L 1.0 M phosphoric acid to stop the reaction. Absorbance was read at 450 nm - 570 nm using a microplate reader (SpectraMax 340; Molecular Devices Corp. Sunnyville California), and analyzed with a 4-parametric logistic curve fit using SOFTmax PRO Software (Version 1.2.0, Molecular Devices Corp. Sunnyville California).

DEXA Analysis

In collaboration with Dr. Hope Weiler (McGill University), rat carcasses were analyzed for fat, lean body mass, bone mineral content (BMC) and bone mineral density (BMD) using dual energy x-ray absorptiometry (DEXA, 4500A; Hologic Inc., Bedford, MA). Animals were scanned after the spleen, stomach, small intestine, colon, cecum and parametrical fat pads had been removed. BMC and BMD will not be reported in this thesis.

Mineral Analysis

Whole femurs and diet samples were wet-ashed using trace element grade nitric acid as previously described (Clegg et al, 1981). After obtaining wet weights, whole femurs and bovine liver reference (standard reference material 1577b, U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) were placed on labeled aluminum foil and dried for 48 hours in an 85°C drying oven. Dry weights were recorded, and then dried

femurs, diet samples and bovine liver reference were placed into individual labeled Digi/PREP tubes (SCP Science, Baie D'Urfe, Quebec). Trace element grade nitric acid (Fisher Scientific, XX, ON) was added to femur, diet and quality control samples as required: Femur (~0.3 - 0.4 g dry) = 3-4 mL acid; Diets (0.5 g dry) = 4 mL; Bovine Liver Reference = (0.2 – 0.5 g dry) = 3-4 mL. An equal amount of nitric acid was added to 2-3 glass tubes to serve as blanks. Each sample was covered loosely with a Digi/PREP tube lid and kept at 85°C in a Digi/PREP (SCP Science, Baie D'Urfe, Quebec) until femurs and diets were completely dissolved. Once cooled, samples were diluted with Millipore water accordingly: Femur = 25 mL for Zinc Deficient Rats and 100 mL for Control/Diet-Restricted Rats; Diet = 10-15 mL for Zinc Deficient and 20 mL for Control/Diet-Restricted Rats; Bovine Liver Reference = 20 mL for 0.2 g. Serum samples were not treated with acid digestion, and were diluted with Millipore water accordingly: Severe Zinc Deficiency = 400 µL serum + 400 µL water; Control/Diet-Restricted = 200 µL serum + 800 µL water. Zinc concentrations were determined by atomic absorption spectroscopy using a Spectra AA-30 Spectrophotometer (Varian Canada, Georgetown, ON).

Statistical Analysis

Data was analyzed by ANOVA using the general linear models procedure (SAS software release 8.2, SAS Institute). To determine fiber effects over time, data for the BASE, 4-CEL, 4-PRE, 8-CEL and 8-PRE were analyzed using one-way ANOVA. To determine main effects (fiber, zinc and fiber x zinc interaction) at the end of the study, data for CEL (represents 8-CEL, ZD-CEL and DR-CEL),

PRE (represents 8-PRE, ZD-PRE and DR-PRE), ZN (represents 8-CEL and 8-PRE), ZD (represents ZD-CEL and ZD-PRE), and DR (represents DR-CEL and DR-PRE) were analyzed using two-way ANOVA. Feed efficiency was analyzed using two-way ANOVA with repeated measures. When necessary, data was normalized by log transformation for statistical analysis, but nontransformed means are reported. Significant differences between means were determined using Duncan's multiple range test. Differences were considered significant at $p < 0.05$.

VI. MAIN STUDY

RESULTS

Total Feed Intake

Over Time: At 4 weeks, there was no difference in total feed intake. At 8 weeks, the total feed intake of 8-PRE was ~8% lower compared to 8-CEL (Figure 13).

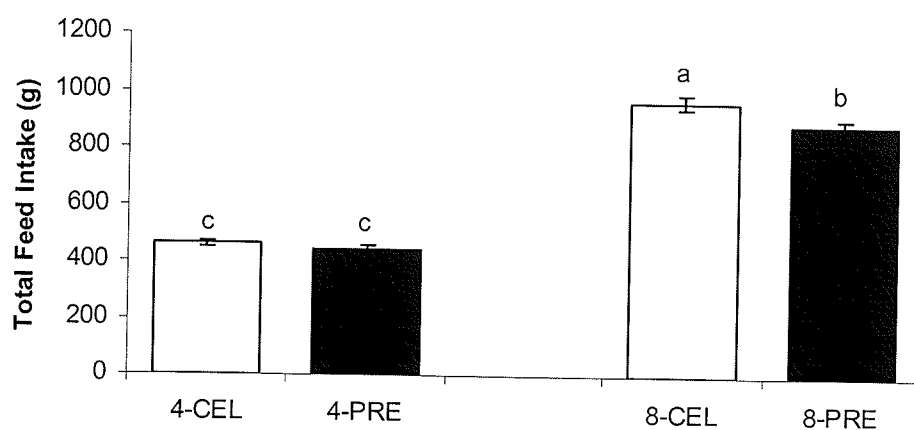


Figure 13: Total Feed Intake over Time

The p value < 0.0001. Values are means $\pm$ SE, n=10 per group. Data were analyzed by one-way ANOVA and statistical differences among means ($p \leq 0.05$) are indicated by lowercase letters. 4-CEL = 4 week cellulose; 4-PRE = 4 week prebiotic; 8-CEL = 8 week cellulose; 8-PRE = 8 week prebiotic.

End Point: At 8 weeks, the total feed intake of PRE was ~7% lower compared to CEL. The total feed intake of ZN was 6% higher compared to ZD and 10% higher compared to DR (Figure 14).

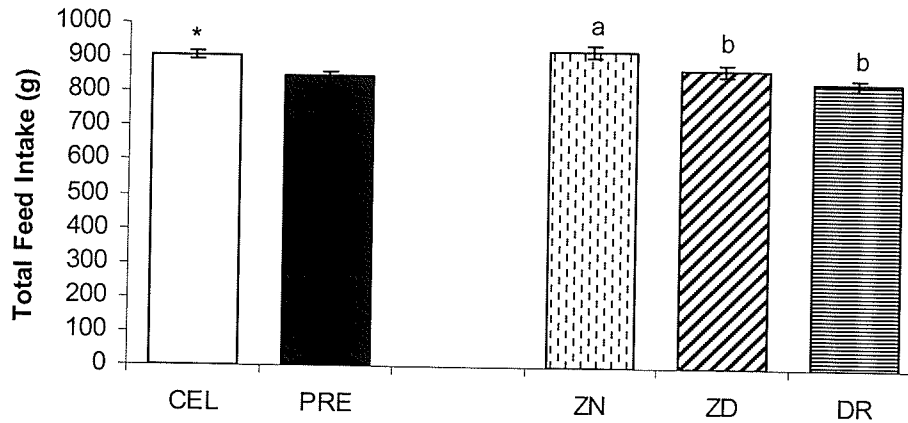


Figure 14: Total Feed Intake of 8 Week Groups

Fiber Effect $p = 0.0006$; Diet Effect $p = 0.0003$. As there was no interaction between fiber and dietary treatment (zinc or diet restriction), but there was a main effect of fiber and a main effect of dietary treatment, data were pooled to show means of main effects only. CEL represents 8-CEL, ZD-CEL and DR-CEL ($n=30$); PRE represents 8-PRE, ZD-PRE and DR-PRE ($n=30$); ZN represents 8-CEL and 8-PRE ($n=20$); ZD represents ZD-CEL and ZD-PRE ($n=20$); DR represents DR-CEL and DR-PRE ($n=20$). Values are means $\pm$ SE. Data were analyzed by two-way ANOVA and statistical differences among means ($p \leq 0.05$) are indicated by an asterisk (fiber effect) or lowercase letters (dietary effect). CEL = cellulose; PRE = prebiotic inulin; ZN = zinc normal; ZD = zinc deficient; DR = diet restricted.

Termination Body Weight

Over Time: Body weight increased as the age of the rats increased. There was no difference between CEL and PRE groups at week 4 or week 8 (Figure 15).

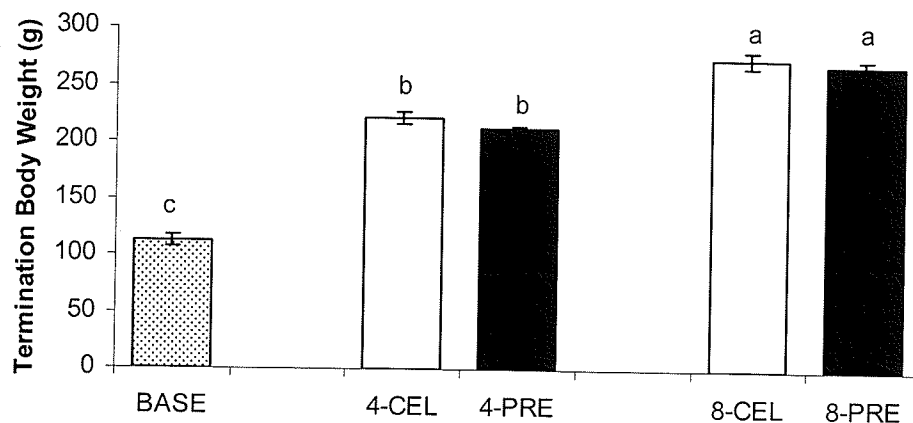


Figure 15: Termination Body Weight over Time

The p value < 0.0001 . Values are means $\pm$ SE. Data were analyzed by one-way ANOVA and statistical differences among means ($p \leq 0.05$) are indicated by lowercase letters. BASE= baseline ($n=8$); 4-CEL = 4 week cellulose ($n=10$); 4-PRE = 4 week prebiotic ($n=10$); 8-CEL = 8 week cellulose ($n=10$); 8-PRE = 8 week prebiotic ($n=10$).

End Point: At termination, there was no difference in body weight between CEL and PRE. ZN weighed 11% and 10% more compared to ZD and DR, respectively (Figure 16).

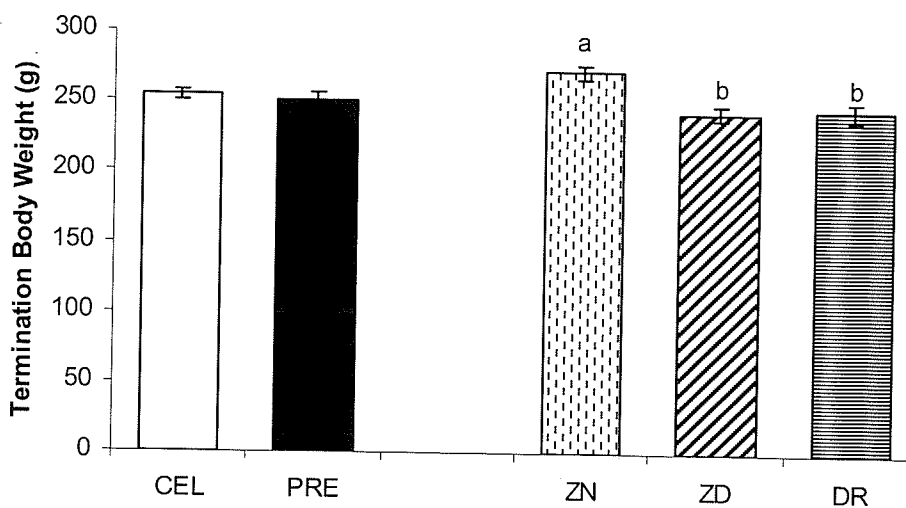


Figure 16: Termination Body Weight of 8 Week Groups

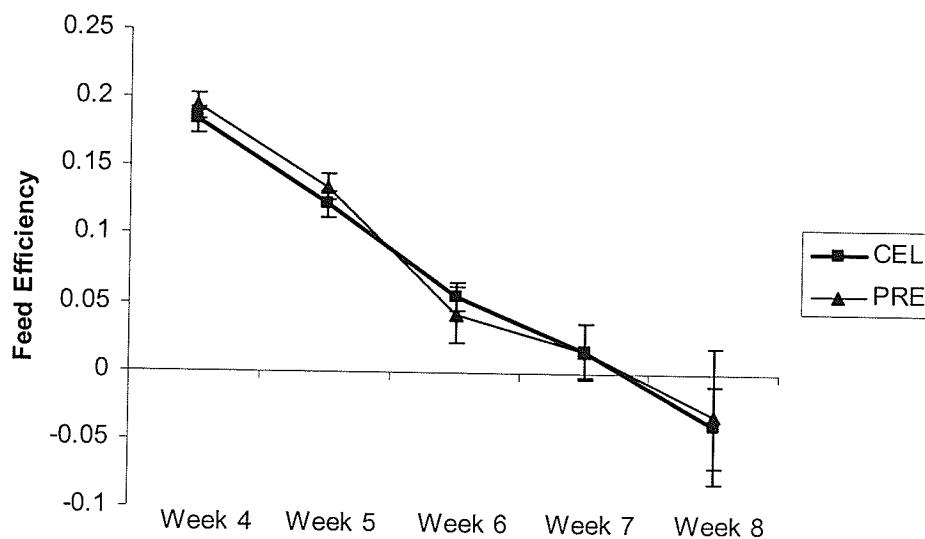
Diet Effect $p = 0.0006$. Statistical analysis and abbreviations as described in Figure 14.

Feed Efficiency

There was no difference in feed efficiency between CEL and PRE (Figure 17a).

Overall the feed efficiency of ZD was lower than DR, which was lower than ZN (Figure 17b).

a) Fiber Effect



b) Diet Effect

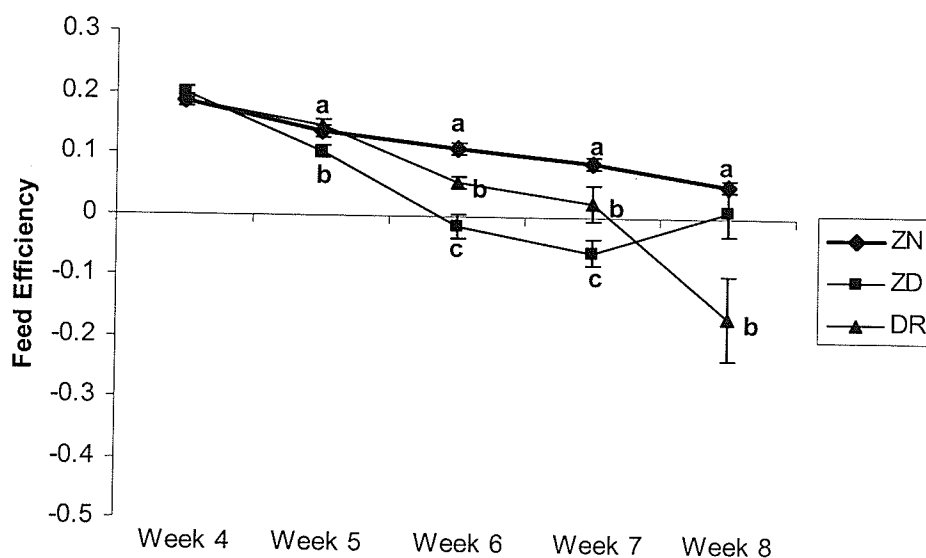


Figure 17: Feed Efficiency over Time of 8 Week Groups

Diet Effect $p < 0.0001$. Statistical analysis as described in Figure 14, except data was analyzed by two-way ANOVA with repeated measures. Feed efficiency was calculated as g of weight gain per week/g of feed intake per week.

Body Composition

a) Parametrical Fat Pads

Over Time: There was no difference between CEL and PRE groups at week 4.

At week 8, 8-PRE was 31% lower compared to 8-CEL (Figure 18).

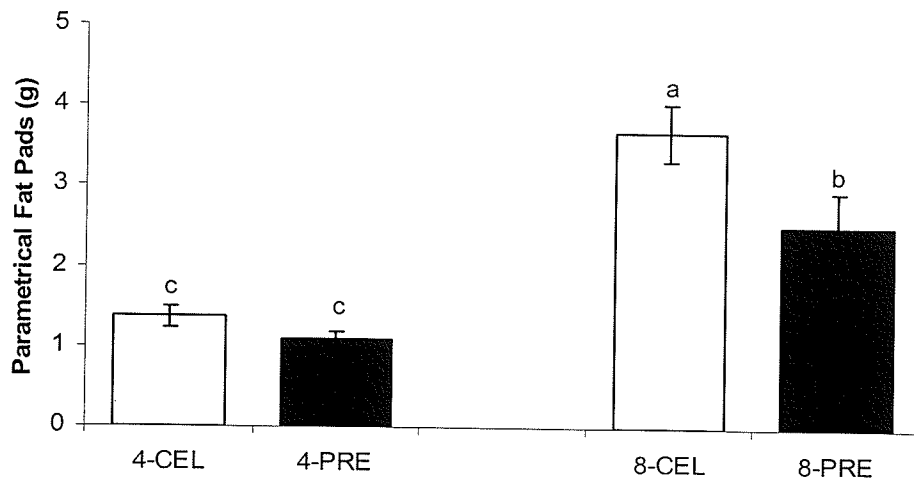


Figure 18: Parametrical Fat Pads Over Time

The p-value < 0.0001. Statistical analysis and abbreviations as described in Figure 13.

End Point: There was no difference between CEL and PRE. ZN was 49% and 53% higher compared to ZD and DR, respectively (Figure 19).

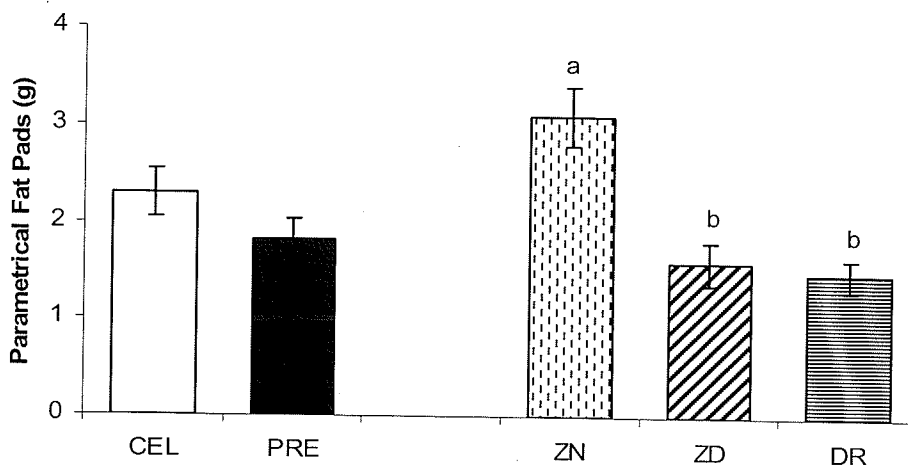


Figure 19: Parametrical Fat Pads of 8-Week Groups

Diet Effect p < 0.0001. Statistical analysis and abbreviations as described in Figure 14.

b) Adipose Tissue as Determined by DEXA

Over Time: There was no difference between CEL and PRE groups at week 4.

The total percent of adipose tissue was 19% lower in 8-PRE compared to 8-CEL (Figure 20).

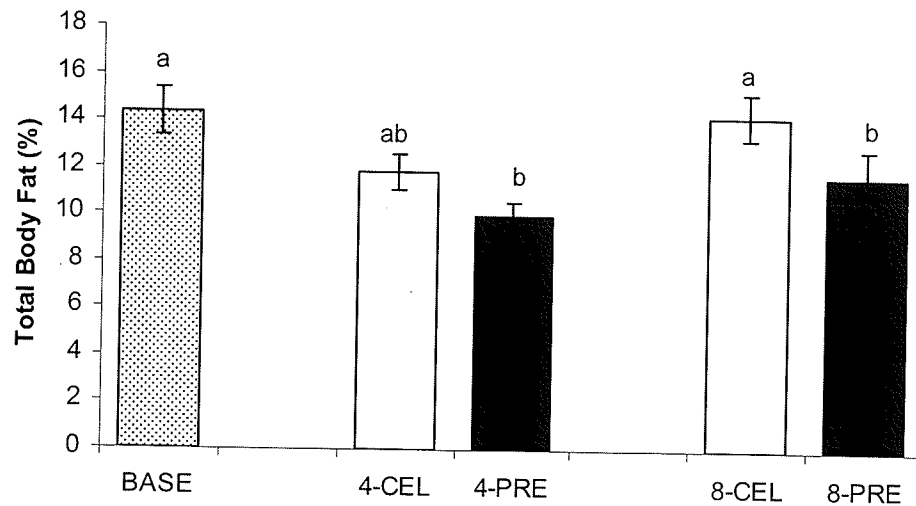


Figure 20: Adipose Tissue Over Time

The p value = 0.05. Statistical analysis and abbreviations as described in Figure 15.

End Point: There was no difference between CEL and PRE. ZN was 26% higher compared to ZD and 31% higher compared to DR (Figure 21).

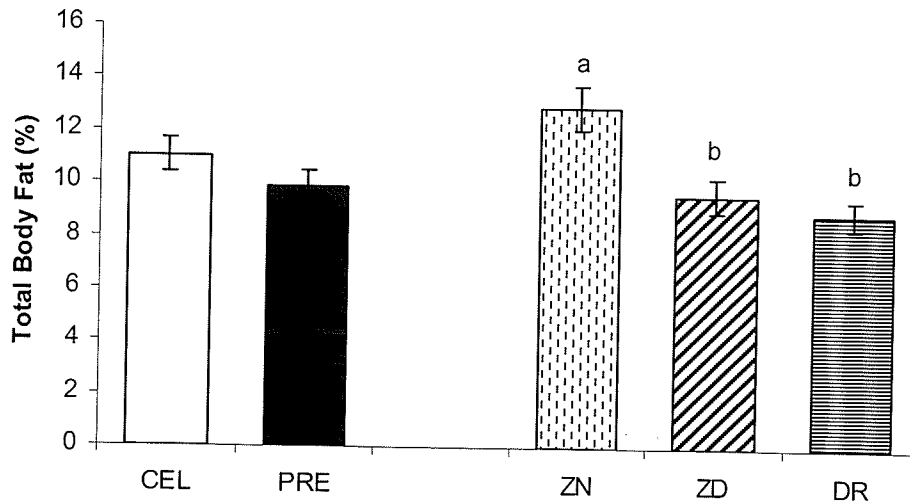


Figure 21: Adipose Tissue of 8 Week Groups

The p value = 0.0003. Statistical analysis and abbreviations as described in Figure 14.

c) Lean Non-Fat Mass as Determined by DEXA

Over Time: There was no difference between CEL and PRE groups at week 4 or week 8 (Figure 22).

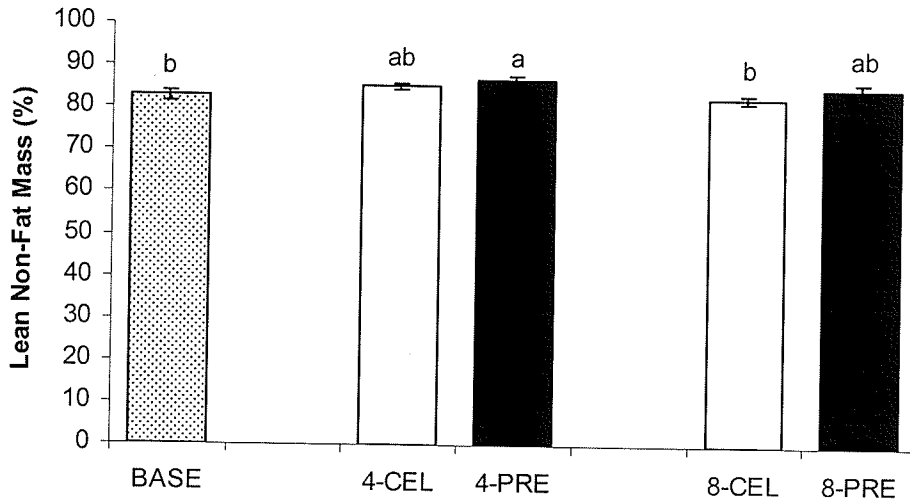


Figure 22: Lean Non-Fat Mass Over Time

The p value = 0.008. Statistical analysis and abbreviations as described in Figure 13.

End Point: There was no difference between CEL and PRE. ZN was 4% lower compared to both ZD and DR (Figure 23).

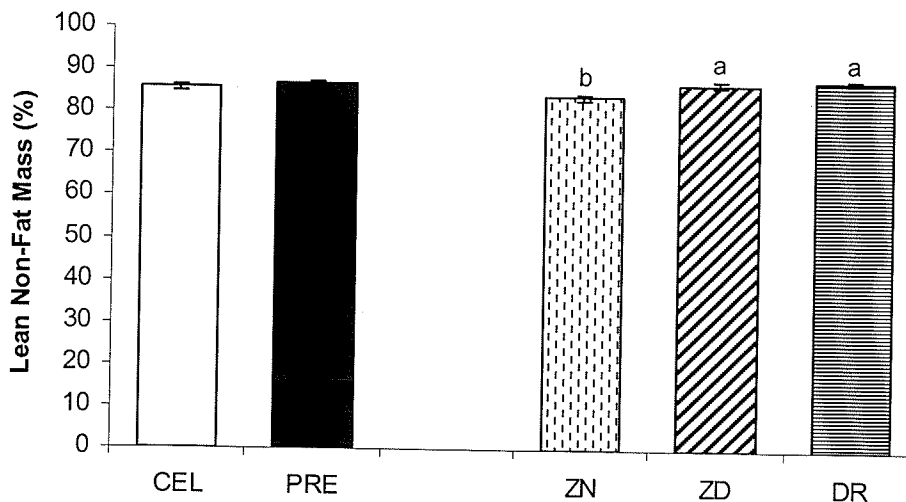


Figure 23: Lean Non-Fat Mass of 8-Week Groups

The p value = 0.0003. Statistical analysis and abbreviations as described in Figure 14.

Serum Zinc

Over Time: There was no difference between CEL and PRE at week 4 or week 8 (Figure 24).

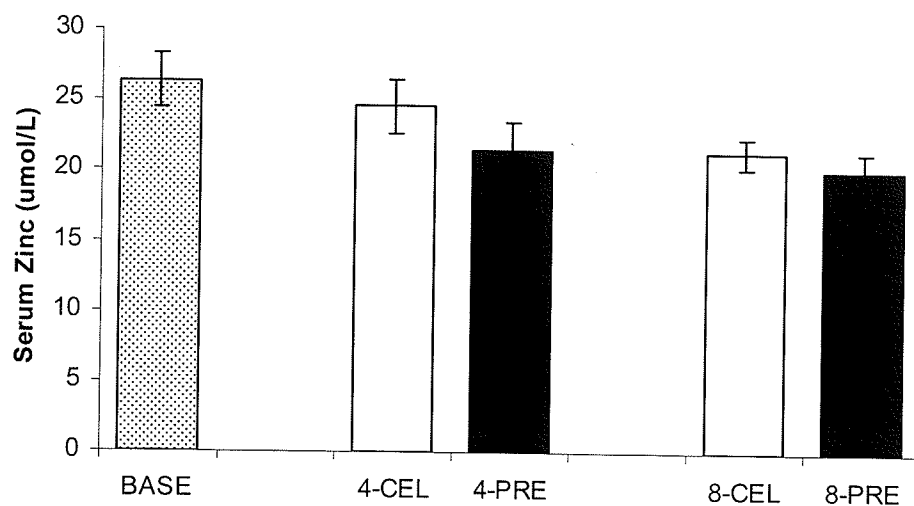


Figure 24: Serum Zinc Over Time

Statistical analysis and abbreviations as described in Figure 13.

End Point: There was no difference between CEL and PRE. Serum zinc was 85% lower in ZD compared to both ZN and DR (Figure 25).

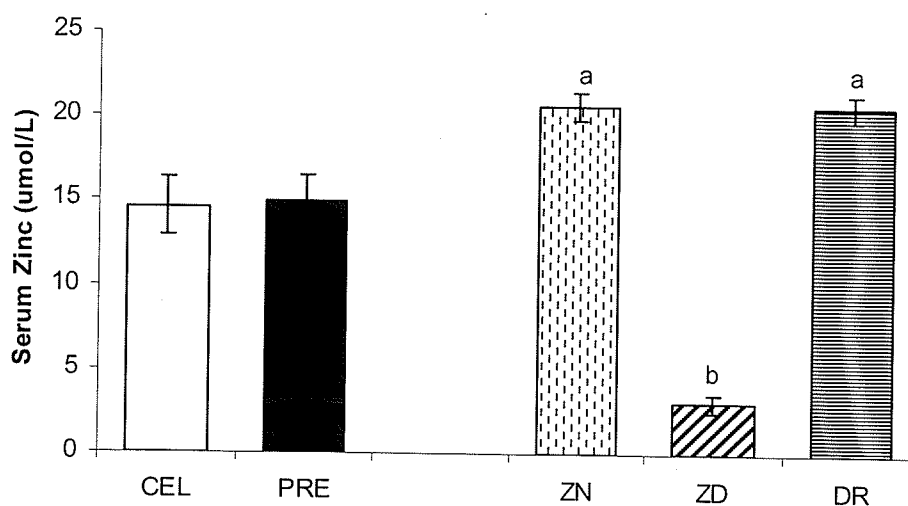


Figure 25: Serum Zinc of 8-Week Groups

Diet Effect $p < 0.0001$. Statistical analysis and abbreviations as described in Figure 14.

Femur Zinc

Over Time: Femur zinc of PRE was 13% lower than CEL at week 4. There was no difference between CEL and PRE at week 8 (Figure 26).

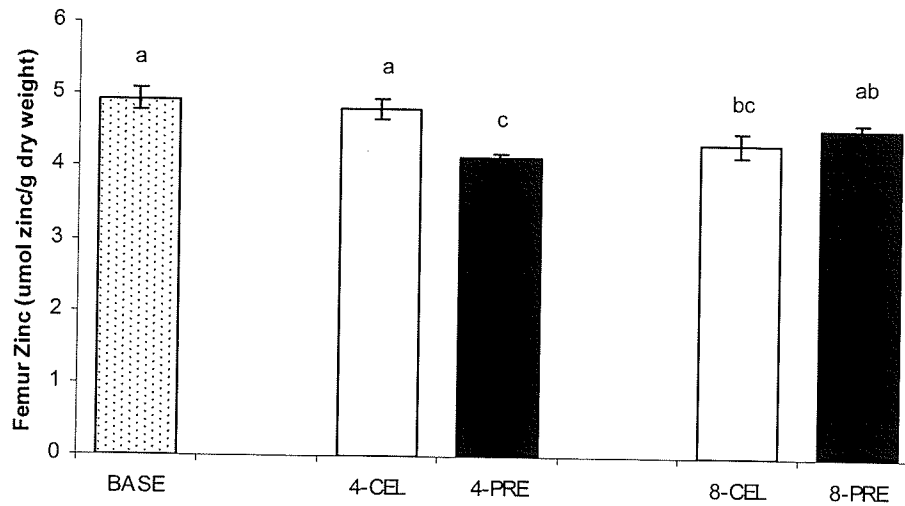


Figure 26: Femur Zinc Over Time

The p value = 0.0006. Statistical analysis and abbreviations as described in Figure 13.

End Point: ZD-CEL was 27% lower than DR-CEL and 35% lower than 8-CEL.

ZD-PRE was 35% lower than 8-PRE and 39% lower than DR-PRE. There was

also a significant fiber x diet interaction; DR-PRE was 21% higher than DR-CEL.

There was no difference between 8-CEL and 8-PRE or ZD-CEL and ZD-PRE

(Figure 27).

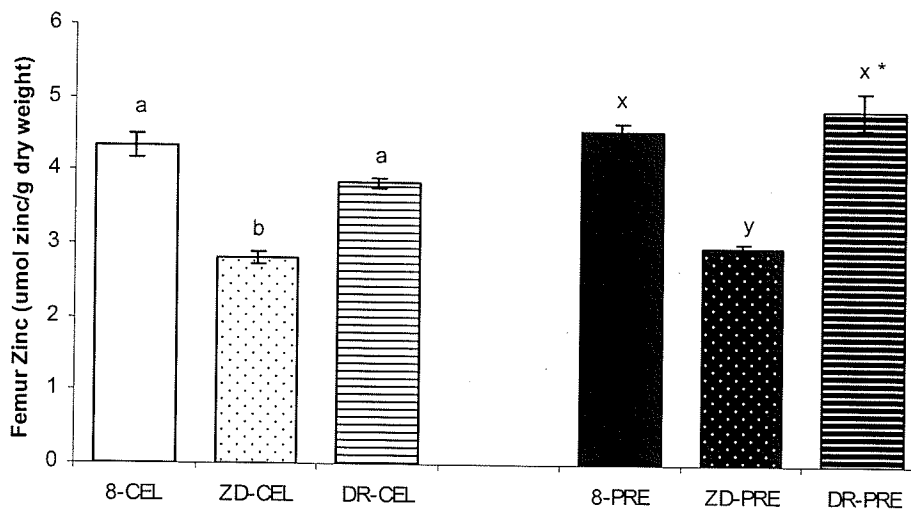


Figure 27: Femur Zinc of 8-Week Groups

Fiber x Diet Interaction $p = 0.01$. Statistical analysis and abbreviations as described in Figure 14.

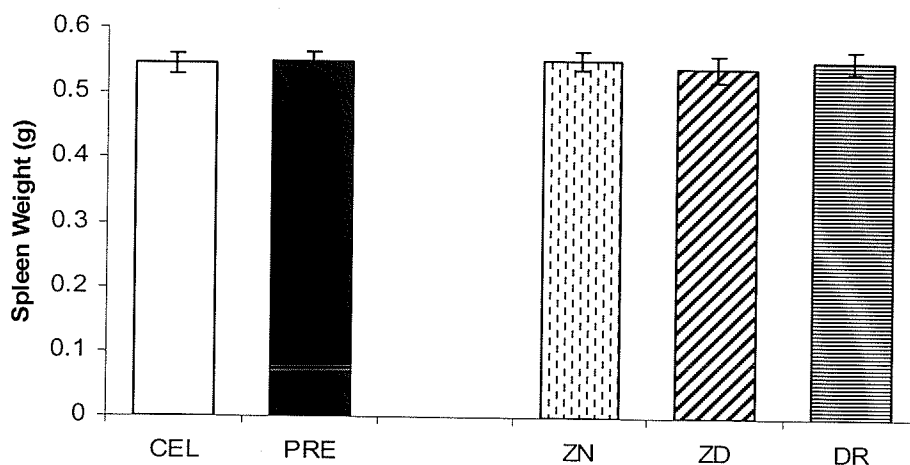
Organ Weights/Length

a) Spleen

Over Time: There was no difference between CEL and PRE at week 4 or week 8 (results not shown).

End Point: There was no difference between CEL and PRE, or among ZN, ZD or DR (Figure 28a). However, when adjusted for body weight, the spleen of ZN was 9% less than ZD and 10% less than DR (Figure 28b).

a)



b)

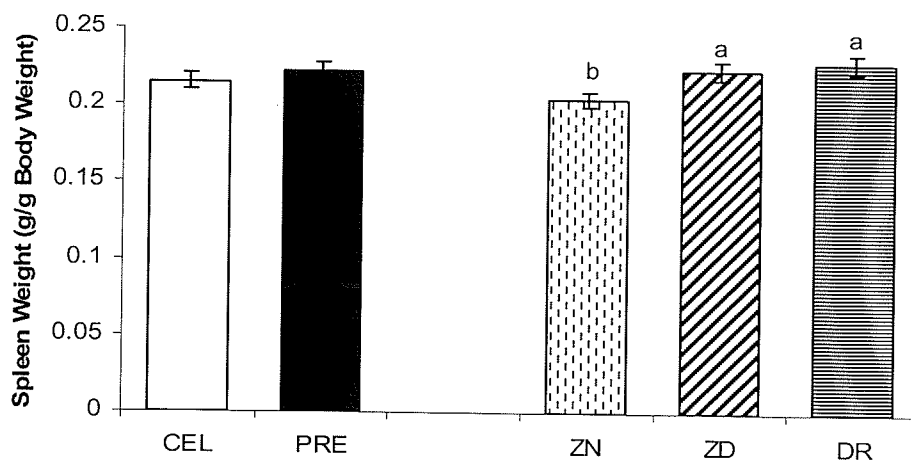


Figure 28: Spleen Weight for 8-Week Groups

Diet Effect $p = 0.02$. Statistical analysis and abbreviations as described in Figure 14.

b) Small Intestine Weight

Over Time: There was no difference between CEL and PRE at week 4. PRE was 6% higher compared to CEL at week 8 (Figure 29).

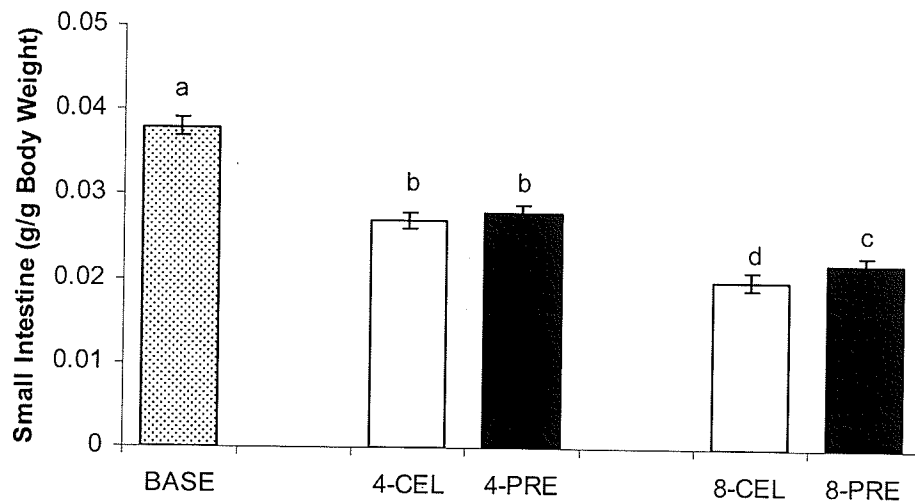


Figure 29: Small Intestine Weight over Time

The p-value < 0.0001. Statistical analysis as described in Figure 15.

End Point: The small intestine weight of PRE was 6% higher than CEL. There was no difference among ZN, ZD or DR (Figure 30).

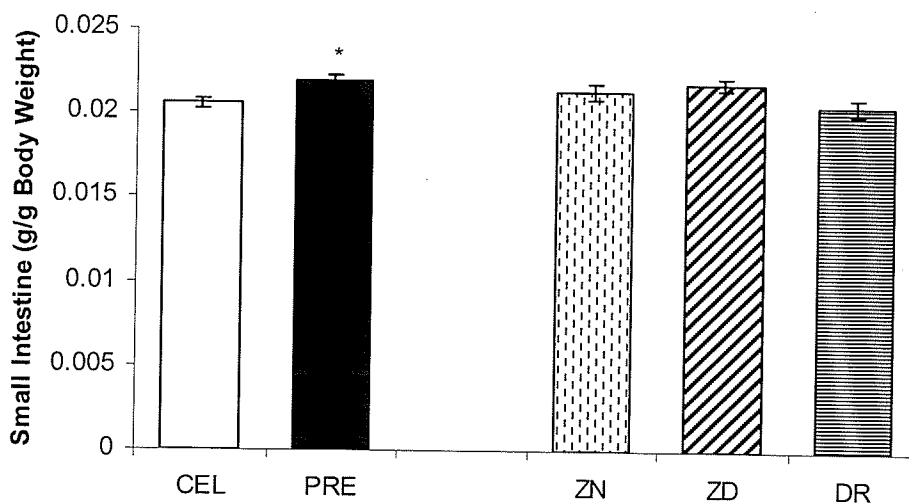


Figure 30: Small Intestine Weight of 8-Week Groups

Fiber Effect p = 0.01. Statistical analysis as described in Figure 14.

c) Small Intestine Length

Over Time: There was no difference between CEL and PRE groups at week 4 or week 8 (Figure 31).

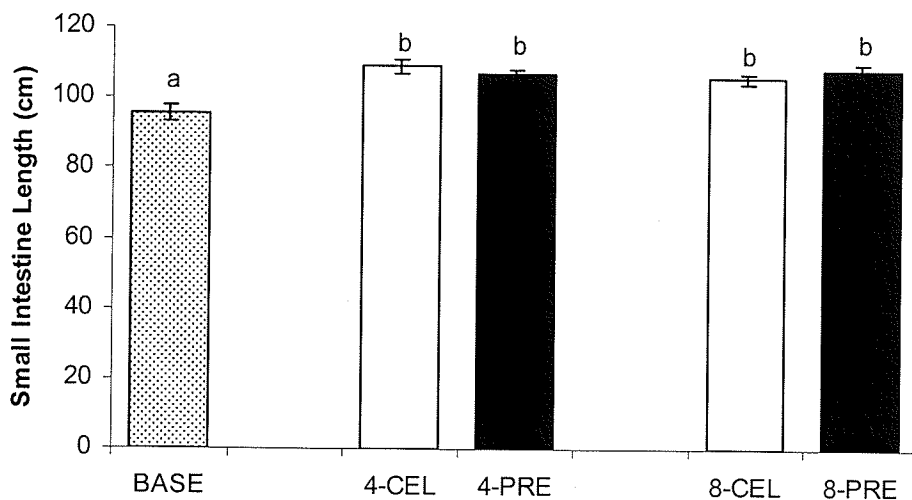


Figure 31: Small Intestine Length over Time

The p-value < 0.0001. Statistical analysis as described in Figure 15.

End Point: The small intestine length of PRE was 4% longer than CEL. There was no difference among ZN, ZD or DR (Figure 32).

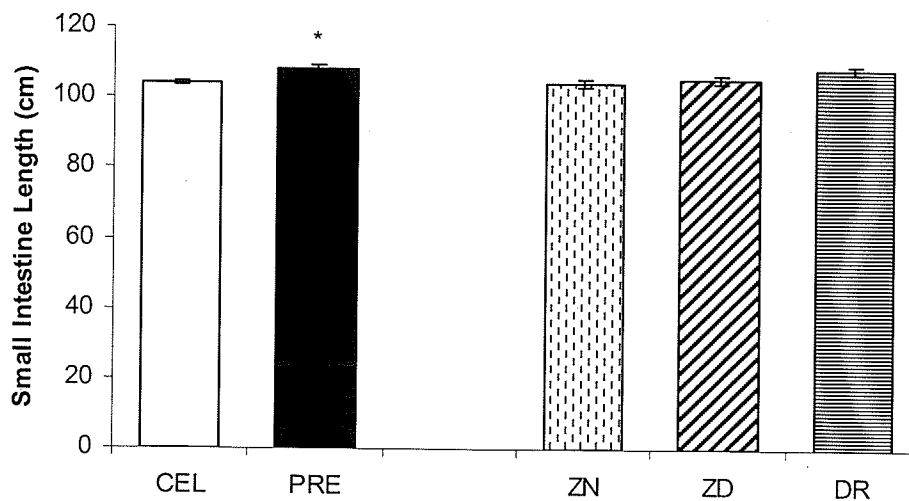


Figure 32: Small Intestine Length of 8-Week Groups

Fiber Effect p = 0.003. Statistical analysis as described in Figure 14.

Phenotyping

- There was no change in the proportion of immune cells in the spleen, MLN or PP after 4 weeks of feeding inulin or cellulose (results not shown).

TCR $\alpha\beta$ ⁺ Cells

- After 8 weeks, the proportion of TCR $\alpha\beta$ ⁺ cells in the PP was 15% higher in DR animals compared to ZN and 14% higher compared to ZD (Figure 33). There was no change in the spleen or MLN.

TCR $\alpha\beta$ ⁺CD4⁺ Cells

- The proportion of TCR $\alpha\beta$ ⁺CD4⁺ cells in the spleen was 4% higher in PRE compared to CEL (Figure 34a). There was no change in the MLN or PP.

TCR $\alpha\beta$ ⁺CD8⁺ Cells

- The proportion of TCR $\alpha\beta$ ⁺CD8⁺ cells in the spleen was 10% lower in PRE compared to CEL (Figure 35a). In the PP, the proportion of cells was 15% lower in PRE compared to CEL; DR was 13% higher than ZD and 10% higher than ZN (Figure 35b). There was no change in the MLN.

TCR $\gamma\delta$ ⁺ Cells

- There was no change in the proportion of TCR $\gamma\delta$ ⁺ cells CD4⁺/ CD8⁺ subsets in the spleen, MLN or PP (Figure 36, Figure 37, Figure 38).

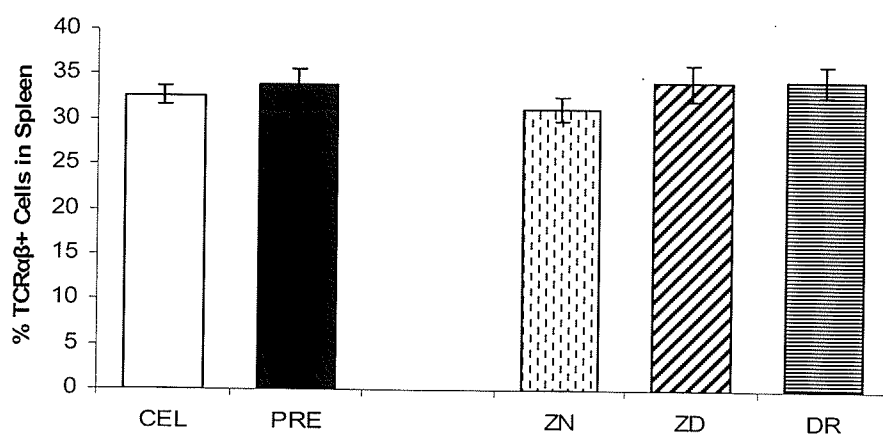
CD45RA⁺ Cells

- In the MLN, the proportion of CD45RA⁺ cells was 8% lower in PRE compared to CEL (Figure 39b). The proportion of CD45RA⁺ cells in the spleen was 7% higher in ZN compared to both ZD and DR (Figure 39a). In the PP, the proportion of cells was 4% higher in both ZN and ZD, compared to DR (Figure 39c).

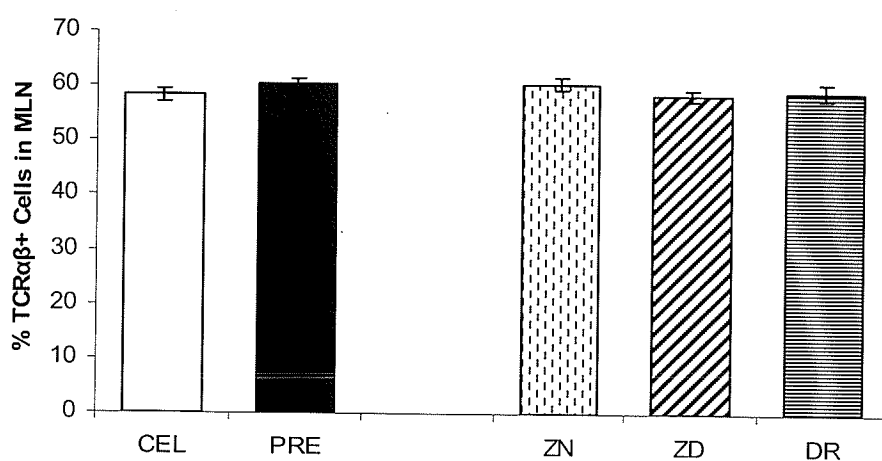
OX62⁺ Cells

- The proportion of OX62⁺ cells in the PP was 24% higher in PRE compared to CEL (Figure 40). There was no change in the spleen or MLN.

a) Spleen



b) MLN



c) PP

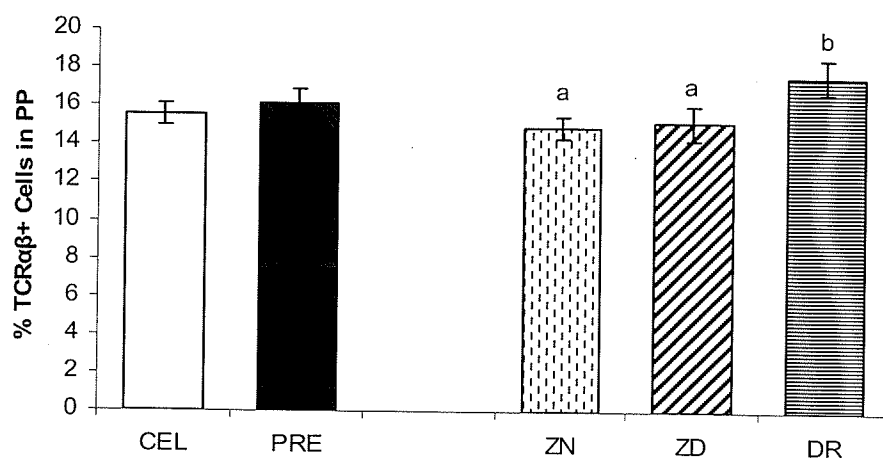
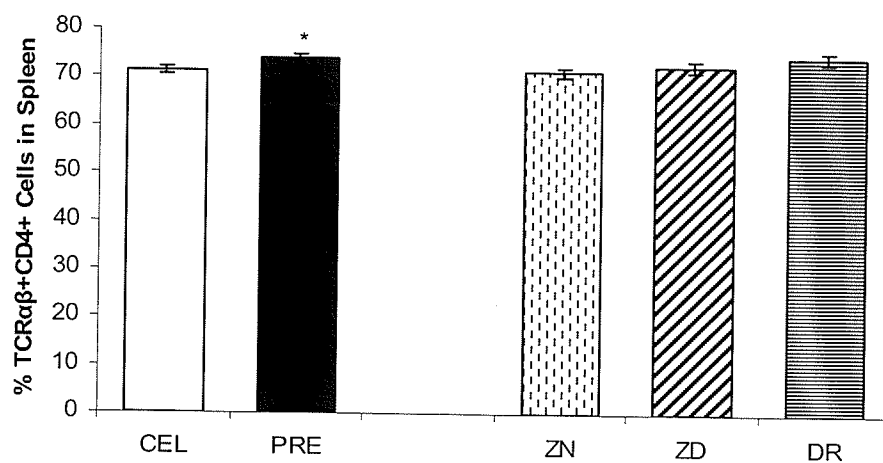


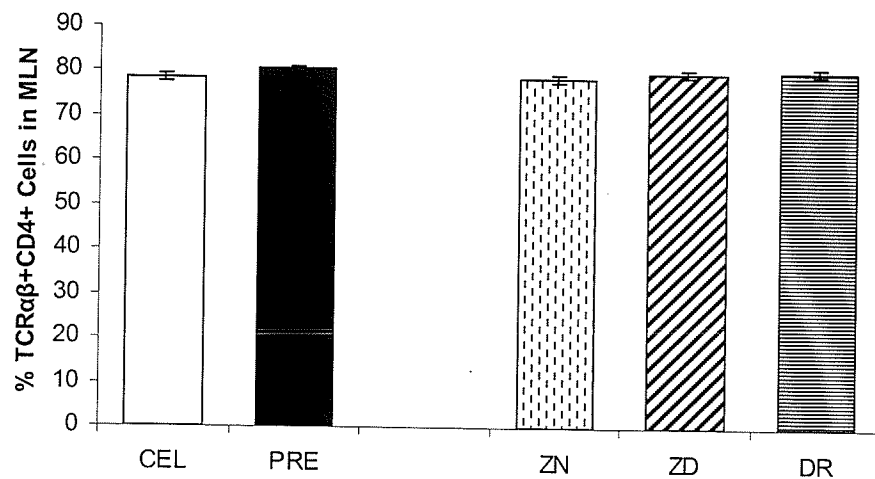
Figure 33: TCRαβ⁺ Cells for 8 Week Groups

Diet Effect (PP) $p = 0.05$. Statistical analysis as described in Figure 14.

a) Spleen



b) MLN



c) PP

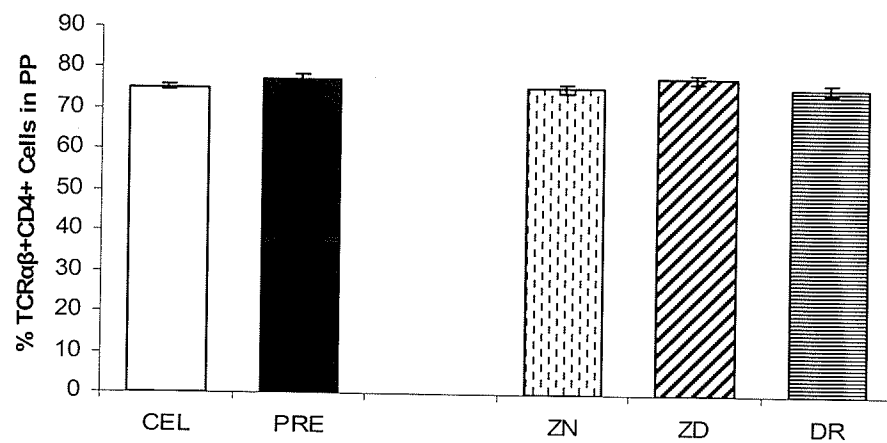
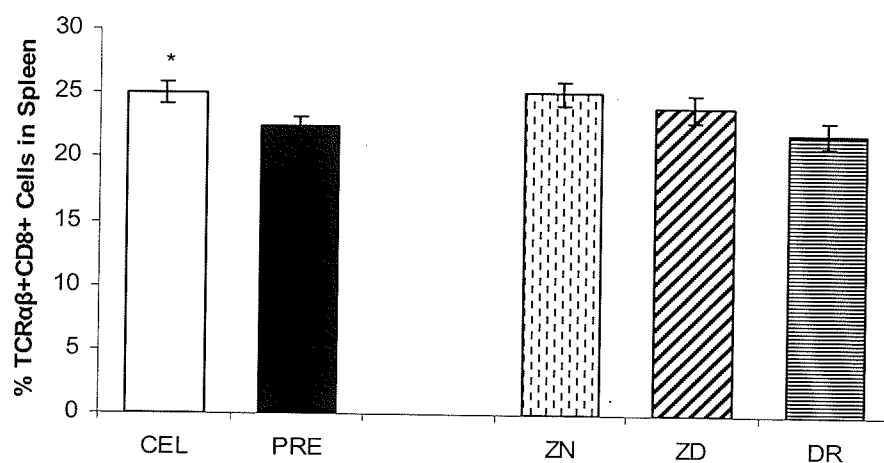


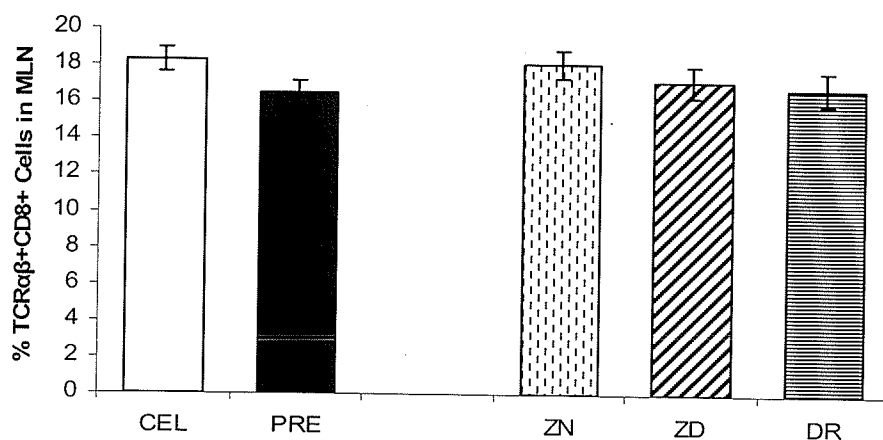
Figure 34: TCRαβ+CD4+ Cells for 8 Week Groups

Fiber Effect (spleen) $p = 0.02$. Statistical analysis as described in Figure 14.

a) Spleen



b) MLN



c) PP

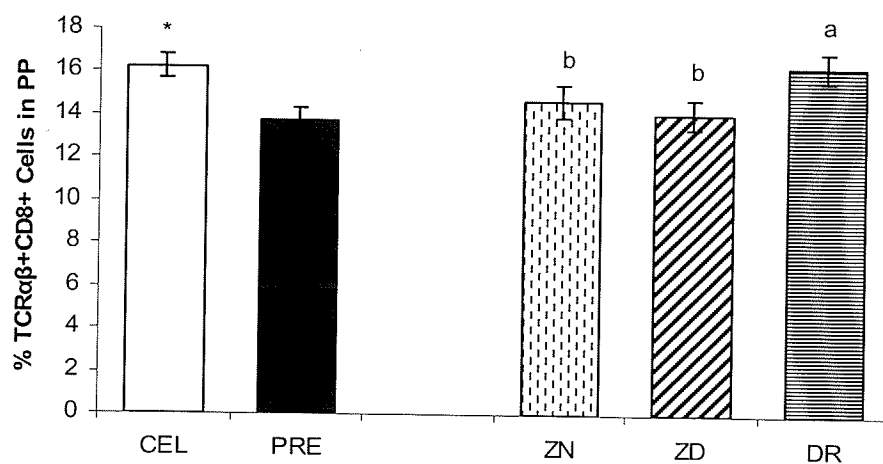
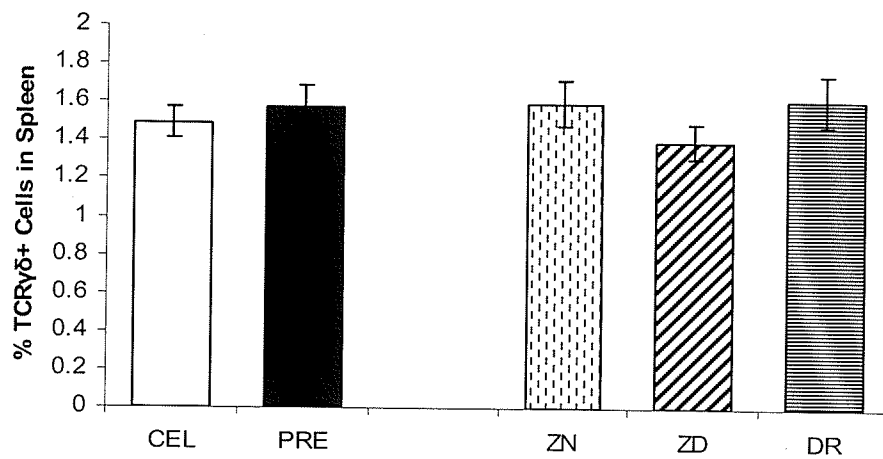


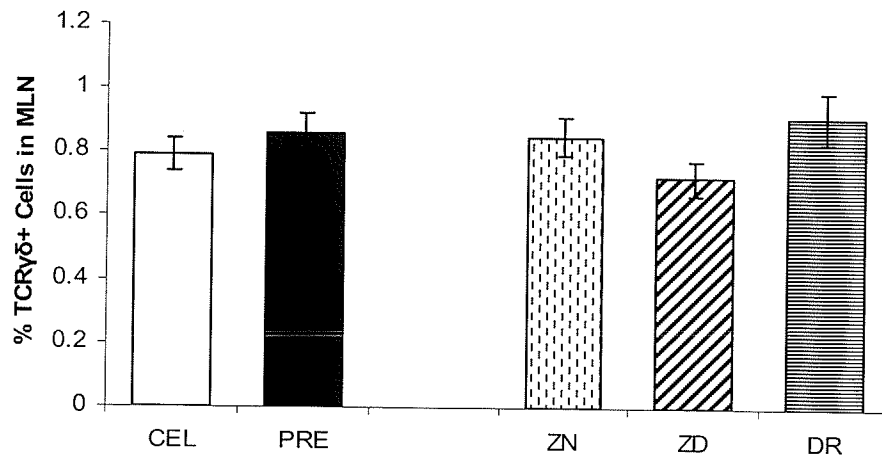
Figure 35: TCRαβ⁺CD8⁺ Cells for 8 Week Groups

Diet Effect (spleen) $p = 0.02$; Fiber Effect (PP) $p = 0.006$; Diet Effect (PP) $p = 0.03$. Statistical analysis as described in Figure 14.

a) Spleen



b) MLN



c) PP

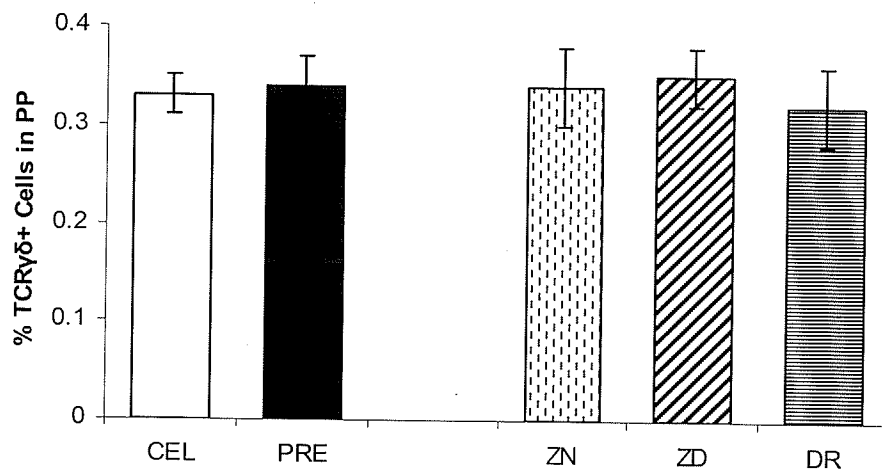
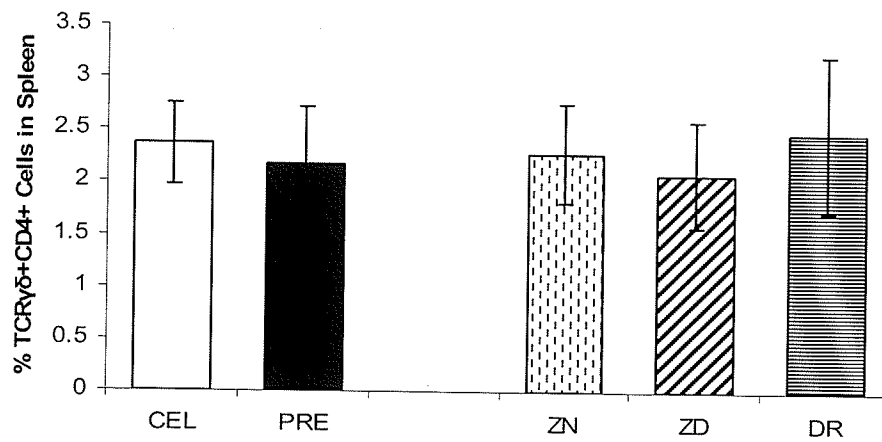
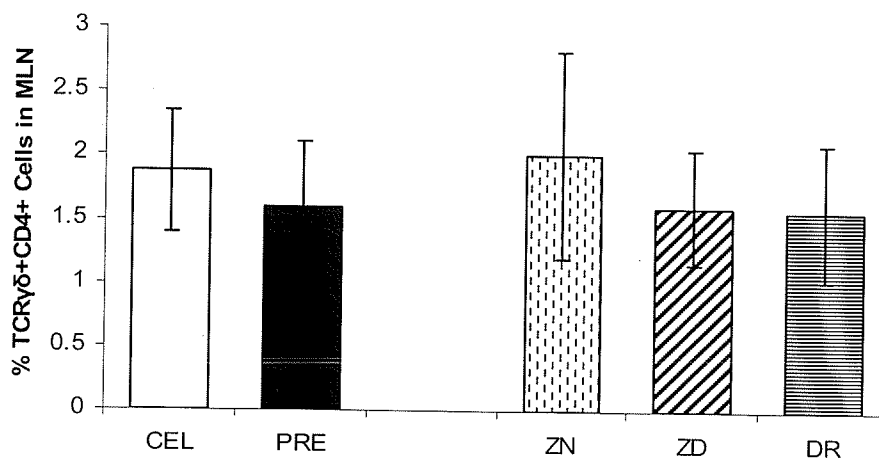


Figure 36: TCRγδ⁺ Cells for 8 Week Groups
Statistical analysis as described in Figure 14.

a) Spleen



b) MLN



c) PP

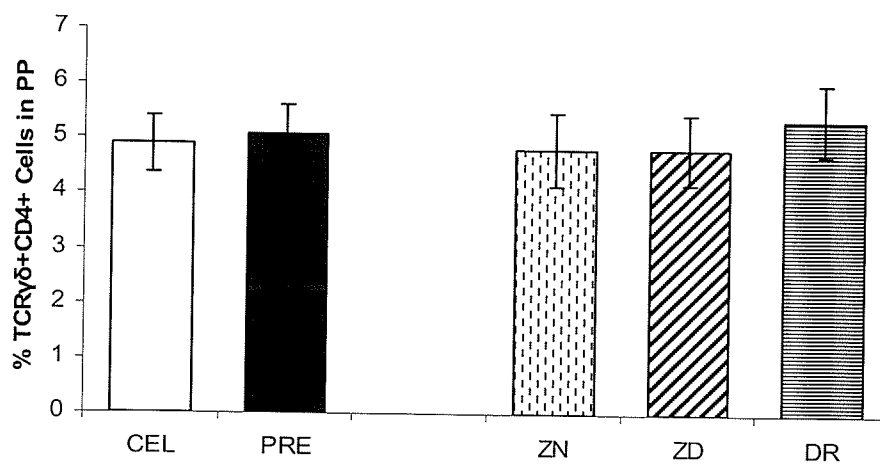
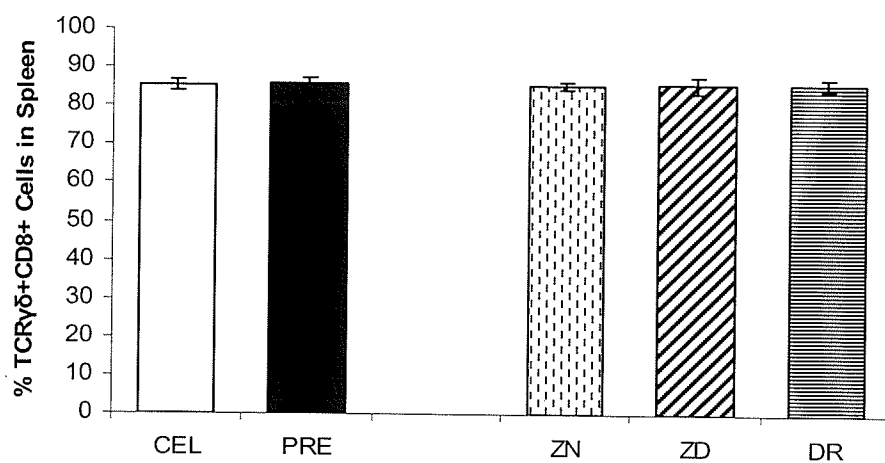
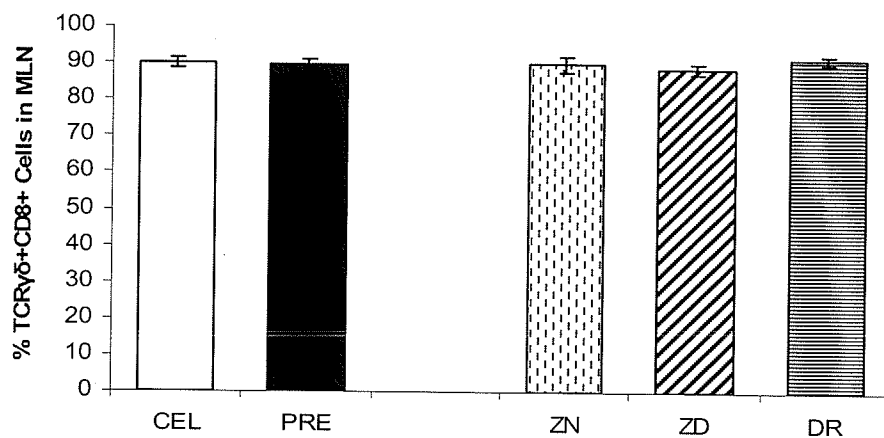


Figure 37: TCRγδ+CD4+ Cells for 8 Week Groups
Statistical analysis as described in Figure 14.

a) Spleen



b) MLN



c) PP

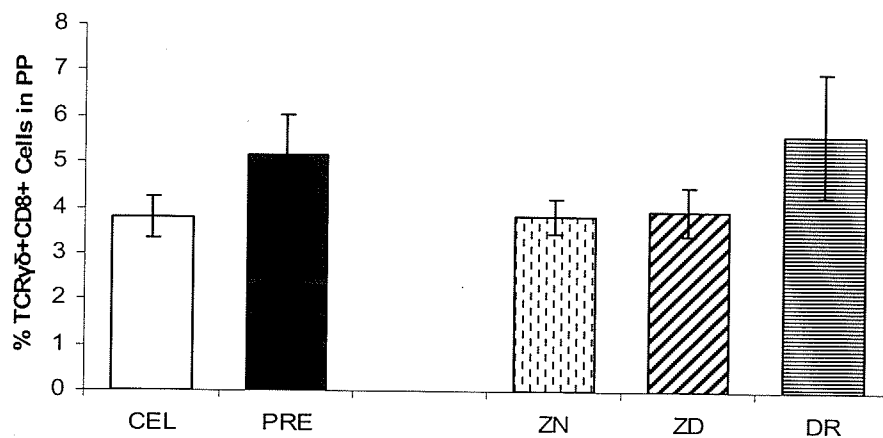
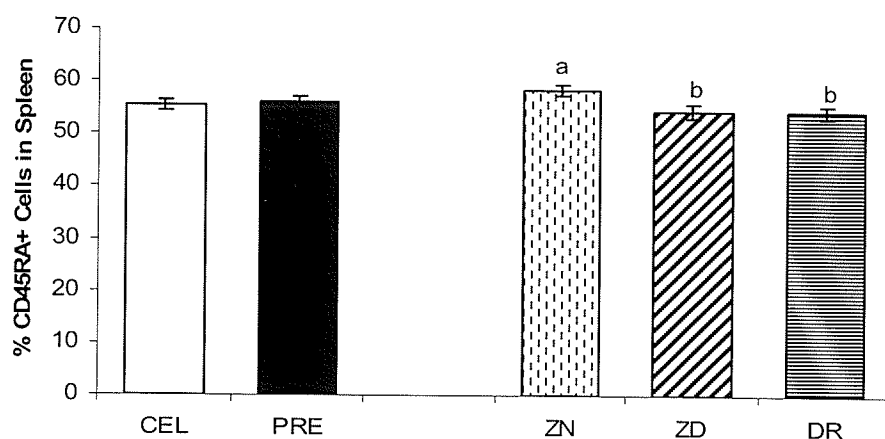
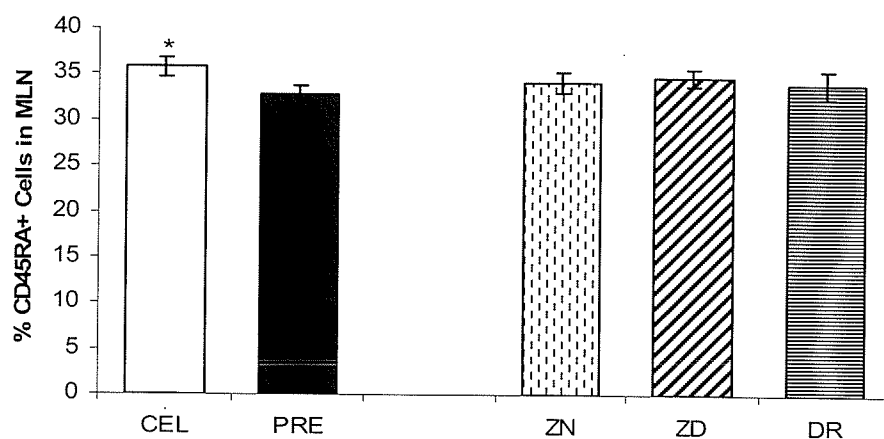


Figure 38: TCR $\gamma\delta$ +CD8+ Cells for 8 Week Groups
Statistical analysis as described in Figure 14.

a) Spleen



b) MLN



c) PP

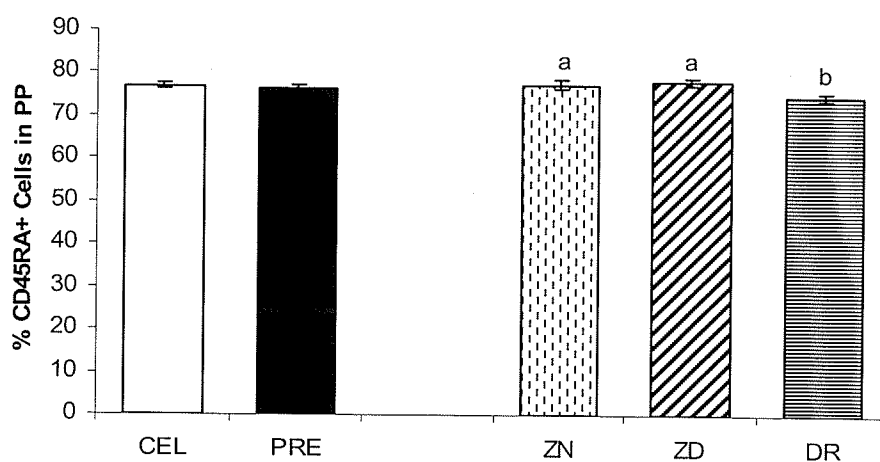
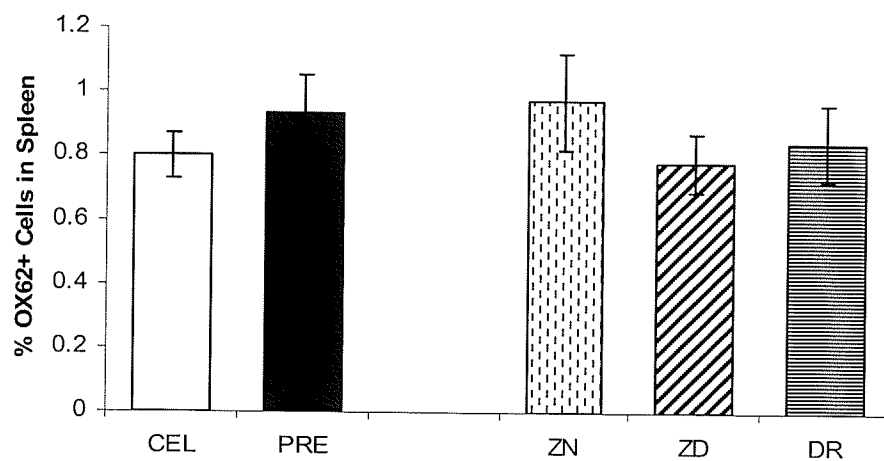


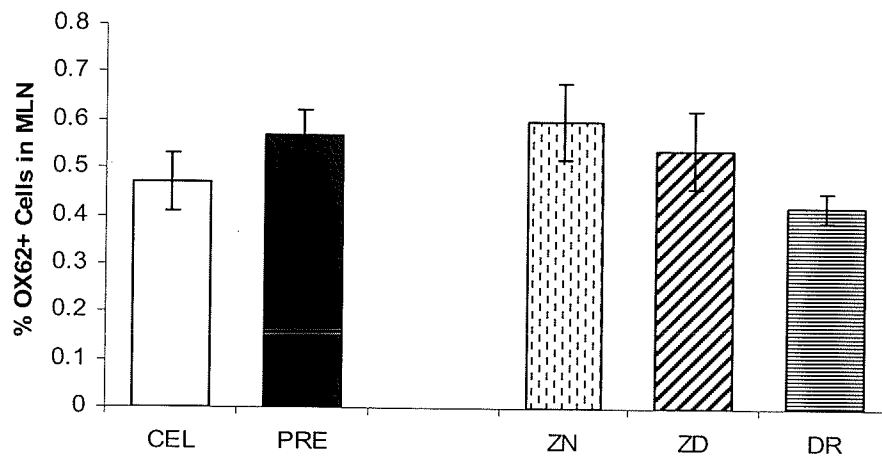
Figure 39: CD45RA+ cells for 8 Week Groups

Diet Effect (spleen) $p = 0.02$; Fiber Effect (MLN) $p = 0.03$; Diet Effect (PP) $p = 0.04$. Statistical analysis as described in Figure 14.

a) Spleen



b) MLN



c) PP

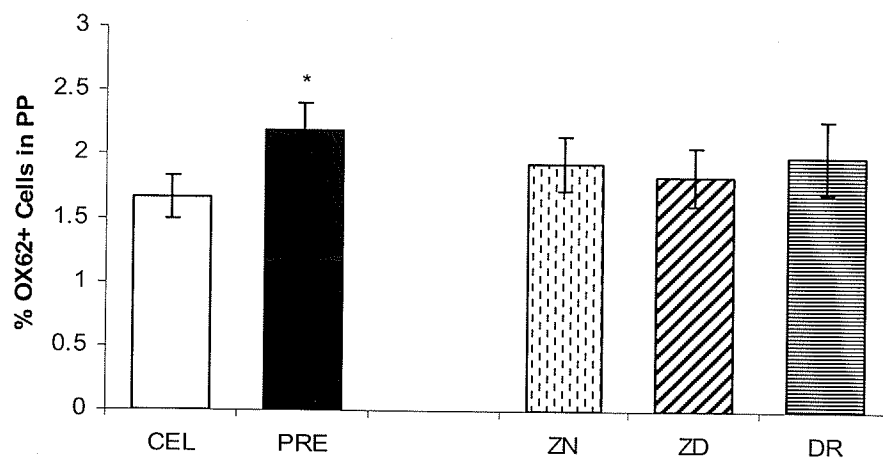


Figure 40: OX62+ cells for 8 Week Groups

Fiber Effect (PP) $p = 0.01$. Statistical analysis as described in Figure 14.

CYTOKINES

- Data regarding the cytokine secretion from PP cells is shown in Tables 4, 5, 6 and 7 in the appendix. The cells did not appear to be stimulated by ConA because the cytokines released from stimulated cells are similar to those released by unstimulated cells.
- Cytokine secretion from unstimulated cells are reported in tables 4 & 6.

Interleukin 2

Over Time

- In the MLN, IL-2 was 31% higher in 8-PRE than 8-CEL (Figure 41b). There was no difference between PRE and CEL at week 4 or week 8 in the spleen.

End Point

- In the spleen, IL-2 was 38% higher in PRE compared to CEL (Figure 42a). In the MLN, IL-2 was 58% higher in PRE compared to CEL (Figure 42b). There was no difference among ZN, ZD or DR in the spleen or MLN.

Interferon gamma

Over Time

- In the spleen, IFN- γ was 54% higher in 8-PRE compared to 8-CEL (Figure 41c). In MLN, 4-PRE was 70% lower than 4-CEL (Figure 41d), however, there was no difference between 8-PRE and 8-CEL.

End Point

- In the spleen, IFN- γ was 39% higher in PRE compared to CEL (Figure 42c). In the MLN, IFN- γ was 46% higher in PRE compared to CEL (Figure 42c). There was no difference among ZN, ZD or DR in the spleen or MLN.

Interleukin 10

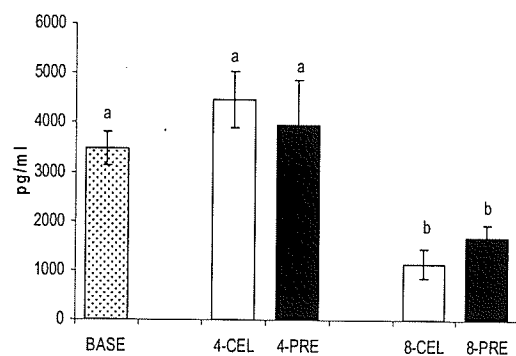
Over Time

- In the MLN, IL-10 was 53% lower in 4-PRE compared to 4-CEL (Figure 41f), however there was no difference between 8-PRE and 8-CEL. In the spleen, there was no difference between PRE and CEL at week 4 or 8.

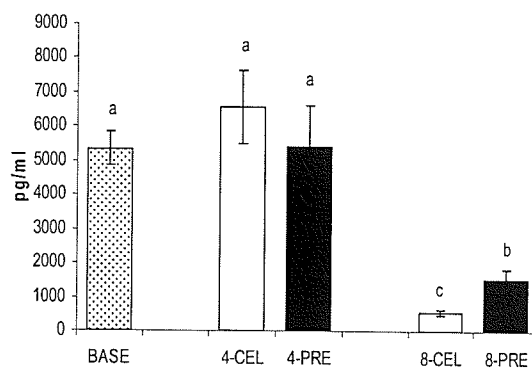
End Point

- In the spleen, IL-10 was 30% higher in PRE compared to CEL (Figure 42e). In the MLN, IL-10 was 56% higher in PRE compared to CEL (Figure 42f). There was no difference among ZN, ZD or DR in the spleen or MLN.

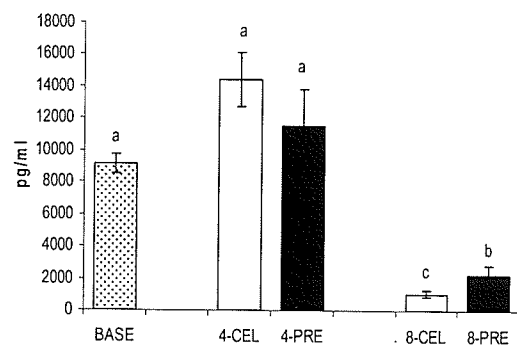
a) IL-2 Spleen



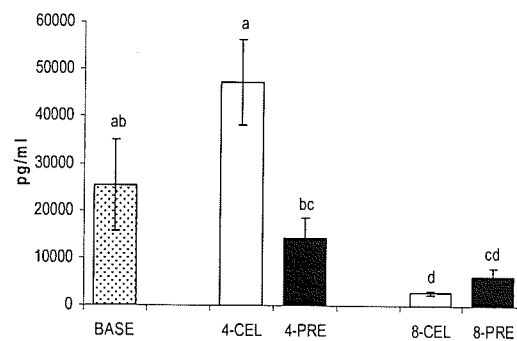
b) IL-2 MLN



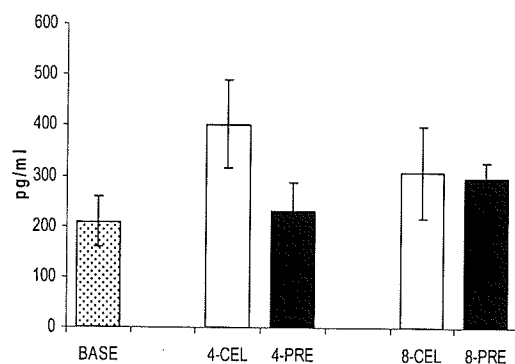
c) IFN-γ Spleen



d) IFN-γ MLN



e) IL-10 Spleen



f) IL-10 MLN

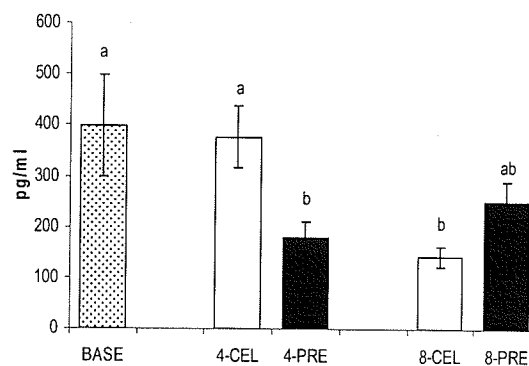
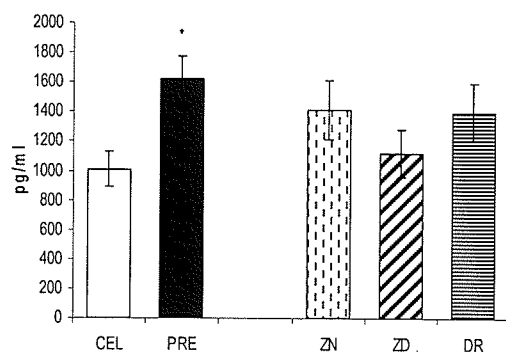


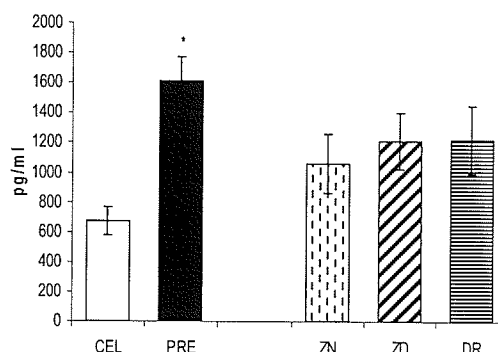
Figure 41: Cytokines Over Time

IL-2 in spleen $p < 0.0001$, IL-2 in MLN $p < 0.0001$, IFN- γ in spleen $p < 0.0001$, IFN- γ in MLN $p < 0.0001$, IL-10 in MLN $p = 0.007$. Statistical analysis as described in Figure 15.

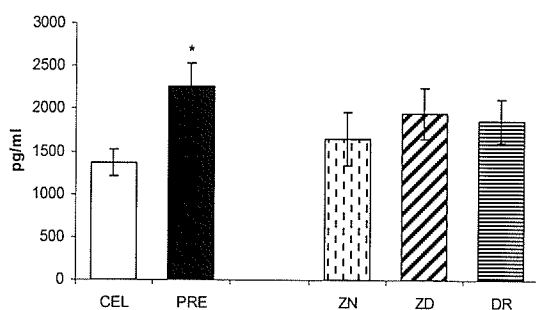
a) IL-2 Spleen



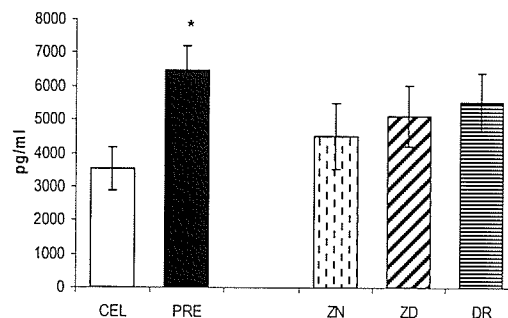
b) IL-2 MLN



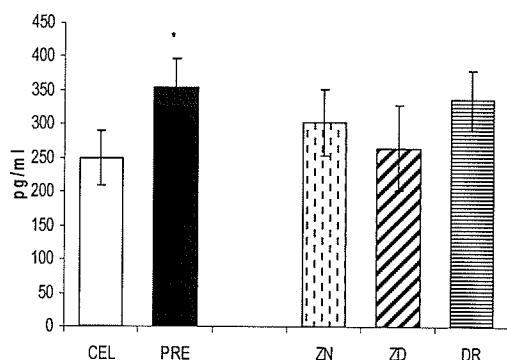
c) IFN-γ Spleen



d) IFN-γ MLN



e) IL-10 Spleen



f) IL-10 MLN

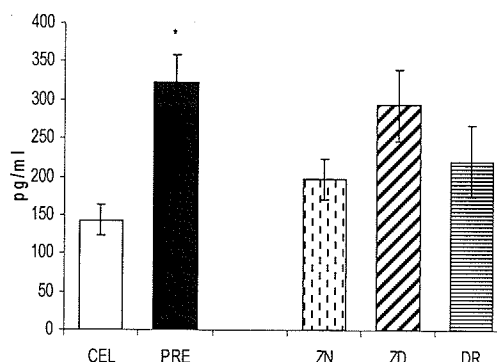


Figure 42: Cytokines for 8-Week Groups

Fiber Effect: IL-2 in spleen $p = 0.006$, IL-2 in MLN $p < 0.0001$, IFN- γ in spleen $p = 0.008$, IFN- γ in MLN $p = 0.005$, IL-10 in spleen $p = 0.01$, IL-10 in MLN $p < 0.0001$. Statistical analysis as described in Figure 14.

Serum Immunoglobulin G1

Over Time: There was no difference between CEL and PRE at week 4 or 8 (Figure 43).

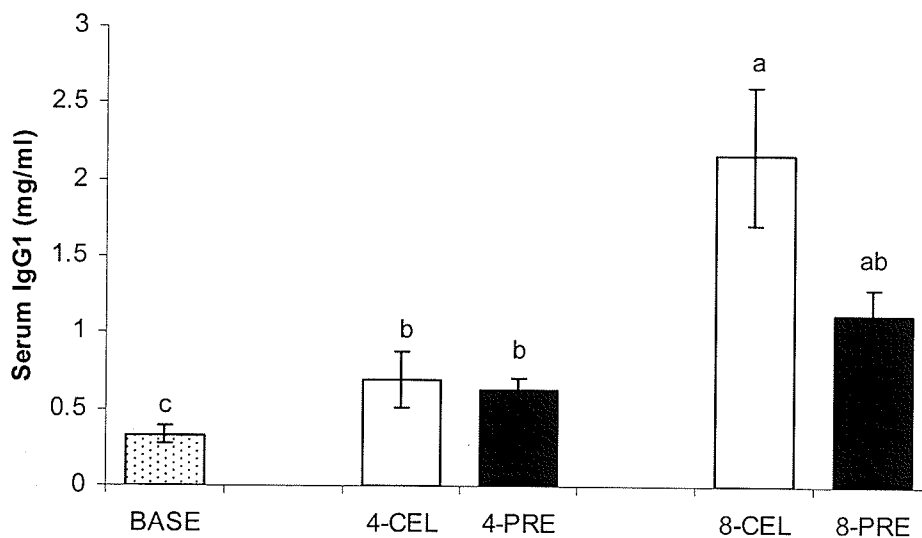


Figure 43: Serum IgG1 Over Time

The p-value <0.0001. Statistical analysis as described in Figure 15.

End Point: There was no difference between CEL and PRE or among ZN, ZD or DR groups (Figure 44).

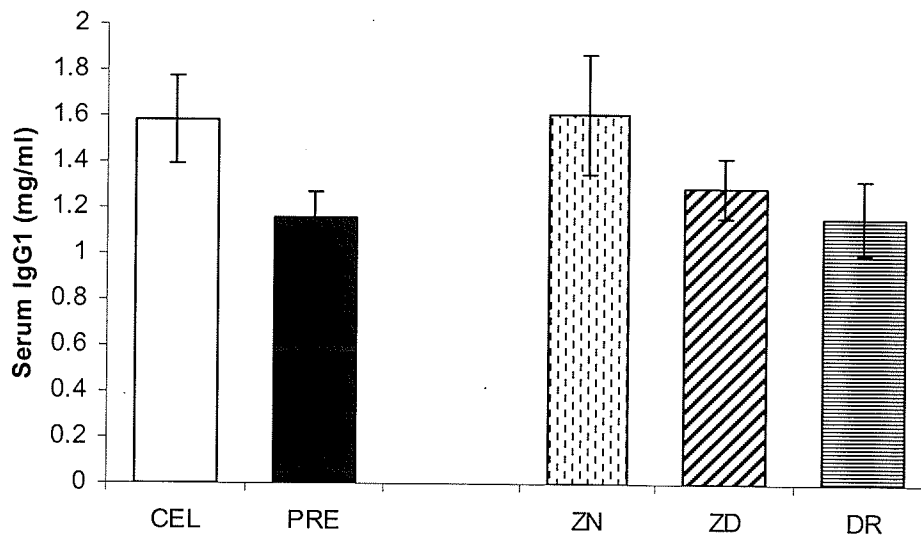


Figure 44: Serum IgG1 for 8 Week Groups

Statistical analysis as described in Figure 14.

Serum Immunoglobulin G2a

Over Time: There was no difference between CEL and PRE at week 4 or 8

(Figure 45).

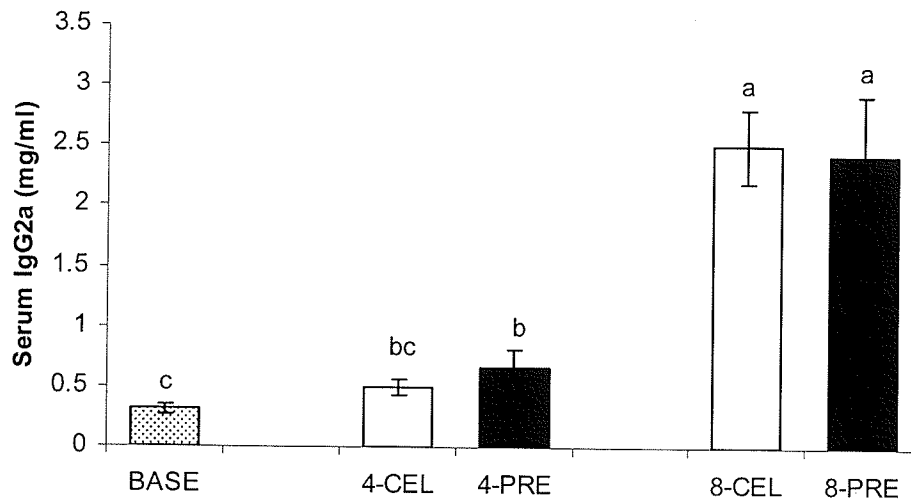


Figure 45: Serum IgG2a Over Time

The p-value <0.0001. Statistical analysis as described in Figure 15.

End Point: There was no difference between CEL and PRE. Serum IgG2a of ZD was 33% lower than ZN and 39% lower than DR (Figure 46).

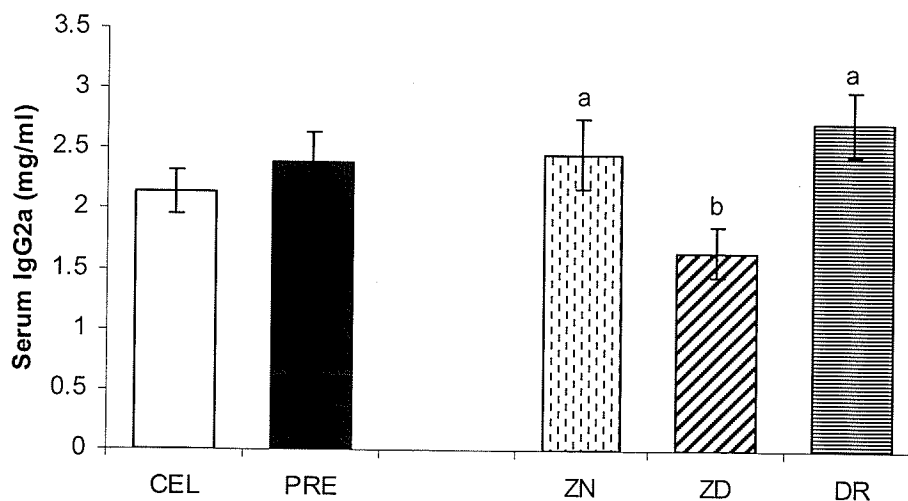


Figure 46: Serum IgG2a for 8-Week Groups

Diet Effect $p = 0.002$. Statistical analysis as described in Figure 14.

VII. MAIN STUDY

DISCUSSION

FEED INTAKE, BODY WEIGHT AND FEED EFFICIENCY

Fiber Effect

At week 4, there was no difference in total feed intake (Figure 13), body weight (Figure 15) or feed efficiency (Figure 17) between CEL and PRE. At week 8, the total feed intake of PRE was 8% lower compared to CEL (Figure 14), but there was no difference in body weight or feed efficiency. Similarly, in the preliminary inulin study, inulin fed animals consumed 16% less diet than cellulose fed animals (Figure 9), yet there was no difference in body weight (Figure 10) or feed efficiency (Figure 11).

Cani et al (2004) fed rats a control diet (5% cellulose) or control diet supplemented with 10% oligofructose, long-chain inulin or Synergy 1 (mix of inulin and oligofructose). At the end of 3 weeks, total feed intake was significantly higher in control animals compared to all experimental groups, but there was no difference in body weight. However since the experimental diets contained 15% total fiber compared to 5% fiber in the control group, it is difficult to determine if the results are due to prebiotic fibers or simply consuming a high fiber diet. The findings in our studies demonstrate that either inulin or a mix of soluble/insoluble fiber has an effect on feed intake, since the total amount of fiber in both control and experimental diet was 10%.

Bacteria in the large intestine can completely degrade inulin to yield SCFAs and lactate, which are absorbed and used for energy. The metabolic pathways that use SCFAs and lactate originating from intestinal carbohydrate fermentation are still not completely understood, but it has been estimated that 1 g of inulin provides ~1.5 kcal energy (Roberfroid, 2005).

Cellulose is only partially metabolized in the colon. Kelleher et al (1984) have shown that in humans ~38% of ingested cellulose may be digested. In rats, as much as 50% of cellulose may be digested (Yang et al, 1969). In rats, the principle site of microbial fermentation occurs in the cecum, whereas in humans fermentation occurs in the colon. Interestingly, Yang et al (1969) found that cellulose was partially digested by rats whether the cecum was present or not, although cecectomy did result in a lower amount of cellulose digestion. Rats also practice coprophagy, which allows for further cellulose digestion. It should be noted that the use of hanging wire-mesh floor caging does not prohibit coprophagy (Canadian Council on Animal Care, 1984). This is because rats produce two kinds of feces: the ordinary firm dark pellets, and a softer, lighter pellet that is not dropped by the animal, but is eaten directly from the anus (Findlay, 1998). It is thus difficult to determine the extent of coprophagy in the rat. Fajardo & Hornicke (1989) have estimated that when fed a nutritionally complete diet, rats re-ingest 0-11% of their feces.

The amount of energy available from cellulose digestion is still unclear, however since inulin is completely fermented, it can be assumed that inulin provides more energy to the host compared to cellulose. Thus, perhaps

cellulose fed animals consumed more diet to meet their caloric requirements. Taking this into account, the 16% feed difference in the preliminary study, compared to the 8% feed difference in the main study may be due to the higher inulin dose consumed in the preliminary study.

Another potential explanation for the difference in feed intake is that prebiotic fibers, such as oligofructose, have been shown to influence the glucagon-like peptide-1 (GLP-1) hormone which is involved in appetite regulation (Delzenne et al, 2005). However, Cani et al (2004) found that after 3 weeks of treatment, oligofructose and Synergy 1 (mix of inulin and oligofructose) led to an increase in proglucagon-GLP-1, whereas long-chain inulin had no major effects on GLP-1 amide.

Other researchers have found conflicting results; Verghese et al (2002) fed rats a control diet (5% cellulose) or a control diet supplemented with 10% long chain inulin for 13 weeks. Body weight gains tended to be lower ($p < 0.08$) in the inulin group compared to the control group, although the inulin group tended to consume more food ($p < 0.08$). Juskiewicz & Zdunczyk (2004) fed weanling rats diets containing either 4% cellulose, 4% inulin or a mix of 4% cellulose and 4% inulin. At the end of 4 weeks, there was no difference in feed intake, but the body weight gain of the inulin group (167 g) was significantly higher compared to the mix group (156 g). There was no difference between cellulose (164 g) and inulin, or cellulose and mix. Roller et al (2004) found that supplementation of a high fat control diet (2% cellulose) with 10%

inulin/oligofructose mix did not affect feed intake or final body weight in rats.

However, conflicting results are most likely due to differences in study design.

Diet Effect

In the present study, 7-week-old female Sprague-Dawley rats were fed a zinc deficient diet for 4 weeks. At the end of the study, zinc normal (30 mg/kg zinc) animals consumed 6% more diet (Figure 14) and had a 10% higher body weight (Figure 16) compared to zinc deficient (1 mg/kg zinc) and diet restricted animals.

A reduction in feed intake and stunted growth is a well documented effect of zinc deficiency. It is not known exactly why zinc deficiency causes a decrease in feed intake, but it may be due to the impaired release of neuropeptide Y, a known stimulator of appetite and food consumption (Levenson, 2003).

Interestingly, force feeding a zinc-deficient diet by stomach tube is fatal to rats (Flanagan, 1984). Thus, a reduction in feed intake may be an adaptation to limit growth in order to conserve limited zinc stores for critical functions (Cousins, 1996).

Most zinc research with young adult rats has been done using males, and these studies have found larger differences in feed intake and body weight compared to our study with female rats (Takeda et al, 2005a; Takeda et al, 2005b; Stallard & Reeves, 1997). For instance, Keller et al (2000) fed 7-week old male Sprague-Dawley rats a zinc adequate (20 mg/kg zinc) or zinc deficient diet (1-2 mg/kg zinc) for 3 weeks. A pair-fed group was also included. The mean daily intake of the control animals was 23% higher than ZD and the pair-fed

group. At the end of the depletion phase, the control animals weighed ~30% more than ZD and ~19% more than pair-fed animals. Interestingly, the pair-fed group weighed ~15% more than ZD, even though they consumed the same amount of diet.

Windisch (2003) looked at the development of zinc deficiency in young adult female rats (~11 weeks-old). The animals were fed restricted amounts (8 g/day) of a diet containing either 58 mg zinc /kg diet (control) or 2 mg zinc /kg diet (zinc deficient) for up to 29 days. After 4 weeks, there was no difference in energy intake or body weight between control and zinc-deficient animals. Since food intake was restricted, the control animals could be considered a pair-fed group. In studies with male rats, pair-fed animals generally weigh more than zinc-deficient animals, even though they consume the same amount of diet (Hosea et al, 2003; Keller et al, 2000; Stallard & Reeves, 1997). In my study, even though there were no differences in feed intake or body weight between female ZD and DR animals, the feed efficiency of DR animals was generally higher than ZD (Figure 17b). This demonstrates the importance of zinc in growth.

Why is there a more pronounced effect in male rats? In general, male rodents are used for zinc nutritional studies because of their faster growth rate and relatively uniform response to nutrient or dietary changes, compared to females (Panemangalore & Bebe, 1997). By approximately week 9 of age, male Sprague Dawley rats continue to gain weight, while the females begin to plateau (Figure 49). As shown in our preliminary zinc study (Figure 3), young adult ZD

rats do not lose weight over time, they just stop growing. Thus, since female weight begins to plateau, the effects of zinc deficiency are less pronounced.

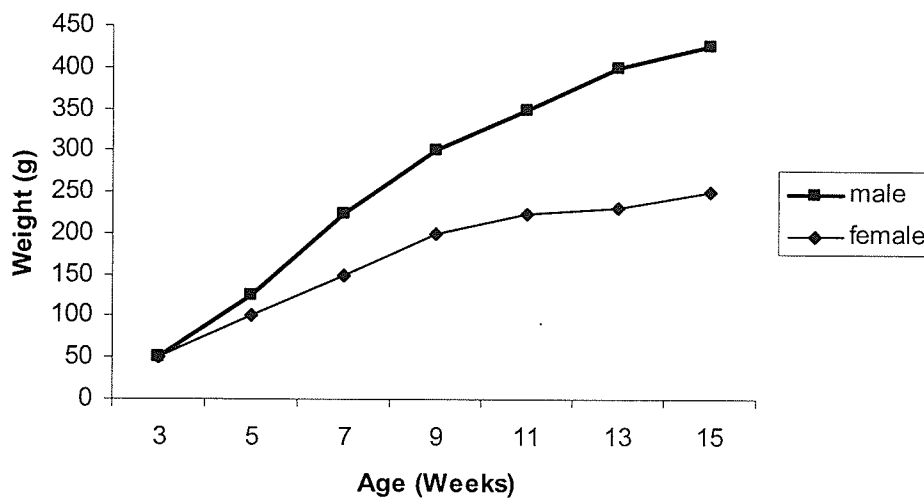


Figure 49: Sprague Dawley Growth Chart

(Adapted from

http://www.charlesriverlabs.biz/flex_content_area/documents/rm_rm_c_sprague_dawley_rat.pdf)

BODY COMPOSITION

Fiber Effect

At week 8, the parametrical adipose tissue weight of 8-PRE was 31% lower compared to 8-CEL (Figure 18), and the total percent adipose tissue was 19% lower in 8-PRE compared to 8-CEL (Figure 20). There was no difference in lean non-fat mass (Figure 23). Cani et al (2004) found that after 3 weeks of treatment with a control or prebiotic supplemented diet (oligofructose, inulin, or Synergy 1), the epididymal adipose tissue weight was ~30% higher in control animals, compared to oligofructose and Synergy rats. However, there was no significant difference between control and inulin fed rats. Perhaps no difference was found in inulin fed rats because their study length was only 3 weeks, and we did not see changes until 8 weeks of feeding.

Thus, in animals consuming a prebiotic diet, there was a decrease in adipose tissue, while lean non-fat mass was conserved.

Diet Effect

The parametrical adipose tissue weight of ZN animals was 49% and 53% higher compared to ZD and DR, respectively (Figure 19). The total percent of adipose tissue in ZN animals was 26% higher compared to ZD and 31% higher compared to DR (Figure 21).

Interestingly, when body weight is taken into account, the lean non-fat mass of ZN was 4% lower compared to both ZD and DR (Figure 23). O'Leary et al. (1979) reported increases of between 31 and 90% in the weights of individual muscles in rats fed severely Zn-deficient diet. Furthermore, Giugliano et al.

(1984) found a marked increase in muscle Zn with a similar loss from bone, indicating that Zn can be redistributed from bone to allow the growth of other tissues. McCarter et al. (1982) studied the lateral omohyoideus muscle structure in rats maintained on a 40% calorie restriction and found no difference in muscle mass or fiber number between restricted and ad libitum diets. In life-span studies with Fischer rats, a 60% caloric restriction delayed the decline of the gastrocnemius muscle mass (Yu et al, 1982). Thus, it appears that when diet intake is limited due to diet restriction or zinc deficiency, there is a decrease in adipose tissue, while muscle mass is conserved.

SERUM ZINC

Fiber Effect

There was no difference in serum zinc between CEL and PRE (Figure 25). These findings are similar to Coudray et al (2006), who also looked at the effect of inulin intake on zinc status. Male Wistar rats of 4 different ages (2, 5, 10, and 20 months) were assigned to a control group or a group administered 3.75% inulin in their diet for 4 days followed by 7.5% inulin for 26 days. Plasma Zn concentration was not affected by inulin intake, but was decreased with increasing age.

Diet Effect

Serum zinc concentration was 85% lower in ZD compared to both ZN and DR (Figure 25). Similarly, in 7-week-old male Sprague Dawley rats, serum zinc was 79% lower in ZD compared to control after consuming a zinc deficient diet for 3 weeks. However, serum zinc of DR animals was also 21% lower compared to control (Stallard & Reeves, 1997). In 3-week-old male Sprague-Dawley rats, serum zinc was 83% lower in ZD compared to CTL and 15% lower in DR compared to CTL after a 3 week experimental period (Hosea et al, 2006a). It is unknown why we found no difference in serum zinc between ZN and DR animals. Perhaps it is a gender effect. Since female rats are smaller and need less zinc for growth, they may be able to utilize the zinc from the DR diet more efficiently than male rats can.

FEMUR ZINC

At week 4, the femur zinc of PRE was 13% lower than CEL, however by week 8 there was no difference between PRE and CEL (Figure 26). It is unknown why there was a 13% difference in femur zinc at week 4. One potential explanation is that less dietary zinc was absorbed in animals consuming PRE, compared to CEL. Inulin has been shown to influence intestinal function by stimulating bowel movements and increasing stool frequency (Gibson, et al. 1995, Menne, et al. 1997). Perhaps an increase in stool frequency did not allow enough time for adequate zinc absorption to occur in the intestine, and therefore zinc was released from bone to maintain serum zinc stores. After 8 weeks, the animals may have adjusted to the PRE diet and therefore zinc absorption returned to normal.

At the end of the study, ZD-CEL had ~30% lower femur zinc concentration than both DR-CEL and 8-CEL, and similarly ZD-PRE was ~35% lower than both 8-PRE and DR-PRE (Figure 27). It has been previously demonstrated that femur zinc decreases in diet-restricted animals, but is still higher compared to zinc-deficient animals. Hosea et al (2004) investigated the effects of dietary zinc deficiency and diet restriction on bone development in growing rats. Three-week old male Sprague Dawley rats were fed a zinc-deficient diet or a control diet either *ad libitum* or pair-fed to the ZD group. At the end of 3 weeks, ZD rats had a 64% lower femur zinc concentration than DR rats, and DR rats had a 15% lower zinc concentration than CTL rats. Perhaps we did not see any difference between DR and control animals because our rats were older and thus had

adequate zinc stores built up, and therefore were more resistant to femoral changes. Or, as described under “serum zinc” it may be a gender effect.

Interestingly, there was also a significant fiber x diet interaction; Femur zinc of DR-PRE was 21% higher than DR-CEL (Figure 27). Raschka & Daniel (2005) found that femur zinc was increased by 8% in male adolescent Sprague Dawley rats when fed Synergy1 (inulin oligofructose mixture) for 15 days. However, Coudray et al (2006) found that tibia zinc was not affected by inulin intake.

To our knowledge, this is the first study to investigate feeding prebiotics to zinc-deficient and diet restricted animals. It seems that during diet restriction, inulin helps conserve femur zinc levels. However, during a severe zinc deficiency, there is no zinc available in the diet, so inulin is not beneficial. In rats fed 7.5% inulin, Coudray et al (2006) found a positive correlation between Zn absorption and cecal amounts of acetate, propionate, and butyrate (SCFAs), which confirms the involvement of inulin-fermentation products in intestinal Zn absorption. SCFAs lower the pH of the lumen, which may increase mineral solubility and enhance absorption. Furthermore, SCFAs may also form complexes with zinc and increase their uptake by intestinal cells. Butyrate can induce cell growth and thus increase the absorptive surface area of the gut (Coudray et al, 2006). Perhaps inulin would be beneficial during a moderate zinc deficiency, since the prebiotic could increase absorption of the limited zinc in the diet.

ORGAN WEIGHT/LENGTH

Spleen

There was no difference in spleen weight among ZN, ZD or DR animals (Figure 28a). However, when adjusted for body weight, the spleen of ZN was 9% less than ZD and 10% less than DR (Figure 28b). Hosea et al (2003) found contradictory results; three-week-old male Sprague Dawley rats were fed a zinc-deficient or a control diet either *ad libitum* or pair-fed to the ZD group (energy restricted, ER). After 3 weeks, spleen weights were significantly lower in ZD and ER compared to control. However, when adjusted for body weight, the spleen of ZD and ER animals was not different from control. These findings are similar to our lean non-fat mass results. Perhaps they can be explained in the same way: older animals are more resilient to changes caused by zinc deficiency and diet restriction, and are more likely to conserve muscle (in this case tissue).

Small Intestine

The SI weight of PRE was 6% higher than CEL (Figure 30), and the SI length of PRE was 4% longer than CEL (Figure 32). These results agree with previous studies, which have shown that oligofructose and fructooligosaccharides increase the length and weight of the small intestine in the rat (Tokunaga et al, 1986; Ohta et al, 1998). Kleessen et al (2003) found that feeding inulin-type fructans to germ free rats inoculated with human feces resulted in an increase in villus height and crypt dept, a thicker epithelial mucus layer and an increase in goblet cells, compared to control animals. It can be

assumed that these changes are caused indirectly by the fermentation of inulin-type fructans by intestinal bacteria. The end products of inulin fermentation include SCFA, which stimulate colonic blood flow and supports the epithelial cell mass by increasing the delivery of oxygen and nutrients (Sakata, 1997). Furthermore, butyrate is the prime energy substrate for intestinal cells and can enhance the growth of normal cells (reviewed by Bird et al, 2000). Thus, if more cells are being produced, the weight of the intestine will increase.

PHENOTYPING

T-Lymphocytes

The majority of T-cells in circulation and lymphoid organs express the $\alpha\beta$ T-cell receptor (Dyugovskaya et al, 2003). In our study with 11-week-old animals, the highest proportion of total $\text{TCR}\alpha\beta^+$ cells was in the MLN (~60%), followed by the spleen (~35%) and the PP (~16%) (Figure 33). Similarly, Stepankova et al (1998) found that 8-week-old conventionalized rats (AVN, F89, Prague) had the highest proportion of total $\text{TCR}\alpha\beta^+$ cells in the MLN (~60%) followed by the spleen (~45%) and PP (~23%). T-cells express the $\alpha\beta$ TCR commonly with the co-receptor of either CD4 (~70%) or CD8 (~20%) (Dyugovskaya et al, 2003). This agrees with our findings, since in the spleen, MLN and PP, the proportion of $\text{TCR}\alpha\beta^+\text{CD4}^+$ and $\text{TCR}\alpha\beta^+\text{CD8}^+$ cells was ~70-80% and ~15-23%, respectively (Figure 34 and Figure 35).

T cells expressing the $\gamma\delta$ TCR are a minor T-cell population in circulation and lymphoid organs. The highest proportion of $\text{TCR}\gamma\delta^+$ cells was found in the spleen (1.5%) followed by MLN (0.8%) and PP (0.3%) (Figure 36). These findings are similar to results by Stepankova et al (1998), which found that 8-week old rats had the highest proportion of $\text{TCR}\gamma\delta^+$ cells in the intraepithelial compartment of the intestine (10.6%), followed by the spleen (2.8%), MLN (1.6%) and PP (0.6%). As previously shown, rat $\gamma\delta$ T cells in the spleen are almost exclusively of the $\text{CD4}^-\text{CD8}^+$ phenotype (Kuhnlein et al, 1994). This is similar to our findings, since of the ~1.5% $\text{TCR}\gamma\delta^+$ cells in the spleen; ~85% were $\text{CD8}^+\text{TCR}\gamma\delta^+$ (Figure 38a).

$\gamma\delta$ T cells are a minor T-cell subset present in the spleen and lymph nodes, but they represent a major subset within the epithelia of intestine and skin. In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells do not recognize antigen in the context of classical major histocompatibility complex, and as such do not express the coreceptors CD4 and CD8 (as mentioned previously, $\gamma\delta$ T cells in the rat do express CD8⁺). The functions of $\gamma\delta$ T cells are not well understood, but they seem to play a key role in oral tolerance and have anti-inflammatory and immunosuppressive activities. In murine models, intestinal $\gamma\delta$ intraepithelial lymphocytes have been shown to play a protective role in disease conditions associated with tissue damage, such as inflammatory bowel disease (Holtmeier & Kabelitz, 2005).

Fiber Effect

PRE had a higher proportion of TCR $\alpha\beta$ ⁺ CD4⁺ cells in the spleen, MLN and PP, however only the spleen reached statistical significance (Figure 34). Conversely, PRE had a lower proportion of TCR $\alpha\beta$ ⁺ CD8⁺ cells in all tissues, yet only the spleen and PP reached significance (Figure 35). This is contrary to what other researchers have found: Roller et al (2004) fed ~14-week-old male Fischer rats a high fat/low fiber (2% cellulose) diet supplemented with 10% Synergy 1 (inulin and oligofructose mix). At the end of 4 weeks, there were no significant differences in CD4⁺ or CD8⁺ T-cells in the spleen or MLN compared to control animals. In another study, female mice were fed a control diet with 10% cellulose or a diet with cellulose replaced completely by oligofructose or inulin, or partially (2.5%) with oligofructose. At the end of 6 weeks, there were no differences in

CD4⁺ or CD8⁺ T-cells in the spleen or thymus (Kelly-Quagliana et al, 2003). Manhart et al (2003) looked at the influence of 10% dietary FOS on PP lymphocyte numbers in healthy and endotoxemic female mice. The FOS diet increased the total cell yield in healthy and endotoxemic mice. However, T-cells were only increased in LPS-challenged mice fed the FOS diet. The increase of CD4⁺ cells was more pronounced than that of CD8⁺ cells.

It should be noted that the amount of fiber used in a study may be important. In most prebiotic studies, the experimental diet is supplemented with a prebiotic, therefore the total fiber content is higher than the control diet. It then must be considered that the immune effects could be due to a high fiber diet, instead of the prebiotic in question. Lim et al (1997) showed that different fibers had different effects on immune cell and function. For instance, 5% cellulose increased the proportion of CD8⁺ T-cells in the MLN of male Sprague-Dawley rats, compared to diets containing pectin, konjak manna and chitosan.

The diets in the study by Kelly-Quagliana et al (2003) had similar amounts of fiber, but the experimental diets had no source of insoluble fiber (i.e. cellulose) for fecal bulking. As observed in our preliminary study, a 10% inulin diet with no insoluble fiber was not well tolerated by the rats and resulted in diarrhea. Similarly, Hosono et al (2003) reported that mice fed a 7.5% FOS diet suffered from diarrhea during the initial week of the experiment. Since prebiotics work by being fermented in the colon, diarrhea would hinder their effectiveness, since they would not be in the colon long enough to be metabolized by bacteria.

However, Kelly- Quagliana et al (2003) did not mention any problems with dietary intolerance.

There was no difference in the proportion of total TCR $\gamma\delta^+$ cells or CD4 $^+$ /CD8 $^+$ subsets between CEL and PRE in the spleen, MLN or PP (Figure 36, Figure 37 and Figure 38). The effect of prebiotic fibers on TCR $\gamma\delta^+$ cells has not been previously investigated in these tissues. Since TCR $\gamma\delta^+$ cells are found in abundance in the epithelia of the mucosa, future studies should analyze intraepithelial lymphocytes.

Diet Effect

The effect of zinc deficiency and diet restriction on T-cells has been investigated in the weanling rat. Dowd et al (1986) fed weanling Wistar rats (gender unknown) either a zinc-deficient diet (1 mg Zn/Kg), or a control diet pair-fed to the zinc-deficient animals. After 4 weeks there was a significant decline in the absolute number of splenic T cells in ZD animals, but no change in the proportion of T helper (W3/25-positive) or cytotoxic (Ox8-positive) T-cells. In a more recent study, three-week-old male Sprague Dawley rats were fed a zinc-deficient diet or a control diet either *ad libitum* or pair-fed to the ZD group for 3 weeks. At termination, ZD rats had 40-63% fewer splenic TCR $\alpha\beta^+$ CD4 $^+$ and TCR $\alpha\beta^+$ CD8 $^+$ cells compared to both energy restricted and control animals. Energy restricted animals had 39% fewer splenic TCR $\alpha\beta^+$ CD4 $^+$ compared to control (Hosea et al, 2004). In contrast, our study with 7-week-old female Sprague Dawley rats showed no difference in the total proportion of TCR $\alpha\beta^+$ or the CD4/CD8 subtypes in the spleen or MLN (Figure 33, Figure 34 and Figure

35). Since our animals were older, they may be more resistant to the immune effects of zinc deficiency. Furthermore, since female rats were used and they stop gaining weight ~9 weeks of age, the effects of zinc deficiency may be less pronounced, compared to male rats.

Very little is known about the impact of zinc deficiency and diet restriction on mucosal immunity. Interestingly, in the PP, the proportion of total $\text{TCR}\alpha\beta^+$ cells and $\text{TCR}\alpha\beta^+\text{CD8}^+$ cells was 15% and ~10% higher, respectively, in DR animals compared to ZN and ZD (Figure 33 and Figure 35). Unlike the immunodeficiencies observed in severe protein energy malnutrition, moderate diet restriction is associated with increased T-cell function (reviewed by Yoshida et al, 1999). Furthermore, moderate diet restriction has been shown to delay the onset of T-lymphocyte-dependent autoimmune disease and increase lifespan in rodents (Jolly, 2004).

There was no difference in the proportion of total $\text{TCR}\gamma\delta^+$ cells or $\text{CD4}^+/\text{CD8}^+$ subsets among dietary groups (Figure 36, Figure 37 and Figure 38). This is similar to findings by Hosea et al (2003); three-week-old male Sprague Dawley rats were fed a zinc-deficient or a control diet either *ad libitum* or pair-fed to the ZD group for 3 weeks. At termination, few cells in the spleen were $\text{TCR}\gamma\delta^+$ (~1.69%) and they did not differ due to dietary treatment. To our knowledge, the effect of zinc deficiency and/or diet restriction on $\text{TCR}\gamma\delta^+$ cells in the MLN or PP has not been previously investigated.

B-Lymphocytes

In our study with 11-week-old animals, the highest proportion of CD45RA⁺ cells was in the PP (~75%), followed by the spleen (~55%) and the MLN (~33%) (Figure 39). Stepankova et al (1998) used Ig polyclonal antibodies for B-cell detection in 8-week-old rats and similarly found the highest proportion of Ig⁺ cells in the PP (~72%) followed by the spleen (~51%) and MLN (~31%).

Fiber Effect

In the MLN, the proportion of CD45RA⁺ cells was 8% lower in PRE compared to CEL (Figure 39b). There was no fiber effect on the proportion of CD45RA⁺ cells in the spleen or PP. Similarly, in female mice fed oligofructose or inulin for 6 weeks, there was no difference in B-cells in the spleen (Kelly-Quagliana et al, 2003). However, Manhart et al (2003) found that both healthy and endotoxemic female mice fed 10% FOS had increased B-cells in PP compared to control fed animals. The effect of prebiotics on B-cells in the MLN has not been previously studied. It is unknown why the B-cells in the MLN were altered, while the spleen and PP were unchanged. As previously described, when immune cells mature in the PPs, they leave via the MLN to enter systemic circulation (blood, spleen) and then return via the MLN to the lamina propria regions of the gut (Schley & Field, 2002). Thus, the MLN can be considered a gateway for the intestinal and systemic immune systems. If only the PP lymphocytes are affected, it can be assumed that the dietary treatment was not sufficiently long enough to affect the MLN and spleen. Conversely, if only the

spleen lymphocytes are altered, it can be thought that the effects in the PP and MLN have subsided.

Diet Effect

In the spleen, the proportion of CD45RA⁺ cells was 7% lower in both ZD and DR animals compared to ZN (Figure 39a). Similarly, King and Fraker (1991) found a decline in the proportion of splenic B cells (5-8%) in zinc deficient adult female mice. Unlike our findings, the researchers found no difference in the proportion of B-cells in zinc normal and diet restricted animals.

In the PP, the proportion of B cells was 4% higher in both ZN and ZD, compared to DR (Figure 39c). The effect of zinc deficiency or diet restriction on PP lymphocytes has not been previously investigated. Manhart et al (2000) fed young adult mice a protein deficient diet and found that after 7 days, B-cells dropped by 86% in the spleen, and 38% in the PP, compared to controls. However, since a protein deficiency is more severe than a diet restriction, these results may not be comparable to our study.

Dendritic Cells

Fiber Effect

In the PP, the proportion of OX62⁺ cells was 24% higher in PRE compared to CEL (Figure 40c). There was no significant difference in the MLN or spleen. The effect of inulin on dendritic cells has not been previously investigated. Lindsay et al (2006) investigated the effect of fructooligosaccharides on mucosal dendritic cell function in patients with moderately active Crohn's disease. In the lamina propria, the percentage of IL-10 positive dendritic cells increased, as determined by flow cytometry of dissociated rectal biopsies.

This is the first study to show that prebiotic fibers can increase the proportion of dendritic cells in the PP. An increase in the numbers of dendritic cells has been associated with increased immunological tolerance (Viney et al, 1998). Tolerance is the immune unresponsiveness to dietary antigens and indigenous bacterial antigens to maintain immunological homeostasis (Makala et al, 2004). Prebiotics may increase dendritic cells indirectly through the modulation of indigenous bacteria. For instance, Smits et al (2005) have demonstrated that the probiotic lactobacilli was able to prime DCs to promote the development of Treg cells, which mediate tolerance by inhibiting the proliferation and cytokine production of effector T cells.

Diet Effect

There was no effect of ZD or DR on the proportion of OX62⁺ cells in the spleen, MLN or PP (Figure 40). To our knowledge, no one has looked at the effect of ZD on the proportion of dendritic cells. However, recently Kitamura et al (2006) have demonstrated that zinc homeostasis affects DCs, through the involvement of zinc transport proteins during lipopolysaccharide-induced upregulation of MHCII proteins and co-stimulatory molecules.

In a study with energy-deficient mice, Zhang et al (2002) found no difference in the proportion of DCs in the spleen or lymph nodes, and the DCs retained antigen-presenting capacity, compared to control animals. However, during starvation, Abe et al (2003) found that the total number of spleen DCs were fewer in starved mice than in control. Furthermore, the capacities of DCs to induce IFN- γ and stimulate T cells decreased due to starvation.

CYTOKINES

Cytokines are the chemical messengers of the immune system. Their major role is to regulate the development and behavior of immune effector cells (Benjamini, 2000). T-cell derived cytokines include IL-2, IFN- γ and IL-10. IL-2 stimulates the growth and differentiation of T-cells, B-cells and NK cells. IFN- γ is involved with the activation, growth and differentiation of T cells, B cells, macrophages and NK cells. Furthermore, IFN- γ upregulates MHC expression on antigen presenting cells, such as dendritic cells. IL-10 stimulates or enhances proliferation of B-cells, thymocytes, and mast cells. Along with TGF- β , IL-10 also stimulates IgA synthesis and secretion by B cells (Goldsby et al, 2003).

Fiber Effect

At week 4, IL-2, IFN- γ and IL-10 were lower in 4-PRE than 4-CEL in both the spleen and MLN (Figure 41). However, this only reached significance with IL-10 and IFN- γ in the MLN. In contrast, at week 8, IL-2, IFN- γ and IL-10 were higher in 8-PRE than 8-CEL, which only reached significance with IFN- γ in the spleen and IL-2 in the MLN (Figure 41). These results show that it takes up to 8 weeks of consuming PRE to increase the cytokine secretion of cells isolated from the spleen and MLN.

At the end of 8 weeks the cytokines IL-2, IL-10 and IFN- γ were higher in PRE than CEL in both the spleen and MLN (Figure 42). In female mice fed prebiotics for 6 weeks, it was demonstrated that mitogen stimulated PP cells had increased secretions of IL-10 and IFN- γ in a dose-dependent manner. In splenocytes, the pattern of IFN- γ secretion was similar to PP (Hosono et al,

2003). In rats fed a high fat diet with prebiotics for 4 weeks, mitogen stimulated PP cells had increased secretions of IL-10, and the production of IL-10 and IFN- γ was correlated. However, the dietary treatment did not modulate cytokine production in the spleen or MLN (Roller et al 2004). Our findings demonstrate that PRE can stimulate both Th1 (IL-2, IFN- γ) and Th2/Th3 (IL-10) responses.

Diet Effect

Neither zinc deficiency nor diet restriction had an effect on cytokine secretion in the spleen or MLN (Figure 42). In another study conducted by our lab (Hosea et al 2006b), weanling male Sprague Dawley rats were assigned to one of three dietary treatments for 3 weeks: zinc deficient (ZD, <1 mg zinc/kg), control (CTL, 30 mg zinc/kg), or diet-restricted (DR, 30 mg zinc/kg). ZD splenocytes produced 54% less IL-2 compared to DR and CTL, and 71% less IFN- γ compared to DR in response to ConA. However, IL-10 concentrations were not different among dietary treatment groups. Perhaps we saw conflicting results due to the age (weanling vs. young adult) and gender (male vs. female) of the animals used in this study.

SERUM IMMUNOGLOBULIN G

Fiber Effect

There was no significant difference in serum IgG1 or IgG2a between PRE and CEL at week 4 or week 8 (Figure 43 and Figure 45). Similarly, in mice fed FOS for 6 weeks, Hosono et al (2003) found no difference in serum IgG2a. However, by week 6, serum IgG1 was lower in both 2.5% and 7.5% FOS supplemented rats, compared to controls (Hosono et al, 2003). In our study, there was a trend for serum IgG1 to be lower in PRE, but this did not reach significance (Figure 44).

Diet Effect

There was no significant difference in serum IgG1 among ZD, ZN or DR animals (Figure 44). Serum IgG2a of ZD was ~35% lower than both ZN and DR (Figure 46). Similarly, in weanling mice, Neyestani & Woodward (2005) found no difference in serum IgG2a between control and diet restricted animals. However, serum IgG1 was higher in restricted animals, compared to control. The suckling rodent absorbs IgG directly into the bloodstream until gut closure occurs at ~ 20 days of age. Thus, a decline in serum IgG after weaning can be attributed to turnover of milk-derived immunoglobulin. The researchers suggest that the high IgG1 levels sustained by diet restricted animals may be a biologically trivial consequence of decreased turnover, especially since a prolonged half-life is reported for IgG in the blood of malnourished humans. To our knowledge, serum IgG2a has not been previously measured in zinc deficiency. The IgG2a subclass

reflects a Th1 immune response and zinc deficiency is known to inhibit type 1 responses, therefore, our results appear logical.

VII. SUMMARY AND CONCLUSIONS

Major Research Findings and Implications

Fiber Effects:

- At week 8, the total feed intake of PRE was 8% lower compared to CEL, but there was no difference in body weight or feed efficiency. Furthermore, animals consuming PRE had less adipose tissue, while muscle mass was conserved. Consequently, PRE may help maintain a healthy body weight and body composition.
- The small intestine weight of PRE was 6% higher than CEL, and the small intestine length of PRE was 4% longer than CEL. These findings demonstrate that PRE is important for intestinal health.
- In PRE animals, the proportion of $\text{TCR}\alpha\beta^+ \text{CD4}^+$ cells was 4% higher in the spleen, the proportion of OX62^+ cells was 24% higher in PP and the cytokines IL-2, IL-10 and IFN- γ were 40-70% higher in both the spleen and PP, compared to CEL. Conversely, the proportion of $\text{TCR}\alpha\beta^+ \text{CD8}^+$ cells was ~10% lower in the spleen and PP, and the proportion of CD45RA^+ cells was 8% lower in the MLN of PRE compared to CEL. Thus, oral administration of PRE for 8 weeks can stimulate immune function in healthy young adult female rats. PRE elevated CD4^+ T-cells (T-helper cells), OX62^+ cells (dendritic cells) and the cytokines IL-2, IFN- γ and IL-10. PRE also reduced CD8^+ T-cells (mature T-cells, which can include both T suppressor and cytotoxic cells) and CD45^+ cells (B-cells). Therefore, it seems that PRE

increased the cells and cytokines involved with initiation of immune responses, while decreasing the proportion of effector cells.

Diet Effects:

- Zinc deficiency can be achieved in young 7-week-old female rats by consuming 1 mg zinc/kg diet for 4 weeks, as determined by the 85% drop in serum zinc and ~30% drop in femur zinc concentrations, as compared to ZN and DR animals.
- In the spleen, the proportion of CD45RA⁺ cells was 7% lower in both ZD and DR animals compared to ZN. Serum IgG2a of ZD was ~35% lower than both ZN and DR. Thus, ZD in young adult female rats is not as detrimental to the immune system as compared to ZD in weanling rats. The only immune marker that was specifically hindered by ZD was serum IgG2a.
- In the PP of DR animals, the proportion of total TCR $\alpha\beta$ ⁺ cells and TCR $\alpha\beta$ ⁺CD8⁺ cells was 15% and ~10% higher, respectively, compared to ZN and ZD. The results obtained from DR animals are interesting since DR seemed to have more effect on immune function than ZD. However, the body weight impairment of DR animals in this study (10%) was less than reported in other studies (20-40%). Consequently, in this study with young adult female rats, a 10% loss in body weight over 4 weeks may actually be beneficial for immune function.

Combined Fiber & Diet Effects:

- The femur zinc concentration of DR-PRE was 21% higher than DR-CEL; thus, it appears that PRE helps conserve femur zinc levels in DR animals.
- PRE is not protective against immune dysfunction caused by severe ZD in otherwise healthy, young adult female rats. However, the severe ZD was not particularly damaging with regards to immune markers measured in our study. Perhaps there would be protective effects of PRE in weanling rats or if a longer ZD period was used. PRE may be protective in a moderate ZD state, since it is hypothesized that PRE would increase absorption of the limited zinc.

Strengths

- Gender. The majority of zinc studies use male rats; however, ZD is more common in females in the human population. We have shown that symptoms of ZD, such as reduced feed intake and growth, are less pronounced in female rats compared to males, while markers of ZD such as reduced serum and femur zinc are similar.
- Age. Most zinc research investigating immune function is done using weanling rats. We have shown that severe ZD is less damaging to young adult rats, compared to weanling animals.
- Study length. This is the first study to look at the immune effects of prebiotic fibers in rats for a study period of 8 weeks. Most studies are 4-6 weeks and have found that shorter chain prebiotics, such as oligofructose, are more beneficial for immune function. Long-chain inulin may take longer to ferment and thus changes are not apparent with shorter studies; for instance, we did not see any immune related changes after 4 weeks.
- Fiber. Most experimental diets are supplemented with a prebiotic, thus the changes observed may be due to a higher fiber diet or the prebiotic in question. Our study was designed to have the same amount of total fiber in our CEL and PRE diets. However, it can be noted that differences may be due to a ratio of soluble and insoluble fibers, since our CEL diet contained only insoluble fiber. Few studies have looked specifically at the effect of long-chain inulin on immune function; most research has used oligofructose, fructooligosaccharides or some combination.

Future Directions/Limitations

- Gender and age are important characteristics to consider when designing a zinc study, since both aspects seem to influence the animal's response to ZD. If possible, future studies should include male and female animals, and characterize ZD at different times throughout the lifespan.
- Future studies should investigate both systemic (blood, spleen) and gut (PP, IEL, MLN) immunity, since we have shown that different tissues react differently to dietary treatments.
- The rat is not the best model for the human colon, because the principle site of microbial fermentation occurs in the caecum, whereas in humans fermentation occurs in the colon. The pig may be a better model for the human colon; however pigs utilize both the colon and the caecum and thus have greater capacity for microbial fermentation.
- The Main Study should be repeated, but with marginal ZD (5 mg Zinc/kg diet) to determine if prebiotics are beneficial when there is a limited source of zinc available in the diet. This would also be more applicable to the human situation, because severe ZD is rare, whereas mild to moderate ZD is common. Furthermore, the Main Study could be done in reverse, i.e. start with a ZD and then treat with prebiotics.
- Different types of prebiotics should be compared, including oligofructose, long-chain inulin and Synergy 1. It is hypothesized that low molecular weight oligofructose monomers (DP 2-8) are completely fermented in the caecum and the proximal segment of the colon, whereas long-chain inulin

(DP 20-60) can progress along the transverse colon and may reach the distal segments of the colon before being completely fermented (Roberfroid, 2005). Synergy 1, which contains a mix of oligofructose and long-chain inulin, should therefore be fermented along the entire colon. Perhaps oligofructose or Synergy 1 may enhance zinc absorption more so than long-chain inulin because it is fermented closer to the main site of zinc absorption (jejunum).

- Measure IL-10 and TGF- β in the small intestine to better determine Th3 response.
- Histology to determine if inulin reduces intestinal damage caused by zinc deficiency.
- Analyze bacteria levels and SCFA production in the colon to determine if zinc deficiency has any effect on beneficial bacteria and their end-products.
- Further study the dendritic cells, including toll-like receptors, which are how dendritic cells interact with bacteria in the intestine.
- The $\gamma\delta$ T-cells should be measured in the intraepithelial compartment of the intestine.

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XIV. APPENDICES

Table 4: Cytokines Released from Unstimulated Immune Cells over Time

	BASE	4-CEL	4-PRE	8-CEL	8-PRE	<i>F value</i>
Spleen						
IL-2	17.40 ± 4.22 ^a	17.21 ± 2.31 ^a	24.28 ± 4.35 ^a	8.07 ± 2.38 ^b	18.37 ± 4.46 ^{ab}	0.0142
IFN-γ	53.88 ± 12.56 ^a	152.10 ± 50.46 ^a	77.24 ± 21.95 ^a	7.41 ± 2.54 ^b	17.47 ± 4.27 ^b	<0.0001
IL-10	107 ± 20 ^b	901 ± 237 ^a	605 ± 201 ^a	136 ± 35 ^b	207 ± 42 ^b	0.037
MLN						
IL-2	25.98 ± 13.09	20.53 ± 4.37	25.00 ± 6.26	10.96 ± 4.31	7.73 ± 3.65	NS
IFN-γ	61.09 ± 61.09 ^a	17.69 ± 1.46 ^{bc}	56.62 ± 26.56 ^{ab}	10.75 ± 4.19 ^{bc}	4.33 ± 2.72 ^c	0.0074
IL-10	16.78 ± 7.33	38.64 ± 3.96	35.00 ± 7.30	35.87 ± 9.73	37.84 ± 5.69	NS
PP						
IL-2	8.48 ± 2.08	6.67 ± 1.80	3.44 ± 1.14	1.27 ± 0.55	4.61 ± 2.27	NS
IFN-γ	28.03 ± 8.47	12.72 ± 4.66	12.70 ± 4.06	7.50 ± 3.70	2.66 ± 1.17	NS
IL-10	0.89 ± 0.60 ^a	13.04 ± 3.47 ^a	0.19 ± 0.12 ^b	4.89 ± 1.11 ^a	3.73 ± 2.21 ^a	0.0054

Values are means ± SE. Statistical differences among means ($p \leq 0.05$) are indicated by lowercase letters. Abbreviations: BASE = baseline (n=8); 4-CEL = 4 week cellulose (n=8); 4-PRE = 4 week prebiotic (n=10); 8-CEL = 8 week cellulose (n=10); 8-PRE = 8 week prebiotic (n=10).

Table 5: Cytokines Released from Concanavalin A Stimulated Immune Cells over Time

	BASE	4-CEL	4-PRE	8-CEL	8-PRE	<i>F value</i>
Spleen						
IL-2	3479 ± 338 ^a	4478 ± 569 ^a	3945 ± 915 ^a	1150 ± 293 ^b	1675 ± 254 ^b	<0.0001
IFN-γ	9131 ± 633 ^a	14421 ± 1737 ^a	11486 ± 2374 ^a	1039 ± 225 ^b	2249 ± 527 ^c	<0.0001
IL-10	208 ± 50	402 ± 88	230 ± 58	308 ± 92	297 ± 30	NS
MLN						
IL-2	5346 ± 484 ^a	6559 ± 1075 ^a	5409 ± 1206 ^a	554 ± 99 ^b	1519 ± 297 ^c	<0.0001
IFN-γ	25244 ± 9609 ^{ab}	47138 ± 9101 ^a	14192 ± 4200 ^{bc}	2703 ± 469 ^d	6147 ± 1730 ^{cd}	<0.0001
IL-10	398 ± 98 ^a	377 ± 61 ^a	179 ± 30 ^b	141 ± 20 ^b	249 ± 42 ^{ab}	0.0074
PP						
IL-2	143 ± 70	197 ± 60	38 ± 20	53 ± 18	56 ± 19	NS
IFN-γ	1639 ± 803 ^a	696.82 ± 312 ^{ab}	350 ± 187 ^{bc}	10 ± 5 ^d	12 ± 5 ^{cd}	0.0002
IL-10	10.51 ± 6.46	69.49 ± 28.95	17.85 ± 7.30	5.74 ± 2.00	3.51 ± 1.46	NS

Values are means ± SE. Statistical differences among means ($p \leq 0.05$) are indicated by lowercase letters.
 Abbreviations: BASE = baseline (n=8); 4-CEL = 4 week cellulose (n=8); 4-PRE = 4 week prebiotic (n=10);
 8-CEL = 8 week cellulose (n=10); 8-PRE = 8 week prebiotic (n=10).

Table 6: Cytokines Released from Unstimulated Immune Cells of 8-week Groups

	FIBER EFFECT			DIETARY EFFECT			
	CEL	PRE	<i>F value</i>	ZN	ZD	DR	<i>F value</i>
Spleen							
IL-2	6.97 ± 0.97	12.10 ± 2.16	NS	13.22 ± 2.73	9.28 ± 1.11	5.31 ± 1.46	NS
IFN-γ	8.80 ± 2.32	13.40 ± 3.48	NS	12.44 ± 2.68	12.93 ± 3.49	7.16 ± 4.51	NS
IL-10	118 ± 19	179 ± 22*	0.0083	171 ± 28	112 ± 19	157 ± 29	NS
MLN							
IL-2	6.75 ± 1.80	6.56 ± 1.48	NS	9.43 ± 2.79	6.42 ± 1.15	4.11 ± 1.55	NS
IFN-γ	7.87 ± 2.46	5.08 ± 1.41	NS	7.54 ± 2.54	7.79 ± 2.99	3.98 ± 1.66	NS
IL-10	29 ± 4	40 ± 4	NS	37 ± 5	37 ± 5	29 ± 4	NS
PP							
IL-2	2.03 ± 0.45	2.31 ± 0.85	NS	3.03 ± 1.25	1.60 ± 0.35	1.91 ± 0.62	NS
IFN-γ	5.16 ± 1.68	2.06 ± 0.57	NS	5.08 ± 1.97	4.07 ± 1.56	1.57 ± 0.89	NS
IL-10	6.17 ± 1.61	4.10 ± 1.52	NS	4.28 ± 1.25	5.87 ± 2.05	5.26 ± 2.33	NS

As there was no interaction between fiber and dietary treatment (zinc or diet restriction), but there was a main effect of fiber and a main effect of dietary treatment, data were pooled to show means of main effects only. CEL represents 8-CEL, ZD-CEL and DR-CEL (n=30); PRE represents 8-PRE, ZD-PRE and DR-PRE (n=30); ZN represents 8-CEL and 8-PRE (n=20); ZD represents ZD-CEL and ZD-PRE (n=20); DR represents DR-CEL and DR-PRE (n=20). Values are means ± SE. Data were analyzed by two-way ANOVA and statistical differences among means ($p \leq 0.05$) are indicated by an asterisk (fiber effect) or lowercase letters (dietary effect). Abbreviations: MLN= mesenteric lymph nodes; PP = Peyer's patches; NS = not significant; CEL = cellulose; PRE = prebiotic inulin; ZN = zinc normal; ZD = zinc deficient; DR = diet restricted.

Table 7: Cytokines Released from Concanvalin A Stimulated Immune Cells of 8-week Groups

	FIBER EFFECT			DIETARY EFFECT			
	CEL	PRE	<i>F value</i>	ZN	ZD	DR	<i>F value</i>
Spleen							
IL-2	1008 ± 121	1617 ± 156*	0.006	1413 ± 198	1119 ± 158	1396 ± 192	NS
IFN-γ	1367 ± 155	2258 ± 270*	0.008	1644 ± 314	1949 ± 300	1864 ± 262	NS
IL-10	249 ± 41	355 ± 42*	0.013	303 ± 49	265 ± 63	336 ± 43	NS
MLN							
IL-2	673 ± 96	1608 ± 167*	<0.0001	1062 ± 195	1211 ± 192	1223 ± 225	NS
IFN-γ	3529 ± 642	6479 ± 741*	0.005	4516 ± 999	5122 ± 899	5537 ± 869	NS
IL-10	143 ± 20	322 ± 35*	<0.0001	198 ± 27	293 ± 47	220 ± 46	NS
PP							
IL-2	54 ± 11	44 ± 9	NS	54 ± 13	41 ± 9	52 ± 14	NS
IFN-γ	9.75 ± 2.68	7.10 ± 2.01	NS	11.36 ± 3.33	5.44 ± 1.62	8.24 ± 3.27	NS
IL-10	8.49 ± 2.39	1.93 ± 0.7	NS	4.63 ± 1.23	4.09 ± 1.32	6.99 ± 3.51	NS

As there was no interaction between fiber and dietary treatment (zinc or diet restriction), but there was a main effect of fiber and a main effect of dietary treatment, data were pooled to show means of main effects only. CEL represents 8-CEL, ZD-CEL and DR-CEL (n=30); PRE represents 8-PRE, ZD-PRE and DR-PRE (n=30); ZN represents 8-CEL and 8-PRE (n=20); ZD represents ZD-CEL and ZD-PRE (n=20); DR represents DR-CEL and DR-PRE (n=20). Values are means ± SE. Data were analyzed by two-way ANOVA and statistical differences among means ($p \leq 0.05$) are indicated by an asterisk (fiber effect) or lowercase letters (dietary effect). Abbreviations: MLN= mesenteric lymph nodes; PP = Peyer's patches; NS = not significant; CEL = cellulose; PRE = prebiotic inulin; ZN = zinc normal; ZD = zinc deficient; DR = diet restricted.