PROTECTIVE EFFECTS OF HYPERBARIC PRESSURE-PROCESSED WHEY PROTEIN ISOLATE AND POTATO PHYTOCHEMICAL EXTRACTS ON MODELS OF GUT INFLAMMATION

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A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy (Human Nutrition).

This thesis is dedicated to my beloved

parents, João Carlos and Neusa

my sister, Vanessa

my brother, Rodrigo

who always supported and gave me strength

throughout these years in Canada

"Perhaps the real heart within us is not just a pump.

Perhaps the real heart within us is about love and faith.

Perhaps the physical body is not who we really are.

Perhaps we are these invisible souls walking around,

and the body is just an instrument or metaphor

for something we are trying to learn"

Dr. Jeff Rediger, 2010

TABLE OF CONTENTS

ABSTRACT

Inflammatory bowel disease (IBD) is a chronic debilitating disorder characterized by various forms of chronic mucosal and/or transmural intestinal inflammation, which increases the synthesis of the hepatic acute phase proteins contributing to muscle wasting and impaired growth. For the present thesis, *in vitro* and *in vivo* models of gut inflammation were used to study the antioxidant and anti-inflammatory effects of native and pressurized whey protein isolate (WPI) and polyphenols, as these nutritional supplements could be used to ameliorate complications of IBD. The antioxidant, anti-inflammatory and anabolic effects of WPI were examined in piglets with dextran sulfate sodium (DSS)-induced colitis that were fed a moderate protein deficiency diet that contained either native WPI (nWPI), pressurized WPI (pWPI) or skim milk (SM) as the protein source plus a well nourished SM control group. Although piglets receiving both forms of WPI diets gained less absolute weight than their SM counterparts, pWPI-fed piglets gained more lean mass than nWPI piglets, and were the only protein restricted group to gain a high lean/fat ratio comparable to well-nourished controls. Histopathology scores in descending colon (DC) were lowest in both WPI groups. The pWPI-fed piglets had the lowest myeloperoxidase activity in DC reflecting low neutrophil infiltration and also had the highest ferric reducing antioxidant power (FRAP) activity in DC and the highest serum peroxynitrite scavenging capacity. Pro-inflammatory cytokines in DC were most elevated in SM piglets, and lowest in pWPI. Control-, nWPI- and pWPI-fed piglets had the lowest caspase-3 abundance in DC signifying anti-apoptotic effects as compared to SM group. As an *in vitro* model of gut inflammation, Caco-2 cells were exposed with H_2O_2, that received either pre- or post-treatment with WPI hydrolysates. WPI hydrolysates were associated with decreased interleukin (IL)-8 secretion, lower intracellular reactive oxygen species (ROS) and increased FRAP activity; however, those parameters were all significantly improved when hydrolysates of pressurized WPI were used. In another study, polyphenolic-rich potato extracts were subjected to gut digestive enzymes and microbial metabolism in a simulated human gut model and the antioxidant and anti-inflammatory effects of the

polyphenolic digests were tested in Caco-2 cells exposed to H_2O_2. Treatment of H_2O_2-stimulated Caco-2 cells with digests of polyphenolic-rich potato extracts was associated with decreased IL-8 release, lower ROS production and higher FRAP activity. Overall, the present thesis showed results regarding the antioxidant and anti-inflammatory effects of pressurized WPI and polyphenolic extracts that indicate the potential for these nutraceutical agents towards the treatment of IBD.

RÉSUMÉ

Les maladies inflammatoires de l'intestin (MII) sont des troubles chroniques et débilitants caractérisés par diverses formes d'inflammation chronique de la muqueuse et/ou transmurale, ce qui augmente la synthèse des protéines hépatiques de phase aigüe qui contribuent à l'atrophie musculaire et à l'altération de la croissance. Pour la présente dissertation, des modèles *in vitro* et *in vivo* d'inflammation intestinale ont été employés pour étudier les effets antioxydants et anti-inflammatoires de polyphénols et d'isolats de protéines de lactosérum (WPI) natives et pressurisées, puisque ces suppléments nutritionnels pourraient être utilisés afin d'améliorer les complications des MII. Les effets antioxydants, anti-inflammatoires et anaboliques des WPI ont été examinés dans un modèle de colite induite par administration de dextran sodium sulphate chez le porcelet. Des porcelets ont été soumis à une alimentation modérément déficiente en protéines contenant des WPI natives (nWPI), pressurisées (pWPI) ou du lait écrémé (SM) comme source de protéines; et un groupe de porcelets contrôle a été soumis à une alimentation suffisante contenant du lait écrémé. Bien que les porcelets recevant les deux formes d'alimentations WPI ont démontré un gain de poids absolu moindre que leurs homologues SM, les porcelets du groupe pWPI ont gagné plus de masse maigre que les porcelets nWPI et étaient le seul groupe sous restriction protéique à gagner un ratio de maigre/gras élevé et comparable aux contrôles bien nourris. Les résultats de l'histologie pathologique du côlon descendant (DC) ont été les plus bas chez les deux groupes WPI. Dans le DC, les porcelets nourris de pWPI ont eu les taux d'activité de myeloperoxidase les plus bas, reflétant un bas taux d'infiltration par les neutrophiles, ainsi que la puissance antioxydante réduisant les ions ferriques (FRAP) la plus élevée, et les taux sériques les plus élevés de capacité de balayage peroxynitrite. Les cytokines pro-inflammatoires dans le DC étaient le plus élevées chez les porcelets SM, et le moins élevées chez les pWPI. Les porcelets contrôles, nourris de nWPI et pWPI ont montrés la présence en abondance de caspase-3 la plus basse dans le DC, signifiant un effet anti-apoptotique comparativement au groupe SM. Dans un modèle *in vitro* d'inflammation intestinale, des cellules

Caco-2 étaient exposées au H_2O_2 avant ou après être traitées avec des hydrolysats de WPI. Les hydrolysats de WPI étaient associés à une diminution de la sécrétion d'interleukine (IL)-8 et des taux intracellulaires d'espèces réactives de l'oxygène (ROS), ainsi qu'une augmentation de l'activité FRAP. De plus, ces paramètres étaient tous améliorés de façon significative quand les hydrolysats de WPI pressurisées étaient employés. Dans une autre expérimentation, des extraits de pomme de terre riches en polyphénols étaient soumis à l'action d'enzymes digestives intestinales et au métabolisme microbien dans un modèle intestinal humain simulé. Les effets antioxydants et anti-inflammatoires des produits digestifs phénoliques étaient administrés à des cellules Caco-2 exposées au H_2O_2. Le traitement des cellules stimulées au H_2O_2 par les produits digestifs des extraits de pommes de terre riches en polyphénols était associé à une diminution de la sécrétion d'interleukine (IL)-8 et des taux de ROS, ainsi qu'une augmentation de l'activité FRAP. Globalement, la présente dissertation démontre des résultats concernant les effets antioxydants et anti-inflammatoires des WPI pressurisées et des extraits polyphénoliques, indiquant un usage potentiel de ces agents nutraceutiques pour le traitement des MII.

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STATEMENT OF ORIGINALITY

1. Claims of Original Research

This thesis has demonstrated for the first time that:

1. Pressurized whey protein isolate (WPI) exerted anabolic, antioxidant and anti-inflammatory effects and decreased disease severity both systemically and in the descending colon (DC) in a piglet model of pediatric ulcerative colitis (UC).

2. The mechanism by which WPI suppressed the inflammatory response is partly mediated via the caspase-3 apoptotic pathway in the DC of piglets with induced UC.

3. Hydrolysates of pressurized WPI showed better antioxidant and anti-inflammatory effects than native WPI as exhibited by decreased IL-8 secretion and reduced intracellular reactive oxygen species generation in H_2O_2-treated Caco-2 cells.

4. The bioaccessibility and biotransformation of polyphenolics from polyphenol-rich potato extracts was demonstrated in the colonic compartment of a dynamic simulated human gut model involving digestive enzyme and bacterial metabolism.

5. Extracts of a polyphenol-rich potato cultivar exposed to digestive enzyme and bacterial metabolism via the simulated human gut model protected against fecal water toxicity in Caco-2 cells and exerted significant antioxidant and anti-inflammatory effects in both non-stimulated and H_2O_2-stimulated Caco-2 cells treated with fecal water.

2. Research Manuscripts in Preparation for Publication:

1. **Piccolomini, A.**, Antunes, J., Rodrigues, A., Marilene, P., Vassilyadi, P., Weiler, H., Kubow S., Wykes, L. The impact of milk protein quality and quantity on anabolism and inflammation in a piglet model with dextran sulfate sodium-induced colitis. In preparation.

2. **Piccolomini, A.**, Iskandar, M., Lands, L., Kubow. Whey protein isolate hydrolysates inhibit oxidative stress and hydrogen peroxide-induced IL-8 secretion in intestinal epithelial cells. In preparation.

3. **Piccolomini, A.**, Sabally, K., Nassar, A., Khairallah, J., Ekbatan, S., Prakash, S., Donnelly, D., Kubow, S. Potato-derived polyphenolic extracts that undergo simulated human gut digestion decrease oxidative stress and the inflammatory response in Caco-2 cells. In preparation.

3. Abstracts Published and Patent Application:

1. **Piccolomini, A.**, Kubow, S., Donnelly, D., Annamalai, A., Riciardi, P., Nassar, A., Sabally, K. (2008). Differential antioxidant capacity among twelve potato cultivars. Abstracts (Oral Sessions). *Hortscience* 43(4): 1082.

2. **Piccolomini, A.**, Kubow, S., Donnelly, D., Annamalai, A., Riciardi, P., Nassar, A., Sabally, K. (2008). Unraveling the chlorogenic acid and phenolic content among 12 potato cultivars for improved human health and consumer acceptance Abstracts (Poster Sessions). *Hortscience* 43(4): 1222.

3. Nassar, A., Kubow, S., Leclerc, Y., **Piccolomini, A.**, Donnelly, D. (2008). Tribute to the potato. Abstracts (Poster Sessions). *Hortscience* 43(4): 1278.

4. **Piccolomini, A.**, Rodrigues, A., Antunes, J., Paquet, M., Weiler, H., Kubow, S., Wykes, L. (2011). Pressurized whey protein isolate decreases inflammation in descending colon of piglets with dextran sulfate-induced colitis. Abstracts (Poster Sessions). *Applied Physiology, Nutrition and Metabolism* 36: 477.

5. Antunes, J., Rodrigues, A., **Piccolomini, A.**, Paquet, M., Weiler, H., Kubow, S., Wykes, L. (2011). Severe but not moderate protein deficiency increases disease severity while impairing growth and nutritional status of piglets with dextran-sulfate-induced colitis. Abstracts (Poster Competition Finalist). *Applied Physiology, Nutrition and Metabolism* 36: 439.

6. Kubow, S., Donnelly, D., **Piccolomini, A**. (2009). *Compositions and methods for preventing and treating disease and environmentally induced health disorders*. US Provisional Patent Application No. 61290098 filed Dec. 24, 2009.

CONTRIBUTIONS OF AUTHORS

The present thesis involved the collaboration of Dr. Stan Kubow, Dr. Linda Wykes, and Dr. Hope Weiler, School of Dietetics and Human Nutrition, McGill University, Macdonald Campus; Dr. Danielle Donnelly, Plant Science Department, McGill University, Macdonald Campus; Dr. Larry C. Lands, Paediatric Respiratory Medicine, Montreal Children's Hospital.

Dr. Kubow, the candidate's primary supervisor, was the originator of the piglet research project along with Dr. Linda Wykes who was involved in the study design, providing ongoing guidance and feedback in all aspects of the project. In addition, Dr. Kubow conducted weekly meetings with the candidate to monitor progress of the work and set goals for future accomplishments. Each manuscript draft was extensively critiqued and edited by Dr. Kubow, including the literature review.

Dr. Wykes trained the candidate for the piglet surgery, stable isotope infusion experiments, GC-MS analysis and protein synthesis calculations. Dr. Wykes was also responsible for reviewing the manuscript and other materials for the piglet project. Dr. Hope Weiler trained the candidate to use the DXA equipment, comments and suggestions of this project and provided feedback on the abstracts submitted for a Nutrition conference. Dr. Danielle Donnelly provided important contribution for comments and suggestions during the potato project. Dr. Larry Lands granted the permission to use the laboratory facility for the generation of the hydrolysates of whey protein isolate. Dr. Satya Prakash granted the permission to use the simulated human gut model to digest the potato extract.

As the primary author the candidate was responsible for writing the manuscripts, creating the figures and the tables, and running all statistical analyses. The candidate assisted in the study design, diet formulation, conducted the animal surgeries and trial, isotope infusions (including calculations), and tissue sampling

associated with the piglet project. The candidate performed all the experiments in the piglet study including the majority of the biochemical parameters, cytokines analysis, myeloperoxidase activity, antioxidant assays, nitrogen and protein quantifications, and GC/MS analysis for protein synthesis. The candidate was also responsible for carrying out all of the experiments described with cell culture, including generation of *in vitro* whey protein isolate digestates and lyophilisation, antioxidant assays, HPLC analysis of peptide hydrolysates, viability test, IL-8 and ROS assays. The candidate performed all the analysis for the potato trial including sample preparation, calculations, antioxidant assays, and HPLC analysis of digested polyphenols. For the cell culture, the candidate performed basal cell culture maintenance, cell viability determination, cell culture studies involving cell stimulation with H_2O_2 and treatment with digested polyphenols, including antioxidant assays of the cell culture supernatants, IL-8 and ROS assays.

Juliana Antunes helped the candidate in assisting the surgeries, piglet care, diet preparation, infusion of stable isotopes, and tissue sampling during the piglet trial. Dr. Ananda Rodrigues provided assistance in all the activities mentioned before and also helped the candidate to prepare the descending colon tissue for cytokine analysis. In addition, Dr. Ananda Rodrigues performed all the Immunoblots analysis in this thesis. Photios Vassilyadi prepared all the piglets' bone and performed DXA scans. Michele Iskandar provided technical assistance for the whey protein isolate *in vitro* digestion and cell culture study design. Dr. Kebba Sabally provided technical support for the HPLC analysis. Dr. Atef Nassar provided assistance for the statistical analysis in this thesis. Shima Ekbatan and Joelle Khairallah provided the faecal water alone and faecal water with polyphenol digestates from the simulated human gut model.

LIST OF TABLES

TABLE OF FIGURES

Chapter 3 - Manuscript 1

Chapter 4 - Manuscript 2

Chapter 5 - Manuscript 3

LIST OF ABBREVIATIONS

ACN	Acetonitrile
Akt	Alpha serine/threonine-protein kinase
APAF-1	Apoptotic protease activating factor-1
ASR	Absolute synthesis rate
Bad	Bcl-2-associated death promoter
Bax	Bcl-2–associated X protein
BCAA	Branched-chain amino acids
Bcl-2	B-cell lymphoma 2
BMC	Bone mineral content
BMD	Bone mineral density
CA	Caffeic acid
CD	Crohn’s disease
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CGA	Chlorogenic acid
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRP	C-reactive protein
d	Days
DC	Descending colon
DCF	Dichlorofluorescein
DCFH-DA	Dichlorofluorescin-diacetate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
DXA	Dual-energy X-ray absoptiometry
EAA	Essential amino acids
EC	Epicatechin

ELISA	Enzyme-linked immunosorbent assay
FA	Ferulic acid
FADD	Fas-associated protein with death domain
Fas	Apoptosis stimulating fragment
Fas-L	Apoptosis stimulating fragment-ligand
FC	Folin-Ciocalteau reagent
FRAP	Ferric reducing antioxidant power
FSR	Fractional synthesis rate
FW	Faecal water
GC-MS	Gas chromatography-mass spectrometry
GI	Gastrointestinal
GMP	Glycomacropeptide
GSH	Glutathione
GSK-3	Glycogen synthase kinase-3
GST	Glutathione S-transferase
$^{\bullet}OH$	Hydroxyl radical
h	Hour
H&E	Hematoxylin and eosin
H_2O	Water
H_2O_2	Hydrogen peroxide
ha	Hectares
HepG2	Human hepatoma cell line
HFBS	Heat-inactivated fetal bovine serum
HOCl	Hypochlorous acid
i.p.	Intraperitoneal
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin

iNOS	Nitric oxide synthase
JAKs	Janus kinases
kDa	Kilodalton
KGM	Konjac glucomannan
KIC	α-ketoisocaproate
KO	Knockout
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
Lf	Lactoferrin
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTB4	Leukotriene B_4
MAPKs	Mitogen-activated protein kinases
MEM	Minimum Essential Medium Eagle
MeOH	Methanol
Met	Methionine
min	Minutes
mo.	Month
MPO	Myeloperoxidase
MTS	Mitochondrial succinate dehydrogenase
MWCO	Molecular weight cut-off
Na_2CO_3	Sodium carbonate
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NK-1R	Neurokinin-1 receptor
NLRP3	Nod-like receptor family, pyrin domain 3
NLRs	Nod-like receptors
NO	Nitric oxide
NOC	Nitroso compounds
NOD2	Nucleotide-binding oligomerization domain 2

NRC	National Research Council
nWPI	Native whey protein isolate
ORAC	Oxygen radical absorbance capacity
Phe	Phenylalanine
pK_a	Acid dissociation constant
PMA	Phorbol esters
PMNs	Polymorphonuclear leukocytes
PPAR	Peroxisome proliferator-activated receptors
PUFAs	Polyunsaturated fatty acids
PV	Plasma volume
pWPI	Pressurized whey protein isolate
REE	Resting energy expenditure
RONS	Reactive oxygen nitrogen species
ROS	Reactive oxygen species
RP-HPLC	Reverse-phase high performance liquid chromatography
$O_2^{\bullet-}$	Superoxide anion
SB 415286	3-[(3-Chloro-4-hydroxyphenyl)amino]-4-(2-nitrophenyl)-1H-pyrrol-2,5-dione
SCFA	Short-chain fatty acids
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
SM	Skim milk
SOD	Superoxide dismutase
SP	Substance P
TCA	Trichloroacetic acid
TDZD	Thiadiazolidinones
TFA	Trifluoroacetic acid
Th1	T-helper type-1
Th2	T-helper type-2
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumor necrosis factor-alpha

TPTZ	2, 4, 6-tripyridyl-s-triazine
TRAIL	TNF-related apoptosis-inducing ligand
UC	Ulcerative colitis
wk(s)	Week(s)
WP	Whey proteins
WPI	Whey protein isolate
α-la	α-lactalbumin
β-HMB	β-hydroxy β-methylbutyrate
β-lb	β-lactoglobulin

Chapter 1

Introduction, Objectives and Hypotheses

INTRODUCTION

1.1 Rationale and Statement of Purpose

Inflammatory bowel disease (IBD) is a heterogeneous group of disorders characterized by various forms of chronic mucosal and/or transmural inflammation of the intestine (Hyams, 2005). IBD is the generic term used to describe two idiopathic chronic disorders associated with gastrointestinal (GI) inflammation: Crohn's disease (CD) and ulcerative colitis (UC). Currently, research is focused in two major directions: (1) therapeutic approaches towards a cure; and (2) strategies to improve and control disease symptoms (Kaser *et al.*, 2010).

Presently, the main treatments for UC involve medications with long-term side effects (Kim and Ferry, 2002). UC is characterized by chronic intestinal inflammation, which causes neutrophil infiltration, releasing systemically high concentrations of pro-inflammatory mediators such as TNF-α, IL-8, and IL-18 (Sartor, 1994). In addition, the pro-inflammatory state in IBD can increase in 19% the resting energy expenditure (REE) for adult patient with colitis (Klein *et al.*, 1988; Hill *et al.*, 2007) and also elevated REE has been found in pediatric IBD patients as compared to malnourished controls (Azcue *et al.*, 1997). Moreover, energy and protein deficiency was demonstrated to decrease total plasma protein fractional synthesis rates in piglets with colitis (Harding *et al.*, 2008), which reflects an increased hepatic acute-phase response. In this manner, amino acids can be diverted from muscle protein synthesis, leading to long-term muscle wasting and consequent growth failure (Gabay and Kushner, 1999). The chronic inflammatory state observed in IBD has been demonstrated to lead to cytokine-induced catabolism of tissues in order to supply the immune system with energy and amino acids (Bistrian, 1999).

A variety of nutritional supplements including whey proteins (WP) and polyphenols have potent antioxidant and anti-inflammatory effects, which could have therapeutic and preventative properties beneficial at the colonic level. For this reason, nutritional studies are needed to identify the therapeutic utility of

these supplements to reduce disease severity and improve the quality of life for patients with UC.

Many *in vivo* and *in vitro* studies have shown significant protective effects of WP and WP hydrolysates against oxidative stress imbalances associated with a variety of diseases (Appel *et al.*, 1997; Ikeda *et al.*, 1998; Bounous, 2000) including IBD (Meister *et al.*, 2002; Schaafsma, 2007; Kanwar and Kanwar, 2009; Sprong *et al.*, 2010). The antioxidant properties of WP have been investigated in both animal and cell culture models of colonic inflammation (Haversen *et al.*, 2003; Minekawa *et al.*, 2004). In addition, several immune-modulating and anti-inflammatory effects of WP have been suggested in a number of experimental settings and disease conditions (Hanning *et al.*, 1993; Wong *et al.*, 1993; Gill *et al.*, 2000; Sgarbieri *et al.*, 2009), although no human or animal studies to date have studied the anti-inflammatory impact of WP intake in IBD. Moreover, WP have a relatively high content of leucine in comparison to other proteins with high biological value, which could play a role in the putative anabolic effects shown with WP supplementation in human (Lands *et al.*, 1999) and animal (Morifuji *et al.*, 2005) studies. Such anabolic effects of WP intake could be therapeutically useful in UC, which is associated with muscle protein catabolism (Mackenzie *et al.*, 2003). In addition, the loss of epithelial cells in active UC occurs mainly by apoptosis (Calabuig *et al.*, 2009) but the mechanisms underlining this event need to be explored.

Hydrolysates from pressure treatment of whey protein isolate (WPI) have been shown to exert more effective anti-inflammatory, antioxidant, and anabolic effects than native WPI hydrolysates in cystic fibrosis transmembrane conductance regulator (CFTR) mutant respiratory cells (Vilela *et al.*, 2006; Iskandar *et al.*, 2008). Moreover, supplements of pressurized WPI exerted anti-inflammatory properties in humans with cystic fibrosis together with improvement in both nutritional status and lung function (Lands et al., 2010), clinically significant improvements in function in patients with chronic obstructive pulmonary disease (Laviolette *et al.*, 2010), and anti-catabolic effects in colorectal surgical cancer patients (Ball *et al.*, 2011). It has been postulated that hyperbaric

pressure treatment induces conformational changes that can render WPI more susceptible to gastrointestinal digestive enzymes, thereby altering the profile of peptides obtained through enzymatic hydrolysis (Lopez-Fandino, 2006; Vilela *et al.*, 2006), which could lead to an increased release of bioactive peptides. However, there have no studies to date, that have investigated the possible anabolic, antioxidant, and anti-inflammatory effects of pressurized WPI in colitis.

Phenolics compounds exerted antioxidant and anti-inflammatory effects at the intestinal level as shown via *in vitro* human intestinal Caco-2 cell studies (Netsch *et al.*, 2006; Romier-Crouzet *et al.*, 2009) as well as in animal models of dextran sulfate sodium (DSS)–induced colitis (Oz *et al.*, 2005; Kim *et al.*, 2010). Dietary polyphenols down-regulated the intestinal inflammatory response via the modulation of intracellular signaling cascades in intestinal Caco-2 cells (Landolfi *et al.*, 1984; Serafini *et al.*, 1998; Aviram and Fuhrman, 2002; Kim *et al.*, 2005; Romier *et al.*, 2009). Polyphenols inhibited nuclear factor-kappa B (NF-κB) transcriptional activity and the mitogen-activated protein kinase (MAPKs) signaling pathway, and reducing levels of proinflammatory markers such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-8 in inflamed intestinal Caco-2 cells (Netsch *et al.*, 2006; Romier-Crouzet *et al.*, 2009). The antioxidant activity of phenolics compounds has been partly linked to their redox properties, which can absorb and neutralize free radicals, quench singlet oxygen, or decompose peroxides (Cao *et al.*, 1997). To our knowledge, the above cited research studies are the only investigations that have examined for the possible protective effects of polyphenols on intestinal inflammation. In that regard, there are no studies regarding the anti-inflammatory impact of polyphenols after they have undergone intestinal enzymatic digestion and microbial metabolism.

Potatoes are considered a good dietary source of polyphenols (Scalbert and Williamson, 2000). However, only a few studies have examined for differences in the concentrations and profiles of polyphenolics among different cultivars. As only a limited number of potato cultivars have been studied for their polyphenolic content, more studies are needed to identify cultivars that might have relatively greater concentrations of a wide range of polyphenols. Extracts of

polyphenolic mixtures from polyphenolic-rich potato cultivars could be a potentially inexpensive bioactive supplement that could be useful as an antioxidant and anti-inflammatory treatment for patients with UC. The antioxidant and anti-inflammatory potency of polyphenolic extracts can be assessed on a physiological basis by testing polyphenolic digestates after exposure to digestive enzyme(s) and bacterial metabolism via a simulated human gut model.

1.2 Thesis Objectives

1. To investigate the impact of feeding native skim milk protein, native WPI, and pressurized WPI on growth, body composition, protein synthesis, descending colon (DC) histopathology, inflammatory, and antioxidant status in DSS-treated piglets fed an isoenergetic moderately protein-deficient diet.

2. To explore and identify molecular mechanism(s) by which native and pressure-treated WPI might exert anti-apoptotic effects in DC tissue in a piglet model of DSS-induced colitis.

3. To study in human intestinal Caco-2 epithelial cells the potential antioxidant and anti-inflammatory effects of hydrolysates of native and pressure-treated WPI, particularly in relation to IL-8 release, either under basal conditions or induced exogenously by the pro-inflammatory stimulus hydrogen peroxide (H_2O_2).

4. To investigate in cultured Caco-2 cells, either under basal conditions or induced exogenously by the pro-inflammatory stimulus H_2O_2, the antioxidant and anti-inflammatory responses of digestates obtained from extracts of a polyphenol-rich potato cultivar following digestion in a simulated human gut model.

1.3 Hypotheses

1) Pressurized WPI-fed piglets that are moderate protein-deficient will demonstrate more potent anabolic effects than protein- deficient piglets fed native WPI or skim milk protein powder.

2) Piglets fed pressurized WPI will show decreased disease severity in DC demonstrated by reduced pro-inflammatory cytokine levels, increased antioxidant status, and greater suppression of the caspase-3-mediated apoptotic pathway as compared with the well-nourished control piglets or moderately protein-deficient piglets fed native WPI or skim milk protein powder.

3) Hydrolysates of WPI will inhibit secretion of IL-8 and exert antioxidant effects in H_2O_2-stimulated Caco-2 cells, and hydrolysates of pressurized WPI will exert significantly greater antioxidant and anti-inflammatory effects as compared with hydrolysates of native WPI.

4) Potato polyphenol extracts exposed to digestive enzymes and bacterial metabolism, via the simulated human gut model, will promote antioxidant and anti-inflammatory effects including suppressed IL-8 secretion in H_2O_2-stimulated Caco-2 cells.

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Chapter 2

Literature Review

2.1 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is the generic term used to describe two idiopathic chronic disorders associated with gastrointestinal (GI) inflammation: Crohn's disease (CD) and ulcerative colitis (UC). The definitions of CD and UC are based on the location and characteristics of the inflammatory process within the GI tract (Kaser *et al.*, 2010). The inflammation in CD may involve any segment of the digestive tract and is associated with discontinuous transmural lesions of the gut wall. In the condition of UC, inflammation is confined to the colon and rectum, and lesions are continuous extending from rectum and upwards to one or several colonic segments and restricted to the mucosa (Hyams, 2005).

The incidence and prevalence of IBD are greatest in northern latitudes such as in Canada, the UK, and Scandinavia and lower in Croatia and other southern locales; North America and Europe generally have a much greater IBD prevalence than Asia (Loftus and Sandborn, 2002; Loftus, 2004). There are almost 201,000 Canadians living with IBD: 112,000 with CD and 88,500 with UC with over 9,200 new cases diagnosed every year – 5,100 with CD and 4,100 with UC (CCFC, 2008). Therefore, Canada has among the highest reported rates of prevalence and incidence of IBD in the world. It has been noted that in the Canadian population, little vitamin D is produced endogenously during winter because geographically Canada is situated at a latitude above 35° N (Hanley and Davison, 2005). At this zone, the zenith angle of the sun is more oblique, which decreases penetration of UVB into human skin. Dietary intake cannot readily compensate for lack of sun because few foods are naturally rich in vitamin D (Webb *et al.*, 1988). Vitamin D deficiency has been linked with early onset of IBD (Cantorna, 2000; 2010). Vitamin D regulates the development and function of the immune system and early childhood and prenatal changes in vitamin D status affect the resultant immune response and the development of autoimmune diseases such as IBD (Ardizzone *et al.*, 2011; Levin *et al.*, 2011). IBD can be diagnosed at any age, but has a typical age of onset in the twenties (CCFC, 2008). Among the pediatric population there is less data available in this age

group but overall CD was more common than UC in all Canadian provinces (CCFC, 2008). The latest estimative from 1998 to 2000 counted for about 3,300 children under 20 with CD and 1,600 children under 20 with UC in Canada, for a total of 4,900 people (Bernstein *et al.*, 2006).

The treatment of UC involves medications that decrease the abnormal inflammation in the colon lining and thereby control the symptoms, with the goal of maintaining this induced remission (CCFC, 2008). Medical options are centered on oral or topical aminosalicylates therapy for people with mild to moderate symptoms. Sulfasalazine or corticosteroids are used for moderately to severely active disease. However, due to long-term side effects these should not be used for maintenance therapy. Immune modifiers include 6-mercaptopurine and azathioprine work by causing a reduction in lymphocyte count, and another type of drug known as infliximab which acts as tumor necrosis factor-alpha (TNF-α) antagonist can be used to replace these drugs once acute symptoms come under control (Kim and Ferry, 2002).

2.1.1 Gut Health and IBD

Approximately $2x10^{14}$ bacteria reside in the GI tract, and this tremendous amount of bacteria challenge the mucosal immune system, which needs to protect the intestinal mucosa against pathogenic agents (Peppelenbosch and Comalada, 2006). IBD results when there is an imbalance in mucosal immune system responsiveness tilting towards an exaggerated or uncontrolled reaction against the commensal flora (Sturm *et al.*, 2008). Resident bacterial flora has been suggested to be an essential factor in driving the inflammatory process in IBD (Shanahan, 2001a). Patients with UC have increased intestinal mucosal secretion of immunoglobulin (Ig)G type antibodies against a broad spectrum of commensal bacteria (Macpherson *et al.*, 1996). Immunoinflammatory responses mediated by IgG can damage the intestinal mucosa since, unlike normal IgA responses, they activate a cascade of inflammatory mediators such as interleukin (IL)-1, TNF-α, and lymphotoxin (Brandtzaeg *et al.*, 1989). Moreover, patients with IBD have greater amounts of bacteria attached to their epithelial surfaces than do

healthy people (Swidsinski *et al.*, 2002). These bacteria are from diverse genera and some of them, such as *Bacteroide fragilis* and *Escherichia coli*, were identified within the epithelial layer, and in some instances, intracellularly (Swidsinski *et al.*, 2002). So, unrestrained activation of the intestinal immune system by elements of the flora could be a key event in the pathophysiology of IBD.

Several species of commensal microflora, including some anaerobes, invade the mucosa after induction of colitis in rats and various species of *Bacteroides* appear to be especially active in inducing transmural inflammatory lesions (García-Lafuente *et al.*, 1997). These anaerobes induce a mild granulocyte response as well as a widely diffused infiltration of mononuclear cells, which is associated with an accumulation of collagen in gut tissue (Casellas *et al.*, 1998). So, some anaerobes have the potential to induce diffuse fibrinogenic responses when invading the intestinal wall (Casellas *et al.*, 1998). In humans with UC, direct interaction of commensal microflora with the intestinal mucosa stimulates inflammatory activity in the gut lesions (Guarner and Malagelada, 2003; Conte *et al.*, 2006).

2.1.2 Inflammation and IBD

Colitis is considered to be partly caused by lymphocytes that are activated by the cytokines secreted from activated macrophages (Okayasu *et al.*, 1990). The expression of many inflammatory cytokine genes such as IL-1, TNF-α, IL-6, and IL-8 are regulated by the transcription factor, nuclear factor-kappa B (NF-κB). In the course of colonic inflammation, the colonic mucosa shows increased expression of these molecules (Sartor, 1994). This activity leads to amplification of the inflammatory cascade and secretion of more inflammatory mediators, destructive enzymes, and free radicals that cause tissue injury and are implicated in the pathogenesis of colitis (Elson, 1996). Nod-like receptors (NLRs) are intracellular pattern recognition receptors that are responsible for detecting invading pathogens and activating the innate immune response. Upon recognition of microbial components, some NLRs form cytoplasmic, multiprotein

complexes known as inflammasomes that serve as platforms for the recruitment, cleavage, and activation of inflammatory caspases. Nucleotide-binding oligomerization domain (NOD)2 and NOD-like receptor, pyrin domain (NLRP)3 are two of the best characterised NLRs, and mutations of these receptors have been linked to mice with colitis. NOD2 recognises the bacterial peptidoglycan-derived molecule muramyl dipeptide and activates the NF-κB pathway to induce an inflammatory response (Bauer *et al.*, 2010). Inflammasome activation of caspase-1 is essential for the maturation and secretion of IL-1β and IL-18 (Zaki *et al.*, 2011). Either independently or in synergy with IL-12, high levels of IL-18 via induction of interferon-gamma (IFN-γ), can lead to a rapid activation of the monocyte/macrophage system with an up-regulation of innate immune capabilities leading to colonic inflammation (Sivakumar *et al.*, 2002; Ishikura *et al.*, 2003; Takagi *et al.*, 2003).

TNF-α is an important indicator of colitis as activated macrophages and T cells are abundant in intestinal mucosa in patients with IBD. However, reported data on the expression of TNF-α in colitis is contradictory. A British group found elevated serum TNF-α concentrations and in stools of children with active UC (Murch *et al.*, 1991; Braegger *et al.*, 1992). In contrast, another study failed to detect differences in serum levels between children with and without colitis (Hyams *et al.*, 1991), which was likely due to a lower sensitivity of the assay used for detection of TNF-α.

Colonic tissue homogenates from subjects with colitis have been shown to contain extremely high concentrations of IL-8 (Izzo *et al.*, 1993). The source of IL-8 in the mucosa is still under investigation because of the possible contribution of epithelial cells to its production. Some human studies using *in situ* hybridization to localize the cellular origin of IL-8 in active colitis lesions identified macrophages, neutrophils, and epithelial cells, but other studies failed to find IL-8 mRNA expression in the intestinal epithelium (Mazzucchelli *et al.*, 1994; Grimm *et al.*, 1996). Serum levels of IL-8 are a poor specific marker of IBD clinical activity because elevated IL-8 concentrations are also linked with other types of inflammations such as in cardiovascular disease (Apostolakis *et al.*, 2009). The

dominant role of IL-18 as a pro-inflammatory cytokine involved in T-helper type-1 (Th1)-mediated diseases is supported by its ability to directly stimulate TNF-α gene expression and synthesis from $CD3^+/CD4^+$ and NK (natural killer) cells. Enhanced TNF-α production leads to subsequent production of IL-1β and IL-8 from the $CD14^+$ population in peripheral blood (Puren *et al.*, 1998). Therefore, elevated serum or colon tissue levels of the above cytokines are important indicators of colitis seen in animal models and humans.

Glycogen synthase kinase-3 (GSK-3), a serine–threonine protein kinase involved in glycogen metabolism, plays an important role in the regulation of many cellular functions, including the control of cell division and apoptosis (Ali *et al.*, 2001). It has been proposed that GSK-3 can influence the activity of the NF-κB transcriptional factor (Frame and Cohen, 2001). The effects of the inhibitors of GSK-3, thiadiazolidinones (TDZD)-8 and 3-[(3-Chloro-4-hydroxyphenyl)amino]-4-(2-nitrophenyl)-1H-pyrrol-2,5-dione (SB 415286), that can substantially reduce the systemic inflammation associated with endotoxic shock *in vivo*, have been investigated on the acute colitis provoked by TNBS in rats (Whittle *et al.*, 2006). Administration of the GSK-3 inhibitor TDZD-8 provoked a dose-dependent reduction in colonic inflammation induced by intracolonic TNBS. The results showed reduced colonic inflammation, decreased MPO activity, and lowered TNF-α levels. In conclusion, these findings demonstrate that inhibitors of the activity of GSK-3 reduce colonic inflammation and tissue injury in a rat model of acute colitis. The mechanisms underlying this anti-inflammatory action may be related to down-regulation of NF-κB activity, involved in the generation of pro-inflammatory mediators (Whittle *et al.*, 2006).

Nitric oxide (NO), synthesized from L-arginine by NO synthases, has a double function in the body in normal or pathological conditions (Nathan, 1992). NO exerts pro-apoptotic effects in tumor cells (Drapier and Hibbs, 1986; Drapier *et al.*, 1988), but antiapoptotic effects have been seen in other cell types including cultivated rats hepatocytes (Kim *et al.*, 1997; Saavedra *et al.*, 1997). The same concept of both positive and negative attributes associated with NO has been described in mice with DSS-induced colitis. One study has shown the

exacerbation of DSS-induced colitis following the administration of the NO-donor, NOR3 (Yoshida *et al.*, 2000), whereas subsequent studies showed that NO supplementation ameliorates DSS colitis partly via the down-regulation of endothelial intercellular adhesion molecule-1 (ICAM-1) and P-selectin, and of IL-12 and IFN-γ mRNA expression in colonic tissue (Salas *et al.*, 2002). Differences between the above two studies could be related to differences in the dose of DSS used (4 vs. 8%) and duration of the studies (1 vs. 3 week (wk), which can, in turn, impact disease severity and consequent effects of NO-donor treatment.

2.1.3 Oxidative Stress

Free radicals are unstable chemical entities that contain an unpaired electron in their outer orbital and are in general very reactive (Halliwell, 1991). Free radicals participate in oxidation/reduction reactions with neighbouring compounds in order to regain thermodynamic and electrochemical stability. Oxygen free radicals are continually produced in the mitochondrial electron transport chain as a consequence of the incomplete reduction of molecular oxygen (Turrens and Boveris, 1980). The reactive oxygen species (ROS) that are formed in this process include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$). Normally, most tissues possess sufficient amounts of protective enzymatic (superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase) and nonenzymatic (thiols, ascorbate, α-tocopherol) antioxidants that decompose most of the injurious oxidizing agents that escape into the surrounding environment limiting “bystander” tissue damage. Excess production of ROS may exceed cellular cytoprotective mechanisms and has been shown to be highly toxic to cells (Weiss, 1989). Toxicity occurs by the oxidation of constituent proteins, carbohydrates, lipids, and nucleic acids, thus impairing cellular function and leading to apoptosis (Lih-Brody *et al.*, 1996). A state of oxidative stress exists when there is an imbalance between the relative levels of naturally occurring antioxidant defences and reactive oxygen intermediates (Kehrer, 1993). It has been suggested that ROS plays a role in the pathogenesis of IBD (Otamiri and Sjodahl, 1991).

The inability to efficiently regulate the recruitment and activation of additional phagocytic leukocytes such as polymorphonuclear leukocytes (PMNs), eosinophils, and monocytes will result in a dramatic increase in the production of ROS within the colon via activation of the phagocyte-associated, ROS-producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Pavlick *et al.*, 2002). Immune activation of PMNs and monocytes also results in enhanced formation of the potent oxidant, hypochlorous acid (HOCl) from myeloperoxidase (MPO)-catalyzed oxidation of Cl^- by H_2O_2 (Williams, 1990). HOCl possesses the two oxidizing equivalents of H_2O_2 but is 100–1000 times more toxic than either $O_2^{\bullet -}$ or H_2O_2. HOCl is a chlorinating as well as an oxidizing agent that nonspecifically reacts with sulfhydryls, polyunsaturated fatty acids (PUFAs), deoxyribonucleic acid (DNA), pyridine nucleotides, aliphatic, and aromatic amino acids, and nitrogen-containing compounds in a rapid fashion (Williams, 1990). The uncontrolled overproduction of ROS, as would occur during active episodes of IBD, could easily overwhelm protective mechanisms resulting in oxidative damage to cells and tissues (Conner *et al.*, 1996). Results from human studies have suggested that chronically inflamed intestinal and/or colonic tissue is subjected to significant oxidative stress (Harris *et al.*, 1992; Grisham, 1994). The hydroxyl radical, one of the most reactive and therefore most toxic of reactive oxidants, can be generated by the reductant (e.g., superoxide)-driven Fenton reaction (Freeman and Crapo, 1982):

$$O_2^{\bullet -} + Fe^{+3} \longrightarrow O_2 + Fe^{+2} \quad (1)$$

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + {}^{\bullet}OH + OH^- \quad (2)$$

In the presence of $O_2^{\bullet -}$, ferritin-bound ferric iron (Fe^{3+}) is released as ferrous iron (Fe^{+2}), thereby providing reduced iron for participation in such Fenton reactions (Biemond *et al.*, 1984). The reaction of Fe^{+2} with H_2O_2 resulting in the production of a hydroxyl radical has been shown as an important mediator of colonic tissue injury (Koppenol, 1985). Bleeding occurs during the active phase of UC, which releases both heme and free iron into the GI tract thereby contributing to the pathogenesis and exacerbation of colitis (Simmonds and Rampton, 1993).

2.1.4 Regulatory Mechanisms of Apoptosis in UC

Colonic mucosal alterations shown by reduced crypt size and inflammation have been reported with patients with active UC (Calabuig *et al.*, 2009). These findings suggest that the loss of epithelial cells in active UC occurs mainly by apoptosis in crypts and that the apoptosis stimulating fragment (Fas)/Fas-ligand (L) interaction has shown to be a mediator of the apoptosis (Iwamoto *et al.*, 1996). TNF-α, a cytokine produced by activated macrophages, and activation of the Fas receptor are the major extrinsic mediators of apoptosis. The activation of janus kinases (JAKs) and Fas-associated protein with death domain (FADD) lead to recruitment and activation of caspase-8, which activates caspase-3, leading to a cascade of downstream events such as DNA cleavage and cell membrane changes, finally causing programmed cell death. Caspase-3 can also be activated by a complex of proteins, including caspase-9, apoptotic protease activating factor (APAF)-1, and cytochrome C. The leakage of cytochrome C from the mitochondria is regulated by the B-cell lymphoma (Bcl)-2 family of proteins. An apoptosis defect in mucosal T cells in UC has been attributed to an imbalance of the antiapoptotic mitochondrial protein Bcl-2 and the proapoptotic mitochondrial protein Bcl-2–associated X protein (Bax) (Ina *et al.*, 1999; Peppelenbosch and van Deventer, 2004).

Akt (alpha serine/threonine-protein kinase) plays a critical role in controlling the balance of survival and apoptosis (Franke *et al.*, 1995; Franke *et al.*, 1997). Akt promotes cell survival, inhibits apoptosis by phosphorylating and, inactivating the Bcl-2-associated death promoter (Bad), activating Bcl-2 (Datta *et al.*, 1997), regulating transcriptional factors (Brunet *et al.*, 1999), and caspase-9 (Cardone *et al.*, 1998). Akt activation also protects cells against apoptosis-inducing agents such as FasL (Gibson *et al.*, 1999). Substance P (SP), a member of the tachykinin family, is broadly distributed in the GI tract and studies have indicated a major pro-inflammatory role of SP and the neurokinin-1 receptor (NK-1R) in intestinal colonocytes of different inflammation etiologies (Pothoulakis *et al.*, 1994; Holzer and HolzerPetsche, 1997; Mantyh, 2002). An *in vitro* and *in vivo* study demonstrated the antiapoptotic role of Akt in a colitis model (Koon *et*

al., 2007). Human nontransformed colonocytes stably transfected with NK-1R (NCM460-NK-1R cells) were exposed to SP. The results showed that SP exposure of NCM460-NK-1R colonocytes stimulated phosphorylation of the antiapoptotic molecule Akt and inhibited tamoxifen-induced cell death and apoptosis evaluated by the cell proliferation assay kit. The authors conducted another trial with C57BL/6 mice which were administered 5% DSS dissolved in tap water (H_2O), and treated with daily intraperitoneal **(**i.p.) injection CJ-12,255, a drug known to interfere with the binding of SP with the NK-1R. They showed that CJ-12,255 blocked SP action, promoted Akt activation, decreased colitis severity, and reduced apoptosis. Thus, SP, through NK-1R, possessed antiapoptotic effects in the colonic mucosa by activating Akt, which prevented apoptosis and mediated tissue recovery in different models of colitis (Koon *et al.*, 2007).

2.1.5 Protein Metabolism in IBD

Major loss of body protein mass is commonly found in IBD, which contributes to weight loss (Powelltuck, 1986). Weight loss during IBD occurs especially in subjects with compromised food intake, which is due principally to the combined effects of diminished intake and excessive intestinal losses of nitrogen (Klein *et al.*, 1988).

Nitrogen metabolism is influenced not only by protein nutritional status and net nitrogen intake but also by disease activity (Gassull and Cabre, 2001). Hypoalbuminemia, which is a common feature associated with IBD, can be attributed to decreased synthesis (Mckinley and Reeve, 1969; Rothschild *et al.*, 1972), increased catabolism and maldistribution of albumin between intravascular and extravascular spaces (Steinfeld *et al.*, 1960; Rothschild *et al.*, 1972).

A potential nutritional consequence of UC in children is growth failure, which affects up to 10% of children (Ballinger *et al.*, 2001). In UC, the pathogenesis of growth impairment is multifactorial and includes chronic undernutrition, corticosteroid administration, and the effects of circulating pro-inflammatory cytokines affecting bone metabolism (Mamula *et al.*, 2002). Thus, nutritional intervention in UC could have a valuable role in a multidisciplinary

approach to pediatric IBD management. Nutrition can play an important role in the prevention and treatment of malnutrition, the promotion of growth and development in children, optimization of bone health and prevention of osteoporosis (O'Sullivan and O'Morain, 2006; Ruemmele *et al.*, 2006). In addition, adequate intake of a balanced source of amino nitrogen given orally or enterally combined with a nutritional approach to reduce disease activity could be crucial for the prevention of total body protein depletion in the pediatric UC population (Mallon and Suskind, 2010).

Animal model studies of protein intervention in UC are scarce. However, a study assessed protein synthesis in a macronutrient-restricted piglet model of dextran sulfate sodium (DSS)-induced colitis (Mackenzie *et al.*, 2003). The results showed that DSS treatment was associated with increased synthesis of plasma proteins, compromised weight gain, and muscle protein synthesis, but only when macronutrient intake was inadequate. These results emphasize the importance of adequate nutrition to protect against growth failure and muscle loss in acute colitis (Mackenzie *et al.*, 2003). Another study utilizing the same macronutrient-restricted piglet model of DSS-induced colitis demonstrated that probiotics did not provide additional protective effects relative to adequate nutrition in terms of increased protein synthesis in the colon and decreased colitis severity although probiotics stimulated liver and plasma protein fractional synthesis rate (FSR) (Harding *et al.*, 2010).

2.1.6 Animal Models of IBD

Experimental animal models of IBD have proven to be important tools for detecting potential therapeutic agents and for investigating the mechanisms of pathogenesis (Wirtz and Neurath, 2000; Geier *et al.*, 2007). In general, an appropriate or an optimal animal model should display certain key characteristics: the gut should exhibit pathophysiology, morphological alterations, inflammation, clinical symptoms and signs that are similar or identical to the human IBD (Blumberg *et al.*, 2000; Jurjus *et al.*, 2004).

Animal models of IBD are categorized into five broad classes: gene knockout (KO) models, transgenic mouse and rat models, spontaneous colitis models, adoptive transfer models, and inducible colitis models. Firstly, colitis can be induced in genetically KO models such the IL-2 KO and IL-10 KO. Mice with the disrupted *IL-2* gene developed chronic colitis between 6 and 15 wks after birth. The small intestine of this model was intact, whereas the colon was severely affected with ulcers and wall-thickening. Pathologically, crypt abscesses, mucin depletion, and dysplasia of the epithelial cells, which are the features of human IBD, were observed (Sadlack *et al.*, 1993). IL-2 is an indispensable regulatory cytokine of the immune system that has multiple functions, including the activation of T cells. Colitis in the *IL-2* KO mice is considered to be due to the lack of activation-induced cell death (AICD). Prolonged inflammation in *IL-2* KO mice is presumed to be due to the impairment of AICD, which leads to incomplete activation of T cells due to a deficit of regulatory T cells (Kneitz *et al.*, 1995). The deficit of regulatory T cells induces a Th1 response, which produces the Th1 cytokines such as IFN-γ and TNFα that are known to contribute to colitis (Hibi *et al.*, 2002).

An example of a transgenic colitis model is the IL-7 transgenic mouse, which over-expresses IL-7 mRNA that leads to acute colitis occurring at 1 to 3 wks of age along with an infiltration of neutrophils and $CD4^+$ T cells in the large intestine (Watanabe *et al.*, 1997). The epithelial cell-derived IL-7 was shown to be an essential cytokine for the proliferation and functional regulation mechanism of epithelial cells, intraepithelial lymphocytes, and intramucosal lymphocytes. It has been demonstrated that IL-7 was the substance within the serum of UC patients that influenced the differentiation and proliferation of T cells in the thymus (Hibi *et al.*, 2002). Moreover, it has been suggested that in the acute phase, the excessive secretion of IL-7 induces activation of mucosal lymphocytes, which causes colitis (Watanabe *et al.*, 2003). A model that is known to develop colitis spontaneously is the C3H/HejBir mice, which develop colitis that is limited to ileocecal lesions and the right side of the colon. The colitis occurs spontaneously in the third to fourth wk of life and disappears after 10–12 wks. Ulcers, crypt

abscesses, and regeneration of epithelium are seen, but thickening of the intestinal wall and granulomas are not observed. Increased levels of IFN-γ and IL-2 have been detected in the lamina propria, which suggests a Th1 response in this model of colitis.

Colitis can be also induced experimentally by manipulation of the immune system. CD45 is a general marker of lymphocytes, and its isotype CD45RB is recognized as a marker to distinguish the naive T cell ($CD45RB^{high}$) from the memory T cell ($CD45RB^{low}$) (Birkeland *et al.*, 1992). $CD45RB^{high}$ is involved in the Th1 response whereas $CD45RB^{low}$ is involved in the T-helper type-2 (Th2) response which produces Th2 cytokines such as IL-4 (Lee *et al.*, 1990). Rats or mouse models that only express the $CD45RB^{high}$ T cells have impaired T-cell regulation leading to colitis (Powrie *et al.*, 1993).

2.1.6.1 Inducible Colitis Models

Colitis models are also used whereby the colitis is provoked via exposure to toxic dietary substances or pharmacologic agents. Epithelial and mucosal necrosis and transient inflammation can be induced by luminal instillation of dilute acetic acid (Elson *et al.*, 1995). Advantages of acetic acid-induced injury are its low cost, ease of administration, and widespread availability. However, the nonspecific nature of the initial mucosal injury that precedes the inflammatory response and the minimal and transient damage to the colon makes this model of questionable relevance to IBD (Sharon and Stenson, 1985). Phorbol esters (PMA) are tumor-promoting agents derived from the croton plant that have been used to induce experimental colitis. A single intrarectal instillation of phorbol-12-myristate-13-acetate in an ethanol vehicle into male rats or male rabbits causes colitis within 24 h, with an increased levels of the pro-inflammatory marker MPO in colonic tissue (Fretland *et al.*, 1990). The mechanism of PMA-induced colitis is unclear. However, the addition of ethanol in the PMA mixture at concentrations above 30% is used as a mucosal barrier-breaker allowing PMA to penetrate the gut epithelium to cause widespread acute mucosal damage in the distal colon (Wallace *et al.*, 1985).

Colitis can also be induced by the administration of an enema containing the contact sensitizing allergen trinitrobenzene sulfonic acid (TNBS) in ethanol. In this latter approach ethanol is also used to break the mucosal barrier and allow penetration of TNBS into the bowel wall (Morris *et al.*, 1989). The initial immunologic evaluation after TNBS treatment suggests that TNBS-induced colitis is an imbalanced cytokine response with an over-responsiveness of Th2 cells to release Th2 cytokines (Dieleman *et al.*, 1997; Seibold *et al.*, 2000). The model is simple and inexpensive and is reasonably reproducible. However, lesions can be fairly focal, which can make some immunologic studies difficult, and the chronic lesions are more submucosal than mucosal (Elson *et al.*, 1995).

Carrageenans (poligeenan) are a family of linear sulfated polysaccharides that are extracted from red seaweeds (Weiner, 1991). Carrageenans injure colonic epithelial cells in guinea pigs and destroy intraepithelial tight junctions, leading to enhanced mucosal permeability and the infiltration of anaerobic bacteria causing inflammation (Ling *et al.*, 1988). The advantages of this model are the lengthy period of induction and a well-documented role of luminal bacteria in the pathogenesis of IBD (Kim and Berstad, 1992). An important disadvantage of the carrageenan model is the predilection for the histopathological involvement of the cecum, which makes the relevance to ulcerative colitis uncertain (Hibi *et al.*, 2002).

In addition, colitis is typically induced in animal models by administrating sulfated polysaccharides such as DSS. Continuous administration of 5% DSS in drinking H_2O induces colitis in mice, rats, and hamsters (Okayasu *et al.*, 1990; Cooper *et al.*, 1993). The acute form of DSS-induced colitis is manifested by hematochezia, body weight loss, shortening of the intestine, mucosal ulcers, infiltration of macrophages and neutrophils, obliteration of the crypt lumina, disruption of the epithelial layer, and changes in intestinal microflora (Iwanaga *et al.*, 1994; Hudcovic *et al.*, 2001; Ohtsuka and Sanderson, 2003). Early lesions occur mainly in the ascending colon and over lymphoid aggregates. DSS is found within mucosal macrophages, which contain enlarged lysosomes (Okayasu *et al.*, 1990). Luminal concentrations of *Bacteroides* species, especially *B. distasonis*,

are increased in acute and chronic phases of inflammation (Yamada *et al.*, 1992). However, the role of luminal bacteria in the pathogenesis of these lesions is still unclear at this time.

The DSS model induces reproducible mucosal colonic inflammation in mice by providing an easily administered oral agent that can be given in a cyclic manner to produce chronic lesions. More recently, a piglet model of DSS-induced colitis has been developed (Mackenzie *et al.*, 2003). The piglet has emerged as a relevant model species to study pediatric human nutrition and GI physiology due to the similarity of its gut to the human gut. In terms of protein nutrition, the piglet is similar to humans in terms of amino acid and protein metabolism (Ball *et al.*, 1996). In addition, piglets are considered a pediatric model due to their rapid growth and they also respond rapidly to dietary inadequacies because of their high protein turnover (Wykes *et al.*, 1993).

2.1.7 Pharmacological and Nutritional Anti-Inflammatory Treatments for IBD

Intracolonically administered NF-κB (p65) antisense oligonucleotide was tested in DSS-induced colitic mice in an attempt to alleviate inflammation seen in this animal model of UC. The results showed the effectiveness of NF-κB (p65) antisense oligonucleotides on rectal inflammation when administered on d-0 or -2. However, no effect was observed when antisense oligonucleotides were administered on d-7. These results suggested that NF-κB antisense oligonucleotides have inhibitory effects on DSS-induced colitis when administered in the early phase of inflammation. Therefore, the administration of NF-κB antisense oligonucleotides effectively down-regulated the production of pro-inflammatory cytokines in the colonic mucosa (Murano *et al.*, 2000). Moreover, another study addressed the implication of ROS and reactive oxygen nitrogen species (RONS) in the pathogenesis of IBD using the DSS model of colitis. This study used gene-targeted animals to assess the contributions of three key RONS-related enzymes (nitric oxide synthase (iNOS), SOD, and NADPH oxidase) to gut inflammation and tissue injury in DSS-induced colitic mice. Their findings indicated that either the genetic absence or pharmacologic blockade of

iNOS significantly attenuated the severity of colonic inflammation (Krieglstein *et al.*, 2001). On the other hand, mice that genetically over-express CuZn-SOD exhibited exaggerated inflammatory and tissue injury responses to DSS treatment. The absence of functional NADPH oxidase, resulting from targeted disruption of p47phox, did not alter susceptibility to DSS-induced intestinal inflammation, provided that iNOS remained functional. Using the specific iNOS inhibitor 1400W, it was demonstrated that the combined blockade of iNOS and NADPH oxidase (by genetic deletion of p47phox) was even more effective in protecting mice from DSS colitis than either intervention alone. These results suggested that both iNOS and CuZn-SOD play an important role in the progression of DSS-induced colitis (Krieglstein *et al.*, 2001).

Apart from the pharmacological anti-inflammatory treatment for IBD, nutritional anti-inflammatory and antioxidant supplements have been used as an adjunctive therapy in IBD patients (Geerling *et al.*, 1999; Goh and O'Morain, 2003). Fish oil-derived n-3 polyunsaturated fatty acids inhibit leukotriene B_4 (LTB4), a potent chemo-attractant and pro-inflammatory eicosanoid implicated in the pathogenesis of IBD (Ruggiero *et al.*, 2009). Fish oil was apparently beneficial in patients with UC, but results were controversial (Lorenz *et al.*, 1989; Mccall *et al.*, 1989; Aslan and Triadafilopoulos, 1992; Stenson *et al.*, 1992). Three studies reported clinical improvement after fish oil supplementation, but only Stenson *et al.* (1992) found a significant decrease in LTB4 levels measured in rectal tissue. Differences between the studies could be related to dose, study design, n-3 fatty acid composition of the supplement, and type of assessment used to measure clinical improvement. The intestinal bacterial flora contributes to digestion of nutrients and also plays a role in promoting the development of a barrier against pathogens. Due to the evidence implicating the intestinal bacterial flora in IBD (Guarner, 2005), animal and human trials have been performed to modify the intestinal flora with probiotics as a potential treatment approach for IBD (Jonkers and Stockbrugger, 2003). For example, in IL-10 deficient mice, *Lactobacillus plantarum* 299v prevented onset of disease and reduced established colitis (Schultz *et al.*, 2002). In a clinical trial using subjects with

active UC, 6 of 9 patients given viable *L. plantarum* 299v reached remission, compared with none of 10 patients treated with inactivated bacteria (Niedzielin *et al.*, 2001). Mechanisms of probiotic action are multiple and include bacterial competition, mucosal conditioning, and immune modulation (Shanahan, 2001b). The production of short-chain fatty acid (SCFA) butyrate by probiotic bacteria has been proposed to exert an important nutritional anti-inflammatory effect on IBD. Butyrate is readily absorbed by the intestinal mucosa and is an important substrate for metabolism by colonocytes (Chapman, 2001). A direct anti-inflammatory role for butyrate may be attributable to its inhibition of the transmigration of NF-κB into the nucleus and thereby the subsequent binding of NF-κB to DNA, thus preventing the transcription of pro-inflammatory mediators (Segain *et al.*, 2000; Tedelind *et al.*, 2007). In addition, butyrate inhibits lymphocyte activation and proliferation and MPO activity in neutrophils, which directly causes tissue destruction (Liu *et al.*, 2001).

Another potential anti-inflammatory nutritional supplement to treat UC is glutamine, which is an essential nutrient for the rapidly proliferating cells in the intestinal mucosa. Several studies have demonstrated that glutamine supplementation improves the structural integrity, function and repair of the intestine during catabolic conditions associated with GI disease (Vanderhulst *et al.*, 1993; LeLeiko and Walsh, 1996). However, no study to date has examined the potential of oral glutamine as a therapeutic agent in IBD. One study examined the use of topical glutamine supplementation in patients with pouchitis (inflammation of the ileal pouch). After 21 d of glutamine supplementation, 6 of 10 patients showed significant benefit from the treatment (Wischmeyer *et al.*, 1993).

2.1.8 Staple Isotopes as a Measure of Protein Synthesis

The investigation of protein nutrition and metabolism is fundamental for the understanding of nutritional physiology and metabolic regulation in numerous diseases due to the many important roles of protein, including muscle structure, receptors, transport of molecules, and immune function (El-Khoury, 1999). The two most common methods to measure protein synthesis include (1) the constant

infusion method and (2) the flooding dose method. Both methods are frequently used in animal models to study the role of protein metabolism in several different diseases and disorders, including colitis (Mackenzie *et al.*, 2003; Bradley, 2010). The above methods are used to assess plasma and tissue protein synthesis using isotopic tracers to measure the rate of incorporation of a labelled amino acid into plasma or tissue protein over time, with adjustments for the level of labelling of the precursor pool at the site of protein synthesis (Davis *et al.*, 1999).

In the constant infusion method, a labelled tracer amino acid is infused into the animal at a constant rate in order to rapidly achieve steady-state labelling in the free amino acid pool. Protein synthesis can be determined with a single tissue sample, because the kinetics of free pool labelling can be predicted from knowledge of the relative amounts of the tracee amino acid in the protein-bound and free pools (Reeds and Davis, 1999). Since the length of the labelling period is prolonged, typically 4 to 8 h, this method is well suited for assessment of slow turnover proteins in peripheral tissues, such as skeletal muscles. However, the recycling of tracer can be a problem for the measurement of the synthesis rates of rapid turnover proteins in visceral tissues, such as the liver (Davis and Reeds, 2001). The constant infusion method assumes that there is both an isotopic and metabolic steady state during the tracer infusion. The initial rate of incorporation of label, when the precursor pool enrichment is still rising to plateau, is slower than the final rate of incorporation that is achieved, when the precursor pool has reached isotopic steady state. Therefore, to achieve the appropriate level of labelling in the free amino acid pool more rapidly, a priming dose of the isotopically labelled tracer amino acid is administered at the beginning of the infusion (Davis *et al.*, 1999).

The ideal amino acid for use as tracer in plasma and tissue protein synthesis studies is an essential amino acid which is not limiting in the diet and not extensively metabolized to other compounds (Davis and Reeds, 2001). Phenylalanine is highly soluble, so a higher multiple of the phenylalanine flux can be infused. In addition, phenylalanine is not metabolized or synthesized by

skeletal muscle, simplifying the determination of protein synthesis rates (Matthews, 2007).

2.2 WHEY PROTEINS

Whey proteins (or milk serum proteins) comprise approximately 20% of total milk proteins (Krissansen, 2007). Whey proteins are defined as proteins in milk that remain soluble after acid or rennet casein precipitation (Barth and Behnke, 1997). Acid precipitation yields acid whey, while rennet casein precipitation yields sweet or rennet whey (de la Fuente *et al.*, 2002). Whey proteins are globular proteins that are soluble over a broad pH range (Smithers, 2008).

The amino acid composition is the most important factor in defining food protein quality, followed by the digestibility of the protein and the bioavailability of its amino acids. Due to their amino acid composition, the major bovine milk proteins, i.e., caseins and whey proteins are regarded as complete protein sources of essential amino acids (EAA). However, whey proteins are considered superior to other types of proteins such as casein due to their better digestibility, absorption and closer amino acid profile to human requirements (Boirie *et al.*, 1997; Fruhbeck, 1998; Steijns and Schaafsma, 2009). In addition to study of their nutritive value, milk proteins are extensively being examined as the primary food sources for a variety of biologically-active peptides used in clinical applications such as hypertension, hypercholesterolemia, cancer, and IBD (Sindayikengera and Xia, 2006). The major whey proteins are β-lactoglobulin (β-lb), α-lactalbumin (α-la), serum albumin, immunoglobulins and glycomacropeptide (GMP), while minor proteins include lactoperoxidase, lactoferrin (Lf), β-microglobulin, lysozyme, insulin-like growth factor (IGF), γ-globulins and several other small proteins (Korhonen *et al.*, 1998). A large number of human and animal feeding trials have indicated that whey proteins exert putative health benefits that include anti-inflammatory, immune-enhancement, antioxidative, and anabolic effects (Huppertz *et al.*, 2002).

2.2.1 The Antioxidant and Anti-Inflammatory Effects of Whey Proteins

Several studies have demonstrated that whey protein intake can exert significant protective effects against oxidative stress imbalances associated with a variety of chronic and acute disease conditions including cancer, heart disease, diabetes, and IBD (Chmiel, 1998; Nicodemo *et al.*, 2000; Smithers, 2008). Differences in EAA composition between whole milk proteins with high biological value can greatly affect their biological and nutritional impact. For example, although both casein and whey are proteins with high biological value, several studies have indicated that the comparatively high concentration of cysteine in whey protein stimulates the synthesis of GSH, an intracellular cysteine-containing peptide. This is not true for casein, which does not stimulate GSH (Grey *et al.*, 2003; Tseng *et al.*, 2006). In that regard, Lands and colleagues (1999) have shown that 3-mo. whey protein isolate (20 g) supplementation significantly enhanced intracellular lymphocyte GSH levels in young adults relative to the casein-supplemented controls. There is indication that benefits might accrue from diet-induced stimulation of GSH synthesis during inflammatory conditions. Grimble *et al.* (1992) have suggested that sulphur amino acids, especially, cysteine, play a key role in the amino acid economy of the body under inflammatory conditions. The main metabolic fates of cysteine are in synthesis of GSH and specific acute phase proteins such as albumin and haptoglobin (Malmezat *et al.*, 1998). Several studies have shown that sulphur amino acid requirements are increased in stress situations (Voisin *et al.*, 1996; Vary *et al.*, 1997) and that cysteine supplementation of the diet of septic rats had beneficial effects on recovery of protein status (Vary *et al.*, 1997). Other authors suggested that these latter beneficial effects are associated with an increase in GSH synthesis, since GSH turnover may account for more than 50% of cysteine flux in healthy men (Fukagawa *et al.*, 1996). Another recent study has demonstrated that piglets with DSS-induced colitis supplemented with L-cysteine had markedly improved colon histology including lower inflammation, decreased crypt damage, and increased intestinal regeneration (Kim *et al.*, 2009).

The anti-inflammatory effects of GMP were investigated in a rat model of TNBS-induced colitis. GMP was administered orally to female Wistar rats 3 h post colitis induction. The results showed a decrease in body weight loss, anorexia, colonic damage, colonic alkaline phosphatase activity and IL-1β compared with control rats (Daddaoua *et al.*, 2005). Another study investigated the effect of whey protein in a rat model of DSS-induced colitis. Rats were fed diets containing casein (control), whey protein, or casein plus threonine/cysteine (positive control) for 14 d prior to DSS consumption for 7 d. The authors demonstrated that whey protein and positive control diets decreased colonic expression of IL-1β, calprotectin and iNOS, decreased diarrhea, and increased mucin secretion (Sprong *et al.*, 2010). The anti-oxidative and anti-inflammatory effects of Enprocal, a protein supplement containing a 41% content of whey protein concentrate, were investigated on gut cell proliferation. Caco-2 cells were treated with digested and undigested Enprocal. The results demonstrated a down regulation of TNF-α and IL-8 and upregulation of IL-2 and IL-10 secretion in Caco-2 cells fed digested Enprocal (Kanwar and Kanwar, 2009). These results demonstrated that digested whey protein products exert more potent bioactive properties than whole whey proteins, and promote a more physiological context in the cell culture system. The human colon cancer-derived Caco-2 intestinal epithelial cell system has been widely used as an *in vitro* model of the intestinal epithelium (Quaroni *et al.*, 1979). Caco-2 cell cultures have been used to model IBD. Caco-2 cells exposed to inflammatory mediators such as IL-1β, TNF-α, IFN-γ and lipopolysaccharide (LPS) regulate gut maintenance and defence by secreting and responding to inflammatory mediators and by modulating the intestinal epithelial permeability (Van De Walle *et al.*, 2010). CXCL16 mediates adhesion and phagocytosis of both gram-negative and gram-positive bacteria and is a strong chemoattractant for CXCR6+ T cells. The CXCL16 mRNA and protein expression is up-regulated in intestinal inflammation *in vitro*, suggesting an important role for this chemokine in intestinal inflammation (Diegelmann *et al.*, 2010).

β-lb is currently thought to be an important source of biologically active peptides that are inactive within the sequence of the precursor protein, but can be released by *in vivo* or *in vitro* enzymatic proteolysis. Once released and absorbed, these peptides may play important roles in human health, promoting antihypertensive, antioxidant, and antimicrobial activities (Hernandez-Ledesma *et al.*, 2008). Hernandez-Ledesma *et al.* (2005) investigated the *in vitro* antioxidant activity of hydrolysates of β-lb produced by commercial proteases (pepsin, trypsin, chymotrypsin, thermolysin and corolase). The results demonstrated that a combination of pepsin, trypsin, and chymotrypsin was the most appropriate to produce β-lb hydrolysates having high oxygen radical scavenging activity, measured by oxygen radical absorbance capacity (ORAC) (Hernandez-Ledesma *et al.*, 2005). In addition, Lf binds to cationic metals such as Fe^{+2}, Fe^{+3}, Cu^{+2}, Zn^{+2}, Mn^{+2}, and so could play a role in both stable iron delivery and scavenging of free iron and other minerals that would otherwise catalyze oxidative reactions (Conneely, 2001). Moreover, studies carried out in mice have shown that administration of the whey protein Lf can reduce gastritis and protect gut mucosal integrity during LPS-induced endotoxemia (Fuss and Strober, 1998; Kruzel *et al.*, 1998; 2000).

2.2.2 Anabolic Effects of Whey Proteins

Whey proteins have a relatively high content of leucine in comparison to other high biological value proteins (Table 2.1), which could play a role in the putative anabolic effects shown with whey protein supplementation in human (Lands et al., 1999) and animal studies (Morifuji *et al.*, 2005). Leucine and metabolites of leucine such as α-ketoisocaproate (KIC) have been reported to inhibit protein degradation particularly during periods of increased proteolysis (Nair *et al.*, 1992; Nissen *et al.*, 1996). It has been suggested that the anticatabolic effects of leucine and α-ketoisocaproate are regulated by the leucine metabolite β-hydroxy β-methylbutyrate (β-HMB). Animal studies indicated that β-HMB is synthesized from α-ketoisocaproate almost entirely as a byproduct of leucine metabolism and that approximately 5% of oxidized leucine is converted to β-HMB (Nissen *et al.*,

1994; Vankoevering *et al.*, 1994). Based on these findings, it has been hypothesized that supplementing the diet with leucine and/or β-HMB may inhibit protein degradation during periods of increased proteolysis such as resistance training (Vankoevering *et al.*, 1994). In support of this contention, intravenous leucine infusion has been reported to decrease protein degradation in humans suggesting that leucine may serve as a regulator of protein metabolism (Nair *et al.*, 1992). Moreover, Nissen and colleagues (1996) reported significantly greater gains in fat-free mass and strength in untrained men and women initiating resistance training when administered calcium β-HMB at 1.5 or 3 g/d for 3 to 4 wks. The gains in fat-free mass were approximately 0.4 to 0.7 kg greater than in the placebo groups. A recent study with horses demonstrated that leucine but not whey protein increased insulin levels in response to a glucose load. Thus, leucine supplementation needs further investigation to increase the muscle glycogen synthesis in horses (Urschel *et al.*, 2010).

An animal trial (with in aged rats) has shown that dietary leucine supplementation blunts defects in postprandial muscle protein metabolism (Rieu *et al.*, 2003). Young (9 mo.) and old (21 mo.) adult rats were fed a baseline semi-liquid 18.2 g/100 g standard protein diet for 1 mo. Muscle protein synthesis was assessed *in vivo* 90–120 min using the flooding dose method (^{13}C phenylalanine). After a 10-d feeding period, leucine vs. alanine supplemented meal feeding for 1 h resulted in significantly increased FSR and absolute synthesis rate (ASR) in the old rats, when assessed in either the fasted or the postprandial state. The leucine meal restored FSR and ASR in the old rats but did not further stimulate muscle protein synthetic rates in the young adult rats. The long-term utilization of diets high in leucine-rich proteins may therefore limit muscle protein wasting in protein catabolic states such as aging (Rieu *et al.*, 2003).

In a clinical trial, healthy subjects received whey protein isolate (20 g) supplementation for 3-mo in addition to their regular diets, which were not controlled. This prolonged whey protein isolate supplementation was associated with significantly increased muscle mass, significantly diminished fat mass, and

improved volitional exercise performance, in contrast to negligible effects with the casein control (Lands *et al.*, 1999). Similarly, Morifuji *et al.* (2005) demonstrated that whey protein feeding during prolonged exercise in rats stimulated skeletal muscle to utilize fat as an energy substrate and suppressed hepatic fatty acid synthesis, thereby decreasing accumulation of body fat (Morifuji *et al.*, 2005).

2.2.3 Pressurized Whey Proteins: A Novel Concept

High pressure processing is one of the most successful techniques to non-thermally sterilize foods. Although the primary structure of proteins remains intact during pressure treatment (Mozhaev *et al.*, 1994), high-pressure treatment above 200 MPa can cause changes in the secondary and tertiary structure that can lead to irreversible denaturation of the protein (Hendrickx *et al.*, 1998). The pressurized form of whey protein has been investigated as a food ingredient. In the case of milk proteins, high-pressure treatment increases pH, reduces turbidity, changes appearance and can reduce the rennet coagulating time of milk and increase cheese yield, leading to potential applications in the cheese-making industry (Alting *et al.*, 1997).

A series of animal studies from several different laboratories have recently shown that the bioactivity of whey proteins is enhanced by pressure processing (Kubow *et al.*, 2005). These studies showed that whey protein isolates treated by pressure processing caused changes in the secondary and tertiary structures of whey proteins. The changes in hyperbaric-induced whey protein structure are associated with the release of unique bioactive peptides as well as improved growth, protein digestibility, and antioxidative effects (Kubow *et al.*, 2005; Vilela *et al.*, 2006). For example, Vilela *et al.* (2006) demonstrated that native whey protein isolate was much more resistant to pepsin hydrolysis compared with pressure-treated whey protein isolate. Furthermore, the pressure-treated whey isolate showed a more rapid release of absorbable low molecular weight peptides. Since β-lb is the major protein in whey protein isolate, the high resistance of β-lb to pepsin-mediated hydrolysis is likely a major reason for the relatively lesser digestibility of native whey proteins compared with the pressure-

treated whey proteins. Numerous studies have demonstrated that there is a low accessibility to peptide bonds localized to the interior of the globular structure of β-lb to hydrolytic action of pepsin (Mutilangi *et al.*, 1995; Kinekawa and Kitabatake, 1996; Kitabatake and Kinekawa, 1998; Pantako *et al.*, 2001; Qiao *et al.*, 2004). Recent electrospray ionization mass spectrometry studies have indicated that single cycle pressure treatment at 550 MPa caused a partial unfolding of β-lb, as exhibited by a higher proportion of charges in the charge-state-distribution of pressurized β-lb relative to the native forms (Alvarez *et al.*, 2007). As only the unfolded molecules are susceptible to degradation by proteolytic enzymes (Stauffer, 1989), it is clear that the increased rate of hydrolysis induced by pressure processing is due to the partial unfolding of β-lb caused by pressure treatment. The unfolding of the protein molecule exposes the hydrophobic amino acids buried in the interior to the proteolytic enzymic action.

Zavorsky *et al.* (2007) showed that healthy subjects with short-term intake of pressurized whey protein isolates by showed rapid increases in lymphocyte GSH status within a 2-wk period. The subjects received 45 g of pressurized whey protein supplements in addition to their normal diet but the increase in lymphocyte GSH levels were comparable to those previously observed over a longer 3-mo period of native whey protein isolate supplementation (Zavorsky *et al.*, 2007). Additionally, animals in several studies have shown improved growth when given pressurized versus native whey protein isolates as the dietary protein source (Kubow *et al.*, 2005). It is noteworthy that growth stimulation and other positive functional outcomes were observed in mice and rats fed pressurized whey protein above their normal protein requirements and that these improvements were not observed in animals fed either native whey protein or casein at the same levels of intake. These latter studies suggest the possibility that prior supplementation of pressurized versus native whey protein might be advantageous as an approach to attenuate the impact of short-term protein deprivation on whole body protein status. To assess the impact of feed pressurization on body protein status, a 2-wk feeding trial is likely needed as demonstrated by the above-mentioned Zaworksy *et al.* (2007) study, which

showed differences in lymphocyte GSH status within that period when daily whey protein supplementation occurred. In an animal trial comparing the impact of pressurization on the protective effects of whey, female C57BL/6 mice were fed 20% (v/v) of either native whey or pressurized whey as a protein source for 4-wk, followed by exposure to *Pseudomonas aeruginosa* which is a common infective agent in individuals with cystic fibrosis (CF). Both weight loss and lung bacterial burden were decreased in the mice receiving the pressurized whey. The data clearly suggested that supplementation with pressurized whey was protective; it preserved weight and enhance bacterial clearance following lung infection (Kishta *et al.*, 2009).

A recent open label clinical trial investigated the impact of 1-mo of supplementation with 30 g/d of pressurized whey in 27 CF adults and children. Anthropometric measures, pulmonary function, serum C-reactive protein (CRP), and whole blood glutathione tests were performed. Enhanced nutritional status, as assessed by body mass index, was observed in both adults and children and improved lung function was seen in children receiving supplementation. In addition, the majority of patients with an initially elevated CRP showed a significant decrease when pressurized whey was the supplement, although whole blood glutathione levels were not changed. So, oral supplementation with pressurized whey improved nutritional status and may also have additional beneficial anti-inflammatory effects in patients with CF (Lands *et al.*, 2010). A randomized, double-blind placebo-controlled study investigated the effects of pressurized WPI supplementation (20 g/d) alone or in combination with an exercise training program in patients with chronic obstructive pulmonary disease (COPD) (Laviolette *et al.*, 2010). Pressurized whey supplementation resulted in a significant increase in cycling endurance test time, as well significant improvements in self-reported levels of fatigue while supplementation with the casein placebo did not (Laviolette *et al.*, 2010). In another recent human trial, colorectal surgical cancer patients consumed an oral nutrition drink before surgery, with either glucose alone or pressurized whey protein plus glucose. A constant infusion of D-[6,6-$^{2}H_{2}$]glucose and L-[1-^{13}C]leucine were conducted to

determine glucose rate of appearance and whole body protein turnover in the fasted and fed states. The leucine balance increased in postoperative patients receiving the pressurized whey protein plus glucose drink. Although leucine oxidation doubled, its appearance from protein breakdown decreased and as such was the major determinant of positive balance. The authors concluded that the oral drink based on pressurized whey protein and glucose reduced whole body protein breakdown so a positive protein balance occurred (Ball *et al.*, 2011).

2.3 POTATOES AS A SOURCE OF PHYTOCHEMICALS

Potato (*Solanum tuberosum L.*) is the most important vegetable crop and dietary staple in Canada where the production in 2005 was 4.4 million tons, grown on 165,000 hectares (ha) (FAO, 2006). In addition to being a good dietary source for vitamin C, potassium, and phosphorus, potatoes are an undervalued source of several phytochemicals that may be involved in the protection and prevention of chronic diseases (Robert *et al.*, 2008). In that regard, as potatoes contain significant amounts of several polyphenolic compounds, potato could gain status as a functional food that provides health benefits beyond meeting the needs of basic nutrition (Katan and De Roos, 2004; Leo *et al.*, 2008).

2.3.1 Metabolism of Polyphenols in the Gut

Polyphenols, once they reach the colon, are extensively metabolised by the microflora. In humans, two thirds of the ingested chlorogenic acid (CGA) reaches the colon, where the colonic microflora first hydrolyzes CGA into caffeic acid (CA) and quinic acid (Plumb *et al.*, 1999). Subsequently, the CA is dehydroxylated by bacteria in the colon and after being absorbed, the dehydroxylated CA is β-oxidized to a large extent into benzoic acid (Olthof *et al.*, 2001; Olthof *et al.*, 2003). A recent human study has identified bacteria belonging to the genera *Bidobacterium* and *Lactobacillus* to be involved in the release of bioactive hydroxycinnamic acids, such as CGA and CA in the human colon (Couteau *et al.*, 2001). The CA that is absorbed intact is methylated into ferulic acid (FA) in the liver (Masri *et al.*, 1964) where it is further metabolized into vanillic acid (Teuchy

et al., 1971). FA and CA are also transformed by the microflora into 3-hydroxyphenylpropionic acid and 3-hydroxyhippuric acid, respectively (Booth *et al.*, 1957). The flavonoid glycosides such as rutin are not absorbed in the upper part of the intestinal tract but these compounds can be hydrolysed by the microflora into quercetin in the colon (Bokkenheuser *et al.*, 1987).

Indirect evidence of polyphenol absorption through the gut barrier is shown via the increase in antioxidant capacity of plasma following the consumption of polyphenol-rich foods. This has been observed for a wide array of foods rich in polyphenols, especially in red wine (Maxwell *et al.*, 1994; Fuhrman *et al.*, 1995; Serafini *et al.*, 1998). Recovery in urine, after ingestion of given amounts of a particular polyphenol, allows the comparison of relative bioavailability of the different molecules present in diets. The recovery is particularly low for quercetin and rutin, (0.3–1.4%), but greater for CA (27%) (Jacobson *et al.*, 1983). On the other hand, the effects of polyphenols on gut health may not require their absorption through the gut barrier (Santos-Buelga and Scalbert, 2000). They may have a direct impact on the gut mucosa and protect it against oxidative stress or the action of carcinogens. For example, poorly absorbed wine and tea polyphenols given orally to rats limited DNA oxidative damage in colonic cells and reduced the number of tumours in rats treated with azoxymethane (Giovannelli *et al.*, 2000). Intestinal colonic anti-inflammatory activity was observed with oral supplementation of quercetin in a rat model of chronic experimental colitis. The anti-inflammatory effects were associated with suppressed activation of the p38 mitogen-activated protein kinase (MAPK) pathway modulating cyclooxygenase (COX)-2 and iNOS expression (Camacho-Barquero *et al.*, 2007).

2.3.2 Antioxidant and Anti-Inflammatory Effects of Polyphenols in Gut Health

Polyphenolic compounds are secondary plant metabolites characterised by antioxidative activities and they are found in numerous plant species, including potato (Brown, 2005). Polyphenols are characterized by cyclic rings with hydroxyl substitutions at various positions that readily react with the damage-causing free radicals that frequently attack cells (Duthie *et al.*, 2000). Polyphenolic compounds

are distributed mostly between the periderm and cortex tissues of the potato (Reeve *et al.*, 1969). Potatoes contain a diverse mixture of antioxidants, which exhibit multiple antioxidant activities including superoxide scavenging capability, ferrous ion chelating effects, and a strong reducing capacity (Singh and Rajini, 2004; Brown, 2005; 2008). Polyphenolic compounds provide a large portion of the antioxidant action of potatoes in addition to ascorbic acid (RiceEvans *et al.*, 1997), which is involved in scavenging and neutralizing free radicals, decomposing lipid peroxides, and quenching singlet oxygen (Cao *et al.*, 1997). Polyphenolic compounds such as CGA, CA, FA, and rutin have a strong antioxidant capacity and have been shown to cause inhibition of certain types of cancer in animal and cell culture models (Miccadei *et al.*, 2008). Moreover, potato peel extracts containing a high polyphenolic content protected human and rat erythrocytes against oxidative damage and prevented H_2O_2-induced morphological alterations (Singh and Rajini, 2008).

The major polyphenol found in potatoes is CGA that can constitute up to 90% of the total phenolic content of potato tubers (Friedman, 1997). CGA and CA are free-form phenolics while FA, which is linked to cell wall polysaccharides, is a bound-form phenolic (Nara *et al.*, 2006). Taking the sum of bound and free phenolic compounds as total phenolic compounds, there is suggestive evidence that about 40% of the total phenolic content in potatoes is present in the bound form (Boivin *et al.*, 2009). The bound form of polyphenolics are unaffected by the upper GI digestion but can exhibit physiological bioactivity at the colonic level (Nara *et al.*, 2006).

Previous work has investigated the effect of the flavonoid epicatechin (EC) and CGA on the regulation of apoptotic and survival/proliferation pathways in a human hepatoma cell line (HepG2) (Granado-Serrano *et al.*, 2007). EC and CGA had no effect on apoptosis but enhanced the intrinsic cellular tolerance against oxidative insults either by activating survival/proliferation pathways or by increasing antioxidant potential in HepG2. Polyphenol extracts from various plants were added directly to Caco-2 cells as a pre-treatment before or simultaneously with the inflammatory stimuli (IL-1b alone or IL-1b + IFN-γ + TNF-

α mix): *Punica granatum* (pomegranate fruit peel), *Saccharum officinarum* (sugar cane stem), and *Quercus robur* (oak heartwood). The final concentrations of polyphenolic extracts varied from 2 to 500 µg/mL. The highest dose of polyphenolic extract inhibited NF-kB transcriptional activity and reduced IL-8 secretion (Scalbert *et al.*, 2002; Romier-Crouzet *et al.*, 2009; Romier *et al.*, 2009). Pure polyphenol compounds (0.1 to 370 µM) also reduced IL-8 secretion in rats with DSS-induced colitis (Romier *et al.*, 2009). Such quantities are all realistic intestinal concentrations since it was estimated that 500 mg of polyphenols would give a local gut concentration of 300 µM when ingested (Scalbert and Williamson, 2000). For example, after rutin was administered orally to rats (at 10 mg/kg), the majority of rutin accumulated in the large intestine, reaching a concentration of 220 µM in the form of quercetin (Kim *et al.*, 2005). In humans, the anti-inflammatory effects of quercetin reduced the risk of cardiovascular disease (Landolfi *et al.*, 1984). Quercetin at relatively high concentrations blocked both the COX and lipoxygenase (LOX) pathways (Guardia *et al.*, 2001). In addition to the free-form phenolic compounds such as CGA and CA, bound-form phenolics (such as FA), that are ester-linked to cell wall polysaccharides, are also present in significant concentration in certain potato cultivars, and could contribute radical-scavenging activities in the gut (Nara *et al.*, 2006).

Since native polyphenols will have different structures and functions than their metabolites following exposure to digestive enzymes and bacterial metabolism (Han *et al.*, 2007), it is conceivable that the degree of antioxidant and anti-inflammatory protection associated with polyphenolic intake will be altered due to different physiological and metabolic effects of the metabolites. However, to date, no studies have specifically investigated the impact of bacterial or enzymatic metabolism of polyphenols on their antioxidant and anti-inflammatory properties, particularly in relation to colonic cells.

2.3.3 Organic Acids in Gut Health

Organic acids in potato tubers consist of approximately 0.4-1.0% of fresh weight (Lisinska and Leszczynski, 1989). The major organic acids are represented

mainly by ascorbic, benzoic, citric, fumaric, malic, oxalic, and succinic acids. Organic acids are biologically important for their role in different metabolic pathways such as the Kreb Cycle (Alcock, 1965). Organic anions are absorbed in the digestive tract and metabolised in various tissues, especially the liver and intestine, and finally produce carbon dioxide (CO_2) and energy (Wolffram *et al.*, 1994). Organic acids contribute to the acidity and pH of potato juice, depending on the concentration and acid dissociation constant (pK_a). Therefore, organic acids influence flavour directly by their tartness, and affect colour by inhibiting of darkening and enzymatic browning produced before and after cooking (Silva *et al.*, 1991).

Citric acid is a major organic acid that complexes with oxidant metals and has a synergistic reducing effect along with ascorbic acid (Galdon *et al.*, 2010). Malic acid is an important indicator of produce freshness because its concentration increases during storage (Lisinska and Aniolowski, 1990). The bioavailability of digested organic acids of fruit and vegetables were investigated using Caco-2 cells (Sabboh-Jourdan *et al.*, 2011). Firstly, banana and sweet potato were digested utilizing a human digestion model which simulated oral, gastric and pancreatic digestions. In sequence, Caco-2 monolayers were exposed to the digestates and malic and oxalic acids were absorbed more than citric acid. The incomplete release of organic acids from banana and sweet potato under gastric and small intestine conditions demonstrated that a significant fraction reached the colon. So, organic acids in the colon could have positive effects on fermentation processes and modulation of microbial metabolism in the large intestine (Sabboh-Jourdan *et al.*, 2011). This study showed the importance of digestion in increasing the bioavailability of organic acids. However, to date, no study has investigated the protective effect of organic acids synthesized by colonic bacteria in a model of gut inflammation.

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Table 2.1 Amino acid composition of egg, soybean, skim milk, casein, and whey protein isolate (g/100g protein).

Amino Acid	Egg[1]	Soybean[2]	Skim Milk[3]	Casein[4]	Whey Protein Isolate[5]
Leucine*	8.5	9.6	8.7	8.9	12.3
Isoleucine*	5.9	5.8	4.2	4.6	6.3
Valine*	6.8	6.7	4.8	5.5	5.3
Cysteine	2.5	1.6	0.6	0.9	2.3
Methionine*	3.2	2.4	1.8	2.3	2.3
Cys + Met	5.7	4.0	2.4	3.2	4.6
Phenylalanine*	5.2	2.5	4.8	6.6	3.5
Tyrosine	3.9	1.4	4.5	3.6	3.6
Phe + Tyr	9.1	3.9	9.3	10.2	7.1
Tryptophan*	1.7	1.1	1.5	1.0	0.9
Threonine*	5.8	5.1	4.5	4.1	5.6
Lysine*	6.9	8.6	8.1	7.8	8.9
Histidine*	2.6	3.0	3.0	2.5	2.2
Arginine	6.1	4.4	3.3	3.4	2.4
Alanine	5.4	4.2	3.0	2.8	5.4
Aspartic Acid	9.6	9.3	7.8	7.6	11.2
Glutamic Acid	12.2	18.1	23.2	21.8	14.3

Amino Acid	Egg[1]	Soybean[2]	Skim Milk[3]	Casein[4]	Whey Protein Isolate[5]
Glycine	3.2	3.7	1.8	1.7	1.5
Proline	2.9	6.8	9.6	9.3	5.1
Serine	7.6	5.7	4.8	5.5	6.8

*Essential amino acids [1]Lunven and Marq (1973); [2]Smiricky *et al.*, (2002); [3]Csapó-Kiss *et al.*, (1995); [4]Sindayikengera (2006); [5]Vitalus (2004).

Chapter 3

Manuscript 1

THE IMPACT OF MILK PROTEIN QUALITY AND QUANTITY ON ANABOLISM AND INFLAMMATION IN A PIGLET MODEL OF DEXTRAN SULFATE SODIUM-INDUCED COLITIS

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Running title: Milk proteins and piglets with dextran sulfate sodium-induced colitis

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3.1 ABSTRACT

Inflammatory bowel disease (IBD) is a chronic debilitating condition with a high prevalence in Canada. Inadequate protein intake due to food avoidance and anorexia could adversely affect growth, inflammatory status, and disease severity in the pediatric population. Intake of whey protein isolate (WPI) is associated with improved antioxidant status, and high pressure processing of WPI increases anti-inflammatory bioactivity. The present study investigated the impact of differences in dietary milk protein quality and quantity on growth, body composition, colonic histopathology, protein synthesis, and inflammation in a piglet model of dextran sulfate sodium (DSS)-induced colitis. Thirty-two female piglets were randomized to receive 12 days of complete isoenergetic liquid diets by gastrostomy. Three groups were supplied 50% of their protein requirement: as pressurized WPI (pWPI), native WPI (nWPI), and skim milk (SM) and a control group was provided with 100% of its protein requirement (SM). Protein synthesis was studied on day 12 with a 6-h infusion of tracer L-[ring-$^{2}H_{5}$]phenylalanine. Hematoxylin and eosin sections of descending colon (DC) were graded while blinded to group assignment. Although piglets that received both WPI diets gained less absolute weight than their SM counterparts, pWPI-fed piglets gained more lean mass than nWPI piglets, and were the only protein-restricted group to gain a high lean/fat ratio comparable to well-nourished controls. Protein FSR in DC was not different among groups. However, histopathology scores were lowest in both WPI groups. The pWPI-fed piglets had the lowest myeloperoxidase activity in DC, reflecting low neutrophil infiltration. In addition, pWPI-fed piglets had the greatest total antioxidant activity in DC as measured by the FRAP assay and the greatest serum peroxynitrite scavenging capacity. Proinflammatory cytokines (TNF-α, IL-8, and IL-18) in DC were most elevated in SM piglets, and lowest in pWPI. In addition, control-, nWPI- and pWPI-fed piglets had the lowest caspase-3 abundance compared with the SM group. At the clinical level, pWPI-fed piglets had less severe diarrhea compared with other protein restricted groups. In conclusion, pWPI showed anabolic, antioxidant, and anti-inflammatory effects and decreased disease severity in this pediatric piglet model of IBD.

3.2 INTRODUCTION

Inflammatory bowel disease (IBD) is a heterogeneous group of disorders characterized by various forms of chronic mucosal and/or transmural inflammation of the intestine (Hyams, 2005). In humans, IBD is a chronic disease affecting millions of people worldwide (Su *et al.*, 1999). IBD can be diagnosed at any age, but the North American Pediatric IBD Consortium estimated that the beginning of IBD is in the first 12 months of life in 1% of patients (Heyman *et al.*, 2005). The etiology of IBD is still unknown. However, some studies have shown an alteration of immune balance in the intestinal mucosa in a genetically predisposed pediatric population (Podolsky, 2002; Bouma and Strober, 2003; Bernstein *et al.*, 2006).

Growth failure, muscle wasting and hypoalbuminemia commonly presented in IBD are associated with protein deficiency and increased demand for the acute phase response (Ballinger, 2002). Our group has been studying the piglet model to investigate the effect of protein and protein-energetic malnutrition alone or with concurrent inflammation on protein kinetics (Jahoor *et al.*, 1999; Mackenzie *et al.*, 2003). The piglet is ideal for amino acid stable isotope studies due to its well-known protein and amino acid metabolism. The piglet amino acid requirements and the digestive system are similar to humans, so this animal is an appropriate model to investigate pediatric protein metabolism during gastrointestinal (GI) disorders (Kararli, 1995).

We have studied the effect of inflammation and macronutrient restricted diets on the protein synthesis of piglets with dextran sulfate sodium (DSS)-induced colitis. Macronutrient restricted piglets showed increased synthesis of plasma proteins, particularly of albumin, compromised weight gain and muscle protein synthesis as compared to piglets fed an adequate diet (Mackenzie *et al.*, 2003). The increased whole body protein turnover in IBD is associated with elevated metabolic demands including increased energy expenditure and GI losses (Azcue *et al.*, 1997; Ballinger *et al.*, 2001). The greater metabolic demands of the inflammatory process, including the synthesis of acute phase proteins, are proposed to be weakened by an insufficient protein dietary intake

and increased by intestinal protein malabsorption and losses (Campbell *et al.*, 1982; Cabre and Gassull, 2001).

Treatment of IBD involves medication, as well as dietary and lifestyle modifications. In terms of diet therapy, patients with IBD usually avoid milk products regardless of the lack of association of milk products with risk of relapse (Jowett *et al.*, 2004). However, skim milk (SM), provides high quality nutrition and highly bioavailable calcium, iron and zinc which are also frequently deficient in IBD (MacDermott, 2007). Apart from the health benefits of SM, whey protein isolate (WPI) has been shown to exert a wide variety of other health benefits that include anti-oxidative, anti-inflammatory and immune-enhancing effects (Huppertz *et al.*, 2002). Experimental and clinical studies have shown that WPI intake has a significant protective effect against oxidative stress related with some diseases such as cancer, cardiovascular disease, and diabetes (Smithers *et al.*, 1996; Nicodemo *et al.*, 2000; Pfeuffer and Schrezenmeir, 2007). High pressure processing is an efficient technique to non-thermally sterilize foods and also cause changes in the secondary and tertiary structures of whey proteins. Cell culture, animal and human studies have demonstrated a higher bioactivity of pressurised WPI due to the changes in the protein structure, which consequently increase protein digestibility and the antioxidant effects of WPI (Vilela *et al.*, 2006a; Zavorsky *et al.*, 2007; Kishta *et al.*, 2009). For instance, our group demonstrated that short-term intake of pressurized WPI in healthy subjects showed rapid increase in 27% of lymphocyte GSH status within a 2-week period (Zavorsky *et al.*, 2007). However, the possible benefits of WPI supplementation on IBD have not yet been explored, including in a pediatric model.

Therefore our main objective was to investigate the impact of feeding native SM, native WPI (nWPI) and pressurized WPI (pWPI) on growth, body composition, protein synthesis, descending colon (DC) histopathology, antioxidant and inflammatory status in DSS-treated piglets fed an isocaloric moderate protein-deficient diet. Considering the strong evidence from *in vitro* and *in vivo* studies of the health benefits from WPI we hypothesized that pressurized WPI-fed protein-restricted piglets will demonstrate more potent anabolic effects,

and particularly in the DC, show decreased pro-inflammatory cytokine levels, and increased antioxidant status as compared to the moderate protein deficient or well nourished control piglets fed native milk proteins.

3.3 MATERIAL AND METHODS

Experimental protocol

Female piglets, n = 32 (5 - 7 days old; Yorkshire x Landrace (sow) x Duroc (boar); individually housed) were purchased from the Macdonald Campus Farm Swine Complex of McGill University and taken immediately to the Animal Facility of the Macdonald Stewart Building from McGill University. Piglets were randomized into 4 dietary groups (N = 8 / group) and assigned to 3 study diets supplying 50% of protein requirement as either 1) SM, 2) nWPI, or 3) pWPI. The 4^{th} diet group (control) supplied 100% of protein as SM.

Study protocol (Figure 3.3.1) began after removal from the sow (study day 1). Each piglet underwent aseptic surgery under isoflurane anaesthesia to implant catheters into femoral and external jugular veins for tracer infusion and blood sampling, respectively, and into the stomach for enteral feeding and DSS administration (Wykes *et al.*, 1993; Mackenzie *et al.*, 2003; Harding *et al.*, 2008). Enteral feeding was initiated 2 hours post surgery at 60% of the targeted energy intake and increased to 80% on day 2. On day 3, piglets started receiving full energy requirement and continued throughout the study; blood was sampled for baseline measurements; and DSS was started and continued until day 11. Nitrogen balance was assessed on days 9, 10, and 11. On day 12, a 6-h stable isotope infusion study with tracer L-[ring-$^{2}H_{5}$]phenylalanine was conducted to determine protein synthesis rates. Blood was sampled hourly throughout the infusion and tissues were sampled immediately after piglets were euthanized with an intravenous injection of Euthanasol (750 mg sodium pentobarbital; Schering Canada Inc., Toronto, ON, Canada). The study protocol was approved by the McGill University Animal Care Committee in accordance with the Canadian Council on Animal Care Guidelines.

Diet

Piglets received a custom-formulated liquid diet (Table 3.3.1) designed to meet the requirements of all nutrients (except for the three moderately-deficient protein groups) for the young piglet according to the National Research Council (NRC, 1998). The nutrient calculations were based on the average of body weight for 2 different weight ranges: 3 to 5 kg and 5 to 10 kg. The well-nourished control group received 100% of the protein requirement from SM powder (control). In this diet, skim milk powder was used as the main ingredient and protein supplied 27% of energy, carbohydrate 39%, and lipid [as a blend of soybean, safflower, coconut, flaxseed oils (n-6:n-3 ratio 8.2:1)] 35%.

The other three moderately protein-deficient diet groups supplied 50% of protein requirement as SM (1), nWPI (2) or pWPI (3). All 3 moderately-deficient diets had the same macronutrients distribution: 13% of energy supplied as protein, 46% of energy supplied as lactose, and 41% of energy supplied as lipid. Vitamin and mineral mixes were formulated individually for each diet to provide the same total intake of each micronutrient to achieve at least 100% of the NRC requirement (NRC, 1998) accounting for the micronutrient content of other diet ingredients.

WPI was produced by membrane ultrafiltration (Vitalus Nutrition Inc., Abbotsford, BC, Canada). WPI was pressurized in 12.5% (v/v) in double-distilled water and pressurized with an Avure High Pressure Processing System model QFP 215L-600 (Avure Technologies, Columbus, OH) by applying a single cycle of hydrostatic pressure of 550 MPa. This acts to unfold the protein conformation of the whey proteins enabling them to be more susceptible to digestive enzymes (Vilela *et al.*, 2006a).

Amino acid profile of WPI, SM, and NRC diet is shown on Table 3.3.2. Control piglets receiving 100% SM were supplied most of the essential amino acids at 109% of the requirement (Table 3.3.3). Particularly, sulfur amino acids are limiting in the SM group (supplied at 37% of requirement compared to 56% in 57% nWPI and pWPI, respectively).

Intake of vitamins and minerals of each diet achieved 100% of the requirements for piglets (Tables 3.3.4 and 3.3.5, respectively), except zinc with 163% of the requirement. During intestinal inflammation, increased losses of zinc occur due to either malabsorption or endogens secretion of this micronutrient into the small intestine (Hendricks and Walker, 1988).

Diets were mixed each day to be infused via the gastric catheter for a 24 h period (Compat Enteral Feeding Pump; Novartis Nutrition, Minneapolis, MN, USA). The control diet provided an energy intake of 214 kcal/(kg•d) achieving the targeted goal, 14.4 g protein/(kg•d), 20.7 g carbohydrate/(kg•d) and 8.2 g lipid/(kg•d). Piglets receiving SM diet were provided an energy intake of 218 kcal/(kg•d), 7.3 g protein/(kg•d), 25.0 g carbohydrate/(kg•d) and 9.9 g lipid/(kg•d). Finally, piglets receiving the nWPI diet were provided an energy intake of 221 kcal/(kg•d), 7.4 g protein/(kg•d), 25.1 g carbohydrate/(kg•d) and 10.1 g lipid/(kg•d) and the pWPI diet supplied an energy intake of 224 kcal/(kg•d), 7.5 g protein/(kg•d), 25.5 g carbohydrate/(kg•d) and 10.2 g lipid/(kg•d).

Assessment of weight gain, growth and body composition

Piglets were weighed daily and diet infusion rates adjusted accordingly. Growth was assessed by the increase in snout-to-rump length and chest circumference measured at the beginning of the study while under surgical anesthesia and again immediately after death. Body composition assessed by Dual-energy X-ray absoptiometry (DXA) (QDR 11.2 4500A series; Hologic Inc., Waltham, MA, USA) was determined immediately following surgery and on day 11. Piglets were scanned in a prone position, under isoflurane anesthesia, and results were analyzed using infant-whole body software (Kohut *et al.*, 2009). Net change in fat, bone, lean mass, and lean-to-fat ratio were calculated from the change in body composition over the duration of the study. The left femur and tibia were removed during tissue sampling to further bone mineral density analysis. To determine nitrogen balance, urine was collected over 72 hours (Day 9, 10, and 11) in a flask containing 5 mL of 3.8 M H_2SO_4 solution on ice. Feces were collected completely from the cage and dried.

DSS-induced colitis model

The piglet model of DSS-induced colitis, originally described by Mackenzie et al (2003) and adapted by Harding et al (2008), was modified by decreasing the dose and the duration of DSS administration. A DSS solution (200g·L-1, 40,000 MW, ICN Biomedicals Inc, Aurora, OH, USA) was administered twice daily through the gastric catheter at 0.5 g/(kg•d) from study day 3 until the day 11. On day 6 and 7, feces were tested for occult blood with Hemoccult Sensa® Single Slides (Beckman Coulter Inc., Fullerton, CA, USA) to confirm the presence of colitis. This test is based on the oxidation of guaiac by hydrogen peroxide (H_2O_2) to a blue-coloured compound. The heme portion of hemoglobin, if present in the fecal specimen, has peroxidase activity which catalyzes the oxidation of alpha-guaiaconic acid (active component of the guaiac paper) by hydrogen peroxide (active component of the developer) to form a highly conjugated blue quinone compound. In addition, the consistency of feces, characterized as hard, soft, or liquid, was recorded every morning during the entire experimental period.

Histology

Formalin-fixed a 3-cm transversal cut of descending colon proximal segment was embedded in paraffin, cut into 8 µm sections, and stained with hematoxylin and eosin (H&E) using standard slide preparation techniques. The sections were graded by an experienced veterinary pathologist while blinded to group assignment (M.P.). Four features (Table 3.3.6) were graded using the assessment criteria adapted from Dieleman, *et al.* (1998) with a range from 0 to 3 for the amount of inflammation (acute and chronic) and depth of inflammation, and with a range from 0 to 4 for the amount of crypt damage or regeneration. Furthermore, the percentage of each tissue involved was scored for each feature: (1) 1–25%, (2) 26–50%, (3) 51–75%, (4) 76–100%. Each section was then scored for each feature separately by establishing the product of the grade for that feature and the percentage involvement (in a range from 0 to 12 for inflammation and for extent, and in a range from 0 to 16 for regeneration and for

crypt damage). The subtotals of each feature were then summed providing the total histological colitis score (Dieleman *et al.*, 1998).

Biochemical parameters

Total plasma protein and albumin were measured quantitatively using the Vitros 350 (Ortho-Clinical Diagnostics, Markham, ON, Canada) at the Comparative Medicine & Animal Resources Centre, McGill University. Fibrinogen concentration was determined by enzyme-linked immunosorbent assay (ELISA) (Kamiya Biomedical Company, Seattle, USA). Albumin in the descending colon (DC), spiral colon, ileum, and urine was quantified by ELISA (Bethyl Laboratories Inc., Montgomery, TX, USA). Total protein in the intestine segments was assessed by colorimetric detection assay (Thermo Scientific, Rockford, IL, USA). Plasma cortisol was measured by an enzyme immunoassay kit (Assay Designs, Plymouth Meeting, PA, USA).

Cytokines

TNF-α and IL-8 in DC were assessed by ELISA (R&D Systems, Minneapolis, MN, USA). IL-18 concentration was measured by ELISA (eBioscience, Vienna, Austria).

Myeloperoxidase (MPO) activity

MPO is the most abundant protein in neutrophils playing a major role in many inflammatory pathologies. MPO catalyzes the reaction between chloride and H_2O_2 to form hypochlorous acid (HOCl), which is a powerful antimicrobial agent and extremely reactive with biological molecules resulting in neutrophil mediated tissue damage that is characteristic of inflammatory disease (Zhang *et al.*, 2002). MPO activity was quantified by a colorimetric assay (Northwest Life Science Specialties, Vancouver, WA, USA).

Ferric reducing antioxidant power (FRAP) assay

FRAP was used to determine the total antioxidant potential in DC and plasma samples. Briefly, in this assay the electron-donating capacity of the antioxidant was measured by the change in absorbance (abs) at 593 nm when a blue-coloured Fe^{2+}- tripyridyltriazine (Fe^{2+}TPTZ) compound is formed from a colorless oxidized Fe^{3+} form (Benzie and Strain, 1999). A standard curve was prepared from aqueous solutions of 1 mM $FeSO_4.7H_2O$ at different concentrations ranging from 0.1 to 1.0 mM. The reagent was prepared with 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution dissolved in 40 mM HCl at 50ºC, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The working solution was mixture with the 3 solutions in 10:1:1 ratio, wrapped in foil. Once prepared it was immediately incubated for 10 min at 37°C. Into a 96 well plate, was added 30 μl H_2O, 10 μl standards or samples, and 200 μl FRAP working solution. Samples reaction occurred at room temperature for 30 min and abs was taken at 593 nm in a microplate reader (Infinite PRO 200 series, Tecan Group, San Jose, CA, USA).

Oxidative stress

Peroxynitrite, a biomarker of oxidative stress, is formed by the reaction between superoxide and nitric oxide, released simultaneously and continually from a 2.5mmol L^{-1} solution of SIN-1 (3-morpholino-sydnonimine HCl, $C_6H_{10}N_4O_2 \cdot HCl$).

$$O2^{\bullet -}\ (\text{superoxide}) + NO^{\bullet}\ (\text{nitric oxide}) \rightarrow ONOO^{-}\ (\text{peroxynitrite})$$

In this oxidative stress assay, when SIN-1 is injected into a microplate well containing Pholasin® (Knight Scientific Ltd, Plymouth, UK), light of gradually increasing intensity is detected, reaching a peak after a few minutes. If there are antioxidants in the sample capable of scavenging peroxynitrite, such as Vitamin E, then these will compete with the Pholasin® for the peroxynitrite. Any antioxidants in the sample will gradually be consumed, delaying the time at which the maximum peak of light occurs. The time at which peak luminescence occurs after adding SIN-1, is expressed in Trolox equivalent units using 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Sigma-Aldrich, St. Louis, MO,

USA). Automated measurement was carried out on a luminescence microplate reader (Infinite PRO 200 series, Tecan Group, San Jose, CA, USA).

Nitrogen balance

Nitrogen concentration of diet, urine and feces was determined on a Tru-Spec N System (LECO, St Joseph, MI, USA). Nitrogen excretion was considered as the sum of urinary and feces nitrogen and nitrogen balance was calculated by the difference of nitrogen intake – nitrogen excretion. In addition, nitrogen retention was expressed as a percentage and was determined by nitrogen balance / nitrogen intake x 100.

Stable isotope infusion protocol

The tracer solution was prepared by dissolving L-[ring-$^{2}H_5$] phenylalanine (99 % enriched; Cambridge Isotope Laboratories, Cambridge, MA, USA) in sterile saline (9 g/L) for a final concentration of 35 μmol/L, and filtering through a 0.22 μm filter.

A priming dose (50 μmol/kg) of the tracer solution was infused through the femoral catheter followed by a constant infusion rate of 35 μmol (kg•h) for 6 h.

Venous blood samples (3 mL) were collected through the jugular catheter at baseline and hourly thereafter, and immediately separated and placed into in pre-chilled tubes containing Na_2EDTA, and a protease inhibitor to prevent plasma protein degradation (Jahoor *et al.*, 1999; Mackenzie *et al.*, 2003). Blood was centrifuged at 1,500 x g for 3 min, plasma was collected and frozen in liquid nitrogen and stored at -80°C. Whole blood was collected at h 1 and h 5 in heparinized capillary tubes for hematocrit.

After the 6 h sample, piglets were euthanized and tissue samples (liver, DC, spiral colon, ileum, jejunum, longissimus dorsi, and masseter) were collected. Ileum and jejunum sections were immediately immersed in iced physiological saline, rinsed, and mucosa scraped with microscope slides. For histological analysis, a 2-cm cross-sectional cut was made at the proximal section of each segment and fixed in 100 mL/L buffered formalin. All tissues were immediately frozen in liquid nitrogen and stored at -80°C.

Protein analysis

Total protein in plasma was isolated by precipitation with ice-cold 0.6 mol/L trichloroacetic acid (TCA) washed twice and hydrolyzed overnight in 6 mol/L HCl at 110°C. To isolate fibrinogen, an ethanol/saline (1:8) solution was added to plasma and placed in fridge overnight. Samples were then centrifuged at 1,500 x g at 4°C for 10 min. The resulting fibrinogen pellet was dissolved in SDS-PAGE sample buffer. To isolate albumin, proteins were precipitated from the fibrinogen-free supernatant with TCA, centrifuged at 6,740 x g for 7 min, supernatant was discarded and albumin re-solubilized by vortexing in 95% ethanol. The supernatant containing the dissolved albumin was mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing sample buffer. Fibrinogen and albumin were resolved separately by SDS-PAGE on a MINI-PROTEAN II System (Bio-Rad Laboratories, Montreal, QC, Canada). Samples along with pure standards and molecular weight markers were loaded onto the gel and run at 200V for 120 and 45 min, respectively. The appropriate protein bands were excised from the gel and hydrolyzed overnight in 4 mol/L HCl at 110°C.

In addition, tissue free and protein-bound amino acids were isolated. Aliquots from liver, DC, spiral colon, ileum, and jejunum were homogenized in ice-cold 0.6 mol/L TCA and centrifuged at 1,500 x g and 4°C for 10 min. The supernatant containing the free amino acids was removed for analysis as tissue free amino acids, and the protein pellet washed twice with deionised water and hydrolysed (Mackenzie *et al.*, 2003; Harding *et al.*, 2008).

For the longissimus dorsi and masseter muscles the myofibrillar and sarcoplasmic proteins were separated according to the method previously described by Welle *et al.*, (1993) and Welle *et al.*, (2009), respectively. To isolate sarcoplasmic and myofibrillar proteins and free amino acids of both muscle compartments, approximately 100 mg of tissue was homogenized in 4 mL distilled H2O and centrifuged at 1,500 g at 4°C for 10 min. The supernatant was removed and separated into 2 portions in 4 mL glass vials. The free amino acid supernatant from the first 4 mL glass vial was acidified with 1 mL glacial acetic

acid for $^{2}H_5$-phenyalanine enrichment. The myofibrillar protein pellet was washed 3 times with 2 mL cold distilled H_2O and supernatant was discarded. The pellet was dissolved in 2 mL 0.3N NaOH to precipitate non-soluble proteins and the supernatant containing myofibrillar proteins was transferred 4 mL glass vial and acidified with 2 mL 6N HCl. Samples were capped under N_2 and heated at 110 °C overnight. The sarcoplasmic protein supernatant from the second separated 4 mL glass vial was added 10% perchloric acid to precipitate sarcoplasmic proteins. Samples were centrifuged at 1,500 g at 4°C for 10 min and washed 4 times with 2 mL 5% perchloric acid to remove free amino acids. Samples were washed with 2 mL distilled H_2O, centrifuged at 1,500 g at 4°C for 10 min, followed by 2 mL ethanol to remove perchloric acid and transferred to 4 mL glass vial. Finally, samples were dried in Speed Vac (Thermo Savant SC 210A, Waltham, MA, USA). Finally, the dried pellet was acidified with 2 mL 6M HCl, capped under N_2 and heated at 110°C overnight.

Amino acids from total plasma protein, albumin, fibrinogen, and tissue free and bound-amino acids pools were isolated by ion exchange chromatography (Dowex 50WX8, Bio-Rad, Mississauga, ON, Canada), dried, esterified, and derivatized to their n-propyl ester heptafluorobutyramide derivatives using n-propanol and acetyl chloride followed by heptafluorobutyric anhydride (Mackenzie *et al.*, 2003; Harding *et al.*, 2008).

Phenylalanine enrichment was analyzed by methane negative chemical ionization gas chromatography-mass spectrometry (GC-MS) (Agilent Technologies, Model 5975C, Mississauga, ON, Canada), by monitoring the $[M-FH]^-$ ions at mass to charge ratio 383 and 388, plotted against as standard curve of corresponding unlabelled and labelled ions, respectively for albumin, fibrinogen, total plasma proteins, and tissues. Tracer:tracee ratios were calculated from the raw ion abundances and analysis of the tracer and natural abundance of phenylalanine. Baseline measurements were obtained from total plasma proteins at hour 0. Isotopic steady state was confirmed between h 3 and 6, using the hepatic free pool of amino acids.

Protein synthesis calculations

Fractional synthesis rate (FSR) of plasma proteins and of mixed proteins in each tissue were calculated based on the precursor-product relationship (Jahoor *et al.*, 1994) , which relates the rate of increase in product enrichment to the net tracer:tracee ratio of tissue-free phenylalanine at steady state (Ball, 1996; Mackenzie *et al.*, 2003; Harding *et al.*, 2008). Liver-free amino acids were considered the precursor pool for hepatically synthesized plasma proteins (Jahoor *et al.*, 1999; Mackenzie *et al.*, 2003).

FSR of plasma proteins (the total pool, albumin, or fibrinogen) was calculated using:

$$FSR\ (\%/d) = [(Et_2 - Et_1) \times 24 \times 100] / [E_{free} \times (t_2 - t_1)]$$

where Et_2 - Et_1 is the increase in the tracer:tracee ratio of phenylalanine incorporated into the relevant plasma protein pool using slope of the linear regression line during the final 3 h of the infusion ($t_2 - t_1$). E_{free} is the tracer:tracee ratio of liver free phenylalanine at steady state (Jahoor *et al.*, 1994; Jahoor *et al.*, 1999).

The FSR of mixed proteins in each tissue was calculated using:

$$FSR\ (\%/d) = (E_{bound} \times 24 \times 100) / (E_{free} \times 6)$$

where E_{bound} is the net tracer:tracee ratio above baseline of the tissue protein bound phenylalanine at h 6; and E_{free} is the net tracer:tracee ratio of tissue free phenylalanine at isotopic steady state (Wykes *et al.*, 1996; Ljungqvist *et al.*, 1997; Jahoor *et al.*, 1999; Reeds and Davis, 1999). The tracer:tracee ratio of free phenylalanine in each tissue (E_{free}) was assumed to reflect the steady-state tracer:tracee ratio of the phenylalanine pool from which proteins in that tissue were synthesized. The tracer:tracee ratio of free phenylalanine in each tissue at baseline was assumed to be the same as phenylalanine in the total plasma protein pool. Isotopic steady state was achieved in plasma phenylalanine within 1

h of starting the infusion; therefore the tracer:tracee ratio of intracellular phenylalanine was assumed to be constant over the last 5 h of the infusion, and rapidly rising for the first hour. Only one tissue sample was obtained after the 6 h tracer infusion period, so the FSR calculated was assumed to be at the same enrichment throughout the infusion (Wykes *et al.*, 1996).

Absolute synthesis rate (ASR) for each plasma protein was calculated using:

$$ASR\ [mg/kg \bullet d)] = FSR \times conc \times PV$$

where conc is the concentration of the protein in plasma, and PV is the plasma volume calculated using hematocrit measured during the infusion in an average blood volume of 80 mL/kg body (Ramirez *et al.*, 1963).

Immunoblotting analysis

Descending colons were homogenized on ice-cold lyses buffer (T-PER® tissue protein extraction reagent, Thermo Scientific, IL, USA) containing a protease inhibitor cocktail (Complete Mini, Roche, IN, USA). Total protein content in the homogenate was determined by bicinchoninic acid protein assay (BCA Protein Assay Reagent, Thermo Scientific, IL, USA) and the cell lysates were denatured in sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and heated at 95ºC for 5 min. Samples (30-50 µg of total protein) were carried to 10-14% SDS-PAGE, according to the molecular weight of each protein, and transferred to polyvinylidene filters (PVDF Hybond-P, Amersham Pharmacia Biotech, NJ, USA). Rainbow markers (Amersham Pharmacia Biotech, NJ, USA) were run in parallel to estimate molecular weights. Membranes were blocked with 5% nonfat milk in Tween-TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20). Primary antibodies used were anti-Akt (1:1000, Cell Signaling, MA, USA); anti-pAkt Ser473 (1:1000, Cell Signaling, MA, USA); anti-BCl-2 (1:500; Santa Cruz Biotechnology, Inc. CA, USA); anti-caspase-8 (1:50/1:100, Abcam, MA, USA); anti-caspase-3 (1:1000, sc-7148, Santa Cruz Biotechnology, Inc. CA, USA); anti-cleaved caspase-3 (1:1000, Cell Signaling, MA, USA); anti-NOS3 (1:500, Santa Cruz Biotechnology,

Inc. CA, USA); and anti-β-actin (1:2000, Abcam, MA, USA). The PVDF filters were washed 3 times with Tween-TBS, followed by 1 h incubation with appropriate secondary antibody conjugated to horseradish peroxidase. All immunoblots were allowed to react with horseradish peroxidase substrate (ECL-plus; Amersham Pharmacia Biotech, NJ, USA) and then exposed to X-ray film for 1 to 10 min. The images were scanned and the bands were quantified by densitometry, using Image J 1.34s software (Wayne Rasband National Institute of Health, MA, USA).

Statistical analysis

Data were expressed as means ± pooled SEM, and statistical significance assumed at $p < 0.05$. Statistical analysis was performed using SAS for Windows (version 9.2, 2008, SAS Institute Inc., Cary, NC). Least square means were performed using PROC GLM after verification of normality using Shapiro-Wilk and homogeneity of variances using Levene's test, adjusted for Tukey's *post hoc* multiple comparison tests. Changes in variables over time (eg, incorporation of tracer into plasma proteins and gain weight) were assessed with repeated measures ANOVA analysis.

3.4 RESULTS

Weight gain, growth and body composition

All piglets were followed for 12 days and started the study with similar body weights (Table 3.4.1). As expected, control piglets had the highest increment in terms of daily body weight relative to the other moderate protein restricted groups after the 10^{th} day of the study (Figure 3.4.1, $P < 0.05$). The net weight gain of the control piglets consuming 100% of their protein requirement was greater ($P < 0.01$) than all the protein restricted groups. The SM-fed piglets showed a greater ($P < 0.05$) weight gain than the two WPI-fed groups. Length growth measured as percent of initial length was greater ($P < 0.01$) in the control-fed piglets than nWPI-fed piglets although chest growth was unchanged among all piglet groups (Table 3.4.1).

The total lean mass gained was greater ($P < 0.01$) in the control-fed piglets at the end of the study as compared to the protein restricted groups (Table 3.4.2). The fat mass composition was higher ($P < 0.01$) in the SM and nWPI groups relative to the control and pWPI piglets (Table 3.4.2). The bone mineral content followed the same pattern as the net total body weight gain with the control piglets demonstrating higher ($P < 0.01$) bone mineral content relative to the other treatment groups and the SM piglets having greater ($P < 0.01$) bone mineral content as compared to the WPI-fed groups. In terms of the proportions of the body compartments, the lean to fat body ratio was higher ($P < 0.01$) in the control and pWPI groups in comparison to the SM- and nWPI-fed piglets. Analyzing the body composition as a percent net gain, a similar pattern was observed whereby control and pWPI piglets had higher ($P < 0.01$) percent content of lean mass and lower percent fat mass in comparison to the SM and nWPI piglets (Figure 3.4.2, $P < 0.01$). The femur and tibia area, bone mineral content (BMC), and bone mineral density (BMD) measured by DXA are shown in Figure 3.4.3. The area, BMC, and BMD followed the same pattern as assessed in Table 3.4.2. Control- and SM-fed piglets had greater ($P < 0.01$) femur area than the WPI-fed groups. Control-fed piglets showed increased ($P < 0.01$) tibia area as compared to the other moderate protein restricted groups and SM-fed piglets had higher ($P < 0.01$) tibia area than the WPI-fed piglets. The control piglets femur and tibia demonstrated greater ($P < 0.01$) BMC and BMD relative to the other treatment groups and the SM-fed piglets having higher ($P < 0.01$) BMC and BMD as compared to the WPI-fed groups.

Nitrogen balance and hormone levels

Control piglets had greater ($P < 0.01$) N intake, N excretion, and urinary N than the moderate protein restricted groups (Table 3.4.3). Nitrogen balance was higher ($P < 0.01$) in the control group than the other groups despite similar percent N retention among the studied groups. Control-fed piglets had higher ($P < 0.01$) urea concentrations than the moderate protein restricted piglets and no

differences were observed in the serum cortisol concentrations among all piglet groups (data not shown).

Tissue and total plasma protein synthesis and intestine albumin concentration

The rate of incorporation of phenylalanine tracer into the intravascular pools of fibrinogen, total plasma proteins, and albumin was linear (Figure 3.4.4). In terms of muscle FSR, control-fed piglets had greater ($P < 0.01$) masseter (sarcoplasmic) FSR as compared to the moderate protein restricted-fed piglets (Table 3.4.4). Control piglets also had greater ($P < 0.05$) jejunum mucosa FSR as compared to nWPI-fed groups (Table 3.4.5). The effect of quantity and quality of protein did not affect the plasma FSR and ASR nor the plasma concentrations of fibrinogen, total protein, and albumin (Table 3.4.6). The albumin concentration in the ileum mucosa was greater ($P < 0.05$) in the control group as compared to the SM group (Table 3.4.7).

Histology

Hematoxylin and eosin stained sections of the DC were graded to assess severity and extent of inflammation, depth of tissue affected, regeneration, and crypt damage. Scores were compared to a similar group of healthy well nourished piglets (Harding *et al.*, 2008). Interestingly, the control and SM-fed piglets had greater ($P < 0.05$) total histopathology scores as compared to the nWPI- and pWPI-fed piglets (Table 3.4.8). The two histological categories, inflammation and depth affected, showed greater ($P < 0.05$) scores for SM-fed piglets as compared to pWPI-fed piglets (Table 3.4.8). Colon segments from the reference (Harding *et al.*, 2008) and piglets treated with DSS are shown in Figure 3.4.5. Colonic mucosa from reference piglets shows intact epithelium and crypts, a small degree of cell infiltration and the presence of mature cells. Piglets fed with pWPI had the lowest histological score, which was characterized by a lesser degree of cellular infiltration and damage to the mucosa and deep parallel crypts that extended to the lamina propria. nWPI-fed piglets showed a similar histopathology as observed for the pWPI-fed piglets. In contrast, piglets fed either

the SM or control diets had complete crypt destruction ranging from shortening of the crypt to entire crypt damage and epithelium erosion along with severe inflammation due to cell infiltration. The effects of DSS were observed throughout the DC and limited to the mucosal layer.

Characterization of DSS-induced colitis

Colitis was confirmed in all piglets by day 6 or 7 when feces tested positive for occult blood. Fecal consistency that was characterized as hard, soft, and liquid was recorded throughout the study. nWPI- and SM-fed piglets had longer ($P < 0.05$) duration of severe diarrhea as compared to the control- and pWPI-fed piglets (Figure 3.4.6).

Anti-inflammatory and antioxidant parameters

Control-, SM- and nWPI-fed piglets had greater ($P < 0.05$) MPO activity in DC as compared to the pWPI-fed piglets (Figure 3.4.7). SM-fed piglets had greater ($P < 0.05$) concentrations of TNF-α, IL-8, and IL-18 in the DC as compared to pWPI-fed piglets (Figure 3.4.8). The same trend was observed for the antioxidant parameters analyzed whereby the pWPI-fed piglets showed higher ($P < 0.05$) FRAP values as compared to control and SM-fed piglets in the DC. Plasma FRAP values were similar among all groups (Figure 3.4.9). Interestingly, pWPI-fed piglets demonstrated higher ($P < 0.05$) serum total antioxidant capacity, as measured by the quenching of peroxynitrite, in comparison to the SM and nWPI-fed piglets. The SM-fed piglets also had lower ($P < 0.05$) serum peroxynitrite quenching capacity than control piglets (Figure 3.4.10).

Apoptotic pathway

There were no treatment effects on the abundance of Akt, eNOS (Figure 3.4.11, A-C), cleaved caspase-8 p42, and Bcl-2 (Figure 3.4.12, A-C) in the DC of piglets with DSS-induced colitis were all the same; however, the abundance of cleaved caspase-3 was greater ($P < 0.05$) in the SM-fed piglets as compared to the control-, nWPI- and pWPI-fed piglets (Figure 3.4.12, D).

3.5 DISCUSSION

Growth failure associated with the pediatric piglet model of IBD has been previously shown to be exacerbated when concurrent moderate protein and energy deficiency exists together with gut inflammation (Mackenzie *et al.*, 2003; Harding *et al.*, 2010). In the present study, piglets fed the moderately restricted milk protein deficient diets showed reduced net weight gain, thereby indicating that moderate protein deficiency alone can limit growth in the piglet model of IBD. Moderate protein restricted piglets fed WPI, however, showed significantly lower net weight than SM-fed piglets, which indicates that the quality of milk protein could influence the degree of growth failure associated with moderate protein deficiency during colitis. The lower net weight gain of the WPI piglets could possibly be attributable to the relative lack of tryptophan in whey protein, making it the limiting amino acid in the piglets' diet (Lin *et al.*, 1988). In that regard, WPI-fed piglets received 32% of their tryptophan requirements as compared to 46% of the tryptophan requirement met by SM feeding (Table 3.3.3). Piglet feeding studies have demonstrated the importance of adequate amounts of tryptophan to optimize growth performance for piglets weighing 3 to 5 kg as tryptophan deficiency has been documented to limit weight gain in growing pigs (Guzik *et al.*, 2002; Fent *et al.*, 2005). Likewise, a relationship between growth failure in children and the lack of dietary tryptophan has been documented (Golden, 1991; Allen, 1994). Tryptophan deficiency has been also linked to alterations in linear growth during gut inflammation in animal models (Koniaris *et al.*, 1997; Azooz *et al.*, 2000) and in children with IBD (Ballinger, 2002). Tryptophan supplementation in piglets with DSS-induced colitis has been shown to promote growth, reduce colonic inflammation, and decrease the abundance of major pro-inflammatory cytokines including TNF-α, INF-γ, IL-1β, IL-6, IL-8, and IL-17 (Kim *et al.*, 2010). Interestingly, linear growth was significantly lowered only in the nWPI-fed piglets relative to control piglets, which suggests that pressure processing might have improved the growth promoting quality of whey proteins.

Animal and human studies have consistently shown that high-protein feeding in the form of whey protein concentrates and isolates is associated with

decreased adiposity and increased insulin sensitivity (Bouthegourd *et al.*, 2002; Ha and Zemel, 2003; Belobrajdic *et al.*, 2004; Ward *et al.*, 2008). The inhibition of adiposity by whey protein intake has been linked to its high abundance of leucine that is implicated in playing a key role in the shifting energy from adipose tissue to skeletal muscle (Ha and Zemel, 2003). In that regard, leucine appears to inhibit energy storage in adipocytes as shown by suppression of fatty acid synthase abundance and activity and the stimulation of fatty acid oxidation by skeletal muscle (Sun and Zemel, 2007). In the present study, nWPI feeding was not associated with decreased adiposity, whereas the net fat mass gained in pWPI-fed piglets was significantly lower as compared to both SM- and nWPI-fed piglets. It appears that in the presence of moderate protein deficiency and inflammation may have interfered with the fat mass lowering effects of native WPI, which was counteracted by pressure treatment. It is possible that the improved digestibility associated with pressure-treated WPI (Kubow *et al.*, 2005; Vilela *et al.*, 2006b) may have contributed to greater absorption of bioactive amino acids and peptides, which may have increased insulin sensitivity leading to reduced adiposity as compared to the native form. For example, pressurization may have improved the bioavailability of leucine and leucine peptides in whey proteins that have been shown to act as an insulin secretagogues (Maechler *et al.*, 1999). In addition, there may be a potential lesser bioavailability of leucine in nWPI vs pWPI that could have explained the greater fat mass.

In terms of bone mass, the femur and tibia from WPI-fed piglets had a significantly lower area, BMC and BMD than the control and SM-fed piglets. This latter finding could be related to the relative lack of arginine in WP as WPI-fed piglets received 40% of their arginine requirements as compared to 52% of the arginine requirement provided by SM (Table 3.3.3). Previous studies have demonstrated low arginine intake has been associated with decreased intestinal calcium absorption (Kim and Wu, 2004; Wu *et al.*, 2004).

The moderate protein restricted DSS-induced colitis piglets maintained growth and positive nitrogen balance demonstrating that protein synthesis was higher than breakdown although there was a slower growth rate as compared to

control piglets. When protein intake is insufficient, whole-body protein turnover is reduced to decrease nitrogen excretion to allow for sufficient nitrogen retention for growth, which is a metabolic adaption that improves the chances of survival (Wykes *et al.*, 1996). Thus, it seems that the short-term moderate deficiency maintained sufficient positive nitrogen balance to allow the adequate growth of organs and tissues regardless of a potentially increased whole-body protein turnover due to inflammation. The short term duration of this study likely contributed to the lack of observed differences in nitrogen retention among the protein restricted piglets relative to the well-fed controls. Longer-term moderate protein deficiency during inflammation, however, may cause decreased protein absorption since the shortening of intestinal mucosa worsens with gut inflammatory conditions (Buret, 2005). Thus, long-term moderate protein deficiency may lead to negative nitrogen balance compromising growth and other essential body functions, particularly since there is an implication of greater importance of the small intestine in regulating nitrogen or amino acid metabolism than the liver from studies of neonatal piglets receiving parenteral nutrition (Bertolo *et al.*, 1999).

Muscle wasting has been observed in children with IBD (Castaneda, 2002; O'Sullivan and O'Morain, 2006), which may be due to a combination of protein deficiency and gut inflammation. In the piglet model of IBD, it appears that short-term moderate protein deficiency reduced FSR of proteins by approximately 30% in the masseter muscle (Table 3.4.4). The synthesis of masseter muscle proteins appears to be particularly affected by protein deficiency as long-term severe protein deficiency in piglets decreased FSR in the masseter muscle but did not affect the longissimus dorsi (Wykes *et al.*, 1996). The combination of energy deprivation with protein deficiency may aggravate masseter muscle FSR as piglets with DSS-induced colitis fed a moderate protein and energy restricted diet for 2-wks had a masseter FSR that was approximately 43% lower relative to well-nourished control piglets with colitis (Harding *et al.*, 2010). The masseter muscle has approximately 50% of type I fibers and its major characteristic is a high oxidative capacity (Ringqvist *et al.*, 1982). Thus, this type of fiber has a higher

turnover rate compared to other fibers (Rowlerson *et al.*, 2005) and so protein synthesis in this fiber type may be more sensitive to protein inadequacy.

In this study the total plasma protein, albumin and fibrinogen synthesis rates and concentrations of total plasma protein, albumin, and fibrinogen were not affected by moderate protein deficiency. On the other hand, Mackenzie *et al.* (2003) demonstrated increased albumin FSR in short-term moderate protein and energy restricted piglets with colitis despite no observed changes in plasma albumin concentration levels. The contrasting findings may be explained by previous observations that concurrent energy restriction further compromises hepatic plasma protein synthesis associated with protein malnutrition (Birchenallsparks *et al.*, 1985; Merry *et al.*, 1987). The increased albumin synthesis without a corresponding elevation in its plasma concentration shown by Mackenzie *et al.* (2003) also suggested increased loss or catabolism of plasma albumin during colitis. To investigate the possibility of leakage of albumin through the descending and spiral colons, ileum mucosa, and urinary losses, the present study added a well-nourished, non-colitis piglet reference group (Harding *et al.*, 2008) to compare healthy piglets with the DSS-induced colitis piglets. Strikingly, albumin concentrations from colonic tissues and urine were increased as compared to the reference group, which suggests protein losses occurred as a result of colitis. A mechanism of urinary loss of albumin during inflammation has been proposed whereby increased plasma levels of pro-inflammatory cytokines such as TNF-α affect the glomerular permeability of albumin by increasing superoxide production in the glomerular epithelial cells causing an increase in albumin excretion (Ebinc *et al.*, 2008). In addition, Steinfeld *et al.* (1960) and colleagues have studied albumin kinetics measured with ^{131}I-albumin in hypoalbuminemic patients with IBD and demonstrated that increased synthesis of albumin was not sufficient to compensate for the more severely increased rate of removal from circulation through gastrointestinal losses (Steinfeld *et al.*, 1960). In addition, the extravascularization of albumin into interstitial fluid during an acute phase response may contribute to additional albumin losses (Blumenkrantz *et al.*,

1981). It thus appears that losses of albumin in urine and colon may contribute to the compromised albumin balance in IBD.

Disease severity was observed in a greater degree in the control- and SM-fed piglets as exhibited by their high total histological scores showing moderate to severe inflammation, crypt damage, and mucosa destruction. The improved colonic histopathology in piglets receiving WPI might be due in part to those piglets receiving 57% of their cysteine + methionine requirements as opposed to 37% of the cysteine + methionine requirement provided by SM feeding (Table 3.3.3). One study has recently demonstrated that piglets with DSS-induced colitis treated with L-cysteine had markedly improved colon histology including lower inflammation, decreased crypt damage, and increased intestinal regeneration (Kim *et al.*, 2009). Cysteine is the rate-limiting substrate for GSH synthesis, an important intracellular antioxidant that plays an important role in intestinal epithelial cell health and gut function due to the highly oxidant nature (Oz *et al.*, 2007). In addition, cysteine is able to spare methionine between 50 and 80% (Shoveller *et al.*, 2005) and also is a precursor of taurine synthesis (Iwata and Baba, 1982). Both cysteine and taurine are essential for the host defence against oxidative stress (Son *et al.*, 1998a; Son *et al.*, 1998b). Cysteine is also crucial in maintaining gut barrier function as it is important for mucin production (Miller *et al.*, 2002; Noda *et al.*, 2002). The lower degree of colonic histopathology seen in the WP versus SM-fed piglets could also be partly due to their greater threonine intake as piglets fed WP received approximately 62% of their threonine requirements as compared to 44% of the threonine requirement as provided by SM feeding (Table 3.3.3). Dietary restriction of threonine has shown to exacerbate colitis in mouse and rat models of IBD (Shekels *et al.*, 1998; Faure *et al.*, 2005). A large proportion of dietary threonine (up to 60%) is retained by the gut of the healthy pig (Stoll *et al.*, 1998) or human (Fuller *et al.*, 1994) intestine. Threonine is one of the main essential amino acids utilized for mucin production, which acts as both a lubricant and as a physical barrier between luminal contents and the mucosal surface (Shirazi *et al.*, 2000; Einerhand *et al.*, 2002). Because intestinal mucins are particularly enriched in threonine as compared with other

intestinal proteins (Vanklinken, 1995), the high retention of dietary threonine by the gut may reflect the demand for this amino acid in mucin synthesis.

The pro-inflammatory effects in the DSS-treated piglets seen in the present study as shown by the infiltration of inflammatory cells into the mucosa of DC has previously been observed in the DSS-induced colitis piglet model (Hirata *et al.*, 2001). The intake of the pWPI showed significant anti-inflammatory and antioxidant effects in the piglet model of DSS-induced colitis, both systemically and in the DC tissue. Although both nWPI and pWPI-fed piglets demonstrated the lowest degree of total histopathology in the DC, the pWPI-fed piglets relative to all other dietary treatments had the lowest MPO activity, an indirect measure of neutrophil infiltration and inflammation (Klebanoff, 2005). The decreased colonic inflammation shown in the DC of pWPI-fed piglets coincided with lower colonic levels of the pro-inflammatory cytokines TNF-α, IL-8 and IL-18, which likely attenuated colonic damage and inflammation. IL-8 is a neutrophil chemoattractant and activates the immune response (Daig *et al.*, 1996). A pro-inflammatory role has been suggested for IL-8 and its regulatory cytokines IL-1 and TNF-α in terms of mediating neutrophil infiltration of the gut wall in IBD (Yamamoto-Furusho *et al.*, 2008; Matowicka-Karna *et al.*, 2009). IL-18 is another cytokine shown to be induced in DSS-induced colitis models (Desai *et al.*, 2007). IL-18 has been shown to induce an inflammatory response mainly in synergism with IL-12 in the colonic lamina propria (Villani *et al.*, 2009) via induction of infiltration of neutrophils and activation of effector T cells to sustain the inflammatory cascade (Siegmund, 2010). The attenuated IL-18 levels seen with pWPI feeding may have reduced colonic damage, which has been observed in rats with DSS-induced colitis treated with a protein that blocked IL-18 bioactivity (Sivakumar *et al.*, 2002). In another study, mice with DSS-induced colitis model treated with an antimurine IL-18 antiserum demonstrated the neutralization of IL-18 by decreased levels of IFN-γ and TNF-α (Siegmund *et al.*, 2001).

Although intake of native whey did not provide significant anti-inflammatory effects in the piglet model of DSS-induced colitis, other studies have observed the decrease of pro-inflammatory (TNF-α and IL-1β) and down-regulation of the

NF-κB pathway in rats with TNBS-induced colitis provided with the whey protein lactoferrin (Togawa *et al.*, 2002). Pressure processing of WPIs results in changes in the secondary and tertiary structures that are associated with the release of unique bioactive peptides that exert antioxidant and anti-inflammatory effects (Kubow *et al.*, 2005; Vilela *et al.*, 2006b; Kishta *et al.*, 2009). It is thus conceivable that the increased digestibility of pWPI leading to the release more bioactive peptides and amino acids played a role in the greater anti-inflammatory properties of pWPI. As pressurization of WPI is associated with higher intracellular GSH concentrations (Vilela *et al.*, 2006b; Zavorsky *et al.*, 2007), intake of pWPI could have induced decreased inflammatory status and improved gut function during DSS-induced colitis by increasing antioxidant defences via GSH synthesis. A DSS-induced colitis piglet study with cysteine supplementation has shown to attenuate DSS-induced weight loss and intestinal permeability, reduced local chemokine expression and neutrophil influx, and markedly improved colon histology. In addition, cysteine supplemented piglets demonstrated down regulated colonic pro-inflammatory cytokines (TNF-α and IL-6) production at the gene and protein abundance levels (Kim *et al.*, 2009).

The improved histopathology and lower inflammation shown with pWPI feeding is likely partly mediated by an increased antioxidant activity. pWPI was also associated with higher colonic FRAP values as well as an increased serum scavenging capacity of peroxynitrite, which might be related to previous observations regarding increased content of the intracellular antioxidant GSH induced by pWPI (Vilela *et al.*, 2006a; Zavorsky *et al.*, 2007). Oxidative stress has been indicated to play a key pathophysiological role in IBD as shown by increased concentrations of tissue oxidative stress markers and diminished GSH status associated with impaired intestinal GSH synthesis (Ardite *et al.*, 2000). Moreover, antioxidant supplementation of DSS-treated mice was shown to lower diarrhea and mucosal inflammation and circulating TNF-α levels that accompanied the acute colitis as well as normalize GSH status (Oz *et al.*, 2005). The improved peroxynitrite capacity seen with pWPI is particularly significant since suppression of mouse colitis has been observed by reducing levels of

peroxynitrite using a COX-2 inhibitor and peroxisome proliferator-activated receptor (PPAR) ligands (Kohno *et al.*, 2005). In a rat model of DNBS-induced colitis, increased serum peroxynitrite scavenging was associated with reduced colonic MPO activity and systemic TNF-α levels after administration with a green tea extract (Cuzzocrea *et al.*, 2005). In other *in vivo* studies of colonic models of gut inflammation, increased FRAP activity in colonic tissue has been associated with colonic protective effects following intake of food products such as extracts of bark (Nirmal *et al.*, 2011), pomegranate (Espin *et al.*, 2010), and wild flower (Abdollahi *et al.*, 2010). Previous piglet work has shown that restriction in the intake of essential amino acids resulted in an increased frequency of liquid feces (Le Bellego and Noblet, 2002). In that respect, it is interesting to note that the antioxidant and anti-inflammatory effects of pWPI were also associated with significant protective effects with a lesser occurrence of severe diarrhea relative to all the other protein restricted groups.

This study also investigated for possible an anti-apoptotic effects that might be associated with the WPI feeding by examining proteins that have been previously shown to contribute to apoptosis via the caspase-3 pathway in animals exposed to DSS (Rachmilewitz *et al.*, 2002; Sakata *et al.*, 2007; Mennigen *et al.*, 2009). Other animal studies have demonstrated food components such as galectins (Paclik *et al.*, 2008), ginkgo biloba extracts (Kotakadi *et al.*, 2008), and butyrate (Hernandez *et al.*, 2001) can exert anti-apoptotic effects by decreasing the abundance of caspase-3. Both WP-fed and control animals were associated with decreased apoptosis as these groups had significantly lower caspase-3 activation relative to the SM-fed piglets. Future studies are needed to further investigate other pathways leading to apoptosis that might be specifically related to the protective effects associated with WPI intake, which could possibly involve upstream inhibition of calpain and caspase-12 pathways (Kerbiriou *et al.*, 2009) or caspase-10 (Wang *et al.*, 2001).

In conclusion, this study demonstrated the important effects of protein quantity and quality in a pediatric piglet model of IBD. In particular, pWPI feeding of protein-restricted piglets exerted anabolic effects, decreased colonic pro-

inflammatory markers, increased DC and systemic antioxidant status, and WPI-fed piglets showed suppression of the caspase-3 apoptotic pathway. More studies are needed to further investigate the mechanisms involved in the antioxidant and anti-inflammatory effects of WPI, especially pWPI, which has the potential as a potent nutraceutical agent for the treatment of children with IBD.

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Figure 3.3.1 Study protocol of the 12 d study including surgery, adaptation to diet, DSS administration, nitrogen balance, and stable isotope infusion.

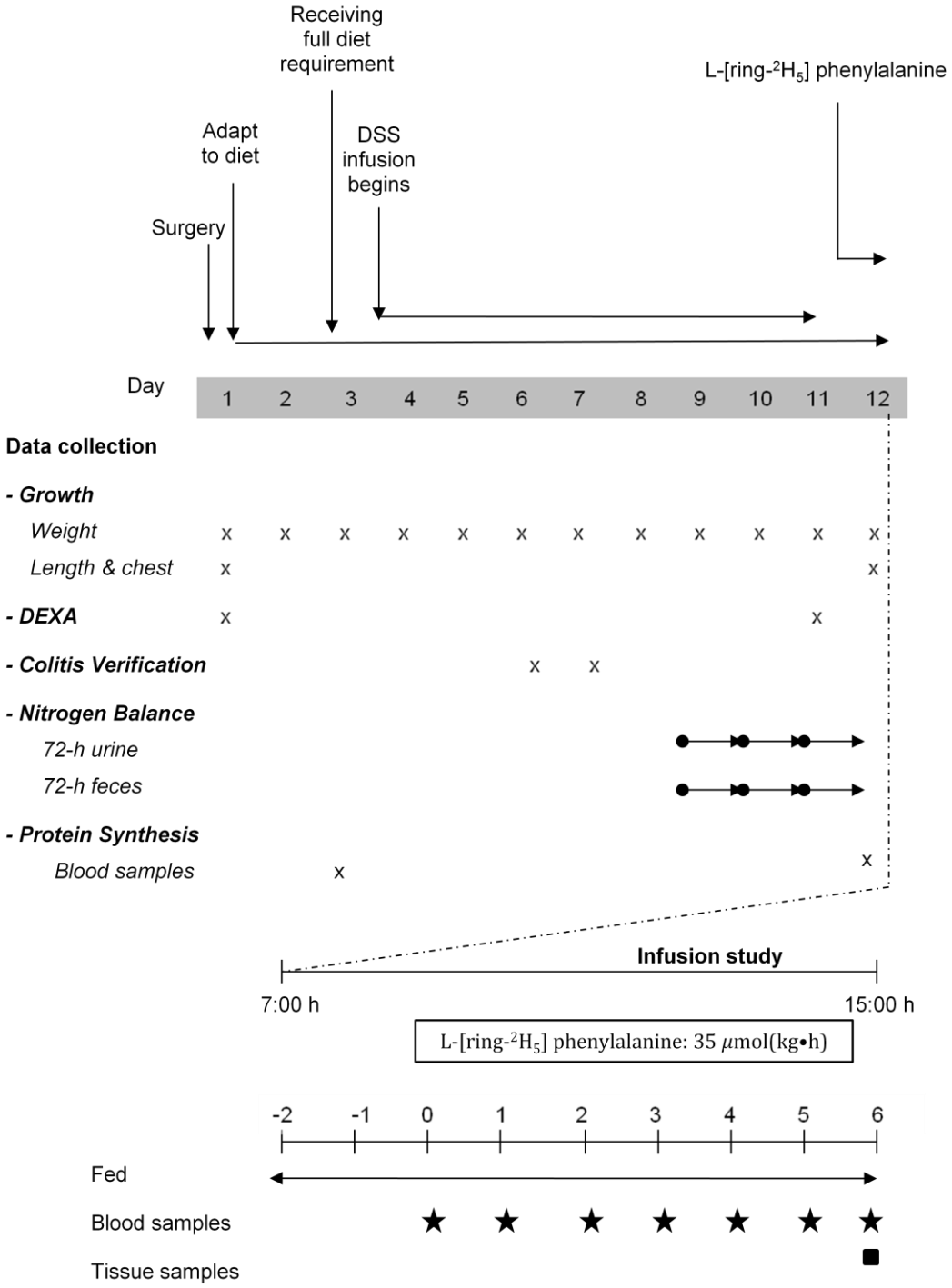


Table 3.3.1 Composition of liquid diets administrated to piglets.

Component in liquid diet as fed	unit	Control[1]	SM[2]	nWPI[3]	pWPI[4]
Energy	kcal/L	824	830	847	859
Protein	g/L	55	28	28	29
Carbohydrate	g/L	79	94	96	98
Lipid	g/L	32	38	39	39
Skim milk powder[5]	g/L	152	77	0	0
Whey protein isolate[6]	g/L	0	0	31	31
Lactose[7]	g/L	0	58	102	103
Soybean oil[8]	g/L	8	10	10	10
Safflower[8]	g/L	15	17	18	18
Coconut oil[8]	g/L	7	9	9	9
Flax seed oil[8]	g/L	2	2	2	2
Vitamin mix[9]	g/L	0.13	0.13	0.14	0.14
Mineral mix[10]	g/L	4.64	8.81	14.42	14.42
Total to dilute to 1L liquid diet	g/L	189	182	187	188

[1]Control = 100% skim milk (SM); [2]SM = 50% SM; [3]nWPI = 50% native WPI; [4]pWPI = 50% pressurized WPI.[5]Parmalat Canada Production and Distribution, Montreal, Canada. [6]INPRO 90.Vitalus Nutrition Inc., Abbotsford, Canada. [7]Lynn Diary Inc., Granton, WI, USA. [8]MP Biomedicals Corporate, Santa Ana, CA, USA. [9]Appendix 1. [10]Appendix 2.

Table 3.3.2 Amino acid profile of whey protein isolate, skim milk, and NRC diet (g/100g protein).

Amino Acids	Whey protein isolate[1]	Skim milk[2]	NRC Diet [3]
Leucine	12.3	8.7	9.2
Isoleucine	6.3	4.2	4.1
Valine	5.3	4.8	4.8
Cysteine	2.3	0.6	NA[4]
Methionine	2.3	1.8	NA
Cysteine + Methionine	4.6	2.4	3.5
Phenylalanine	3.5	4.8	NA
Tyrosine	3.6	4.5	NA
Phenylalanine + Tyrosine	7.1	9.3	8.5
Tryptophan	0.9	1.5	1.2
Threonine	5.6	4.5	3.8
Lysine	8.9	8.1	5.2
Histidine	2.2	3.0	2.7
Arginine	2.4	3.3	6.3
Alanine	5.4	3.0	NA
Aspartic Acid	11.2	7.8	NA
Glutamic Acid	14.3	23.2	NA
Glycine	1.5	1.8	NA
Proline	5.1	9.6	NA
Serine	6.8	4.8	NA

[1]Adapted from Vitalus (2004), [2]Adapted from Csapó-Kiss *et al.*, (1995), [3]Adapted from NRC (1998), [4]Not available.

Table 3.3.3 Amino acid content for each diet in relation to the amino acid requirement in piglets[1]

REQUIREMENTS[1]			DIETS							
	Amt[2]/ day	BW	Control[3]		SM[4]		nWPI[5]		pWPI[6]	
Amino Acids	**g**	**g/(kg•d)**	**g/(kg•d)**	**% Req**	**g/(kg•d)**	**% Req**	**g/(kg•d)**	**% Req**	**g/(kg•d)**	**% Req**
Leucine	4.70	0.83	0.99	119	0.48	58	0.69	83	0.70	84
Isoleucine	2.50	0.44	0.61	139	0.30	68	0.35	80	0.36	82
Valine	3.15	0.55	0.68	123	0.33	60	0.31	57	0.32	58
Cysteine	NA[7]	NA	0.09	NA	0.05	NA	0.13	NA	0.13	NA
Methionine	NA	NA	0.25	NA	0.12	NA	0.13	NA	0.13	NA
Cysteine + Met[8]	2.65	0.46	0.35	75	0.17	37	0.26	56	0.26	57
Phenylalanine	NA	NA	0.49	NA	0.24	NA	0.19	NA	0.20	NA
Tyrosine	NA	NA	0.49	NA	0.24	NA	0.20	NA	0.20	NA
Phe[9] + Tyrosine	4.35	0.77	0.98	127	0.48	62	0.39	51	0.40	52
Tryptophan	0.85	0.15	0.14	95	0.07	46	0.05	32	0.05	32
Threonine	2.90	0.51	0.46	89	0.22	44	0.31	61	0.32	62
Lysine	4.65	0.82	0.80	98	0.39	48	0.50	61	0.51	62
Histidine	1.50	0.26	0.27	106	0.13	51	0.12	47	0.12	48
Arginine	1.90	0.34	0.37	108	0.18	52	0.13	40	0.14	40
Alanine	-	-	0.35	-	0.17	-	0.30	-	0.31	-
Aspartic acid	-	-	0.77	-	0.37	-	0.62	-	0.63	-
Glutamic acid	-	-	2.12	-	1.03	-	0.83	-	0.84	-
Glycine	-	-	0.21	-	0.10	-	0.09	-	0.09	-
Proline	-	-	0.98	-	0.48	-	0.29	-	0.29	-
Serine	-	-	0.55	-	0.27	-	0.38	-	0.39	-

[1]Based on average piglet body weight in range of 3 - 5 and 5 - 10 kg throughout the 12 days of the study (NRC 1998). [2]Amount. [3]Control = 100% skim milk (SM); [4]SM = 50% SM; [5]nWPI = 50% native WPI; [6]pWPI = 50% pressurized WPI. [7]NA = Not available. [8]Methionine. [9]Phenylalanine.

Table 3.3.4 Intake of each vitamin for each diet in relation to vitamin requirement in piglets[1]

REQUIREMENTS[1]				CUSTOM DIETS[2]	
			Amt[3]		
Vitamins	**Amt/day**		**amt/(kg•d)**	**amt/(kg•d)**	**% Req**
Vitamin A	825.00	IU	142.08	142.13	100
Vitamin D3	78.00	IU	13.61	13.61	100
Vitamin E	6.00	IU	1.03	1.03	100
Vitamin K	0.19	mg	0.03	0.03	100
Vitamin B6	0.63	mg	0.11	0.11	100
Vitamin B12	6.88	µg	1.21	1.21	100
Biotin	0.03	mg	0.01	0.01	100
Folacin	0.12	mg	0.02	0.02	100
Niacin	6.25	mg	1.13	1.13	100
Pantothenic Acid	4.00	mg	0.71	0.71	100
Riboflavin	1.38	mg	0.24	0.24	100
Thiamin	0.44	mg	0.08	0.08	100

[1]Based on average piglet body weight in range of 3 - 5 and 5 - 10 kg throughout the 12 days of the study (NRC 1998). [2]Piglets receiving SM, nWPI, and pWPI had the same intake as the control group. [3]Amount.

Table 3.3.5 Intake of each mineral for each diet in relation to mineral requirement in piglets[1]

REQUIREMENTS[1]			CUSTOM DIETS[2]	
		Amt[3]		
Minerals	**Amt/day**	**amt/(kg•d)**	**amt/(kg•d)**	**% Req**
Calcium	3.13 g	0.54	0.54	100
Phosphorus	2.50 g	0.43	0.43	100
Sodium	0.82 g	0.14	0.14	100
Chlorine	0.82 g	0.14	0.14	100
Magnesium	0.15 g	0.03	0.03	100
Potassium	1.08 g	0.19	0.18	100
Copper	2.25 mg	0.39	0.38	100
Iron[4]	37.50 mg	6.52	6.38	100
Manganese	1.50 mg	0.26	0.26	100
Selenium	0.12 mg	0.02	0.02	100
Zinc[5]	37.50 mg	6.52	10.7	163[7]

[1]Based on average piglet body weight in range of 3 - 5 and 5 - 10 kg throughout the 12 days of the study (NRC 1998). [2]Piglets receiving SM, nWPI, and pWPI had the same intake as the control group. [3]Amount. [4]Iron requirements are those of piglets receiving phytate free formula type liquid diet. [5]Zinc requirements are those of piglets receiving phytate free formula type liquid diet. Increased amount provided due to increased losses during inflammatory stress (Hendricks and Walker, 1988).

Table 3.3.6 Histological grading of colitis.

Feature graded	Grade	Description
Inflammation*	0	None
	1	Slight
	2	Moderate
	3	Severe
Depth Affected*	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Regeneration*	4	No tissue repair
	3	Surface epithelium not intact
	2	Regeneration with crypt depletion
	1	Almost complete regeneration
	0	Complete regeneration or normal tissue
Crypt damage*	0	None
	1	Basal 1/3 damaged
	2	Basal 2/3 damaged
	3	Only surface epithelium intact
	4	Entire crypt and epithelium lost

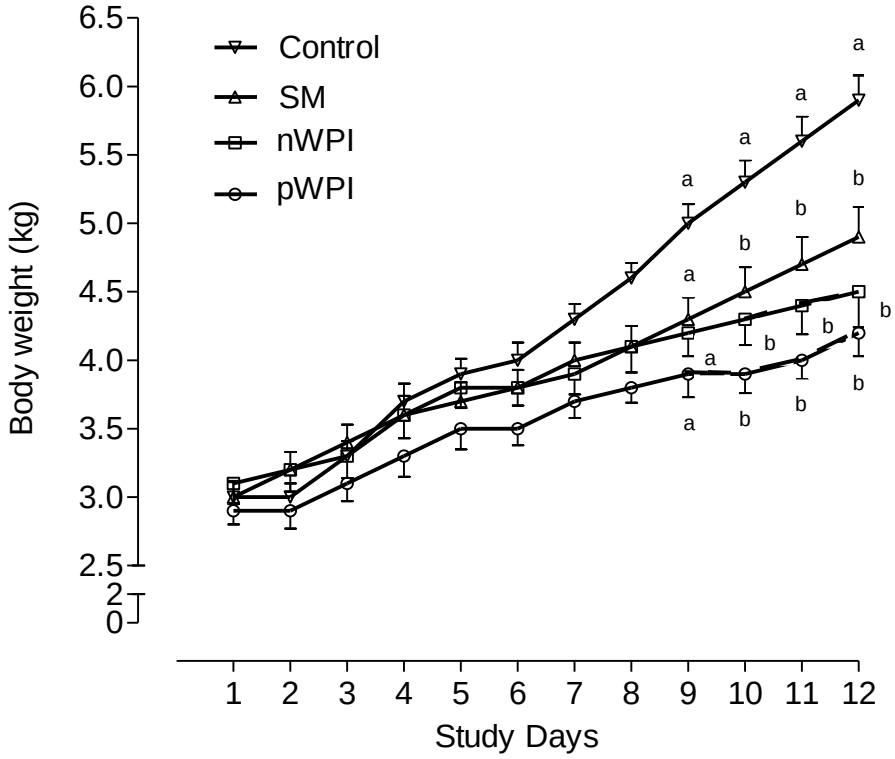
*These features were also quantified as to the percentage involvement by the disease process: (1) 1–25%, (2) 26–50%, (3) 51–75%, (4) 76–100%.

Table 3.4.1 Anthropometric data of piglets with DSS-induced colitis for the 12 days of the study[1]

Anthropometry	Control	SM	nWPI	pWPI	SEM	GLM LSM P Value
Initial weight (kg)	3.0	3.0	3.1	2.9	0.1	ns (0.39)
Final weight (kg)	5.9^{a}	4.9^{bc}	4.5^{bc}	4.2^{c}	0.2	<0.01
Net weight gain (g/(kg•d)	62.4^{a}	47.6^{b}	34.1^{c}	36.7^{c}	2.8	<0.01
Length growth (cm)	13.6^{a}	10.9^{ab}	8.4^{b}	9.9^{ab}	1.1	0.02
Chest growth (cm)	7.5^{a}	5.9^{ab}	4.5^{ab}	4.3^{b}	0.8	0.04
Length growth (% of initial)	30.3^{a}	24.9^{ab}	18.6^{b}	23.2^{ab}	2.8	0.05
Chest growth (% of initial)	24.0	19.0	14.6	14.3	2.8	ns (0.07)

[1]Data are means and pooled SEM, n = 8 per treatment group. Means in a row with superscripts without a common letter differ $P < 0.05$. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Figure 3.4.1 Daily body weight of piglets with DSS-induced colitis for the 12 days of the study[1]



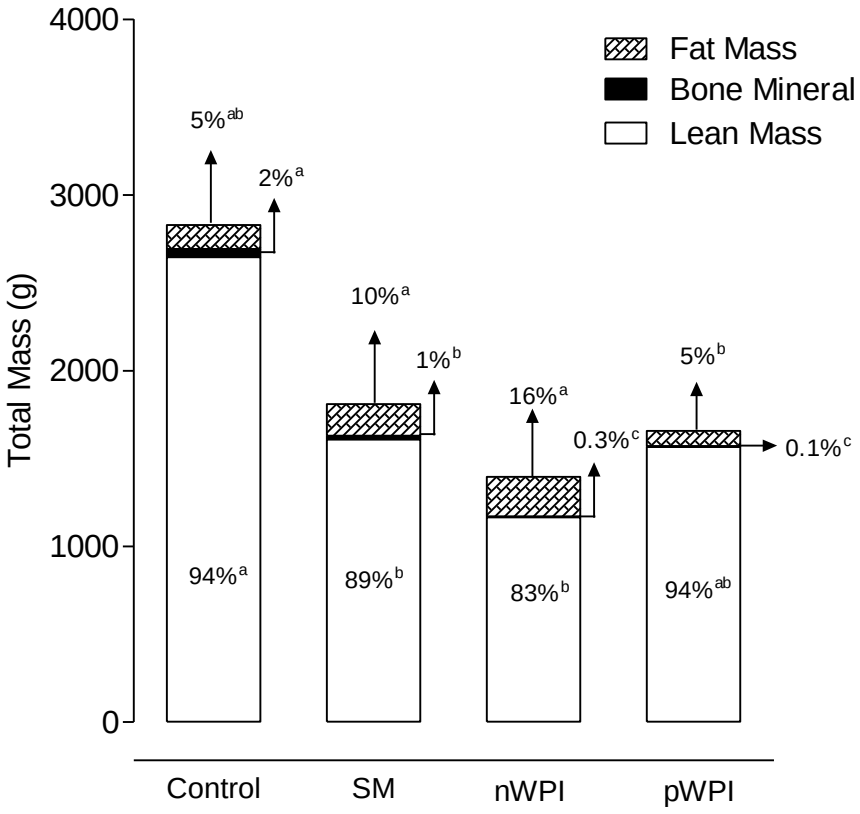
[1]Data are means and SEM, n = 8 per treatment group. Means with superscripts without a common letter differ P < 0.05 (repeated measures ANOVA). Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Table 3.4.2 Body composition of piglets with DSS-induced colitis for the 12 days of the study[1]

Body Composition	Control	SM	nWPI	pWPI	SEM	GLM LSM P Value
Initial (g)						
Lean mass	2870[a]	2786[ab]	3022[a]	2769[b]	135	<0.01
Fat mass	114	109	107	116	14	ns (0.97)
Bone mineral	54	58	61	53	3	ns (0.19)
Lean/Fat ratio	25	26	28	24	6	ns (0.23)
Final (g)						
Lean mass	5515[a]	4395[b]	4187[b]	4247[b]	202	<0.01
Fat mass	251[a]	266[a]	334[a]	206[b]	35	0.03
Bone mineral content	99[a]	78[b]	65[c]	56[c]	3	<0.01
Lean/Fat ratio	22	17	13	21	4	ns (0.09)
Net gain (g)						
Lean mass	2645[a]	1609[b]	1165[b]	1478[b]	167	<0.01
Fat mass	137[ab]	157[a]	227[a]	90[b]	26	<0.01
Bone mineral content	45[a]	20[b]	4[c]	3[c]	2	<0.01
Lean/Fat ratio	19[a]	10[b]	5[b]	16[a]	3	<0.01

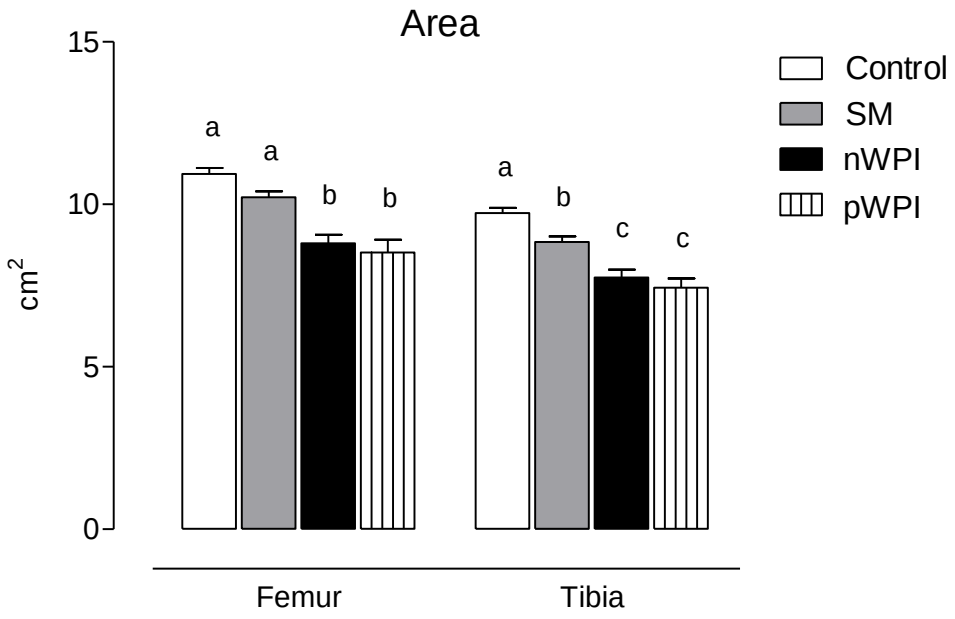
[1]Data are means and pooled SEM, n = 8 per treatment group. Means in a row with superscripts without a common letter differ $P < 0.05$. LSM adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

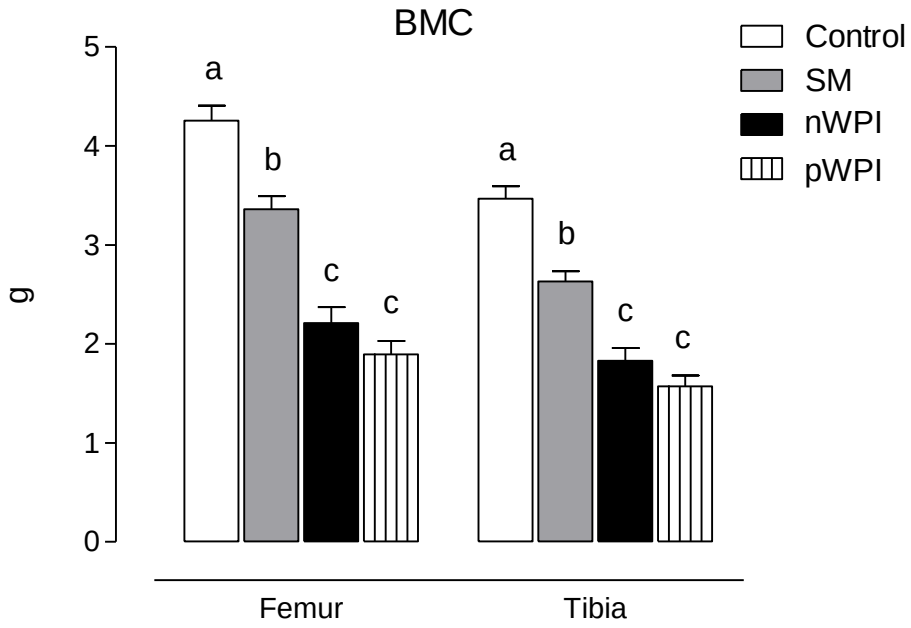
Figure 3.4.2 Net body composition gain in piglets with DSS-induced colitis for the 12 days of the study[1]

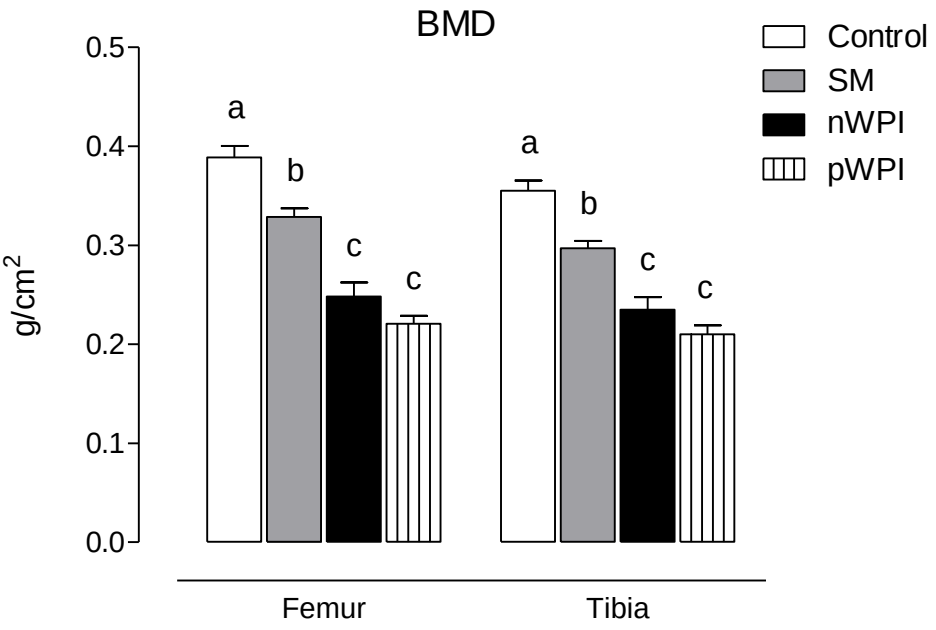


[1]Data are means, n = 8 per treatment group. Means with superscripts without a common letter differ $P < 0.05$. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Figure 3.4.3 Femur and tibia area, bone mineral content and bone mineral density in piglets with DSS-induced colitis[1]







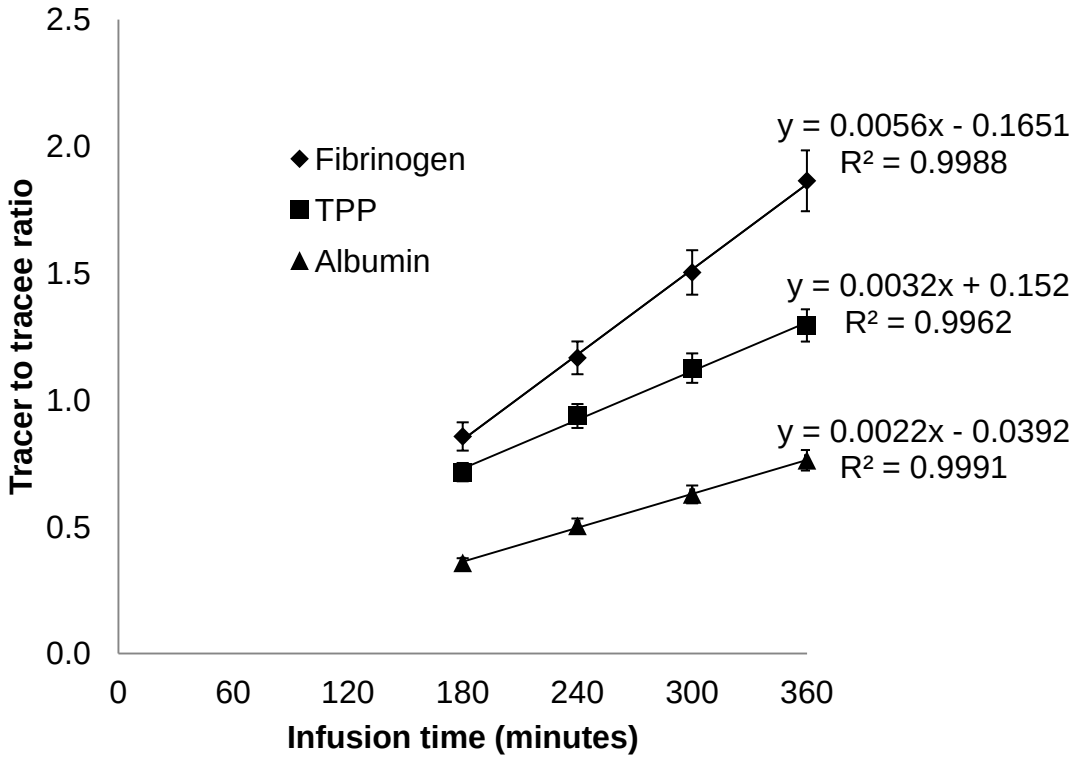
[1]Data are means and SEM, n = 8 per treatment group. Means with superscripts without a common letter differ P < 0.05. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Table 3.4.3 Nitrogen utilization in piglets with DSS-induced colitis[1]

Variables	Control	SM	nWPI	pWPI	SEM	GLM LSM P Value
N intake (g/kg•d)	2.23[a]	1.22[b]	1.14[c]	1.13[c]	0.01	<0.01
Urinary N (g/kg•d)	0.42[a]	0.16[b]	0.22[b]	0.22[b]	0.03	<0.01
Fecal N (g/kg•d)	0.07	0.09	0.05	0.08	0.01	ns (0.22)
N excretion (g/kg•d)	0.49[a]	0.26[b]	0.27[b]	0.30[b]	0.03	0.01
Nitrogen balance (g/kg•d)	1.74[a]	0.95[b]	0.87[bc]	0.82[c]	0.03	<0.01
N retention (%)	78.0	78.4	76.0	73.0	2.40	ns (0.60)

[1]Data are means and pooled SEM, n = 8 per treatment group. Means in a row with superscripts without a common letter differ $P < 0.05$. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Figure 3.4.4 Typical pattern of L-[ring-$^{2}H_5$]phenylalanine tracer incorporation into newly synthesized liver-derived plasma proteins of control piglets with DSS-induced colitis[1]



[1]Data are means and SE of L-[ring-$^{2}H_5$]phenylalanine (n=8) of fibrinogen, total plasma proteins (TPP) and albumin.

Table 3.4.4 Muscle fractional synthesis rates in piglets with DSS-induced colitis[1]

FSR (%/day)	Control	SM	nWPI	pWPI	SEM	GLM LSM P Value
Longissimus dorsi (sarcoplasmic)	15.5	13.3	11.4	12.8	1.7	ns (0.43)
Longissimus dorsi (myofibrillar)	14.1	11.5	10.0	10.3	1.5	ns (0.21)
Masseter (sarcoplasmic)	18.8[a]	14.1[b]	13.0[b]	14.4[b]	1.2	<0.01
Masseter (myofibrillar)	16.0	12.4	11.9	13.9	1.3	ns (0.07)

[1]Data are means and pooled SEM, n = 8 per treatment group. Means in a row with superscripts without a common letter differ P < 0.05. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Table 3.4.5 Visceral tissues fractional synthesis rates in piglets with DSS-induced colitis[1]

FSR (%/day)	Control	SM	nWPI	pWPI	SEM	GLM LSM P Value
Liver	88.0	64.5	75.0	73.1	20.0	ns (0.87)
Jejunum mucosa	139.3[a]	88.2[ab]	82.8[b]	104.3[ab]	14.0	0.03
Ileum mucosa	186.7	160.1	142.5	176.5	22.3	ns (0.53)
Spiral colon	83.8	55.5	54.3	55.9	13.6	ns (0.37)
Descending colon	470	22.6	32.6	41.4	11.8	ns (0.50)

[1]Data are means and pooled SEM, n = 8 per treatment group. Means in a row with superscripts without a common letter differ $P < 0.05$. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Table 3.4.6 Plasma protein synthesis rates and concentrations in piglets with DSS-induced colitis for the 12 days of the study[1]

	Control	SM	nWPI	pWPI	SEM	GLM LSM P Value
Fibrinogen						
FSR[2] (%/d)	72	88	114	150	28	ns (0.23)
ASR[3] (mg/(kg•d))	121	147	185	230	68	ns (0.70)
Concentration (g/L)	2.9	2.9	2.6	2.3	0.3	ns (0.54)
Total Protein						
FSR (%/d)	51	53	67	75	12	ns (0.49)
ASR (mg/(kg•d))	1257	1071	1583	1806	302	ns (0.34)
Concentration (g/L)	41	35	38	40	2	ns (0.06)
Albumin						
FSR (%/d)	40	35	45	45	9	ns (0.86)
ASR (mg/(kg•d))	524	368	550	582	126	ns (0.64)
Concentration (g/L)	22	17	20	21	1	ns (0.12)

[1]Data are means and pooled SEM, n = 8 per treatment group. Means in a row with superscripts without a common letter differ $P < 0.05$. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI. [2]Fractional Synthesis Rate. [3]Absolute Synthesis Rate.

Table 3.4.7 Tissue and urine albumin concentrations in piglets with DSS-induced colitis[1]

Tissue and urine	Control	SM	nWPI	pWPI	SEM	GLM LSM P Value	REF[2]
Total protein (µg/g tissue)							
Descending colon	16	15	14	16	1.0	ns (0.13)	15
Spiral colon	26	25	29	24	2.0	ns (0.51)	24
Ileum mucosa	24[a]	37[b]	28[ab]	30[ab]	3.0	0.04	37
Albumin concentration (ng/g protein)							
Descending colon	209	257	245	222	26	ns (0.57)	182
Spiral colon	206	199	165	198	23	ns (0.70)	123
Ileum mucosa	384[a]	246[b]	378[ab]	316[ab]	39	0.04	150
Urine (µg/kg•d)	2.0	1.3	1.7	1.7	0.4	ns (0.74)	1.0

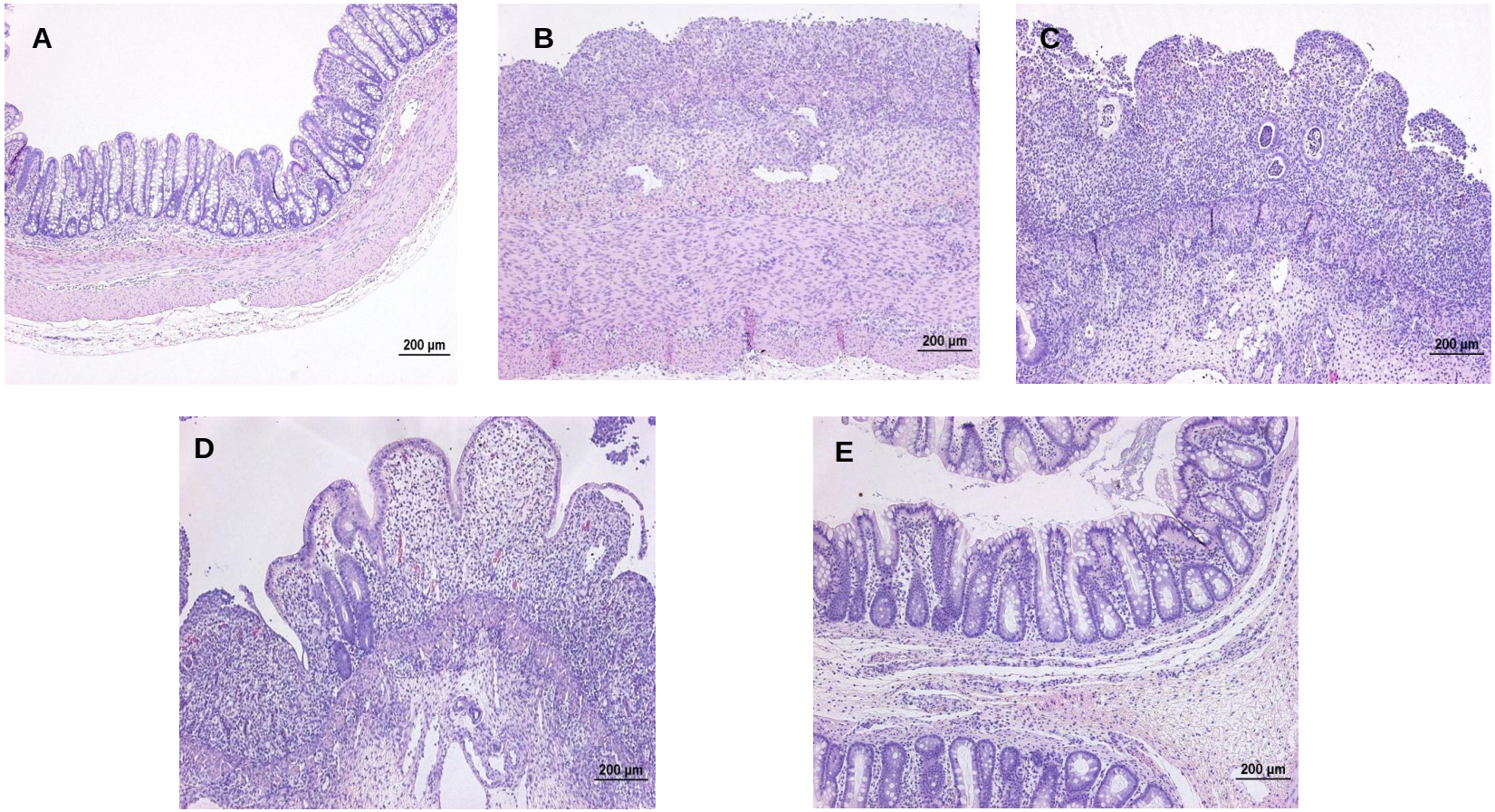
[1]Data are means and pooled SEM, n = 8 per treatment group. Means in a row with superscripts without a common letter differ P < 0.05. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI. [2]Reference (Harding *et al.*, 2008).

Table 3.4.8 Characterization of disease severity and histological impact in the descending colon in piglets with DSS-induced colitis[1]

Descending colon	**Control**	**SM**	**nWPI**	**pWPI**	**SEM**	**GLM LSM P Value**	**REF[2]**
Inflammation							
Severity	2.1	2.6	1.8	1.6	0.3	ns (0.08)	0.6
Extent	3.4^{ab}	3.8^{a}	3.0^{ab}	2.0^{b}	0.3	0.02	0.6
Total	7.1^{ab}	9.9^{a}	5.4^{ab}	3.2^{b}	1.2	0.03	0.4
Depth Affected							
Severity	2.0	2.0	1.8	1.6	0.2	ns (0.43)	0.6
Extent	3.3^{ab}	3.8^{a}	3.4^{ab}	2.0^{b}	0.3	0.01	0.6
Total	6.5^{ab}	7.5^{a}	5.9^{ab}	3.1^{b}	0.8	0.04	0.4
Regeneration							
Severity	2.5	3.0	1.5	1.9	0.4	ns (0.11)	0.0
Extent	3.4	2.5	2.8	2.4	0.4	ns (0.47)	4.0
Total	8.4	7.5	4.1	4.5	1.9	ns (0.11)	0.0
Crypt Damage							
Severity	3.0	3.4	2.5	2.3	0.4	ns (0.44)	0.0
Extent	2.9	3.6	3.1	3.1	0.4	ns (0.61)	4.0
Total	8.6	12.2	7.8	7.2	2.0	ns (0.37)	0.0
Total Score	30.7^{a}	37.0^{a}	23.1^{b}	17.9^{b}	4.5	0.04	0.8

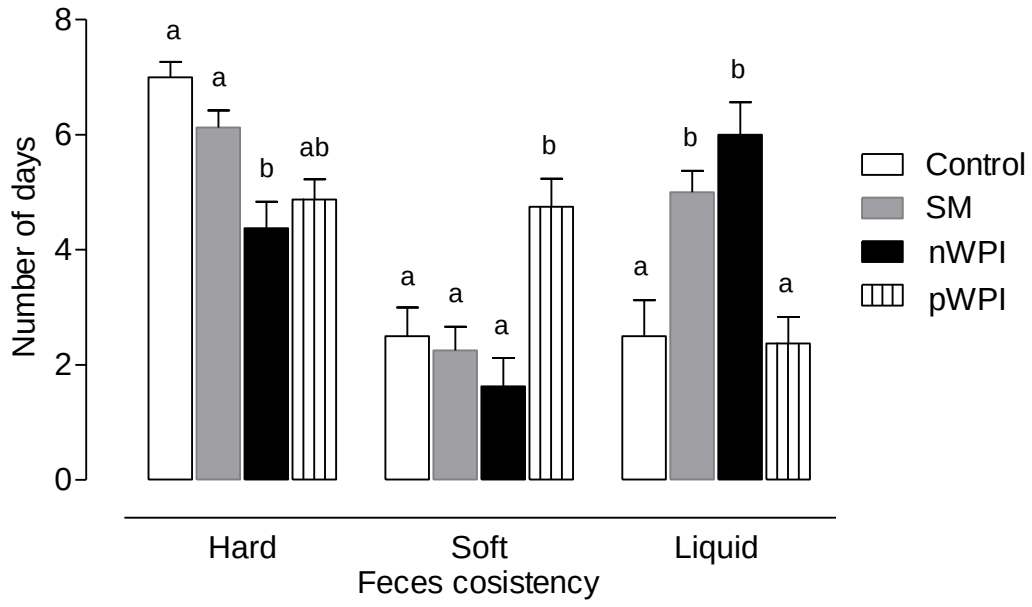
[1]Data are means and pooled SEM, n = 8 per treatment group. Means in a row with superscripts without a common letter differ $P < 0.05$. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI. [2]Reference: healthy piglets (Harding *et al.*, 2008).

Figure 3.4.5 Histological micrographs (hematoxylin and eosin stained; original magnification 200 x) representing the descending colon damage in piglets with DS-induced colitis.



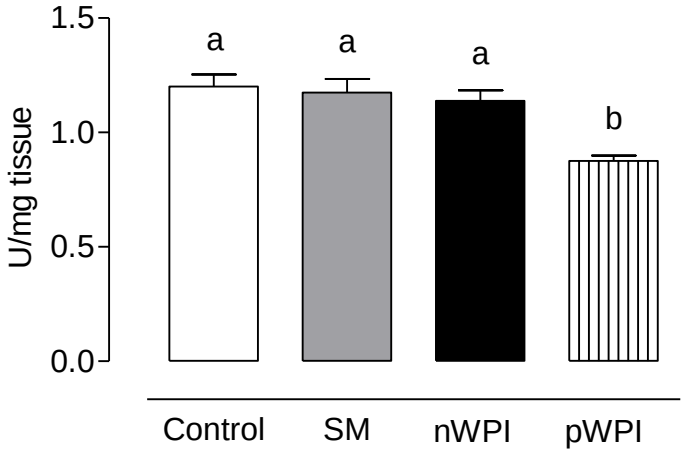
(**A**) Healthy REF piglet (Harding *et al.*, 2008), Range of total histological score (TS) = 0 - 2; (**B**) Control, TS = 14 - 52; (**C**) SM, TS = 28 - 52; (**D**) nWPI, TS = 10 - 38; (**E**) pWPI, TS = 8 - 24.

Figure 3.4.6 Feces consistency in total number of days in the experiment in piglets with DSS-induced colitis[1]



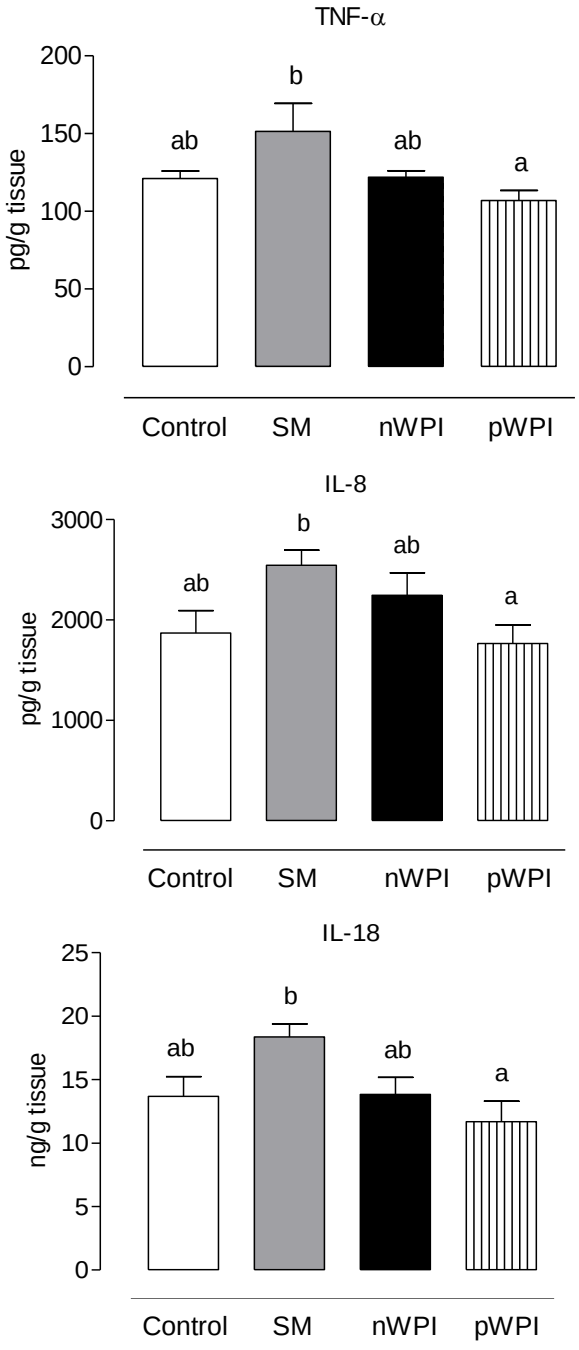
[1]Data are means and SEM, n = 8 per treatment group. Means with superscripts without a common letter differ $P < 0.05$. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Figure 3.4.7 Myeloperoxidase activity in the DC in piglets with DSS-induced colitis[1]



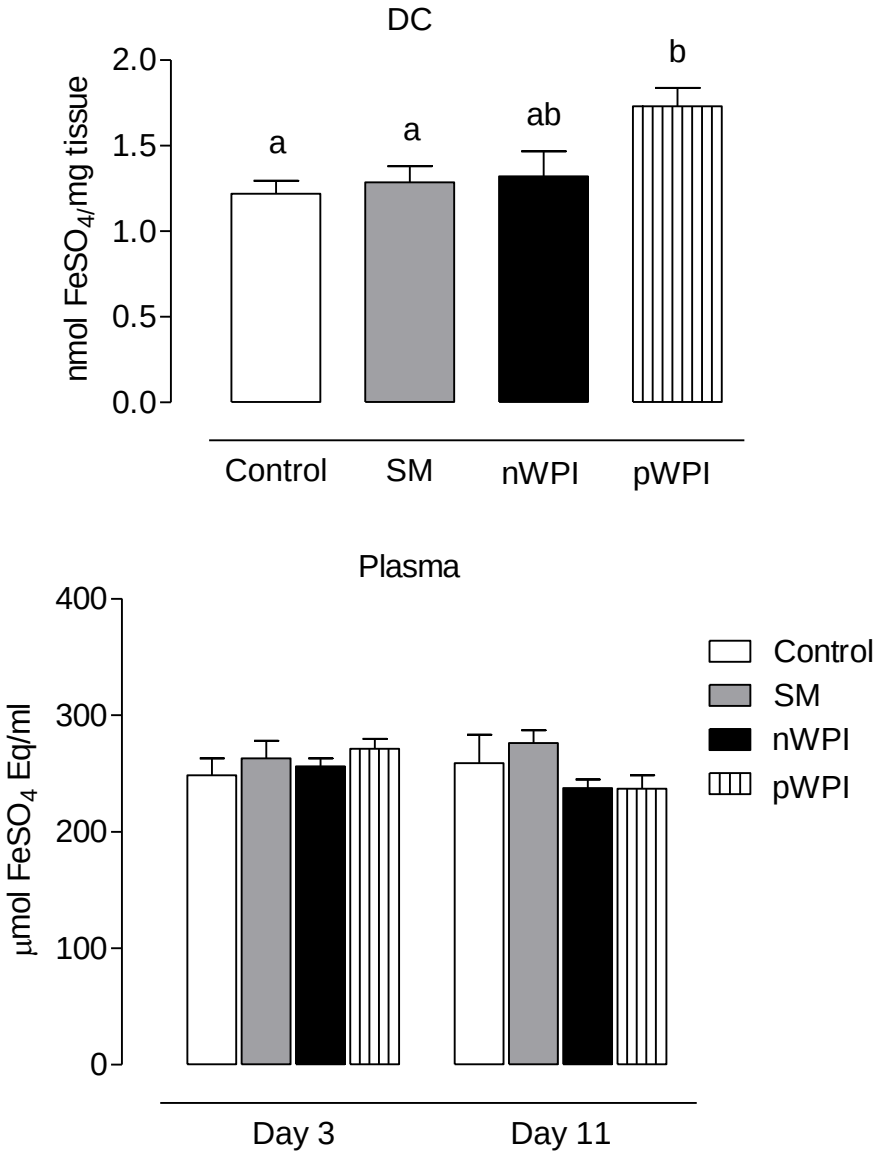
[1]Data are means and SEM, n = 8 per treatment group. Means with superscripts without a common letter differ $P < 0.05$. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Figure 3.4.8 TNF-α, IL-8 and IL-18 concentrations in the DC in piglets with DSS-induced colitis[1]



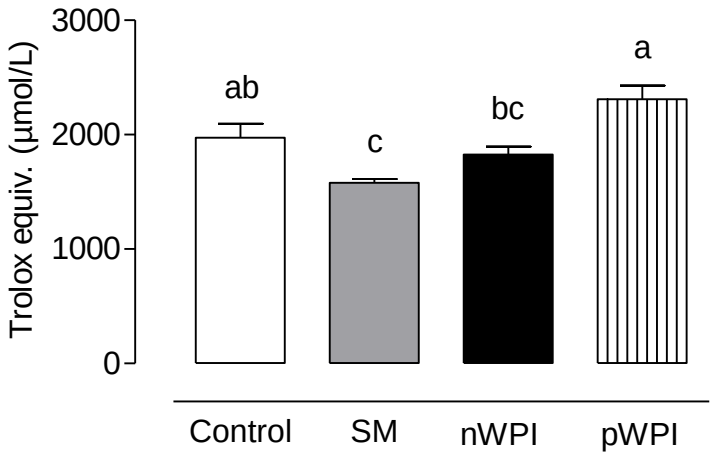
[1]Data are means and SEM, n = 8 per treatment group. Means with superscripts without a common letter differ P < 0.05. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Figure 3.4.9 Ferric reducing antioxidant power (FRAP) assay in DC and plasma samples in piglets with DSS-induced colitis[1]



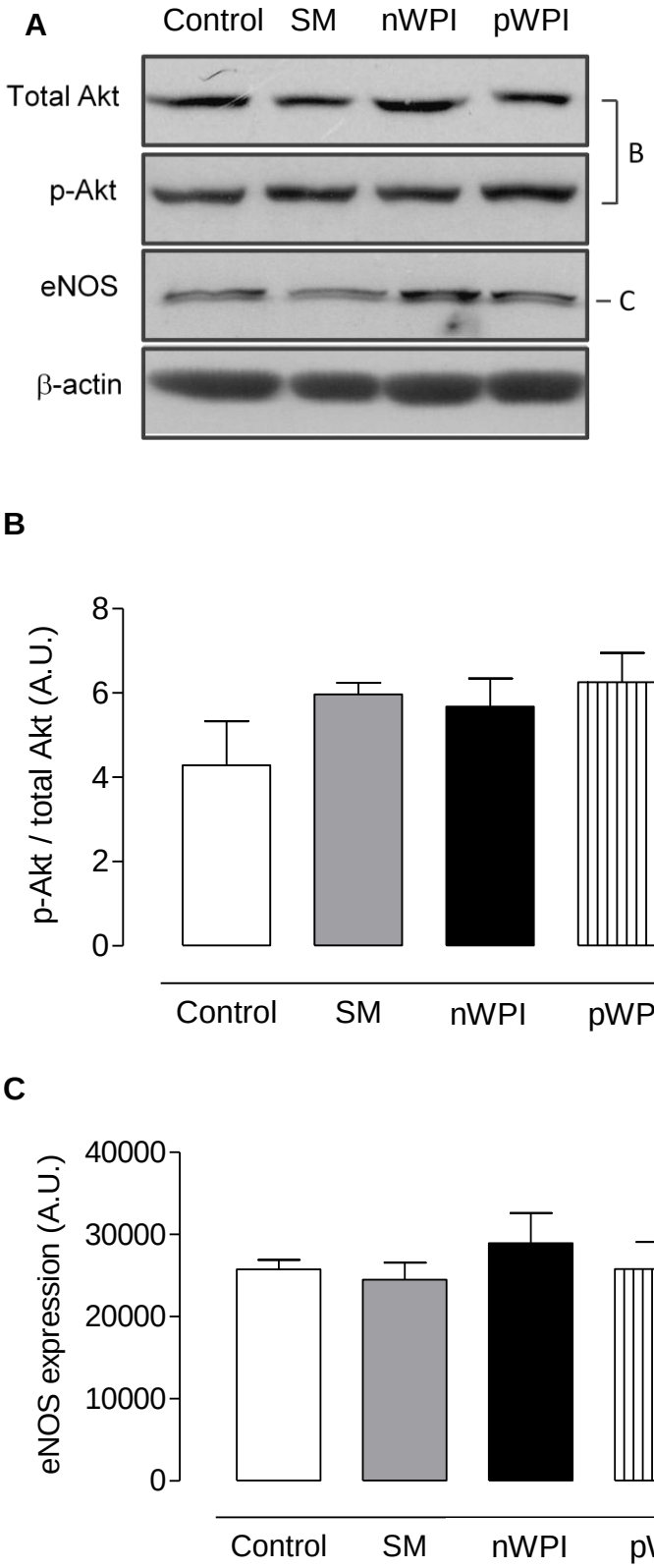
[1]Data are means and SEM, n = 8 per treatment group. Means with superscripts without a common letter differ $P < 0.05$. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

Figure 3.4.10 Serum total antioxidant capacity as measured by quenching of peroxynitrite Pholasin® chemiluminescence[1]



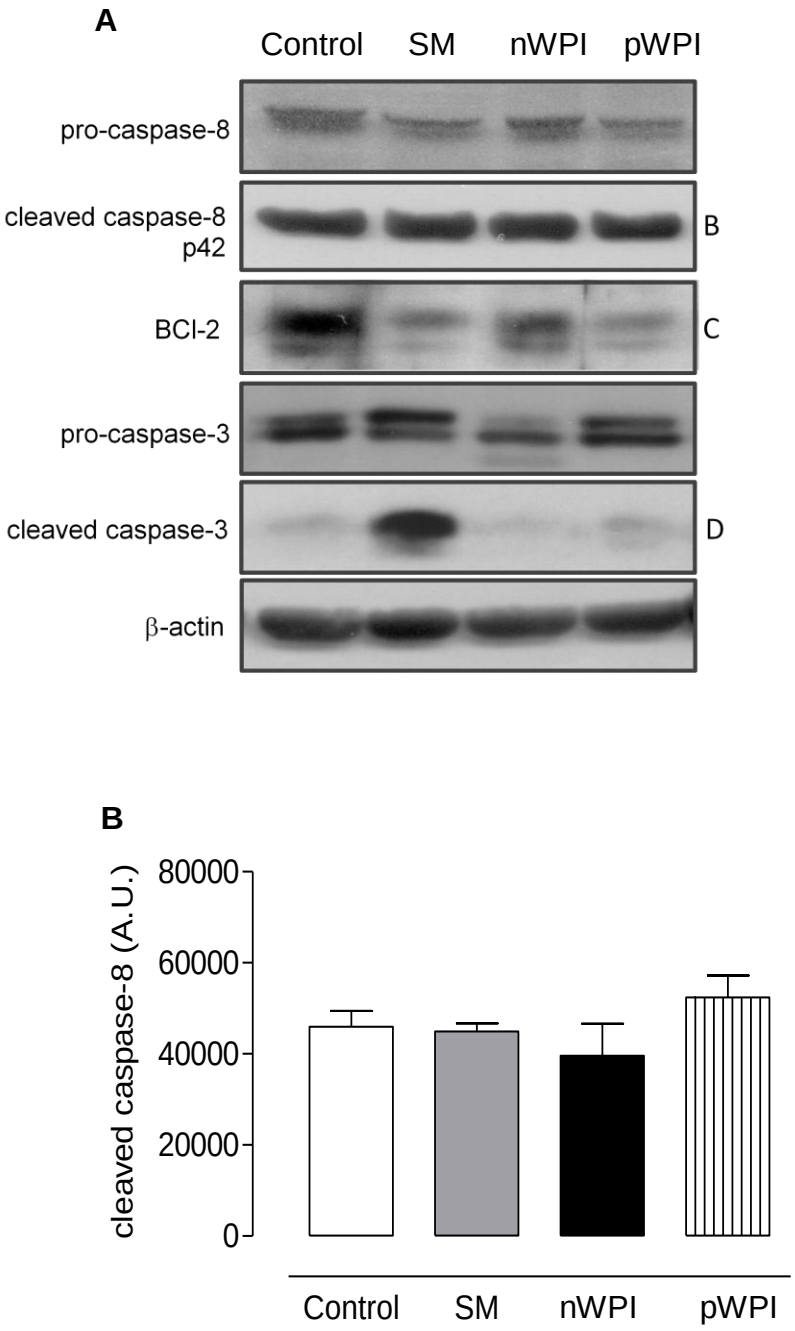
[1]Data are means and pooled SEM, n = 8 per treatment group. Means with superscripts without a common letter differ P < 0.05. Least square means adjusted for Tukey's *post hoc* test. Control = 100% SM; SM = 50% SM; nWPI = 50% native WPI; pWPI = 50% pressurized WPI.

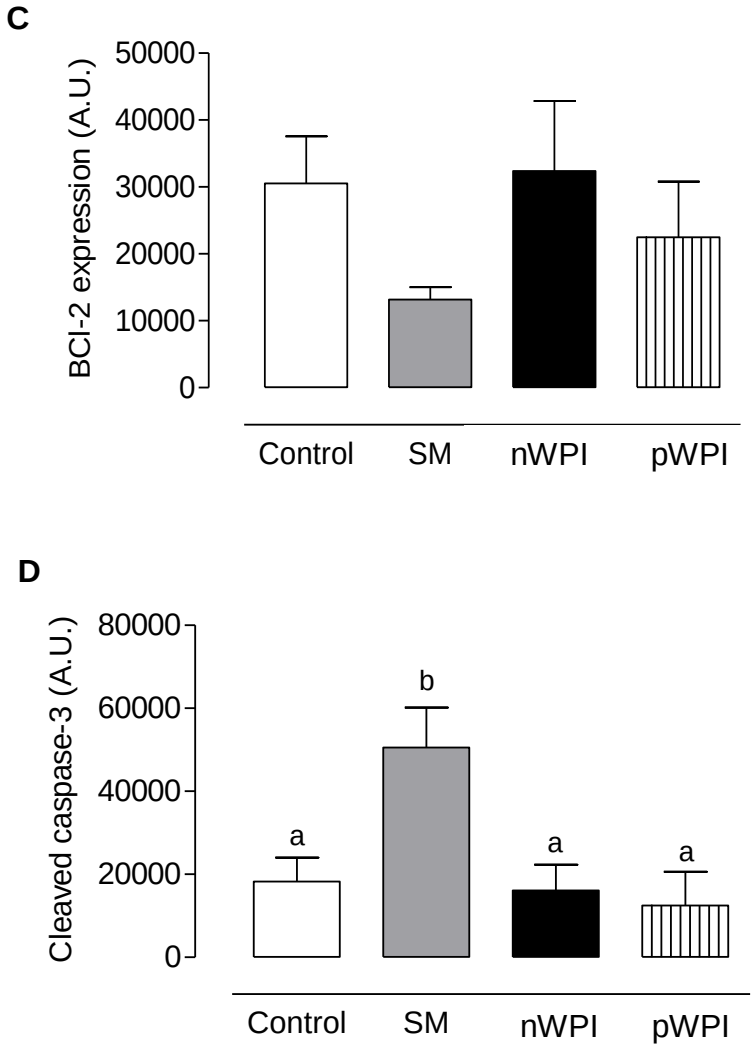
Figure 3.4.11 Effects of different milk proteins in the abundance of Akt and eNOS in the DC of piglets with DS-induced colitis[1]



[1]Data are means and SEM. Homogenates of the DC from Control, SM, nWPI, and pWPI were obtained and the detection of total Akt and pAkt (**B**; n = 6), eNOS (**C**; n = 6) were done by immunoblots. The bands in **A** represent the abundance of proteins in the DC homogenates from piglets. The total Akt, pAkt, and eNOS contents were quantified by scanning densitometry of the bands (expressed in arbitrary units, a.u.). β-actin was loaded as a control.

Figure 3.4.12 Effects of different milk proteins in the abundance of caspase-3, caspase-8, and BCl-2 proteins linked to the apoptosis pathway in the DC of piglets with DS-induced colitis[1]





[1]Data are means and SEM. Homogenates of the DC from Control, SM, nWPI, and pWPI were obtained and the detection of total pro-caspase-8 (n = 6), cleaved caspase-8 p42 (**B**; n = 6), BCl-2 (**C**; n = 6), pro-caspase-3 (n = 6), and cleaved caspase-3 (**D**; n = 6), were done by immunoblot. The bands in **A** represent the abundance of proteins in the DC homogenates from piglets. The total pro-caspase-8, cleaved caspase-8 p42, BCl-2, pro-caspase-3, and cleaved caspase-3 contents were quantified by scanning densitometry of the bands (expressed in arbitrary units, a.u.). β-actin was loaded as a control. Least square means adjusted for Tukey's *post hoc* test.

CONNECTING STATEMENT

The results obtained in Chapter 3 demonstrated that pWPI-fed piglet group was the only milk protein restricted group to gain lean/fat tissue at the high ratio observed with the well nourished controls. This pattern was repeated at the clinical level, as pWPI-fed piglets had less severe diarrhea compared with other protein restricted groups. In terms of histopathology, both pWPI and nWPI-fed piglets showed the least damage in the DC relative to both the SM and well nourished control piglets. Intake of pWPI, however, was shown to have the lowest MPO activity in DC as compared to all the other diet groups signifying the lowest degree of gut inflammation. Moreover, the proinflammatory cytokines (TNF-α, IL-8, and IL-18) in DC were lower in the pWPI-fed piglets in comparison levels in SM piglet group, which had the most elevated levels. Higher antioxidant capacity of DC as assessed via FRAP was also observed in the pWPI-fed piglets when compared with SM and control piglets. Pressurized-fed piglets also demonstrated a higher serum antioxidant capacity in terms of peroxynitrite scavenging when compared with SM-fed piglets. In addition, control-, nWPI- and pWPI-fed piglets had the least caspase-3 abundance compared with the SM group. Thus, intake of pWPI was associated with anabolic, antioxidant, and anti-inflammatory effects and decreased disease severity in a piglet model of pediatric colitis. The following chapter investigates further whether the antioxidant and anti-inflammatory activities of pWPI can be related to direct exposure of colonic cells to dietary peptides released after WPI digestion. Hence, human intestinal Caco-2 cells were used to observe the effects of hydrolysates of nWPI and pWPI on IL-8 secretion and intracellular ROS generation, under basal or stimulated conditions with H_2O_2. In that regard, the antioxidant and anti-inflammatory effects of hydrolysates of nWPI and pWPI were studied in terms of both prevention and recovery of Caco-2 cells exposed to H_2O_2, which is a widely used *in vitro* model of gut inflammation.

Chapter 4

Manuscript 2

WHEY PROTEIN ISOLATE HYDROLYSATES INHIBIT OXIDATIVE STRESS AND HYDROGEN PEROXIDE-INDUCED IL-8 SECRETION IN INTESTINAL EPITHELIAL CELLS

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Running title: Whey protein isolate and Caco-2 human intestinal cells

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4.1 ABSTRACT

A recent study has shown that whey protein hydrolysates exert antioxidative and anti-inflammatory effects in Caco-2 cells exposed to pro-inflammatory stimulus. High-pressure treatment of whey protein isolate (WPI) causes changes in the protein structure that enhances the antioxidant and anti-inflammatory effects of WPI. The aim of this study was to compare the antioxidant and anti-inflammatory effects of the hydrolysates of pressurized WPI (pWPI) vs. native WPI (nWPI) in the Caco-2 intestinal epithelial cell line exposed to H_2O_2-induced oxidative damage. Cells were cultured for 24 h with 0, 500, 1000 and 2000 µg/mL pWPI or nWPI either 1 h before or 1 h after stimulation with H_2O_2 for 1 h. After 24 h, cell viability was measured by the MTS assay, interleukin (IL)-8 secretion by ELISA, intracellular reactive oxygen species (ROS) was assessed by the dichlorofluorescin assay and the antioxidant capacity of the medium was determined by ferric reducing antioxidant power (FRAP) activity. When provided both prior to and after H_2O_2 exposure, nWPI and pWPI inhibited ($P < 0.05$) IL-8 secretion and intracellular ROS generation and increased FRAP activity in a dose-dependent manner. The maximal inhibition of H_2O_2-induced IL-8 secretion was greater ($P < 0.05$) with pWPI (50%) vs. nWPI (30%) hydrolysates, which were provided after H_2O_2 exposure at a concentration of 2000 µg/mL. At the 2000 µg/mL concentration, inhibition of H_2O_2-induced ROS formation reached 76% for pWPI hydrolysates, which was greater ($P < 0.05$) than for nWPI hydrolysates (32.5%). Similarly, FRAP antioxidant activity was higher ($P < 0.05$) for pWPI vs. nWPI hydrolysates. These results suggest that pWPI is more efficient than nWPI in alleviating inflammation and oxidative stress in intestinal cells exposed to oxidative injury.

4.2 INTRODUCTION

Gastrointestinal epithelial cells are exposed to oxidative stress, which is thought to contribute to inflammation of the gut mucosa (Pearson *et al.*, 1996). Oxidative stress can be caused by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion, singlet oxygen, or hydroxyl radical (Zhang, 2010) generated both in the lumen and in the intestinal mucosa (Taha *et al.*, 2010). A variety of conditions can induce oxidative stress within intestinal tissues including hemorrhagic shock (Umeda *et al.*, 2009), ischemia (Ocal *et al.*, 2004), drug-induced intestinal injury and enterocolitis (Abraham *et al.*, 2008). Oxidative stress has been implicated as a potential etiological and/or triggering factor for inflammatory bowel disease (IBD) due to the well established detrimental effects of ROS in the gut inflammatory process (Spitz *et al.*, 2004). Excessive oxidative stress has been reported to be associated with mucosal erosions and to play a causative role in a variety of gastrointestinal diseases including colitis (Kruidenier and Verspaget, 2002; Rezaie *et al.*, 2007). H_2O_2 is one of several ROS which causes oxidative stress that contributes towards colonic damage (Zhang, 2010). The reaction of Fe^{+2} with H_2O_2 resulting in the production of a hydroxyl radical has been shown as an important mediator of colonic tissue injury (Koppenol, 1985; Niki, 2010). Bleeding occurs during the active phase of colitis, which releases both heme and free iron into the gastrointestinal tract thereby contributing to the pathogenesis and exacerbation of the disease (Simmonds and Rampton, 1993). H_2O_2 is also generated via a variety of metabolic pathways and its intracellular accumulation can induce dysregulation of normal metabolic processes partly by modifying proteins, lipids, and DNA (Slater, 1984; Chandra *et al.*, 2000). Altered gene abundance has been linked with H_2O_2 generation as H_2O_2 can modify the activity of transcription factors (Aslund *et al.*, 1999). Also, H_2O_2 can also activate transduction pathways such as mitogen-activated protein kinases (MAPKs) (Desikan *et al.*, 1999; Grant *et al.*, 2000) and act as a second messenger (Reth, 2002).

Oxidative stress has been shown to promote interleukin (IL)-8 production in several cell types, including gastric cancer (Taguchi *et al.*, 2005), lung

carcinoma (Zhang *et al.*, 2011) and in the human colon adenocarcinoma Caco-2 cell line (Yamamoto *et al.*, 2003). The secretion of IL-8 is an integral part of the immune response (Baggiolini *et al.*, 1995), particularly in inflammatory processes as IL-8 mediates the recruitment and activation of neutrophils in inflamed tissue (Harada *et al.*, 1994). The dysregulation of IL-8 balance can lead to the type of serious tissue damage that has been reported in IBD (Atreya and Neurath, 2010) and in patients with active colitis (Mahida *et al.*, 1992).

The human colon cancer-derived Caco-2 cell line has been widely used as an *in vitro* model of the intestinal epithelium (Quaroni *et al.*, 1979). Caco-2 cells have been effectively used as an *in vitro* model for many applications including the investigation of toxicants, food mutagens, and their mechanisms (Walle and Walle, 1999; Okada *et al.*, 2000) and testing the protective action of antioxidants and nutraceutical agents (Kameoka *et al.*, 1999; Steijns, 2001). For instance, antioxidant and anti-inflammatory effects of dietary flavonoids have been demonstrated in Caco-2 cells exposed to H_2O_2 (Kameoka *et al.*, 1999) as H_2O_2-induced oxidative stress promotes IL-8 secretion in Caco-2 cells (Yamamoto *et al.*, 2003).

Bioactive proteins and peptides in milk proteins are being used as nutraceuticals to improve human health (Steijns, 2001). Whey proteins (WP), which comprise approximately 20% of the total milk proteins, have been found *in vitro* and *in vivo* studies to exert antioxidant and anti-inflammatory effects (Krissansen, 2007). A recent study has shown that hydrolysates of a dietary protein formula, containing 41% of whey protein concentrate as a key ingredient along with vitamins and minerals, vegetable oils and inulin fibre, exerted immunomodulatory, antioxidative and anti-inflammatory effects in Caco-2 cells by down-regulation of the secretion of pro-inflammatory cytokines (IL-1β and TNF-α) and up-regulation of IFN-γ, lL-2, and IL-10 (Kanwar and Kanwar, 2009).

Cell culture, animal and human studies have indicated that high hydrostatic pressure treatment of WPI increases the antioxidant and anti-inflammatory effects of WPI, which are linked to increased WP digestibility and the greater release of bioactive peptides (Vilela *et al.*, 2006; Zavorsky *et al.*,

2007; Kishta *et al.*, 2009). There exists extensive disulfide linkages in WP that increases their resistance to proteolysis (Alting *et al.*, 2000). The high resistance of most prevalent WP, β-lactoglobulin (β-lb), to pepsin-mediated hydrolysis is considered to be a major factor that impedes WP digestibility (Vilela *et al.*, 2006; Picariello *et al.*, 2010). Various studies have shown a low accessibility to peptide bonds localized to the interior of the globular structure of β-lb to the hydrolytic action of pepsin (Mutilangi *et al.*, 1996; Kinekawa *et al.*, 1998). High hydrostatic pressure treatment of WP can cause changes in their secondary and tertiary structure leading to denaturation (Hendrickx *et al.*, 1998). The most sensitive WP to denaturation induced by high hydrostatic pressure is β-lb, which contains two disulfide bonds and one free sulfhydryl group (Patel *et al.*, 2005). Vilela et al. (2006) has shown that high hydrostatic pressure at 550 MPa causes a major increase in the rate of pepsin-mediated hydrolysis of whey protein isolates (WPI). Pressurization at 550 MPa has been demonstrated to increase the rate of β-lb hydrolysis due to partial unfolding of β-lb as only the unfolded molecules are susceptible to proteolytic degradation and also to expose the hydrophobic amino acids buried in the interior of the molecule to hydrolytic enzymatic action (Alvarez, 2004). Pressure treatment of β-lb above 500 MPa readily facilitates irreversible denaturation mediated by thiol-disulfide interchange leading to the formation of inter- and intramolecular S–S bonds (Patel *et al.*, 2005). The formation of the novel non-native disulfide bonds inhibits the re-folding of the native protein conformation leading to irreversible denaturation (Patel *et al.*, 2005).

A recent *in vivo* study has shown that pressure-processed WPI exerted significantly better anabolic, antioxidant and anti-inflammatory effects than native whey both systemically and in the descending colon tissue of piglets with dextran sulfate sodium-induced colitis including decreased colonic IL-8 tissue concentrations (Piccolomini *et al.*, 2011). In order to study the impact of pressure treatment of WPI on its antioxidant and anti-inflammatory effects in human intestinal epithelial cells, the present work investigated the effects of native and pressure-treated WPI hydrolysates on oxidative damage and inflammation induced by H_2O_2 in intestinal Caco-2 epithelial cells.

4.3 MATERIAL AND METHODS

Hydrostatic pressure treatment and sample preparation for enzymatic hydrolyses

Whey protein isolate were purchased (Vitalus Nutrition Inc., Abbotsford, BC, Canada) and produced by membrane ultrafiltration. Whey protein isolate were pressurized in 12.5% (w/v) solution by applying a single cycle of hydrostatic pressure of 550 MPa. This acts to unfold the protein conformation of the whey proteins enabling them to be more susceptible to digestive enzymes (Vilela *et al.*, 2006). The native whey protein isolate (nWPI) 12.5% (w/v) solution and the pressurized WPI (pWPI) 12.5% (w/v) WPI solutions were lyophilized by placement into vacuum chambers of Flexi-Dry MP Lyophilizer (FTS Systems, Stone Ridge, NY, USA) and freeze-dried under -80°C and 90 MT vacuum in preparation for future in vitro digestibility studies. The time needed to freeze-dry the samples depended on the volume used and varying from 48 to 72 h.

In vitro enzymatic digestion

The enzymatic digestion method was adopted from Vilela *et al.* (2006) with some modifications (Iskandar, 2011). This technique was developed to simulate *in vivo* gastrointestinal digestion of milk proteins in terms of biochemical, physical and mechanical conditions including the enzyme concentration, pH adjustment (i.e., pH 1.9 and then 7.4), temperatures for optimal enzyme activity (37°C), shaking and incubation time (Vilela *et al.*, 2006). Briefly, after lyophilized native and pressurized WPI were diluted in 390 mL of double distilled water to reach a final concentration of 3 mg protein/mL and the pH was adjusted to 1.9 with 12N HCl. The WPI digestion was started by adding freshly prepared pepsin (Sigma-Aldrich, Oakville, ON, Canada) solution to reach a stock solution of 2 mg/mL. Aliquots were taken at 5, 10, and 15 min and subsequently interrupted by raising the pH to 7.4 using 10N NaOH, thereby inactivating pepsin irreversibly. After pepsin inactivation, the pancreatic enzymes tripsin (Sigma-Aldrich, Oakville, ON, Canada) and chymotrypsin Sigma-Aldrich, Oakville, ON, Canada) were dissolved in 10 mM NaH_2PO_4 to reach a final concentration of 5.85 mg/mL and 13.45 mg/mL, respectively and immediately added to the solution. Aliquots were taken

at 30, 45, 60, and 75 min. At the end of the digestion pH was increased to 10.5 to inactivate the pancreatic enzymes. The bottle was immediately chilled in a bucket of ice on a magnetic stirrer under continuous stirring to decrease the temperature to 4°C.

Peptide isolation

After the digestion described above, the hydrolysates from native and pressurized WPI were subjected to an ultrafiltration system implemented by Vilela *et al*. (2006) and Iskandar (2011). Briefly, to remove high molecular weight peptides a membrane filter of 10 kilodalton (kDa) (Millipore, Nepean, ON, Canada) was used in a stirred ultrafiltration membrane reactor (Model 8050, Millipore, Nepean, ON, Canada) under N_2 pressure of 40 psi. The major advantage to use this filter is to retrieve amino acids rich in leucine, tyrosine, phenylalanine, lysine, arginine, proline and aspartate (Agudelo *et al.*, 2004). The filtrate was freeze-dried (Flexi-Dry MP Lyophilizer, FTS Systems, Stone Ridge, NY, USA) at -80°C and 90 MT vacuum for further cell culture experiments. The lyophilized filtrate were stored in sealed tubes flushed under N_2 kept under -20°C.

High-performance liquid chromatography (HPLC) profile

Reagents were HPLC grade and purchased from Sigma Chemical Co. (St Louis, MO, USA). Samples were analyzed using a Varian HPLC system with a tertiary gradient pump, a variable wavelength UV/VIS detector, and an autosampler with refrigerated sample compartment (Varian Canada Inc, Mississauga, ON, Canada). Samples were eluted using a Gemini-NX reverse-phase (RP)-HPLC column (100 x 4.5 mm) (Phenomenex, CA, USA), using a solvent flow rate of 1 mL/min and detection was at 215 nm. Gradient elution was carried out with a mixture of two solvents. Solvent A: 0.05% trifluoroacetic acid (TFA) in 10% aqueous acetonitrile (ACN) and solvent B: 0.05% TFA in 60% aqueous ACN, (v/v) starting with 100% solvent A and reaching 40% solvent A and 60% solvent B in 30min.

Cell culture

The human colonic adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA, USA). All cell culture medium was obtained from Invitrogen (Carlsbad, CA, USA). Caco-2 cells passage numbers 21 to 29 were seeded at a density of 2.5 x 10^5 cells/well cultured in Dulbecco's modified Eagle's medium (DMEM, pH 7.4) with 25 mM glucose, supplemented with 20% heat inactivated fetal bovine serum (HFBS), 1% penicillin-streptomycin and 1% non-essential amino acid solution in a humidified atmosphere containing 5% CO_2 at 37°C. All experiments were carried out in Minimal Essential Medium (MEM) with 2% HFBS and devoid of antibiotics.

Mitochondrial succinate dehydrogenase (MTS) assay

The effects of H_2O_2 on cell survival were measured using the MTS assay (Mosmann, 1983). Caco-2 cells were seeded in 24-well plates at a concentration of 2.5 x 10^5 cells/well. After 24 h of culturing, the cells were treated with different concentrations of H_2O_2 (0 to 2 mM) at 37°C for 1 h. After, cells were washed twice with MEM and incubated for 23 h. Subsequently, 10 µL of MTS reagent (Cayman Chemical, Ann Arbor, MI, USA) was added to each well and incubated for 3 h at 37°C. Finally, 100 µL per well of isopropanol containing 40 mM HCl was added to dissolve the formazan crystals. The absorbance was read at a wavelength of 560 nm using the Wallac Victor^2 1420 Series microplate reader (Harlow Scientific, Arlington, MA, USA). Treatments were compared with untreated control cells. Results are expressed as percentage of cell viability. Each point represents the mean of three experiments.

Enzyme-linked immunosorbent assay

Confluent Caco-2 cells on the 24-well plate were exposed for 1 h to a culture medium containing H_2O_2 (Sigma-Aldrich, Oakville, ON, Canada) ranging from 0 to 2 mM. After H_2O_2 was removed, cells were washed twice with MEM, and allowed to recover for 23 h. The supernatants were collected after 24 h of the treatment to determine IL-8 release using a commercially available ELISA kit (BD

Biosciences, Mississaga, ON, Canada) according to the manufacturer's instructions.

Exposure of Caco-2 cells to WPI and H_2O_2

To investigate the protective effect of WPI on 0.25 mM H_2O_2-stimulated Caco-2 cells were exposed to different concentrations of nWPI or pWPI hydrolysates (0, 500, 1000 and 2000 µg/mL) in different 24-well plates. In this first set of experiments, Caco-2 cells were exposed to different concentrations of nWPI and pWPI hydrolysates for 1 h. WPI was removed, cells were washed twice with MEM, nWPI or pWPI hydrolysates were dissolved in MEM with 0.25 mM H_2O_2 and incubated for 1 h. Subsequently, Caco-2 cells were washed twice and nWPI or pWPI hydrolysates alone were added to the cells for 22 h. In the next day, supernatants were collected for IL-8 determination. To determine the recovery effect of nWPI or pWPI hydrolysates on pre-stimulated Caco-2 cells were exposed to H_2O_2. In this second set of experiments, Caco-2 cells were exposed to concentration 0.25 mM H_2O_2 for 1 h. H_2O_2 was removed cells were washed twice with MEM, nWPI or pWPI hydrolysates were added and allowed to recover for 23 h. The supernatants were collected for IL-8 determination 24 h after the treatment.

Ferric reducing antioxidant power (FRAP) assay

FRAP was used to determine the total antioxidant potential in the supernatant of Caco-2 cells exposed to 0.25 mM in different concentrations of nWPI or pWPI hydrolysates (0 to 2000 µg/mL). Briefly, in this assay the electron-donating capacity of the antioxidant was measured by the change in absorbance (abs) at 593 nm when a blue-colored Fe^{2+}- tripyridyltriazine (Fe^{2+}TPTZ) compound is formed from a colorless oxidized Fe^{3+} form (Benzie and Strain, 1999). A standard curve was prepared from aqueous solutions of 1 mM $FeSO_4.7H_2O$ at different concentrations ranging from 0.1 to 1.0 mM. The reagent was prepared with 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution dissolved in 40 mM HCl at 50ºC, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The working

solution was mixed with the 3 solutions in 10:1:1 ratio, wrapped in foil. Once prepared it was immediately incubated for 10 min at 37°C. In a 96 well plate was added 30 μL H_2O, 10 μL standards or samples, and 200 μL FRAP working solution. Samples reaction occurred at room temperature for 30 min and absorbance was taken at 593 nm in a microplate reader (Infinite PRO 200 series, Tecan Group, San Jose, CA, USA).

Intracellular reactive oxygen species (ROS)

The generation of intracellular ROS was evaluated by oxidation of 2',7'-dichlorofluorescin-diacetate (DCFH-DA) (Invitrogen, Carlsbad, CA, USA). The DCFH-DA was cleaved in the cells to dichlorofluorescin (DCFH) and oxidized by ROS to fluorescent dichlorofluorescein (DCF) (Yokomizo and Moriwaki, 2006). This experiment explored the stimulation of the Caco-2 cells with 0.25 mM H_2O_2 for 1 h and after being washed twice, cells were treated with different concentrations of nWPI or pWPI hydrolysates from 0 to 2000 μg/mL for 23 h. After treatment cells were incubated with 5 μM DCFH-DA in phosphate buffered saline containing 5% (w/v) dimethyl sulfoxide (DMSO) solution for 30 min at 37°C. The fluorescent DCF was monitored by spectrofluorimetry (fluorescence excitation and emission were 485 nm and 530 nm, respectively; Wallac Victor^2 1420 Series spectrofluorimetry reader - Harlow Scientific, Arlington, MA, USA). The values were expressed as percentages of DCF fluorescence intensity to that of untreated control cells.

Statistical analysis

Statistical analysis was performed using SAS for Windows (version 9.2, 2008, SAS Institute Inc., Cary, NC). One-way and two-way ANOVA were performed with Tukey's *post hoc* test to determine significant statistical differences between groups after verification of normality using Shapiro-Wilk. Independent student t test was performed to determine significant statistical differences between treatments (nWPI vs. pWPI).

4.4 RESULTS

Effect of hydrostatic pressure treatment of WPI hydrolysates

Figure 4.4.1 represents the peptide profiles of nWPI and pWPI with molecular weight cut-off (MWCO) less than 10 kDa performed by HPLC analysis. Differences in the peak abundances as well as two new peaks were observed in the pWPI that were not present in the nWPI.

Effects of cell viability and IL-8 response in stimulated Caco-2 cells

Cell viability as measured by the MTS assay following exposure of the cells to different concentrations of H_2O_2 is described in Figure 4.4.2. The higher concentrations of H_2O_2 decreased ($P < 0.05$) Caco-2 cells viability; however, the H_2O_2 concentration of 0.25 mM was associated with a similar number of viable cells as the control treatment. Disturbed integrity of the Caco-2 and detaching cells were also seen at treatment with 2 mM H_2O_2. Figure 4.4.3 represents the IL-8 response in Caco-2 cells exposed to different concentrations of H_2O_2. Increased ($P < 0.05$) IL-8 concentrations relative to control Caco-2 cells was observed in cells treated H_2O_2 concentrations from 0.25 to 2.0 mM with an approximate 300% increase observed in the lowest effective H_2O_2 concentration of 0.25 mM. As the 0.25 mM H_2O_2 dose was not associated with decreased cell viability as opposed to the higher H_2O_2 concentrations (Fig. 4.4.2).

Effects of WPI hydrolysates on cell viability and anti-inflammation

Figure 4.4.4 illustrates the effect of different concentrations of WPI hydrolysates on Caco-2 cell viability in the absence of H_2O_2. No cell toxicity was observed in cells treated with different concentrations of either nWPI or pWPI hydrolysates. The protective anti-inflammatory effects of nWPI and pWPI hydrolysates on H_2O_2- stimulated Caco-2 cells are shown in Figure 4.4.5. Both types of WPI hydrolysates were associated with anti-inflammatory effects although pWPI hydrolysates showed lower ($P < 0.05$) IL-8 release at each of the tested WPI hydrolysates doses. Stimulated Caco-2 cells treated with the highest dose of the hydrolysates of nWPI or pWPI of 2,000 µg/mL demonstrated reduction ($P < 0.05$)

of IL-8 secretion of 26.3% and 37.7%, respectively, as compared with H_2O_2-stimulated Caco-2 cells alone. The Caco-2 cells stimulated with H_2O_2 had reduced ($P < 0.05$) IL-8 concentrations when treated with either type of WPI hydrolysates for 1 h post-stimulation (Figure 4.4.6). The inflammatory response in Caco-2 cells induced by 0.25 mM H_2O_2 after 1 h post-stimulation treatment with the highest dose of nWPI or pWPI hydrolysates showed decreased ($P < 0.05$) IL-8 secretion of 30.4% and 50.1%, respectively.

Effects of WPI hydrolysates on the antioxidant capacity of cell culture medium

The ferric reducing antioxidant power (FRAP) measured in the cell culture medium of Caco-2 cells stimulated with 0.25 mM H_2O_2 after exposure to different concentrations of nWPI or pWPI hydrolysates is shown in Figure 4.4.7. The medium of Caco-2 cells treated with either nWPI or pWPI hydrolysates showed increased ($P < 0.05$) in FRAP activity at all hydrolysates treatment concentrations. In addition, pWPI hydrolysates treated Caco-2 cell culture media showed increased ($P < 0.05$) FRAP activity as compared to nWPI hydrolysates exposed Caco-2 cells at all tested concentrations.

Effects of WPI hydrolysates in the generation of reactive oxygen species (ROS)

The effects of different concentrations of WPI hydrolysates on Caco-2 cells exposed to 0.25 mM H_2O_2 are shown in Figure 4.4.8. Caco-2 cells treated with either type of WPI hydrolysates showed decreased ($P < 0.05$) in intracellular ROS production. Caco-2 cells treated with the highest dose of the hydrolysates of nWPI or pWPI, i.e., 2000 µg/mL demonstrated reduction ($P < 0.05$) in ROS formation of 32.5% and 76.1%, respectively, as compared with Caco-2 cells alone. In addition, Caco-2 cells treated with pWPI hydrolysates had reduced ($P < 0.05$) ROS generation as compared to nWPI hydrolysates treated cells.

4.5 DISCUSSION

The results of the present study have demonstrated that the antioxidant and anti-inflammatory effects of WPI hydrolysates in H_2O_2-stimulated Caco-2 epithelial cells are enhanced by the high pressure processing of WPI. These findings are in agreement with recent observations that intake of pressure-processed WPI significantly improved the antioxidant and anti-inflammatory effects of WPI as seen in the colonic tissue of piglets with dextran sulfate sodium-induced colitis (Piccolomini *et al.*, 2011). It is likely that the changes in the secondary and tertiary structures of pressure-processed whey proteins generates a unique profile of bioactive amino acids and peptides that enhanced their antioxidant and anti-inflammatory effects (Vilela *et al.*, 2006; Zavorsky *et al.*, 2007; Kishta *et al.*, 2009; Piccolomini *et al.*, 2011). Different peptide profiles were noted in hydrolysates resulting from the digestion of pressurized WPI including two new peaks (Figure 4.4.1), which may have contributed to the greater antioxidant and anti-inflammatory effects of pWPI. An altered peptide profile following hydrolysis of pWPI has been previously noted (Vilela *et al.*, 2006) although a different digestion protocol in terms of enzymatic and ultrafiltration methods to better approximate physiological conditions (Iskandar, 2011) was used in the present study that resulted in larger numbers of peptide peaks. Previous work has shown that hydrolysates of pWPI were associated with improved intracellular glutathione (GSH) status (Vilela *et al.*, 2006) that could also partly explain the enhanced antioxidant and anti-inflammatory effects associated with pWPI hydrolysates in the stimulated Caco-2 cell cultures. In this manner, pressure processing of WPI might be comparable to the fermentation of WP that has been shown to enhance the tissue antioxidant effects of WP, which was related to an increased generation of the low molecular weight peptide γ-glutamylcysteine that can induce tissue GSH (Zommara *et al.*, 1996). Whey proteins, which are rich in the cysteine precursor required for GSH synthesis, have shown via *in vitro* (Bomser *et al.*, 2003; Tseng *et al.*, 2006; Vilela *et al.*, 2006) and *in vivo* (Jelen *et al.*, 2004; Rundell *et al.*, 2005; Abdel-Wahhab *et al.*, 2011) studies to improve antioxidant status via increased intracellular levels of GSH (Bounous, 2000). GSH acts as a

major intracellular antioxidant partly by donating sulfhydryl protons to unstable ROS molecules such as H_2O_2 (Blokhina *et al.*, 2003). Thus, the protective effects of WPI hydrolysates in terms of decreasing intracellular ROS generation in the Caco-2 epithelial cells exposed to H_2O_2 could be related to improved intracellular GSH status. In other *in vitro* studies, H_2O_2 at the lowest H_2O_2 concentrations used in the present study caused increased the intracellular oxidation of DCFH indicating intracellular ROS formation in Caco-2 and HepG2 cells (Noda *et al.*, 2001; Alia *et al.*, 2005). Previous findings have shown that Caco-2 cells exposed to similar concentrations of H_2O_2 had attenuated intracellular oxidative stress when treated with the human whey protein lactoferrin (Shoji *et al.*, 2007); however, the present findings indicate that whey protein derived peptides can provide similar antioxidant protection.

Hydrolysates of pressurized WPI have also been shown to contain relatively higher free amino acid levels of alanine, histidine, glutamine, tryptophan, lysine and branched chain amino acids (BCAA) (Vilela *et al.*, 2006). It is conceivable that the combined effects of the above amino acids could have also contributed to the greater antioxidant and anti-inflammatory effects associated with pWPI since pre-treatment with those amino acids was shown to inhibit IL-8 secretion in H_2O_2-stimulated Caco-2 cells (Katayama and Mine, 2007). Treatment with the BCAA valine, isoleucine, and leucine was associated with elevated activities of both GSH S-transferase (GST) and catalase whereas histidine, tryptophan and lysine caused increased in GST activity (Katayama and Mine, 2007). GST has ROS scavenging activity including the breakdown of lipid hydroperoxides (Masella *et al.*, 2005) whereas catalase is involved in the decomposition of H_2O_2 (Chelikani *et al.*, 2004). Histidine treatment in Caco-2 and HT-29 intestinal cells have also been shown to inhibit TNF-α- and H_2O_2-induce oxidative stress and to decrease IL-8 secretion by acting at the transcriptional level (Son *et al.*, 2005). Glutamine has been reported to exert antioxidant effects in the Caco-2 and HCT-8 cell lines exposed to H_2O_2 by preserving cell membrane integrity and viability (Ziegler *et al.*, 2011). Although the intracellular GSH content and antioxidant enzyme activities were not measured in the present work, these

were likely involved with antioxidant and anti-inflammatory effects of both WPI hydrolysates within the Caco-2 cell culture system.

Oxidative stress induced via H_2O_2 has been shown to promote IL-8 production in several cell lines such as gastric and lung carcinoma cells (Josse *et al.*, 2001). In addition, increased IL-8 mRNA abundance has been shown after H_2O_2-induced oxidative stress in Caco-2 epithelial cells (Yamamoto *et al.*, 2003). It has been also demonstrated that H_2O_2 up-regulates IL-8 gene abundance via TNF receptors in the cell membrane signalling the activation of the nuclear factor (NF)-κB pathway and IL-8 release (Okamoto *et al.*, 1993; Mukaida *et al.*, 1994; Li and Verma, 2002). In contrast with the results of the present study, Kanwar and Kanwar (2009) showed no effect on IL-8 production in Caco-2 cells exposed to digestates of whey protein concentrate, although down-regulation of the secretion of other pro-inflammatory cytokines (IL-1β and TNF-α) was reported.

The results from the present study also demonstrated an increased antioxidant capacity in the cell culture medium as measured by the FRAP assay, which could be linked to an improved redox status of the cells. It is conceivable that WPI hydrolysates increased cellular redox status by increasing intracellular GSH synthesis. Cellular redox status is closely regulated the ratio of oxidized glutathione (GSSG) to the reduced form of GSH. The reduced form of GSH is a substrate for GSH peroxidise that catalyzes the reduction of different peroxides such as H_2O_2 to H_2O (Aslund *et al.*, 1999). A high intracellular ratio of GSH/GSSG is crucial for the protection against cell damage (Murphy *et al.*, 2005). When cells are exposed to increased levels of oxidative stress, GSSG will accumulate and the ratio of GSH to GSSG will decrease (Murphy *et al.*, 2005). As shown by a variety of *in vitro* studies (Rahman *et al.*, 2002; Lenzen, 2008), a low GSH/GSSG ratio is linked with elevated levels of pro-inflammatory cytokines since redox status is a key regulator of the inflammatory response (Maccarrone and Brune, 2009).

In this study, a dose-response effect on the viability of Caco-2 cells from the 1 h exposure to H_2O_2 concentrations was seen since decreased cell viability was observed only at the highest H_2O_2 concentration of 2 mM. Similarly, Caco-2

cells exposed to 0.2 mM of H_2O_2 for 30 min showed no effect on cell viability (O'Brien and Aherne, 1999) whereas another study found only a 19% viability as assessed by the MTS assay in Caco-2 cells exposed to 100 mM of H_2O_2 (Ebeler and Min, 2009). Exposure to H_2O_2 at higher concentrations causes cell membrane damage to Caco-2 cells as detected by elevated lactate dehydrogenase leakage, indicating a change in membrane permeability or cell death (Wijeratne *et al.*, 2005). In the present work, cell culture experiments utilized the 0.25 mM concentration of H_2O_2 in order to induce an inflammatory response while maintaining cell viability. In concert with the present findings, Nemeth *et al.* (2007) observed an approximate increase of 350% in IL-8 secretion in Caco-2 cells exposed for 1 h to 1.0 mM concentration of H_2O_2 (Nemeth *et al.*, 2007). Such increased levels of IL-8 secretion upon H_2O_2 exposure has been also seen in other types of cell lines such as dendritic cells (Verhasselt *et al.*, 1998), human bronchial epithelial cells (Pelaia *et al.*, 2004), and mast cells (Jiang *et al.*, 2001).

The present *in vitro* findings indicate that exposure of pressurized and native WPI could provide intestinal health benefits with respect to IBD; however, intake of hydrolysates of WP could also be associated with health promoting properties as described in a number of *in vivo* studies (Poullain *et al.*, 1989; Tomita *et al.*, 1991; Abubakar *et al.*, 1998; Mercier *et al.*, 2004). For example, hydrolysates from lactoferrin have shown antibacterial properties in *Escherichia coli* O111, which has potential commercial value as a food ingredient and for prevention and treatment of gastrointestinal diseases (Tomita *et al.*, 1991). Whey protein concentrates digested with seven different kinds of proteases have demonstrated antihypertensive activity in spontaneously hypertensive rats (Abubakar *et al.*, 1998). Rats fed with WPI hydrolysates demonstrated enhanced growth as a result of greater protein synthesis and lower ureagenesis as compared to rats fed whole proteins or free amino acids (Poullain *et al.*, 1989).

In conclusion, the findings from the present study demonstrated for the first time in H_2O_2-treated Caco-2 cells that hydrolysates of pWPI provides significantly greater antioxidant and anti-inflammatory effects as compared to the

native WPI hydrolysates. Further studies are needed to investigate the specific WPI hydrolysate components involved in the protective effects as well as the mechanisms of action, which could involve down-regulation of the NF-κB pathway.

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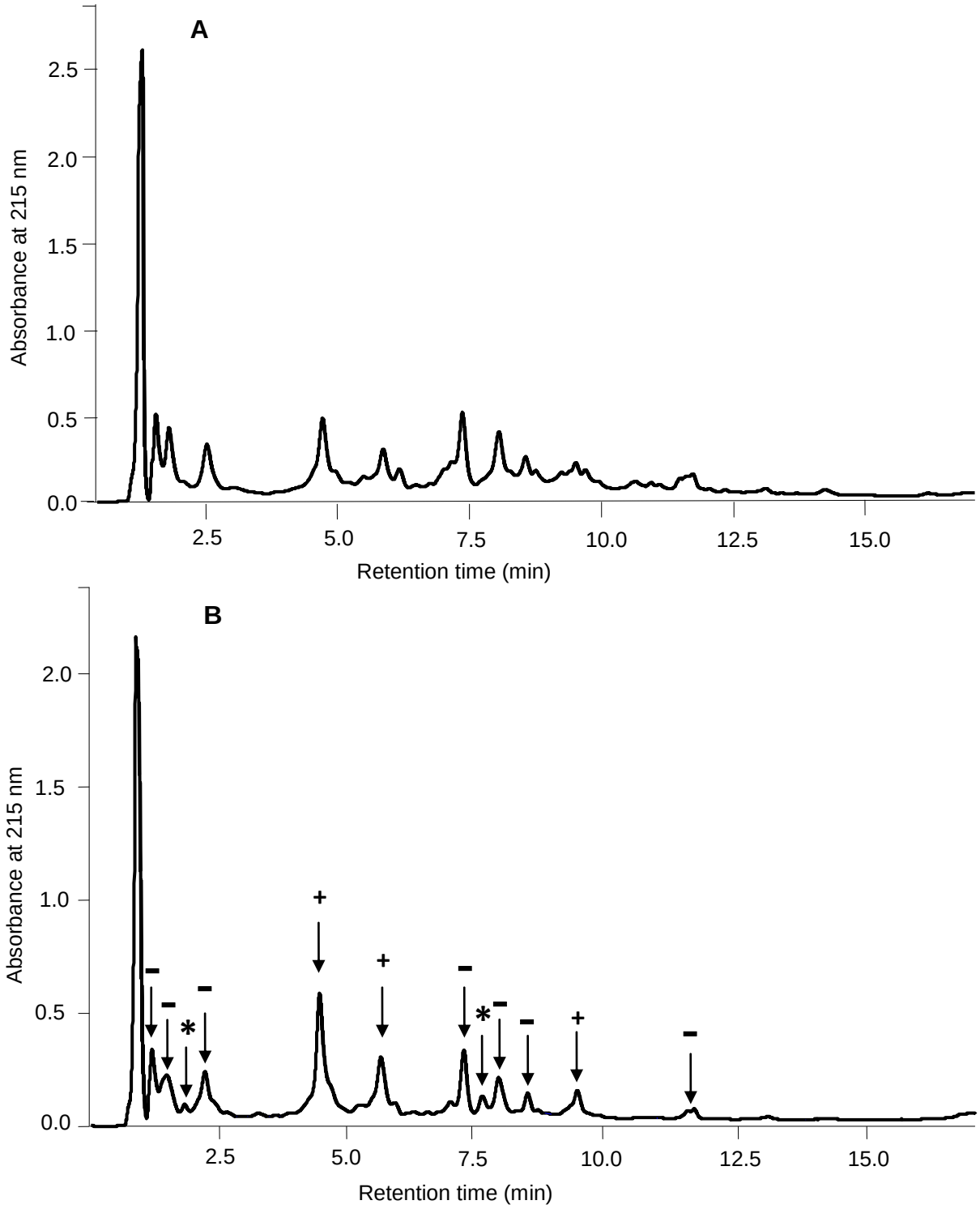
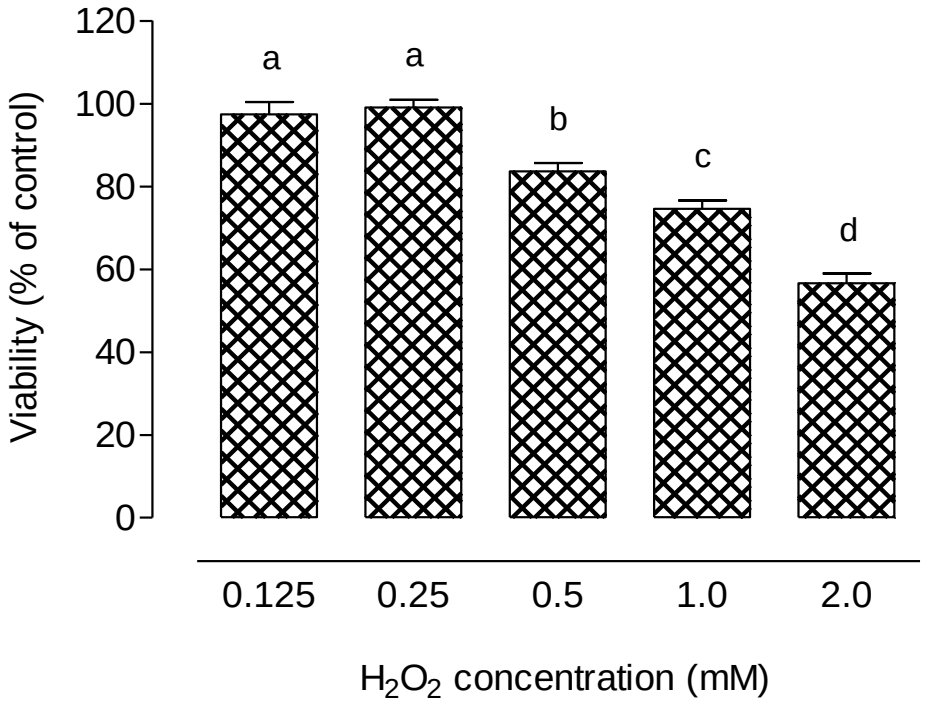
Figure 4.4.1 Chromatogram of peptides from reverse-phase HPLC obtained via UV/VIS detector wavelength of 215 nm.



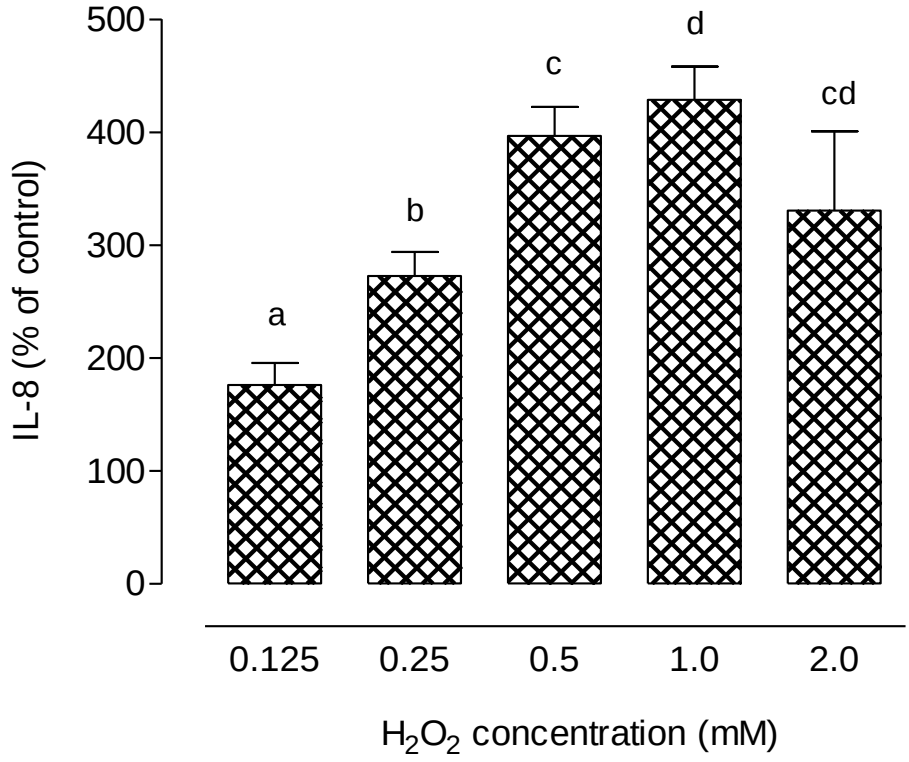
(**A**) Peptides with MWCO less than 10 kDa derived from pepsin, trypsin and chymotrypsin hydrolysis of nWPI. (**B**) Peptides with MWCO less than 10 kDa derived from pepsin, trypsin and chymotrypsin hydrolysis of WPI exposed to hydrostatic pressure at 1-cycle of 550 MPa. The * symbol identifies extra peaks found in pWPH hydrolysates and arrows indicate differences in the relative abundances of peptides as a result of pressure treatment (+, higher abundance, -, lower abundance in pWPI hydrolysates relative to nWPI hydrolysates).

Figure 4.4.2 Effects of different concentrations of H_2O_2 on Caco-2 cell viability.



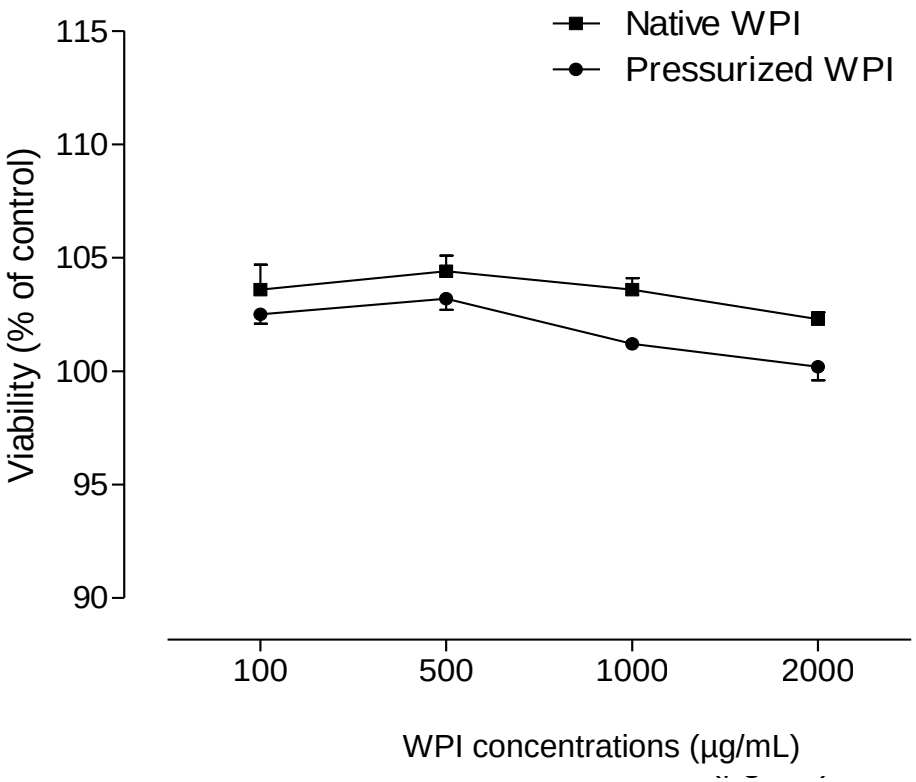
Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 0 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM, and 2.0 mM of H_2O_2 for 1 h. After 1 h, Caco-2 cells were washed twice with MEM and incubated for 23 h. In the next day, supernatant was collected, cells were treated with MTS reagent, incubated for 3 h at 37°C and made permeable with HCl-isopropanol to dissolve the formazan crystals. Optical densities were measured at 560 nm. Values are means ± SE of 3 independent experiments. Survival is expressed as percentage of untreated control cells. Means within each treatment without a common letter differ $P < 0.05$. One-way ANOVA adjusted for Tukey's *post hoc* test. H_2O_2 = Hydrogen peroxide.

Figure 4.4.3 Effect of different concentrations of H_2O_2 on IL-8 secretion of Caco-2 cells.



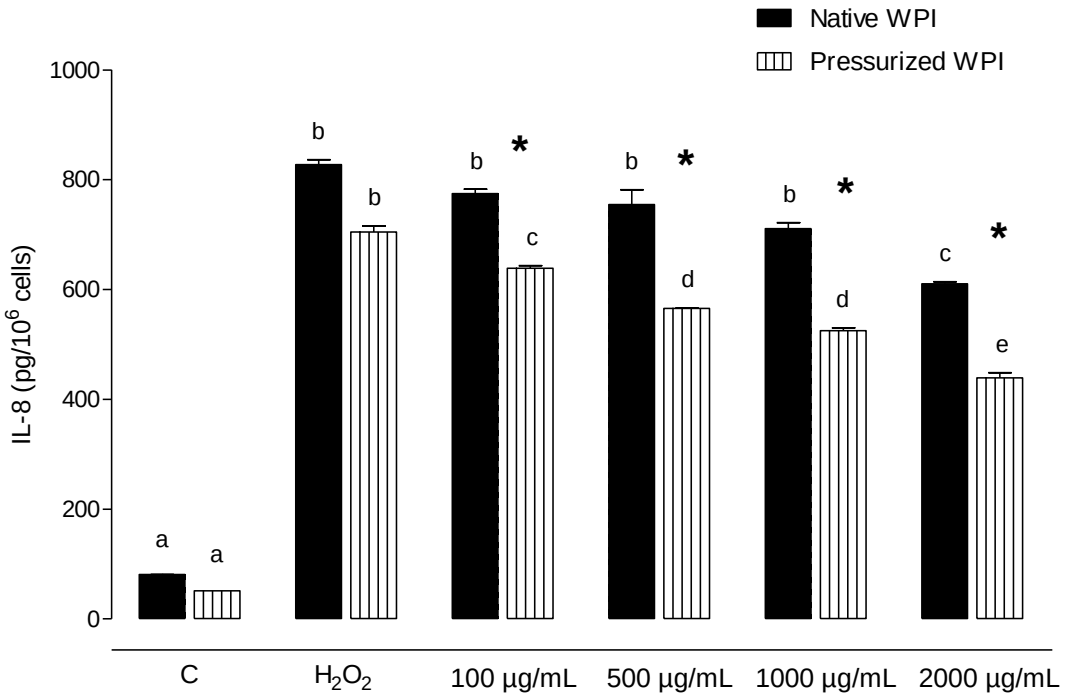
Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 0 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM, and 2.0 mM of H_2O_2 for 1 h. After 1 h, Caco-2 cells were washed twice with MEM and incubated for 23 h. In the next day, supernatant was collected to determine IL-8 release. Values are means ± SE of 3 independent experiments. Means within each treatment without a common letter differ $P < 0.05$. One-way ANOVA adjusted for Tukey's *post hoc* test. H_2O_2 = Hydrogen peroxide.

Figure 4.4.4 Effects of different concentrations of WPI on Caco-2 cell viability.



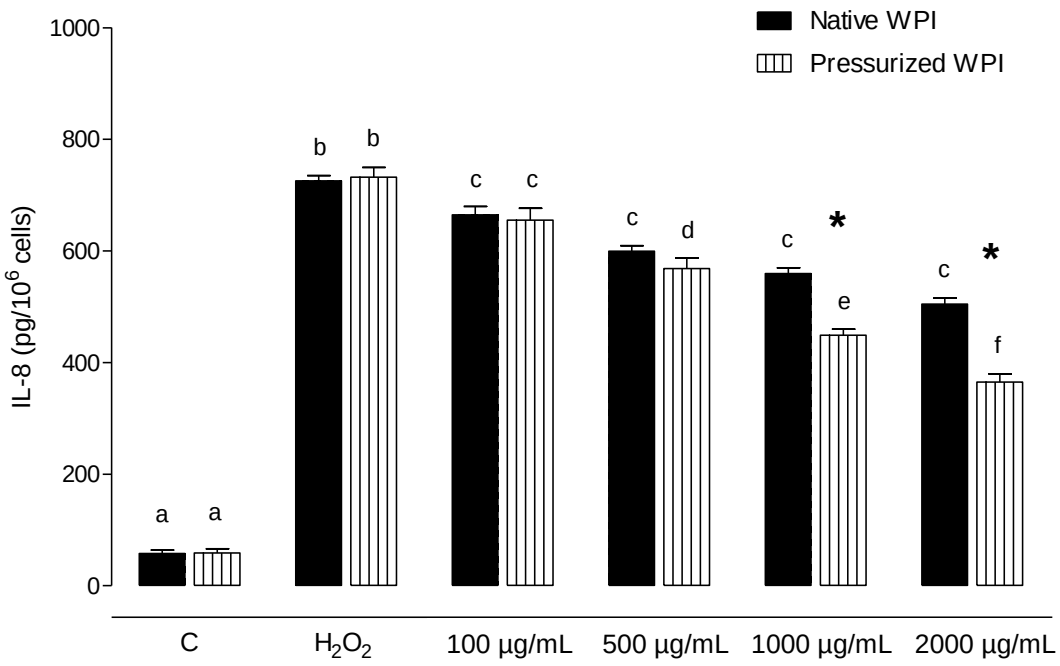
Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 100 µg/mL, 500 µg/mL, 1000 µg/mL, and 2000 µg/mL of either nWPI or pWPI hydrolysates were added for 24 h. In the next day, supernatant was removed, cells were treated with MTS reagent, incubated for 3 h at 37°C and made permeable with HCl-isopropanol to dissolve the formazan crystals. Optical densities were measured at 560 nm. Values are means ± SE of 3 independent experiments. Survival is expressed as percentage of untreated control cells. One-way ANOVA adjusted for Tukey's *post hoc* test. WPI = Whey protein isolate.

Figure 4.4.5 Protective effect of different concentrations of WPI hydrolysates on 0.25 mM H_2O_2 induced IL-8 secretion by Caco-2 cells.



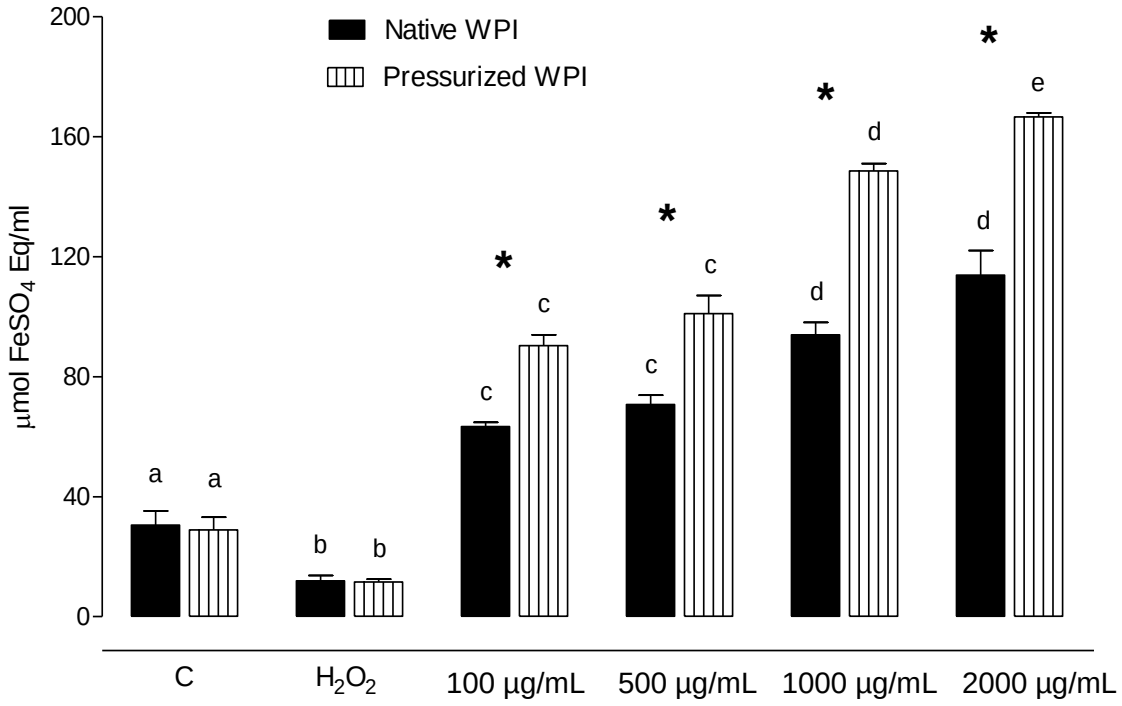
Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 100 µg/mL, 500 µg/mL, 1000 µg/mL, and 2000 µg/mL of either nWPI or pWPI hydrolysates were added for 1 h (pre-incubation). After 1 h, medium was removed, washed twice with MEM and the cells were incubated another 1 h with the same concentrations of WPI hydrolysates with 0.25 mM H_2O_2. Subsequently, Caco-2 cells were washed twice with MEM and nWPI or pWPI hydrolysates alone were added to the cells for 22 h. In the next day, supernatants were collected for IL-8 release. Values are means ± SE of 3 independent experiments. Means within each type of WPI treatment without a common letter differ $P < 0.05$. Two-way ANOVA adjusted for Tukey's *post hoc* test. The * symbol represents the comparison between treatments and significantly differ $P < 0.001$ by independent student t test. C = Control. H_2O_2 = Hydrogen peroxide. WPI = Whey protein isolate.

Figure 4.4.6 Recovery effects of different WPI hydrolysates treatment with pre-stimulation of 0.25 mM H_2O_2 on IL-8 secretion of Caco-2 cells.



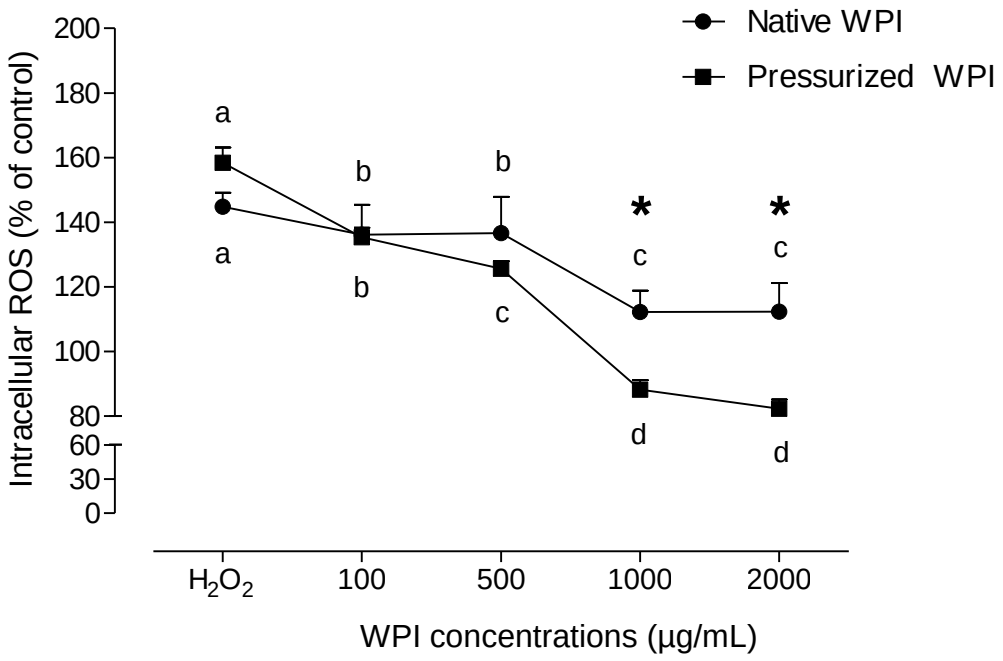
Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 2% HFBS and Caco-2 cells were exposed to 0.25 mM H_2O_2 for 1 h. After 1 h, medium was removed, washed twice with MEM and the cells were incubated with 100 µg/mL, 500 µg/mL, 1000 µg/mL, and 2000 µg/mL of either nWPI or pWPI hydrolysates were added and cells were allowed to recover for 23 h. In the next day, supernatants were collected for IL-8 release. Values are means ± SE of 3 independent experiments. Means within each type of WPI treatment without a common letter differ $P < 0.05$. Two-way ANOVA adjusted for Tukey's *post hoc* test. The * symbol represents the comparison between treatments and significantly differ $P < 0.001$ by independent student t test. C = Control. H_2O_2 = Hydrogen peroxide. WPI = Whey protein isolate.

Figure 4.4.7 The ferric reducing antioxidant power (FRAP) in the supernatant of Caco-2 cells exposed to 0.25 mM H_2O_2 and treated with either nWPI or pWPI hydrolysates.



Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 2% HFBS and Caco-2 cells were exposed to 0.25 mM H_2O_2 for 1 h. After 1 h, medium was removed, washed twice with MEM and the cells were incubated with 100 µg/mL, 500 µg/mL, 1000 µg/mL, and 2000 µg/mL of either nWPI or pWPI hydrolysates were added and cells were allowed to recover for 23 h. In the next day, supernatants were collected for FRAP activity. Values are means ± SE of 3 independent experiments. Means within each type of WPI treatment without a common letter differ $P < 0.05$. Two-way ANOVA adjusted for Tukey's *post hoc* test. The * symbol represents the comparison between treatments and significantly differ $P < 0.001$ by independent student t test. C = Control. H_2O_2 = Hydrogen peroxide. WPI = Whey protein isolate.

Figure 4.4.8 Effects of different WPI hydrolysates treatment on 0.25 mM H_2O_2-induced intracellular ROS generation in Caco-2 cells.



Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 2% HFBS and Caco-2 cells were exposed to 0.25 mM H_2O_2 for 1 h. After 1 h, medium was removed, washed twice with MEM and the cells were incubated with 100 µg/mL, 500 µg/mL, 1000 µg/mL, and 2000 µg/mL of either nWPI or pWPI hydrolysates were added and cells were allowed to recover for 23 h. In the next day, supernatants were removed DCFH-DA in phosphate buffered saline containing DMSO solution for 30 min at 37°C. The fluorescent DCF was monitored by spectrofluorometry (fluorescence excitation and emission were 485 nm and 530 nm, respectively). Values are means ± SE of 3 independent experiments. Means within each type of WPI treatment without a common letter differ $P < 0.05$. Two-way ANOVA adjusted for Tukey's *post hoc* test. The * symbol represents the comparison between treatments and significantly differ $P < 0.01$ by independent student t test. C = Control. H_2O_2 = Hydrogen peroxide. WPI = Whey protein isolate.

CONNECTING STATEMENT

Results obtained from Chapter 4 showed that hydrolysates from nWPI and pWPI have different peptide profiles, which might lead to different potencies of their antioxidant and anti-inflammatory activities. Anti-inflammatory effects of the nWPI and pWPI were demonstrated in terms of suppression of the H_2O_2-induced IL-8 secretion in Caco-2 cells, which was used as an *in vitro* model of colonic inflammation. Likewise, decreased intracellular ROS generation and increased FRAP antioxidant capacity in the cell culture medium were observed in H_2O_2-treated Caco-2 cells receiving nWPI and pWPI hydrolysate treatments. Significantly, H_2O_2-treated Caco-2 cells exhibited a higher degree of antioxidant and anti-inflammatory effects when exposed to pWPI versus nWPI hydrolysates. Thus, the combined results from Chapter 3 and 4 have shown that WPI treatment can exert antioxidant and anti-inflammatory activities in two different models of gut inflammation, which is enhanced by pressure treatment of WPI. Similar to WPI, polyphenols have received considerable research attention regarding their antioxidant and anti-inflammatory properties. Hence, polyphenols are worthwhile to investigate as another nutritional prevention and treatment approach towards gut inflammatory diseases and disorders. Potatoes have been considered a good source of polyphenols with specific cultivars identified to have a particularly rich polyphenolic content. There is emerging evidence that both digestion and colonic bacteria may modify polyphenols in terms of their structure and function that, in turn, may conceivably affect their antioxidant and anti-inflammatory effects on colonic cells. In that regard, a major limitation of previous research involving the functional impact of polyphenols on Caco-2 cell models of inflammation has been that the polyphenols were not exposed to the physiological conditions of digestion and intestinal bacterial metabolism. Hence, the following chapter will examine the antioxidant and anti-inflammatory parameters in H_2O_2-stimulated Caco-2 cells treated with faecal water alone or faecal water with polyphenols exposed to digestive enzyme and bacterial metabolism via a simulated human gut model.

Chapter 5

Manuscript 3

POTATO-DERIVED POLYPHENOLIC EXTRACTS THAT UNDERGO SIMULATED HUMAN GUT DIGESTION DECREASE OXIDATIVE STRESS AND THE INFLAMMATORY RESPONSE IN CACO-2 CELLS

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Running title: Polyphenolic extracts and Caco-2 cells

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5.1 ABSTRACT

The combination of polyphenolic compounds found significant amounts in potatoes may act synergistically to exert antioxidant and anti-inflammatory effects, which could be beneficial for the treatment of gut inflammation. The impact of gut enzymatic and bacterial metabolism of polyphenols on their antioxidant and anti-inflammatory properties in colonic cells exposed to pro-inflammatory stimuli, however, is unclear. The goal of the present study was to evaluate the antioxidant and anti-inflammatory potency of polyphenolic-rich potato extracts in stimulated Caco-2 cells, after the extracts were subjected to digestive processes in a simulated human gut model. In addition, Caco-2 epithelial cells were exposed to various doses (0.1, 0.01, 1 and 10%) of faecal water (FW) with polyphenolics (FW + Phenolics) or without polyphenolics in the presence and absence of 1 h pre-treatment 0.25 mM H_2O_2. Cell viability, IL-8 secretion, intracellular reactive oxygen species (ROS) formation and ferric reducing antioxidant power (FRAP) activity were evaluated. The *in vitro* simulated human digestion demonstrated a high release of phenolics compounds from the potato extract in the colon compartment, which signifies that the majority of the polyphenolics are bioaccessible in the colon and thus capable of exerting antioxidant and anti-inflammatory effects on the large bowel. Caco-2 cell viability was reduced and IL-8 secretion was increased in a dose response manner ($P < 0.05$) by FW exposure, which was also observed in FW-exposed cells that were pre-treated with H_2O_2. In contrast, when FW contained polyphenolics, the cell viability was unaffected and IL-8 release was significantly lowered in Caco-2 cells exposed to either FW or FW + H_2O_2. Also, FW + Phenolics had lower ($P < 0.05$) intracellular ROS generation as well as greater ($P < 0.05$) FRAP activity in the cell culture medium regardless of H_2O_2 pre-exposure. In conclusion, the polyphenolic-rich potato extracts that underwent digestion in a simulated human gut model exerted antioxidant effects and decreased cytotoxicity and inflammation in Caco-2 cells exposed to both FW and FW + H_2O_2, which suggests that polyphenols may have protective effects against colonic inflammation.

5.2 INTRODUCTION

Polyphenols, which are characterized by the presence of more than one phenolic group, have potential benefit for prevention of several chronic diseases including diabetes, cardiovascular disease, and cancer (Liu, 2003). The anti-inflammatory roles of polyphenolics have been suggested to have benefits towards gut health (Scalbert *et al.*, 2002; Van De Walle *et al.*, 2010) as a few studies involving *in vivo* and *in vitro* models of gut inflammation have shown that polyphenol-rich plant extracts can have antioxidant (RiceEvans *et al.*, 1997; Miccadei *et al.*, 2008) and anti-inflammatory effects (McClain *et al.*, 2001; Tedeschi *et al.*, 2004; Cuzzocrea *et al.*, 2005; Netsch *et al.*, 2006). Stimulated Caco-2 cells are a well established model that mimics gut inflammation as seen in colitis (Tanoue *et al.*, 2008) and green tea polyphenol extracts have shown antioxidant and anti-inflammatory effects on IL-1β-stimulated Caco-2 cells by increasing supernatant ferric reducing antioxidant power (FRAP) activity and decreasing TNF-α and IL-8 secretion (Netsch *et al.*, 2006). In another study, oak (*Quercus robur*) and heartwood extracts exerted anti-inflammatory effects in IL-1β-stimulated Caco-2 cells by inhibiting NF-kB transcriptional activity and reducing IL-8 secretion (Romier-Crouzet *et al.*, 2009). In addition, three separate animal studies have demonstrated the capability of rutin and its glycoside quercetin to decrease colonic inflammation in rat models of DSS- and TNBS-induced colitis (Comalada *et al.*, 2005; Kim *et al.*, 2005; Galvez *et al.*, 2006). Moreover, polyphenolic compounds that are not absorbed may protect lipids, proteins, and carbohydrates from oxidative damage during digestion, and spare soluble antioxidants (Bravo, 1998).

The polyphenolic compounds chlorogenic acid (CGA), caffeic acid (CA), ferulic acid (FA), rutin, and other free or bound phenolics are present in significant amounts in potatoes (Giusti *et al.*, 1999). Such phenolics may act synergistically to enhance the antioxidant capacity of potato extracts as recently suggested via the use of potato peel extracts as antioxidants in foods (Kanatt *et al.*, 2005). The release of polyphenols from potato extracts during digestion is important to characterize in order to assess their bioaccessibility for absorption

and for their potential antioxidant and anti-inflammatory effects in the human colon. Also, the biotransformation of polyphenols by human intestinal microorganisms to produce a variety of metabolic end-products has been well characterized (Smith and Macfarlane, 1997). Since polyphenols will have different structures and functions than their metabolites following exposure to digestive enzymes and bacterial metabolism (Han *et al.*, 2007), these metabolites will likely have altered antioxidant and anti-inflammatory effects. To date, however, studies have sparsely investigated the antioxidant and anti-inflammatory properties of polyphenols following their bacterial or enzymatic metabolism, including in relation to colonic cells.

The goals of the present study were to: (a) evaluate the bioaccessibility of polyphenolic-rich potato extracts in the colonic compartment after these extracts were subjected to digestive processes in a simulated human gut model; and (b) to examine for the antioxidant and anti-inflammatory potency of these colonic digests in cultured Caco-2 cells exposed to the pro-inflammatory stimulus of H_2O_2.

5.3 MATERIALS AND METHODS

Caco-2 cell preparation

The human colon cancer-derived Caco-2 intestinal epithelial cell system has been widely used as an *in vitro* model of the intestinal epithelium (Quaroni *et al.*, 1979). Confluent Caco-2 cells form tight junctions and attain many morphological and functional characteristics of enterocytes (Hidalgo *et al.*, 1989). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, pH 7.4) supplemented with 25 mM glucose, 20% heat-inactivated fetal bovine serum (HFBS), 1% penicillin-streptomycin, and 1% non-essential amino acid solution (Jumarie and Malo, 1991). Cells were maintained at 37ºC in a humidified 5% CO_2 atmosphere.

Viability assay

The effect of H_2O_2 on cell survival was evaluated by measuring mitochondrial succinate dehydrogenase activity (MTS test) (Mosmann, 1983). Briefly, Caco-2 cells (passage between 21 and 27-d) were seeded in 24-multiwell plates (Costar, Corning, NY, USA) at a cell density of 2.5 x 10^5 cells/well. After cells reached confluence, they were incubated with serially-diluted H_2O_2 ranging from 0 to 2 mM for 1 h at 37ºC. In sequence, H_2O_2 was aspirated and Caco-2 cells were washed twice with Minimum Essential Medium Eagle (MEM, pH 7.4) supplemented with 2% HFBS and incubated for 23 h. Subsequently, 10 µL of MTT reagent (Cayman Chemical, Ann Arbor, MI, USA) was added to each well and incubated for 3 h at 37˚C in a CO_2 incubator (Midsci, St. Louis, MO, USA). After incubation, the MTS product (formazan) was solubilised in 100 µL of acidified isopropanol (40 mM HCl). The absorbance of each sample was analyzed at 560 nm using the Wallac Victor^2 1420 Series microplate reader (Harlow Scientific, Arlington, MA, USA). Cell survival was expressed as a percentage of the viability of untreated control cells. The experiments were performed three times, with each individual treatment being run in triplicate.

Biochemical assay

Caco-2 cells were incubated in 24-well plates for 24 h at 37ºC. After cells reached confluence, they were incubated for 1 h with different dilutions of H_2O_2 (0 to 2 mM). Cells were washed twice with MEM for the removal of H_2O_2 and incubated for 23 h. Supernatants were then collected, centrifuged at 2,000 x g for 20 min to remove cellular debris, and stored at -80ºC until analysis. Determination of IL-8 release in supernatants was done using an assay kit (BD OptEIA set human IL-8, Biosciences, Mississaga, ON, Canada) and quantities expressed as percentage of control IL-8 levels in untreated control cells. The experiments were performed three times, with each individual treatment being run in triplicate.

Potato polyphenolic extraction

The polyphenolic content and profiles of 12 potato cultivars commonly grown in Canada were previously characterized, which showed large variations in antioxidant capacity and in major polyphenols, particularly CGA, CA, FA, and rutin (Piccolomini *et al.*, 2008a; b; Vunnam, 2010). Further analysis of the same potato cultivars demonstrated consistently high season-to-season polyphenol content and total antioxidant capacity in the potato cultivar 'Onaway' (Vunnam, 2010). 'Onaway' was therefore selected to generate polyphenol-rich extracts for subsequent human simulated gut digestion and cell culture studies. To generate the extract, 0.1 g of lyophilized potato sample was added to 2 mL of 95% methanol (MeOH), vortexed, sonicated for 30 min in cold H_2O, vortexed, and centrifuged at 2,000 x g for 10 min at 4ºC. The supernatant was collected and placed in a 5 mL tube. Thereafter, 1 mL of 95% MeOH was added to the pellet, and the above extraction method was repeated. The supernatants were collected and pooled together. The final total volume of the pooled samples (approx. 3 mL) was immediately frozen in liquid N_2 and lyophilized at -80ºC and 90 MT vacuum in a Flexi-Dry MP lyophilizer (FTS Systems, Stone Ridge, NY, USA). To quantify the polyphenolic content of the freeze-dried powder, 10 mg was solubilised in 1 mL of 95% MeOH and the concentration of polyphenols was determined by HPLC analysis utilizing the same procedure as described below. This polyphenolic-rich powder was utilized for subsequent simulated human gut digestion studies. The proximate composition in 100 g of the 'Onaway' potato cultivar was 73.2% carbohydrates, 6.8% ashes, and 13.4% moisture.

Gut model

It is estimated that the human intake of total phenolics in the average adult American diet is about 1 g/day (Scalbert and Williamson, 2000). To approximate this intake, 130 g of 'Onaway' extract containing 1.4 g of total phenolics was digested in a simulated human GI model called The Computer Controlled Dynamic Human Gastrointestinal Model. This was developed based on the

Simulator of the Human Intestinal Microbial Ecosystem (SHIME) first described by Molly *et al.*, (1993).

The components of simulated human GI model involve five reactors, of which the last three reactors contained microbiota of a different part of the human GI tract, in order of sequence (reactors 1 to 5): the stomach, small intestine (duodenum, jejunum, and ileum), the ascending, the transverse, and the descending colon (Figure 5.3.1) (Martoni *et al.*, 2007). The system is fully computer-controlled and has been validated using enumeration procedures, short chain fatty acids (SCFA) production patterns, enzymatic activities, gas production, and by microorganism-associated activities (Molly *et al.*, 1994; Martoni *et al.*, 2007; Blanquet-Diot *et al.*, 2009). Five healthy, non-smoking individuals with no history of GI disease or antibiotic use in the previous 6 mo. provided faecal samples for the study. Faecal samples were freshly collected, pooled, and readily used to prepare the faecal solution. The system underwent a 2-wk stabilization period to allow bacterial communities from the human fecal diluted samples to grow and stabilize.

Following stabilization, the microbial ecosystem was sustained by feeding 300 mL of sterile medium (set at pH 2 before autoclaving and stored at 37ºC) every 8 h. The composition of the gastrointestinal (GI) nutrient solution was described by Molly et al. (1994), and contained 1 g/L arabinogalactan, 2 g/L pectin, 3 g/L starch, and 1 g/L xylan, 0.4 g/L glucose, 3 g/L yeast extracts, 1 g/L peptone, 4 g/L mucin, and 0.5 g/L cysteine, which are essential for bacterial survival.

The two GI nutrient solutions utilized, for this study were: (a) the control GI nutrient solution described above; and (b) the GI nutrient solution into which the potato extract was incorporated. One day of treatment was followed by a 3 day washout period, in which the system was fed with the control GI nutrient solution without potato extract. Each treatment lasted 24 h and sampling was done every 8 h throughout the treatment day.

For each day of treatment, an aliquot (20 mL) was removed from vessels 3 thru 5, combined, and stored at -80ºC for later analysis and characterization of

polyphenol content. To prevent photodecomposition of the polyphenols, all of the digestive compartments and collection vessels were wrapped in tin foil. After each triplicate treatment, the system was cleaned with saline and 2 M NaOH to remove remaining substances in the vessels. The fermentation vessels were maintained anaerobic by purging the headspace with oxygen-free nitrogen and stirring continuously on magnetic stirrers. The temperature of the simulator was kept at 37ºC. Upon entering vessel 2 (small intestine), pancreatic juice supplemented with bile (12 g/L NaHCO, 0.9 g/ L pancreatin; Sigma, St. Louis, MO, USA) and 6 g/L Oxgall (Difco, Detroit, MI, USA) was added to neutralize stomach acidity. In this way, the pH of the first three vessels was determined by the input of either supply medium or pancreatic juice. Vessels 3, 4, and 5 (representing the colon) were pH-controlled between 5.6 and 5.9, 6.1 and 6.4, and 6.6 and 6.9, respectively. The pH was measured with a probe connected to a pH meter (Fisher Scientific, Ottawa, ON, Canada) and was automatically adjusted by adding 0.2 M NaOH or 0.5 M HCl. Each *in vitro* digestion was performed three times, with each individual treatment lasted 24 h.

Faecal water (FW) preparation

FW represents the portion of colonic content that directly contacts the colonic epithelium cells. FW was prepared with the method previously described with slight modifications (Rieger *et al.*, 1999). The contents of vessel 3, 4, and 5 were pooled and centrifuged at 36,000 x g, 4ºC for 2 h. The supernatant fluid (neutralized to pH 7) and the FW was filter sterilized (Millipore, 0.8/0.2 µm; Billerica, MA, USA) and stored at -20ºC until use in the Caco-2 cell experiments. The pooled samples are representative of 1 day of experiment.

Phenolics HPLC analysis

The following phenolics CGA, CA, FA, and rutin were quantified using HPLC by a previous developed method (Shakya and Navarre, 2006). Initially, samples (50 mg of freeze-dried powder) were extracted in 0.9 mL of extraction buffer (50% MeOH, 2.5% metaphosphoric acid, 1 mM EDTA) in a 2 mL screw-cap tube.

Samples were vortexed for 30 sec and centrifuged at 11,070 x g for 15 min at 4°C. The supernatant was transferred to a 1.5 mL glass vial. The remaining pellet was re-extracted with 0.6 mL of extraction buffer and centrifuged. The supernatants were combined and concentrated in a Speed Vac (Thermo Savant SC 210A, Waltham, MA, USA). The concentrated samples were solubilised with 500 µL of extraction buffer and filtered through 0.45 µm membrane filters (Durapore, PVDF, Fisher Scientific, New Jersey, NY, USA) into 1 mL HPLC glass vials. Samples were kept chilled at all times and shielded from bright light.

Samples were analyzed using a Varian HPLC system with a quaternary gradient pump, a single wavelength UV/VIS detector, and an auto-sampler with refrigerated sample compartment (Varian Canada Inc, Mississauga, ON, Canada). Samples were eluted using a Gemini NX RP-HPLC column (100 x 4.5 mm) (Phenomenex, CA, USA), a solvent flow rate of 2 mL/min and detection was at 280 nm. Solvent gradient of 0-1 min 100% buffer A (10 mM formic acid, pH 3.5, with NH_4OH), 1-5 min 0-30% buffer B (100% methanol with 5 mM ammonium formate), 5-6.5 min 40-70% buffer B, 6.5-8.5 min 70-100% buffer B. The phenolic acids in the samples were identified using standards.

Exposure of Caco-2 cells to FW and H_2O_2

To investigate the cytotoxic effect, Caco-2 cells without or with exposure to a pro-inflammatory stimulus of 0.25 mM H_2O_2 for 1 h (stimulated Caco-2 cells) were treated with FW alone or FW containing digested polyphenols (FW + Phenolics). FW and FW + Phenolics were diluted to 0.01%, 0.1%, 1%, and 10% (v/v) in MEM with 2% HFBS for all experiments. Caco-2 cells were treated with FW or FW + Phenolics for 24 h. After the supernatant was removed, cells were washed twice with MEM with 2% HFBS and either FW or FW + Phenolics were added to the cells for 23 h. The supernatants were collected for IL-8 determination 24 h after the treatment.

Organic acids

Organic acids (ascorbic, citric, malic, and succinic) were identified by HPLC analysis in the undigested 'Onaway' extract, FW, and FW + Phenolics. Briefly, an aliquot of the samples was used for analysis with the Varian HPLC system with a tertiary gradient pump, a variable wavelength UV/VIS detector, and an autosampler with refrigerated sample compartment (Varian Canada Inc, Mississauga, ON, Canada). Samples were eluted using a Gemini-NX reverse-phase HPLC column (100 x 4.5 mm) (Phenomenex, CA, USA). The samples and organic acid standards were eluted with 1 mL of aqueous 0.008 N H_2SO_4 under isocratic conditions at 245 nm for ascorbic acid and at 210 nm for citric, malic, and succinic acids (Schwartz *et al.*, 1962).

Ferric reducing antioxidant power (FRAP) assay

FRAP was used to determine the total antioxidant potential in the supernatant of Caco-2 and stimulated Caco-2 cells exposed to FW or FW + Phenolics. Briefly, in this assay the electron-donating capacity of the antioxidant was measured by the change in absorbance (abs) at 593 nm when a blue-coloured Fe^{2+}-tripyridyltriazine (Fe^{2+}TPTZ) compound is formed from a colorless oxidized Fe^{3+} form (Benzie and Strain, 1999). A standard curve was prepared from aqueous solutions of 1 mM $FeSO_4.7H_2O$ at different concentrations ranging from 0.1 to 1.0 mM. The reagent was prepared with 10:1:1 ratio of 300 mM acetate buffer pH 3.6: 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution: 40 mM HCl at 50ºC, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The FRAP working solution was placed into a foil-wrapped container. Once the reagent was prepared it was immediately incubated for 10 min at 37°C. Into a 96-well plate, 30 µL H_2O, 10 µL standards or samples, and 200 µL FRAP working solution were added. Samples were left to react at room temperature for 30 min then the absorbance was read at 593 nm in a microplate reader (Infinite PRO 200 series, Tecan Group, San Jose, CA, USA).

Determination of total polyphenolics using Folin-Ciocalteu reagent

The total phenolic content of the potato extract was measured spectrophotometrically by the Folin-Ciocalteu colorimetric method (Chirinos *et al.*, 2007; Andre *et al.*, 2009). This assay was used to measure the total phenolic content in the undigested 'Onaway' extract, FW, and FW + Phenolics. Gallic acid was used as a standard to determine the total phenolic content of the sample and the results were expressed in milligrams of gallic acid equivalents per mg/L. Gallic acid standard stock solution was prepared by dissolving 20 mg of gallic acid in 10 mL of MeOH. This standard stock solution was used to prepare a gallic acid standard dilution series with MeOH. Using a micropipette, 100 µL of gallic acid from a dilution series (from 1,000 to 5 mg/L) or samples were transferred into 4 mL microcentrifuge tubes, to which 2 mL of water and 200 µL of 2 N Folin-Ciocalteau reagent (FC reagent; Sigma-Aldrich, Oakville, ON, Canada) were added. The latter solution was left to react for 30 min at room temperature, then 1 mL 1 N Na_2CO_3 aqueous solution was added to this reaction mixture and vortexed for 30 s, and then left to react for a further 60 min at room temperature. The samples were then pipetted into 2.5 mL cuvettes and their absorbance read at 765 nm in a spectrophotometer (Beckman DU 640, Beckman Instruments, Fullerton, CA, USA) from which a standard curve equation was developed. The experiments were performed three times, with each individual treatment being run in triplicate.

Intracellular reactive oxygen species (ROS)

The generation of intracellular ROS was evaluated by oxidation of 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Invitrogen, Carlsbad, CA, USA). The DCFH-DA was cleaved in the cells to dichlorofluorescin (DCFH) and oxidized by ROS to fluorescent dichlorofluorescein (DCF) (Yokomizo and Moriwaki, 2006). In the first trial, Caco-2 cells were treated with different concentrations of H_2O_2 for 1 h, then washed twice with MEM, and were allowed to recover for 23 h. In the second trial, stimulated Caco-2 cells were washed twice, then treated with FW or FW + Phenolics for 23 h. After treatment, cells were incubated with 5 µM DCFH-

DA in phosphate buffered saline containing 5% (w/v) dimethyl sulfoxide (DMSO) solution for 30 min at 37°C. The fluorescent DCF was monitored by spectrofluorometry (fluorescence excitation and emission were 485 nm and 530 nm, respectively; Wallac Victor^2 1420 Series spectrofluorometry reader - Harlow Scientific, Arlington, MA, USA). The values were expressed as percentages of DCF fluorescence intensity compared with untreated control cells. The experiments were performed three times, with each individual treatment being run in triplicate.

Statistical analysis

Data were reported as the mean and standard errors. Two-way ANOVA (SAS for Windows, version 9.2, 2008, SAS Institute Inc., Cary, NC) was performed with Tukey's *post hoc* test to determine significant statistical differences between groups after verification of normality using Shapiro-Wilk. Statistical significance was declared at $P < 0.05$.

5.4 RESULTS

Identification of polyphenolic compounds by reverse-phase HPLC

Figure 5.4.1 represents the polyphenolics profile of: (A) undigested 'Onaway' potato extract, (B) FW and (C) FW + Phenolics. In panel A, the major polyphenols, neo-chlorogenic acid, CGA, CA, FA, and rutin are identified in potato extract. Panel B shows the absence of polyphenolic compounds in FW. In panel C, new peaks representing protocatechuic acid, crypto-chlorogenic acid, and quercetin are present in the colon compartment after simulated human gut metabolism of the potato extract, in addition to the major phenolic compounds found in panel A. In addition, the peak representing rutin was not observed in the chromatogram of Panel C. Differences in the peak abundances as well as six new peaks were observed in the FW with phenolics (Panel C) that were not present in the FW alone (Panel B). The undigested 'Onaway' extract (A), FW alone (B), and FW + Phenolics (C) had total phenolic concentrations of 320.5, 52.4, and 292.8 gallic acid equivalents (mg/L), respectively. Table 5.4.1 shows

the polyphenolic concentrations of potato extract and in FW + Phenolics. Apart from rutin, all the polyphenolic concentrations were greater in FW + Phenolics as compared to those seen in the potato extract in the GI nutrient solution used in the Dynamic Human Gastrointestinal Model.

Identification of organic acids by RP-HPLC

Organic acids identified from undigested 'Onaway' extract (A), FW (B) and FW + Phenolics (C) are shown in Figure 5.4.2. Panel A shows that only ascorbic acid was present in the potato extract, whereas in panels B and C, citric, succinic, and malic acids were found in addition to ascorbic acid. Organic acid concentrations for FW and FW + Phenolics were: 0.2 µmol/L and 9.1 µmol/L (ascorbic acid); 53.6 µmol/L and 402.8 µmol/L (citric acid), 7.0 µmol/L to 8.8 µmol/L (malic acid); and 135.5 µmol/L and 57.3 µmol/L (succinic acid), respectively.

Effect of FW treatments on Caco-2 cell viability

The effects on cell viability with different FW treatments on control and stimulated Caco-2 cells are shown in Figure 5.4.3. Panel A demonstrates reduced ($P < 0.05$) Caco-2 cell viability of 43% and 84% when treated with 1% and 10% FW, respectively, compared with untreated control cells. The 10% FW treatment reduced cell viability and so was excluded from further experiments. Caco-2 cells treated with different concentrations of FW + Phenolics had unaffected cell viability. Panel B shows reduced ($P < 0.05$) Caco-2 cell viability of 50% and 83% when exposed to 1% and 10%, respectively, compared with untreated control cells. Caco-2 cells stimulated with H_2O_2 and then treated with FW + Phenolics showed no adverse effect on viability.

Effect of FW treatments on the inflammatory response in Caco-2 cells

Figure 5.4.4 represents the effects on IL-8 secretion with different FW treatments on control and stimulated Caco-2 cells. Panel A shows increased ($P < 0.05$) IL-8 release of 210%, 338%, and 786% in Caco-2 cells treated with FW at 0.01%, 0.1%, and 1%, respectively, while Caco-2 cells treated with FW + Phenolics had

lesser relative to the cells treated with FW at the 0.01%, 0.1%, and 1% doses, but still showed significantly increased secretion compared with untreated control cells. Panel B shows that Caco-2 cells pre-treated with H_2O_2 and thereafter exposed to varying concentrations of FW (0.01%, 0.1%, and 1%) all had increased ($P < 0.05$) IL-8 secretion as compared with control untreated cells. The exposure of H_2O_2-stimulated Caco-2 cells to both 0.01% and 0.1% FW + Phenolics was associated with lowered ($P < 0.05$) IL-8 release relative to cells treated with H_2O_2 alone. Also, exposure to 0.01%, 0.1% and 1.0% FW + Phenolics was associated with lowered ($P < 0.05$) IL-8 concentrations as compared to each of the respective 0.01%, 0.1% and 1.0% FW treatments.

Effect of FW treatments on the antioxidant capacity of Caco-2 cell medium

The ferric reducing antioxidant power (FRAP) measured in the supernatant of control and stimulated Caco-2 cells exposed to FW or FW + Phenolics is shown in Figure 5.4.5. Panel A shows that Caco-2 cells exposed to FW + Phenolics had increased ($P < 0.05$) cell culture medium antioxidant capacity when cells were treated with 1% and 10% FW, respectively, as compared cells treated with the respective treatments of 0.1% and 1.0% FW alone and relative to the control cells. Panel B shows that stimulated Caco-2 cells had increased ($P < 0.05$) cell culture medium antioxidant capacity when treated with 10% FW, as compared to control cells. FW + Phenolics had increased ($P < 0.05$) cell culture medium antioxidant capacity at the 0.01%, 1%, and 10% FW concentrations relative to the respective treatments of 0.01% 1%, and 10% FW alone and as compared to the control cells.

Effect of FW treatments on the intracellular ROS generation in Caco-2 cells

Figure 5.4.6 shows intracellular ROS generation in different FW treatments in control and stimulated Caco-2 cells. Panel A shows Caco-2 cells treated with 0.1%, 1%, and 10% FW + Phenolics had reduced ($P < 0.05$) intracellular ROS generation as compared with the Caco-2 cells exposed to 0.1% and1% FW alone. The 0.1%, 1%, and 10% of FW + Phenolics Caco-2 treated cells had

reduced ($P < 0.05$) intracellular ROS production in comparison to the untreated control cells. Panel B shows H_2O_2-stimulated Caco-2 cells treated with 0.1%, 1%, and 10% of FW + Phenolics had reduced ($P < 0.05$) intracellular ROS formation as compared with Caco-2 cells receiving FW treatment alone at each of the respective FW concentrations of 0.1%, 1%, and 10% as well as in comparison to the cells treated only with H_2O_2. Also, 0.1%, 1%, and 10% of FW + Phenolics treatment was associated with lowered ($P < 0.05$) intracellular ROS production in comparison to control cells.

5.5 DISCUSSION

The present study has demonstrated several novel findings using the combination of the dynamic human simulated gut model and Caco-2 cell culture as assessment tools to study the protective effects of polyphenolic-rich potato extracts on colonic cell cytotoxicity and inflammation. Firstly, exposure of the extracts to simulated human digestion that mimics the digestive and microbial metabolism of the human gut resulted in a high bioaccessibility of polyphenols in the colon together with an altered polyphenolic profile. Secondly, this study showed that polyphenolic compounds taken from the colonic compartment of the gut model could protect against the cytotoxicity of FW on Caco-2 cells. Thirdly, the digested polyphenolic extracts obtained from the colon vessels also had a protective effect against inflammation and oxidative stress in Caco-2 cells induced by both FW and the combination of H_2O_2 and FW.

Based on the total phenolic content observed post in vitro simulated human digestion, there appears to be high percentage (125%) release of phenolic compounds from the matrix of the potato extract. The higher release of phenolic compounds in the colon compartment relative to the original starting concentrations in the GI nutrient solution could be related to gut microfloral-mediated release of free phenolic acids from the bound form of phenolics that may be present within the potato extract matrix. As much as 40% of the total phenolic content in potatoes has been reported to be present in the bound form (Chu et al., 2002). Gut microbial metabolism has been suggested to lead to an

increase in the bioaccessibility of non-extractable polyphenolics that are bound to dietary fibers, resistant starches and polysaccharides (Visioli et al. 2011). This finding therefore signifies that a majority of the polyphenols contained in the potato extract are bioaccessible in the colon and thus capable of exerting antioxidant and anti-inflammatory effects on the large bowel. This result is in agreement with recent work showing that the majority of polyphenols from almond skin powder survive *in vitro* gastric and duodenal digestive enzymatic conditions (Mandalari *et al.*, 2010). In this study, marked changes were observed in the HPLC phenolic acid profiles with the disappearance of rutin and the appearance of new polyphenolic components in terms of quercetin, protocatechuic acid and crypto-chlorogenic acid. In addition to the polyphenols that were identified, microbial and digestive enzyme metabolism likely could account for some of the unidentified HPLC peaks seen in the FW. Previous studies have demonstrated that biotransformation of polyphenols leads to a variety of metabolites produced via digestive enzyme (Mandalari et al., 2010) and gut microbial (Russell *et al.*, 2008) activities. Polyphenols such as CGA, CA, FA, and rutin have poor intestinal absorption and so reach the colon where they undergo extensive metabolism via colonic flora (Gee and Johnson, 2001) before being absorbed (Olthof *et al.*, 2001; Olthof *et al.*, 2003). For instance, intestinal bacteria converted CGA into metabolites that reach the circulation (Olthof *et al.*, 2003), which could account for the appearance of crypto-chlorogenic acid and the major increase of neo-chlorogenic acid in the FW (Plumb *et al.*, 1999). Rutin can be transformed into quercetin by colonic microflora in the colon (Bokkenheuser *et al.*, 1987), which likely accounts for the disappearance of rutin together with the appearance of significant concentrations of quercetin in the FW (Table 5.4.1).

The polyphenolic compounds CGA, CA, FA, and quercetin identified in FW in the present study have also shown to be major polyphenolics within human FW (Gill *et al.*, 2010). The observed FW values generally corresponded with or were somewhat higher than seen in previous studies of FW from healthy humans; however, polyphenolic FW concentrations in humans likely vary according to factors such as diet and distinct gut microflora among individuals (Karlsson *et al.*,

2005). Ferulic acid has been shown to be present in human FW at a concentration of 10 µg/mL (Karlsson *et al.*, 2005), which is comparable to 6 µg/mL FW concentration observed in the present findings. The quercetin concentrations of 3.4 µg/mL observed in FW in the present work, however, were about 5-fold higher than those previously measured in FW of healthy adults, which showed mean quercetin concentrations of 0.63 µg/mL (Jenner et al., 2005). Raspberry supplementation of healthy adult individuals significantly increased CA concentrations in the FW from 3.7 µg/mL to 5.1 µg/mL (Gill *et al.*, 2010), which is about 1.5-fold lower than the CA concentration of 8.9 µg/mL in FW seen in the present study. The SHIME and other gastrointestinal models have previously been utilized to monitor the behaviour of human gut microbiota with different polyphenols (Verstraete *et al.*, 2008; van Duynhoven *et al.*, 2011); however, there has been relatively little research regarding the use of these *in vitro* gastrointestinal models to study bioaccessibility and the generation of polyphenolic metabolites from the colonic microbiota (van Duynhoven *et al.*, 2011). The production of polyphenol metabolites generated by bacterial metabolism within the SHIME model has been well correlated polyphenol fecal metabolites seen with intake of polyphenols in humans. For instance, the bacterial metabolites of catechins measured in healthy individuals consuming a bolus of tea polyphenols had a high correlation with the same amount of metabolites produced from tea polyphenols after processing via the SHIME model (Kemperman *et al.*, 2010). Thus, the previous studies with the SHIME model supports the concept that Dynamic Human Gastrointestinal Model used in the present study is applicable to study the polyphenolic end-products produced from the bacteria metabolism in the colonic compartments.

In agreement with previous studies, FW exerted cytotoxic effects on the Caco-2 cells, which has been related to the production of oxidative damage (Venturi *et al.*, 1997). The aqueous phase of human feces in the form of FW is known to contain many compounds that interact with colonic cells leading to the pathogenesis of colon cancer (Rafter *et al.*, 1987a; Rafter *et al.*, 1987b) and IBD (Montgome *et al.*, 1968; Martini and Brandes, 1976). Bile acids in FW have been

shown to cause oxidative stress and DNA damage via the NF-κB pathway (Payne *et al.*, 1998) and have also been implicated as promoters in colon cancer (Zheng *et al.*, 1996; Payne *et al.*, 1999; Dvorak *et al.*, 2009). One study showed that bile acids, to which *Escherichia coli* are naturally exposed, induced abundance of specific stress response genes such as *micF*, *osmY*, and *dinD*, which possibly caused cell membrane perturbation, oxidative stress, and DNA damage in the *E. coli* (Bernstein *et al.*, 1999). In addition, secondary bile acids, which have been converted by gut microflora in the colon, have shown to induce cytotoxicity and genotoxicity in Caco-2 cells exposed to FW (Lapre and Vandermeer, 1992; Lee *et al.*, 2005). Secondary bile acids cause single-stranded DNA breaks and FW-induced intestinal DNA damage was reported to directly correlate with bile acid concentrations (Venturi *et al.*, 1997). Secondary bile acids have recently been shown to exert adverse effects on epithelial barrier function, an endpoint thought to be related to tumor promotion (Rowland *et al.*, 2008). FW contains a range of bioactive components derived from dietary sources that could modulate the pathogenic effects of FW including polyphenols, organic acids, and SCFA (de Kok and van Maanen, 2000; Pearson *et al.*, 2009). The present study showed that the colonic digests of polyphenolic-rich potato extracts protected against the cytotoxic effects of FW on Caco-2 cells. It has been suggested that transition metal ions in FW such as ferrous ion can catalyze the formation of ROS, which can thus cause DNA damage and cell death in colon cells (Yeh et al., 2007). As polyphenolic compounds exert strong ferrous ion-chelating effects (Morel *et al.*, 1993), it is possible that this chelating property could provide cytoprotective effects mediated by this antioxidant protection. The ability of CGA, CA, FA, rutin, and quercetin to have strong affinity for ferrous ions is important because ferrous ions catalyze many processes leading to the generation of ROS (Iwahashi *et al.*, 1990; Ahn and Chen, 1998; Kono *et al.*, 1998).

Exposure to both FW either alone or together with H_2O_2 caused a major pro-inflammatory response in the Caco-2 cells. The pro-inflammatory effects of FW alone coexisting with reduced levels of phenolics have been found in patients with ulcerative colitis and treatment with human anti-TNF-α have improved

disease activity (Evans *et al.*, 1997). Moreover, H_2O_2-induced oxidative stress has been well characterized to promote IL-8 secretion in Caco-2 cells (Yamamoto *et al.*, 2003). Significant anti-inflammatory effects were seen when the FW contained polyphenols, which is in line with previous observations that the pro-inflammatory effects in Caco-2 cells exerted by either FW or TNF-α were inhibited by treatment with individual polyphenolic compounds including gallic acid, quercetin, and resveratrol (Schneider *et al.*, 2008; Romier-Crouzet *et al.*, 2009). For instance, Schneider *et al.*, (2008) have shown no inflammatory response in Caco-2 cells exposed to 0.5 µg/mL of quercetin for 4 h and then incubated with one NF-κB activator, either IL-1β, TNF-α, or LPS for 24 h. In the present study, the quercetin concentration in FW was 3.4 µg/mL, which suggests that this polyphenolic compound could be partly involved in the protective effects against H_2O_2-induced inflammation. Another study has demonstrated the anti-inflammatory effects of CGA and CA in terms of reduced IL-8 secretion in Caco-2 cells stimulated with TNF-α or H_2O_2 (Zhao *et al.*, 2008). The above studies have indicated that the decreased IL-8 levels were related to polyphenol modulation of the NF-κB pathway. The inhibition of the NF-κB pathway has been shown by polyphenols such as quercetin to block the IL-1β receptor and block the phosphorylation of IκB-α. Phosphorylation of IκB-α is needed to release the subunits p50 and p65 that migrate to the cell nucleus to activate the NF-κB pathway (Romier *et al.*, 2007). The anti-inflammatory effects of polyphenolic compounds naturally present in human FW has been also demonstrated in terms of inhibition of the pro-inflammatory COX-2 pathway in colonic HT-29 cells (Karlsson *et al.*, 2005). The analysis of phenolic compounds in FW in the latter study showed that the concentrations of cinnamic acids ranged from 0.02 to 44.0 µg/mL among different individuals whereas in the present study ranged from 0.8 to 79.2 µg/mL (Table 5.4.1) It is also possible that polyphenol metabolites in the FW generated by microfloral metabolism exerted significant anti-inflammatory effects. Russel et al. (2008) showed that H_2O_2 stimulated colonic fibroblast cells had a decreased prostanoid inflammatory response following exposure to human fecal derived microbial by-products of FA.

The FW + Phenolics treatment of Caco-2 cells was associated with a marked decrease in intracellular ROS formation together with an enhanced FRAP activity in the cell culture medium. The significant concentrations of quercetin, FA, CA and protocatechuic acid seen in the FW likely contributed to the observed antioxidant activities, particularly as such polyphenolics have been shown to prevent oxidative cell damage induced by H_2O_2 (Nakayama *et al.*, 1993). Quercetin treatment has been demonstrated to result in a 45% decrease in ROS formation in Caco-2 cells exposed to H_2O_2 (Yokomizo and Moriwaki, 2006). Likewise, quercetin and quercetin glycosides present in apple juice extracts have been associated with decrease ROS formation in stimulated Caco-2 cells (Janzowski *et al.*, 2006). Similar antioxidant activity of FA has been seen in Caco-2 cells stimulated with TNF-α (Andreasen *et al.*, 2001). FA has been shown to act as a potent antioxidant because of its ability to scavenge peroxyl radical (Castelluccio *et al.*, 1995) and other radicals due to its phenolic nucleus and unsaturated side chain to form a resonance-stabilized phenoxy radical (Graf, 1992). CGA is a potent antioxidant which has observed to scavenge ROS (Truscott *et al.*, 1999), decrease DNA damage (Shibata *et al.*, 1999) and inhibit colonic and hepatic carcinogenesis in hamsters and rats (Mori *et al.*, 1986; Tanaka *et al.*, 1990). Protocatechuic acid has been shown to reduce LDL oxidation in intestinal Caco-2 epithelial cells (Giovannini *et al.*, 2002).

Other antioxidant compounds apart from polyphenolics might have also contributed to the improved antioxidant and anti-inflammatory activities associated with the FW + Phenolics treatment. In that regard, significant concentrations of ascorbic acid and succinic, citric and malic organic acids were present in the FW + Phenolics. Ascorbic acid is an efficient antioxidant which scavenges toxic ROS generated in colonic cells stimulated with H_2O_2 (Arrigoni and De Tullio, 2002). Furthermore, ascorbate has been shown to exert antioxidant actions by causing reduction in superoxide anion production measured by chemiluminescence in rats with acetic acid-induced colitis and human ulcerative colitis biopsy specimens (Millar *et al.*, 1996). Citric acid is a major organic acid that complexes with oxidant metals and has a synergistic

reducing effect along with ascorbic acid (Galdon *et al.*, 2010). Succinic acid has been shown to reverse brain ischemia in rats by inhibiting mitochondrial complex I, a protein responsible for the formation of hydroxyl radicals during brain ischemia (Piantadosi and Zhang, 1996). The anti-inflammatory effects of organic acids have been studied in probiotics as a management treatment option in IBD (Fedorak and Madsen, 2004). For instance, the cecal content from healthy pigs has been shown to increase the production of succinic acid by probiotic preparations containing *Bifidobacterium*, *Enterococcus*, or *Lactobacillus* (Sakata *et al.*, 1999*).*

In conclusion, this study has shown that there is significant bioaccessibility of polyphenols from potato extracts in the colon as demonstrated by the major release of polyphenols into the colonic compartments following digestion of potato extracts in a dynamic simulated human gut model. This study also demonstrated the colonic compounds generated from digestion and microbial metabolism of the potato extracts exerted significant antioxidant and anti-inflammatory effects in Caco-2 cells that were exposed to FW in both non-stimulated and stimulated conditions. The present findings suggest that the consumption of a polyphenolic-rich diet might have a possible preventive role in this chronic debilitating disease.

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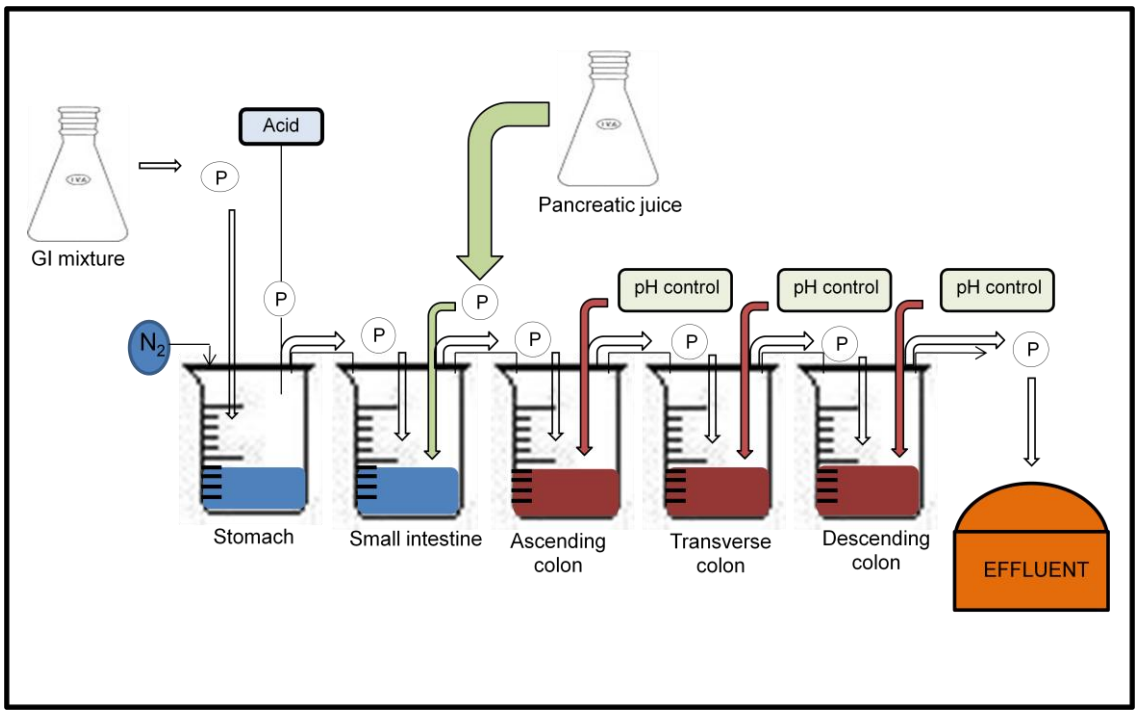
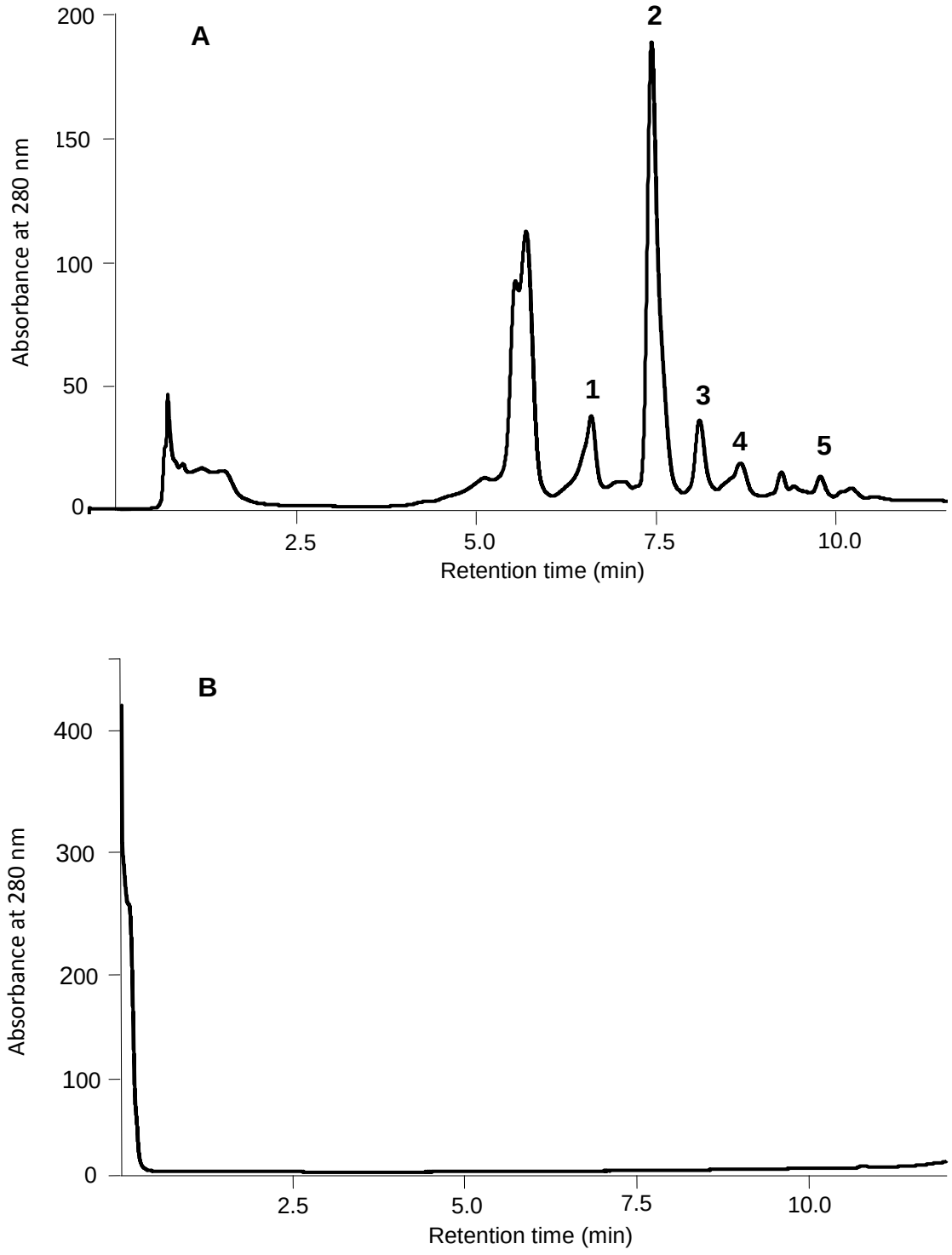
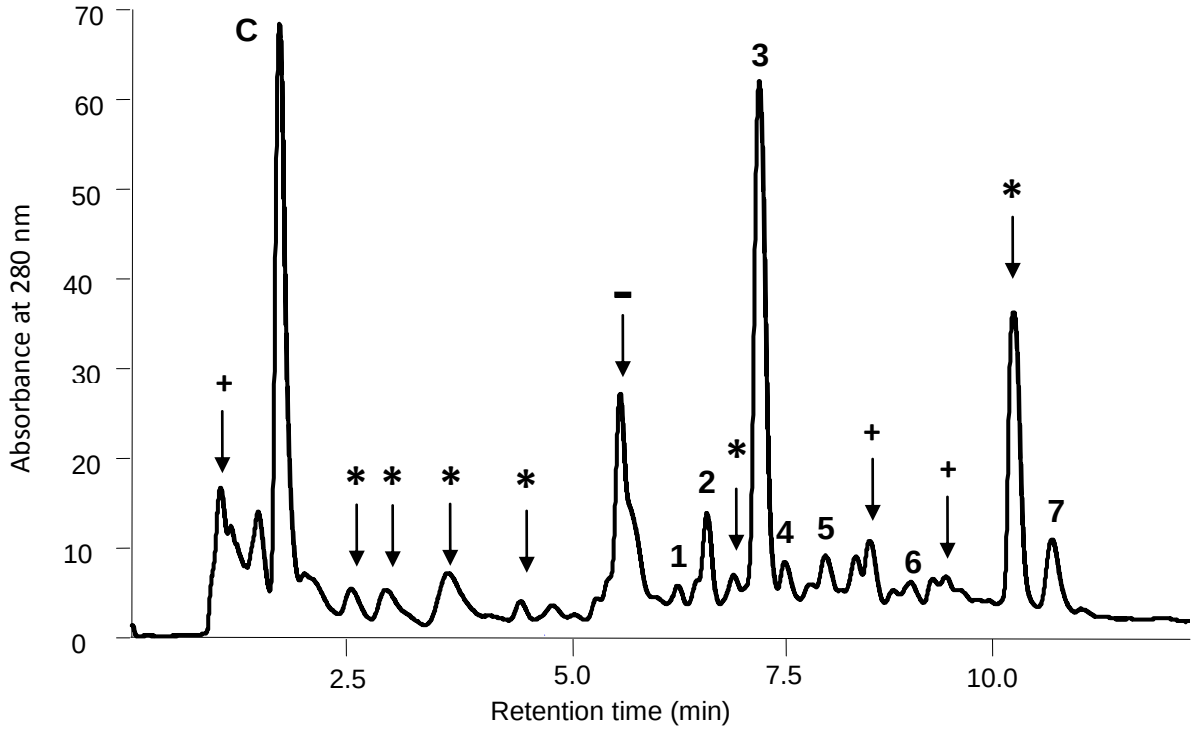
Figure 5.3.1 The Computer Controlled Dynamic Human Gastrointestinal Model.



Adapted from Martoni et al., 2007

Figure 5.4.1. Chromatogram of polyphenols from reverse-phase HPLC obtained via UV/VIS detector wavelength of 215 nm.





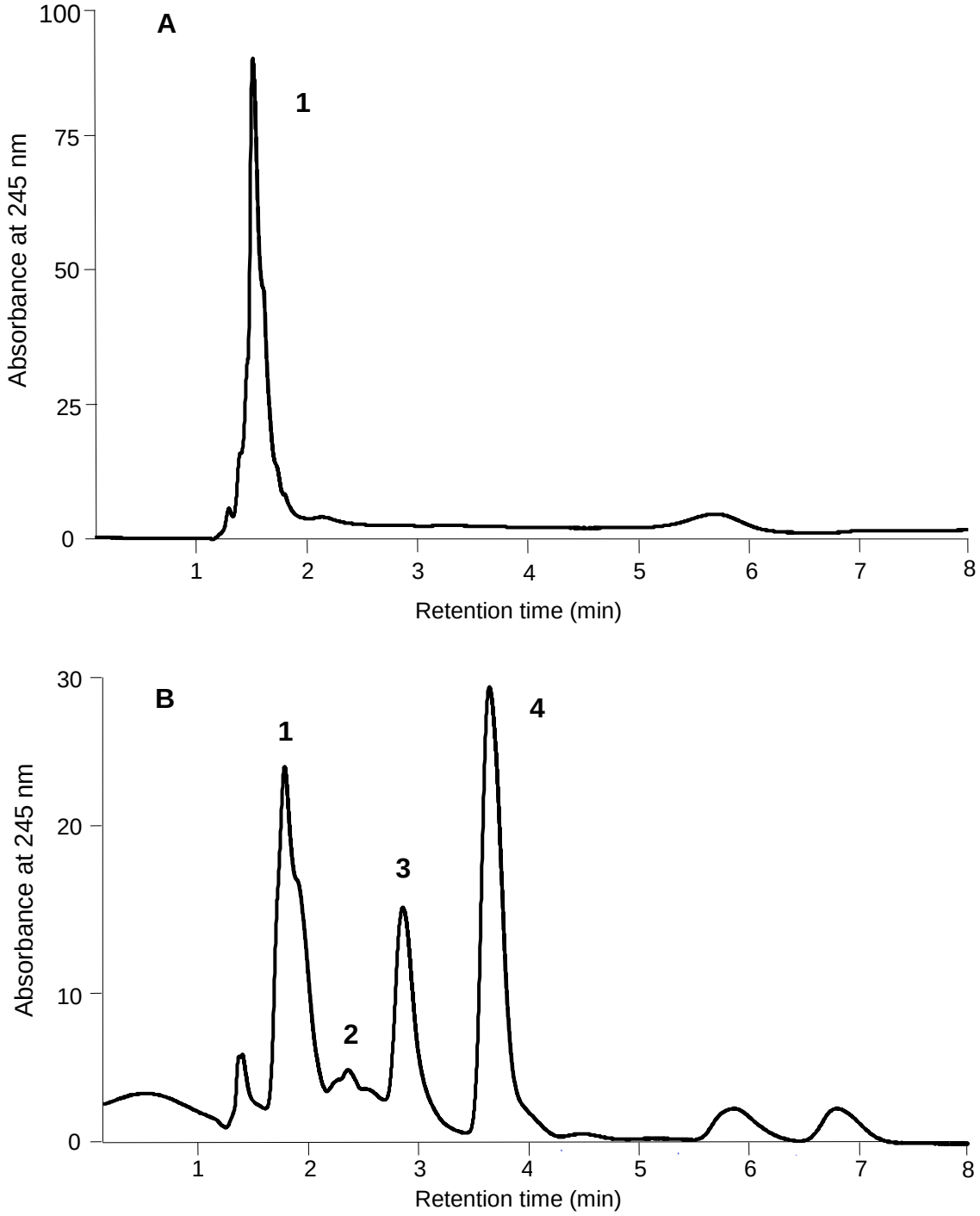
(**A**) Polyphenolic profile of undigested 'Onaway' extract: (1) Neo-chlorogenic acid, (2) Chlorogenic acid, (3) Caffeic acid, (4) Ferulic acid, (5) Rutin. (**B**) Polyphenolic profile of FW alone. (**C**) Polyphenolic profile of "digested" 'Onaway' extract after digestion: (1) Protocatechuic acid, (2) Neo-chlorogenic acid, (3) Chlorogenic acid, (4) Crypto-chlorogenic acid, (5) Caffeic acid, (6) Ferulic acid, (7) Quercetin. The * symbol identifies extra peaks found in FW + Phenolics and arrows indicate differences in the relative abundances of Phenolics in FW (+, higher abundance, -, lesser abundance in FW with phenolics relative to undigested 'Onaway' extract). Each graph represents the mean of three replicates.

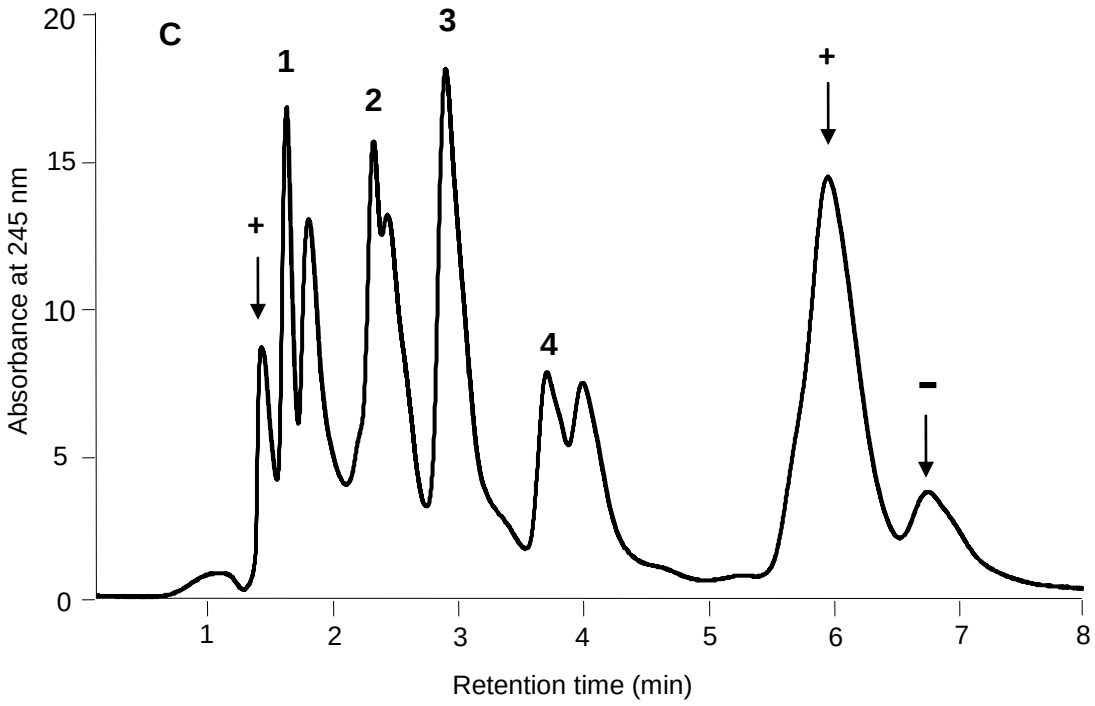
Table 5.4.1 Polyphenolic concentrations of potato extract in the nutrient solution and in FW + Phenolics.

	Potato extract in nutrient solution (µg/mL)	FW + Phenolics (µg/mL)
Protocatechuic acid	ND	0.8
Neo-chlorogenic acid	14.4	27.7
Chlorogenic acid	68.6	79.2
Crypto-chlorogenic acid	ND	1.3
Caffeic acid	8.0	8.9
Ferulic acid	4.8	6.4
Rutin	7.9	ND
Quercetin	ND	34.3

ND = Not detectable. FW = Faecal water.

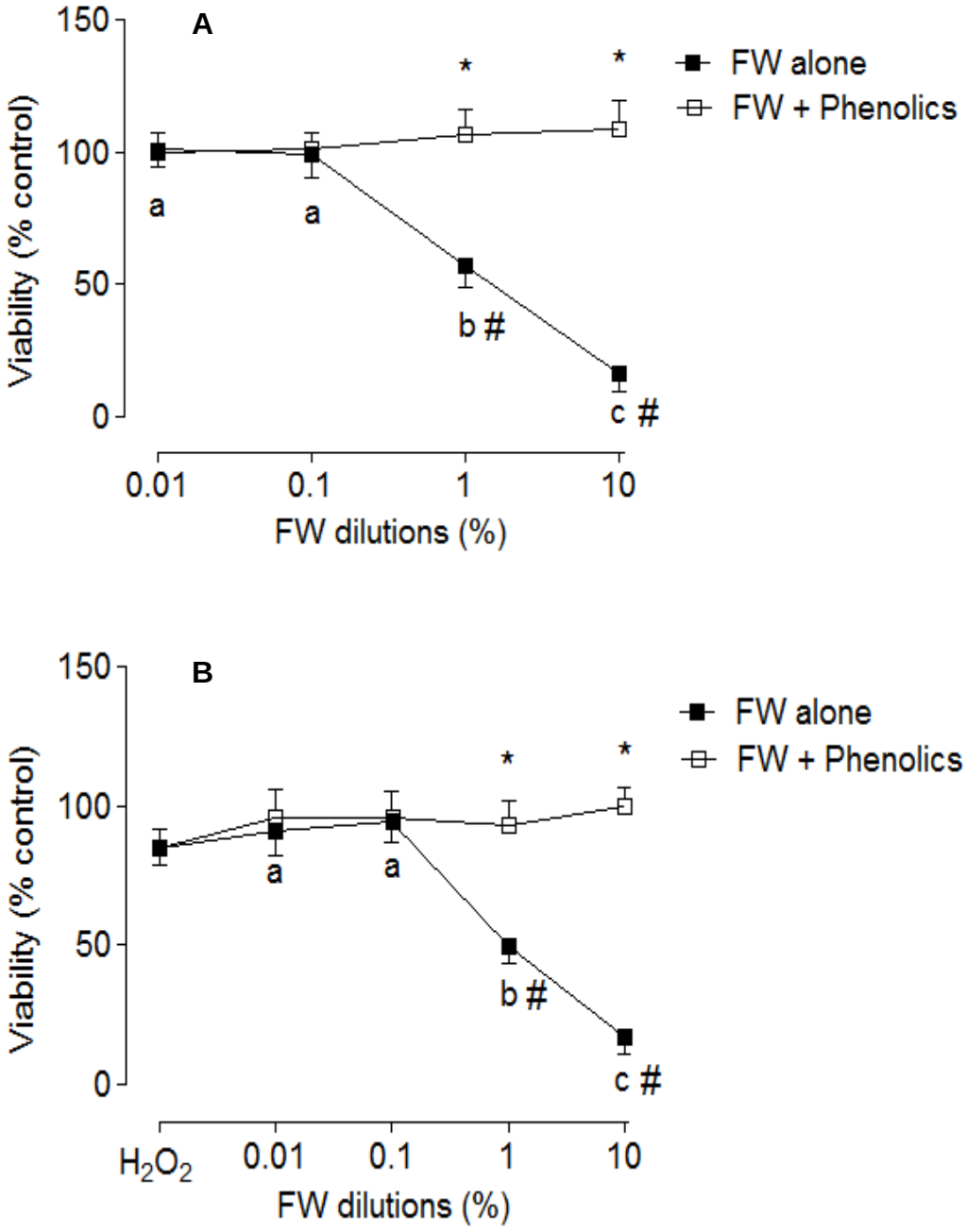
Figure 5.4.2 Chromatogram of organic acids from reverse-phase HPLC obtained via UV/VIS detector wavelength of 245 nm.





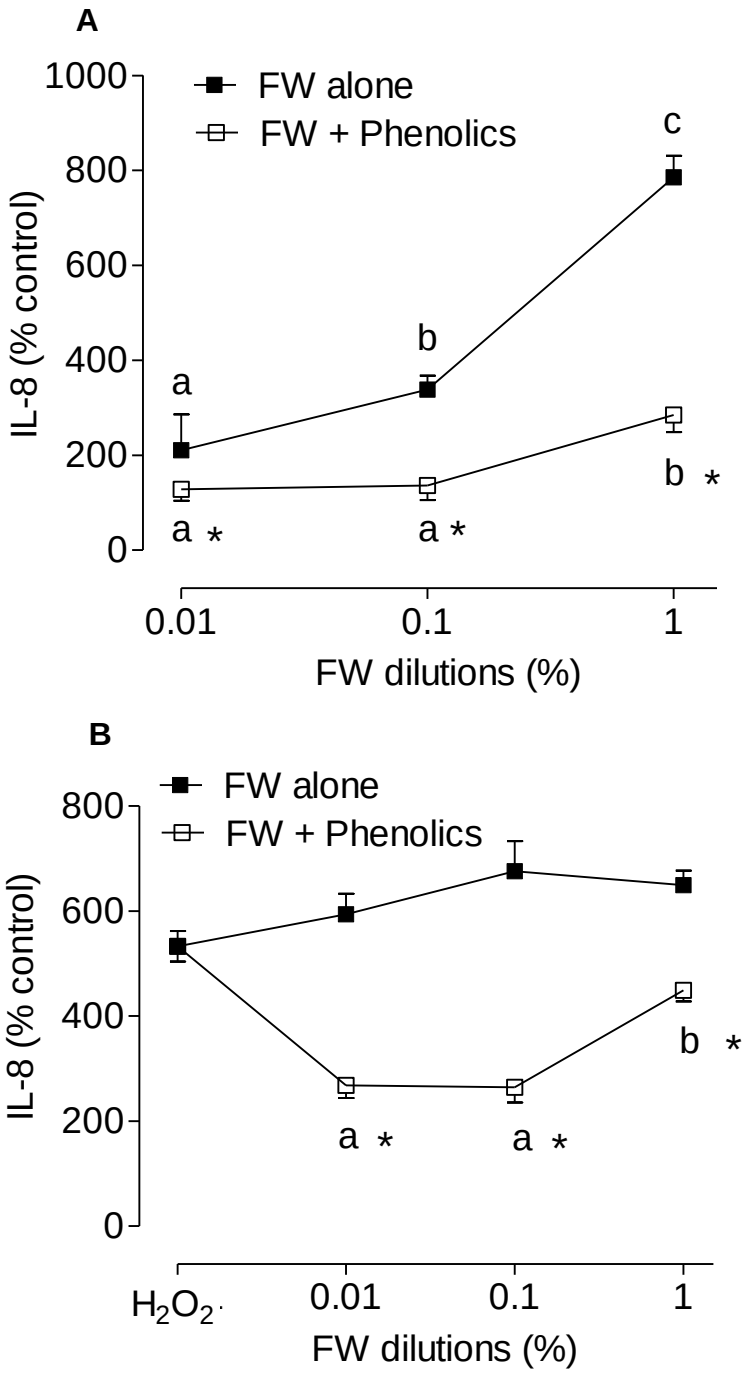
(**A**) Organic acid profile from undigested 'Onaway' extract: (1) Ascorbic acid. (**B**) Organic acid profile from FW alone. (**C**) Organic acid profile from FW + Phenolics extracts: (1) Ascorbic acid, (2) citric acid, (3) succinic acid, (4) malic acid. The arrows indicate differences in the relative abundances of organic acids in FW (+, greater abundance, -, lesser abundance in FW + Phenolics relative to FW alone). Each graph represents the mean of three replicates.

Figure 5.4.3 Effects of different FW treatments on Caco-2 cell viability.



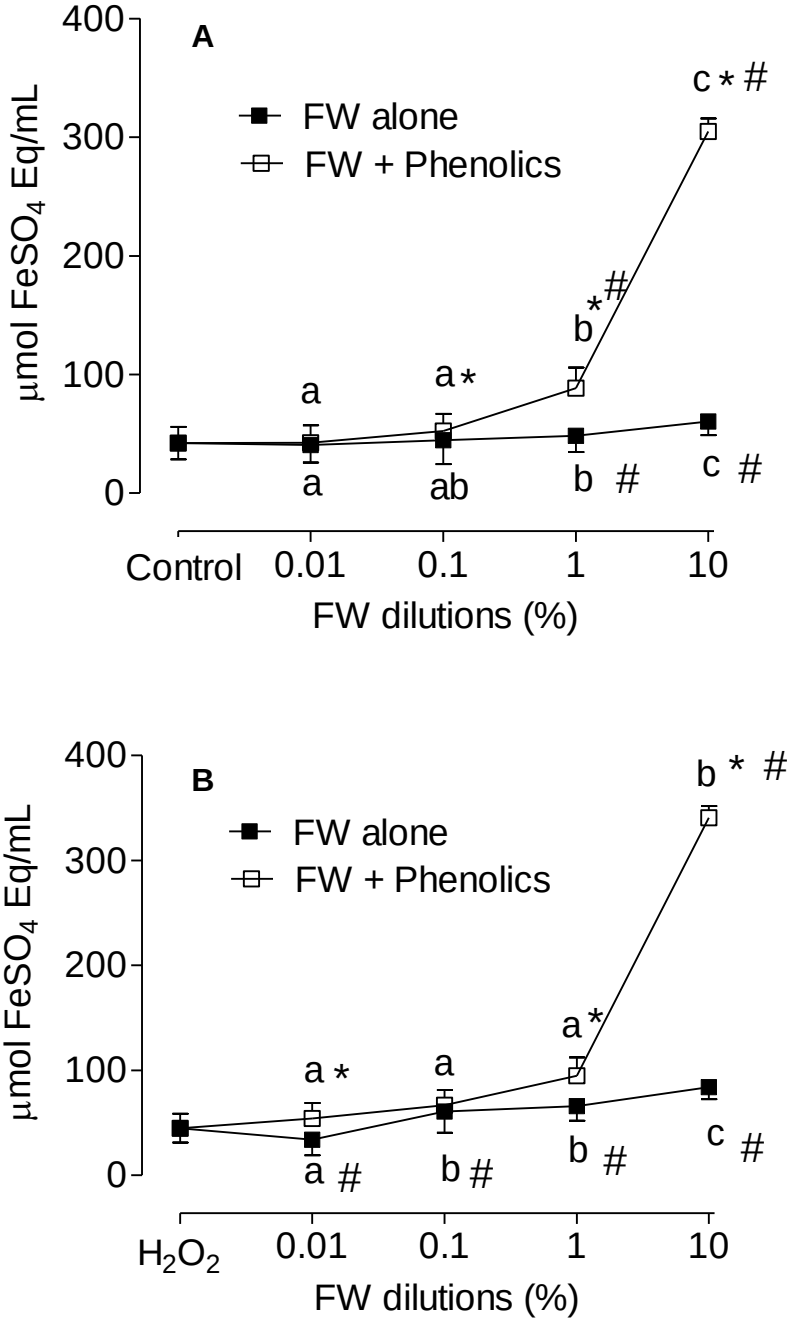
(**A**) Caco-2 cells were treated with FW or FW + Phenolics for 24 h. (**B**) Stim. Caco-2 cells were incubated with 0.25 mM H_2O_2 for 1 h and then treated with FW or FW + Phenolics for 23 h. Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 0 %, 0.01 %, 0.1 %, 1.0 %, and 10.0 % of either FW or FW + Phenolics for 24 h (**A**) or 23 h (**B**). In the next day, supernatant was collected, cells were treated with MTT reagent, incubated for 3 h at 37°C and made permeable with HCl-isopropanol to dissolve the formazan crystals. Optical densities were measured at 560 nm. Values are means ± SE of 3 independent experiments. Means within each treatment without a common letter differ ($P < 0.05$). Means with the symbols (*) indicate significant differences ($P < 0.05$) between treatments at the same dose. Two-way ANOVA followed by Tukey's *post hoc* test for comparison of group means. FW = Faecal water. H_2O_2 = Hydrogen peroxide.

Figure 5.4.4 Effect of different treatments of FW on IL-8 release of Caco-2 cells.



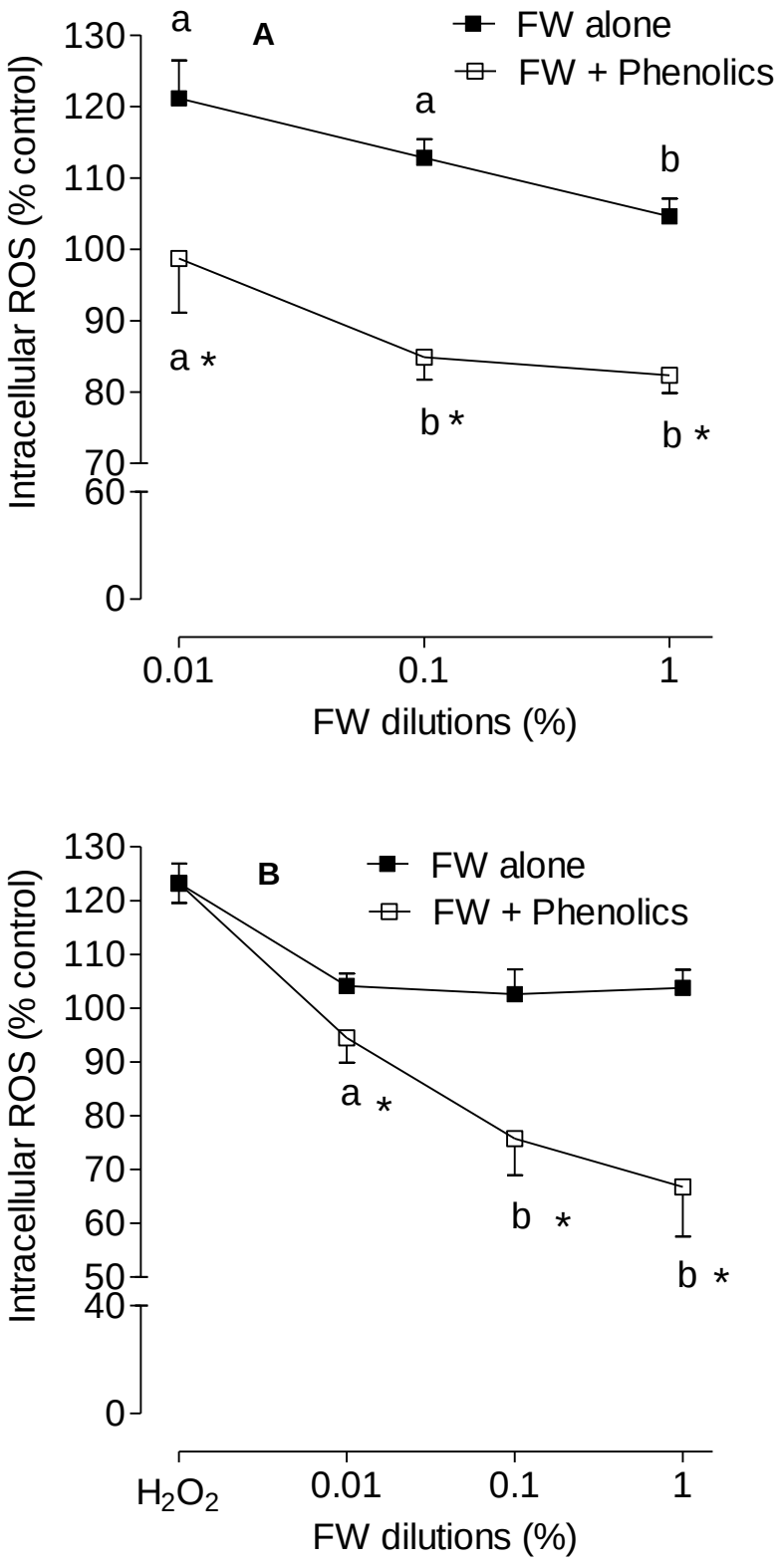
(**A**) Caco-2 cells were treated with FW alone and FW + Phenolics. (**B**) Stim. Caco-2 cells were incubated with 0.25 mM H_2O_2 for 1 h and then treated with FW or FW + Phenolics for 23 h. Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 0 %, 0.01 %, 0.1 %, 1.0 %, and 10.0 % of either FW or FW + Phenolics for 24 h (**A**) or 23 h (**B**). In the next day, supernatant was collected for IL-8 release using an ELISA kit (BD, OptEIA set human IL-8). Values are means ± SE of 3 independent experiments. Means within each treatment without a common letter differ ($P < 0.05$). Means with the symbols (*) indicate significant differences ($P < 0.05$) between treatments at the same dose. The symbol (#) indicates significant differences ($P < 0.05$) with untreated control cells. Two-way ANOVA followed by Tukey's *post hoc* test for comparison of group means. FW = Faecal water. H_2O_2 = Hydrogen peroxide.

Figure 5.4.5 The ferric reducing antioxidant power (FRAP) in the supernatant of Caco-2 cells with and without H_2O_2 stimulation, exposed to different FW treatments.



(**A**) Caco-2 cells were treated with FW alone and FW + Phenolics. (**B**) Stim. Caco-2 cells were incubated with 0.25 mM H_2O_2 for 1 h and then treated with FW or FW + Phenolics for 23 h. Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 0 %, 0.01 %, 0.1 %, 1.0 %, and 10.0 % of either FW or FW + Phenolics for 24 h (**A**) or 23 h (**B**). In the next day, supernatant was collected for determination of FRAP activity. Values are means ± SE of 3 independent experiments. Means within each treatment without a common letter differ ($P < 0.05$). Means with the symbols (*) indicate significant differences ($P < 0.05$) between treatments at the same dose. The symbol (#) indicates significant differences ($P < 0.05$) with untreated control cells. Two-way ANOVA followed by Tukey's *post hoc* test for comparison of group means. FW = Faecal water. H_2O_2 = Hydrogen peroxide.

Figure 5.4.6 Effects of different treatments of FW in the intracellular ROS generation in Caco-2 cells.



(**A**) Caco-2 cells were treated with FW alone and FW + Phenolics. (**B**) Stim. Caco-2 cells were incubated with 0.25 mM H_2O_2 for 1 h and then treated with FW or FW + Phenolics for 23 h. Caco-2 cells were seeded in 24-well plates at 2.5 x 10^5 cells/well and incubated in DMEM containing 20% HFBS. After 24 h the medium was replaced by MEM containing 0 %, 0.01 %, 0.1 %, 1.0 %, and 10.0 % of either FW or FW + Phenolics for 24 h (**A**) or 23 h (**B**). In the next day, supernatants were removed, added DCFH-DA in phosphate buffered saline containing DMSO solution for 30 min at 37°C. The fluorescent DCF was monitored by spectrofluorimetry (fluorescence excitation and emission were 485 nm and 530 nm, respectively). Values are means ± SE of 3 independent experiments. Means within each treatment without a common letter differ ($P < 0.05$). Means with the symbols (*) indicate significant differences ($P < 0.05$) between treatments at the same dose. The symbol (#) indicates significant differences ($P < 0.05$) with untreated control cells. Two-way ANOVA followed by Tukey's *post hoc* test for comparison of group means. FW = Faecal water. H_2O_2 = Hydrogen peroxide.

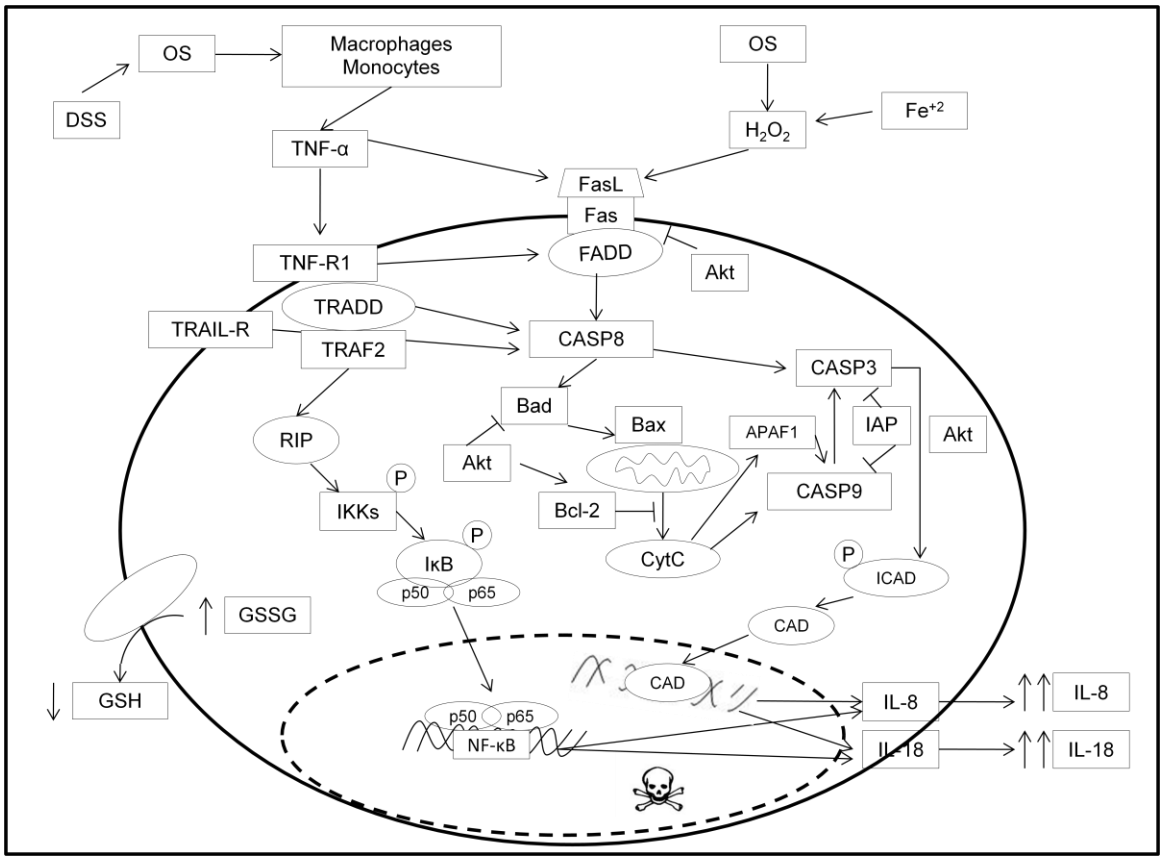
Chapter 6

Final Conclusion

6.1 GENERAL SUMMARY AND CONCLUSION

Inflammatory bowel disease (IBD) is a chronic debilitating condition with a high prevalence in Canada (Su *et al.*, 1999). Inadequate protein intake due to food avoidance and anorexia could adversely affect growth, inflammatory status, and disease severity in the pediatric population (Ballinger, 2002). The inflammatory response has been suggested via various metabolic pathways (Whittle, 2004; Castrillo and Gonzalez, 2011) including the NF-κB and capase-3 pathways (Mukaida *et al.*, 1994; Montaner *et al.*, 2008) and a summarized overview is presented in Fig. 6.1. Unfortunately, the main treatment of IBD involves the use of medications with long-term side effects (Kim and Ferry, 2002). For this reason, new nutritional strategies focusing on the amelioration of the chronic consequences from this disease should be investigated.

Figure 6.1 Proposed molecular mechanism of two main inflammatory pathways[1].



[1]The inflammatory response of colonic cells have been described as having: Interleukin (IL)-8 and IL-18 releases hyperresponsiveness to tumor necrosis

factor-alpha (TNF-α) (Abreu-Martin *et al.*, 1995); increased activation of nuclear factor-kappa B (NF-κB) (Wang *et al.*, 2009); overexpression of apoptosis stimulating fragment-ligand (FasL)/Fas (Fantini and Pallone, 2008); increased activation of caspase-3 pathway (Sanchez-Alcazar *et al.*, 2006); and low glutathione (GSH) content (Sido *et al.*, 1998).

The present thesis study demonstrated the important effects of protein quantity and quality in a pediatric piglet model of dextran sulfate sodium (DSS)-induced colitis in which whey protein and skim milk protein were provided as the protein source in moderate protein deficient diets. The thesis work showed that there was decreased disease severity in the descending colon (DC) in piglets with colitis fed either native or pressurized WPI relative to piglets fed either moderate protein deficient or well nourished diets containing skim milk protein as the protein source. Although anabolic, antioxidant, and anti-inflammatory effects of whey proteins have been shown in other *in vitro* and *in vivo* studies of gut inflammation (Daddaoua *et al.*, 2005; Hernandez-Ledesma *et al.*, 2005; Kanwar and Kanwar, 2009; Kim *et al.*, 2009), the present thesis showed that piglets with colitis fed pressurized whey protein isolate (WPI) showed superior anabolic and anti-inflammatory effects relative to native WPI feeding. In that regard, pressurized WPI feeding in this IBD model was associated with a decreased fat mass to lean body mass ratio whereas both native WPI and skim milk protein feeding under moderate protein deficient conditions showed an increased ratio of fat to lean body mass. It is possible the enhanced anabolic and anti-inflammatory of pressure-treated WPI could be related to its improved protein digestibility (Kubow *et al.*, 2005; Vilela *et al.*, 2006b) that may have contributed to greater absorption of bioactive peptides as well as amino acids such as leucine. Interestingly, bone mass was significantly lower in the WPI-fed piglets than the control and SM-fed piglets, which could be explained by the lack of arginine in WPI as arginine regulates intestinal calcium absorption (Wu *et al.*, 2004; Kim and Wu, 2007). The plasma protein concentrations and the plasma protein fractional synthesis rates (FSR) were not affected by moderate protein deficient diets. This results contrasts with the findings of Mackenzie *et al.* (2003) who demonstrated

an increased albumin FSR in short-term moderate protein and energy restricted piglets with colitis despite unaltered changes in plasma albumin concentration levels. These contrasting results might be also explained from previous studies showing that concurrent protein and energy restriction is needed to compromise hepatic plasma protein synthesis associated with protein malnutrition (Birchenallsparks *et al.*, 1985; Merry *et al.*, 1987).

Disease severity was observed in a greater degree in the control- and SM-fed piglets as exhibited by their higher total histological scores showing moderate to severe inflammation, crypt damage, and mucosa destruction. The improved colonic histopathology in piglets receiving WPI might be due to the higher amounts of cysteine associated with whey protein. Cysteine is the rate-limiting amino acid for glutathione (GSH) synthesis, an important intracellular antioxidant that plays an important role to decrease colonic inflammation (Oz *et al.*, 2007). In addition, the anti-inflammatory effects of pressurized WPI were shown in the descending colon (DC) as exhibited by decreased levels of the pro-inflammatory cytokines TNF-α, IL-8 and IL-18 as compared to the SM-fed piglets. Also, decreased myeloperoxidase levels in DC tissue were only identified in pressure WPI-treated piglets. At the clinical level, pressurized-fed piglets had less severe diarrhea compared with other protein restricted groups. In addition, pressurized WPI-fed piglets had higher colonic FRAP values as well as an increased serum scavenging capacity of peroxynitrite. These effects could be explained due to the pressure processing of WPI that results in changes in the secondary and tertiary structures of whey proteins leading to the release of unique bioactive peptides following digestion that exert antioxidant and anti-inflammatory effects (Kubow *et al.*, 2005; Vilela *et al.*, 2006b; Kishta *et al.*, 2009). In that respect, previous observations have described increased intracellular concentrations of the antioxidant GSH induced by pressurized WPI (Vilela *et al.*, 2006a; Zavorsky *et al.*, 2007). Finally, control-, native- and pressurized-fed piglets had lower caspase-3 abundance in DC as compared to SM group, which could play a protective role in IBD. In summary, intake of pressurized WPI was associated

with anabolic, antioxidant, and anti-inflammatory effects and decreased disease severity in a piglet model of pediatric colitis.

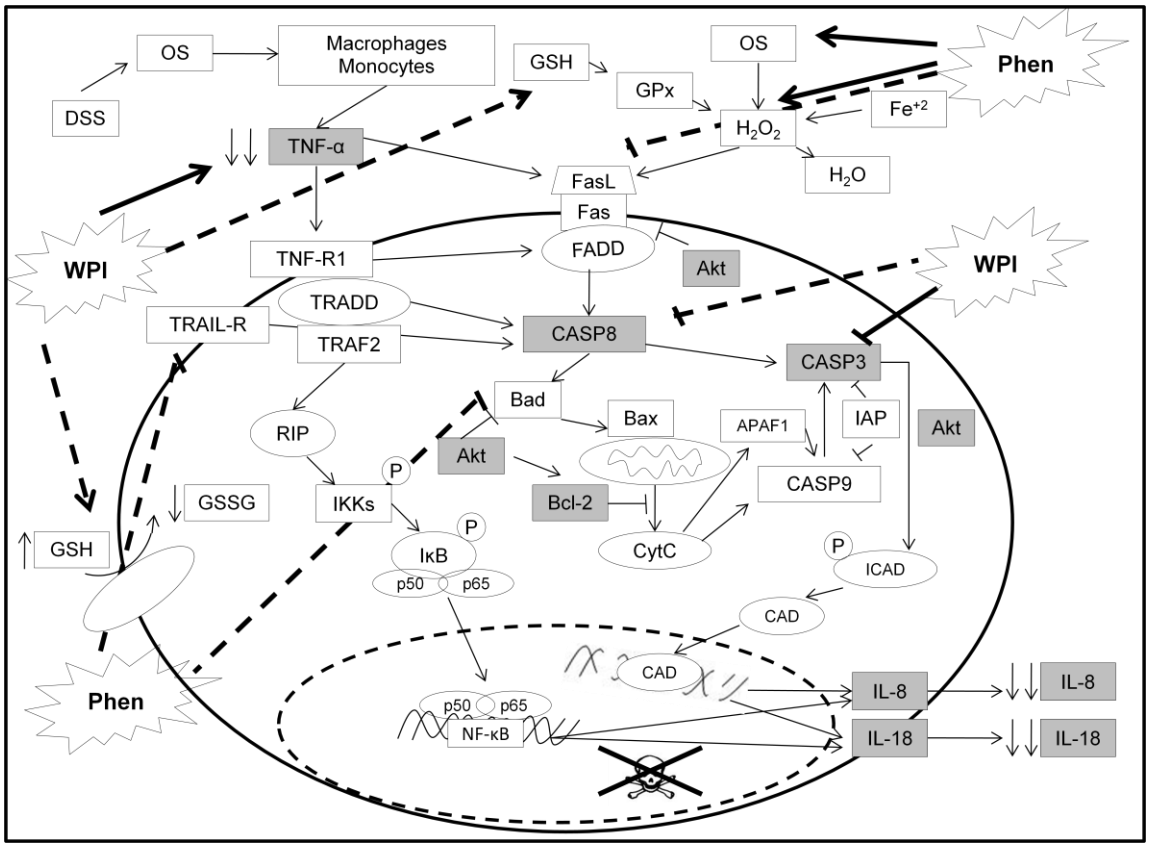
The thesis work further investigated the antioxidant and anti-inflammatory activities of pressure-treated WPI by studying the direct exposure of stimulated colonic cells to dietary peptides and amino acids released after WPI digestion. The human intestinal Caco-2 epithelial cells were exposed to hydrolysates of WPI to evaluate IL-8 secretion and intracellular reactive oxygen species (ROS) generation, under basal or stimulated conditions with hydrogen peroxide (H_2O_2). *In vitro* studies have shown increased IL-8 mRNA abundance after H_2O_2-induced oxidative stress in Caco-2 epithelial cells (Yamamoto *et al.*, 2003). The mechanism involved relates to the up-regulation of IL-8 gene abundance by H_2O_2 via TNF receptors in the cell membrane, which signals the activation of the NF-κB pathway and IL-8 release (Okamoto *et al.*, 1993; Mukaida *et al.*, 1994; Li and Verma, 2002). The results of this study showed that both types of WPI hydrolysates exerted antioxidant and anti-inflammatory effects as shown by decreased IL-8 secretion and reduced intracellular ROS generation, and increased supernatant FRAP activity. However, pressure-treated WPI hydrolysates showed these biological effects to a significantly greater extent, which further confirms the greater efficacy of pressure-treated versus native WPI as a possible treatment for IBD.

Similar to WPI, polyphenols have received considerable research attention regarding their antioxidant and anti-inflammatory properties (RiceEvans and Miller, 1997; Scalbert *et al.*, 2002; Van De Walle *et al.*, 2010). Potatoes have been considered a good source of polyphenols (Giusti *et al.*, 1999). The ‘Onaway’ potato was selected to generate polyphenolic-rich extracts based on a previous study which showed the higher antioxidant activity and a relatively high polyphenolic content for this potato cultivar in comparison to several other potato cultivars (Vunnam, 2010). In this thesis work, the Onaway potato polyphenolic extracts were subjected to digestive processes in a simulated human gut model and the bioactivity of the digests from the colonic compartments was investigated in cultured Caco-2 cells exposed to the pro-inflammatory stimulus of H_2O_2. To our

knowledge, this was the first *in vitro* study whereby Caco-2 cells were exposed to digested polyphenols obtained from the colonic compartment of a dynamic simulated human digestion model as opposed to the typical use of non-digested polyphenols in this regard (Netsch *et al.*, 2006; Romier-Crouzet *et al.*, 2009). In this respect, the digestion of the polyphenol rich potato extracts was closer to the enzymatic and microbial conditions encountered in the human gut that can generate a variety of secondary polyphenolic metabolites. The results showed significant antioxidant and anti-inflammatory effects of the potato derived polyphenolics obtained from the colonic compartment of the simulated human gut model. Caco-2 cells treated with faecal water (FW) with the polyphenolics had reduced IL-8 secretion and decreased intracellular ROS formation, and increased supernatant FRAP activity as compared to Caco-2 treated with FW alone. Moreover, the presence of polyphenolic compounds was associated with a complete inhibition of the cytotoxicity of FW to Caco-2 cells.

In conclusion, we have shown the protective effects of pressure processed WPI and potato polyphenolic extracts in two different models of gut inflammation. The proposed down-regulation of the main molecular mechanisms is shown in Fig. 6.2.

Figure 6.2 Proposed molecular mechanisms involved in the antioxidant and anti-inflammatory effects of WPI and Polyphenols (Phen)[1].



[1]Boxes in gray refer to proteins that were measured. Solid lines are measured pathways. Dotted lines are proposed mechanism of action from WPI and polyphenols but not measured in the current thesis. Considering the results from all the studies, we suggest that the inhibition of IL-8 and IL-18 release could be explained by the effects of WPI in reducing in the DC concentration of TNF-α and in down-regulating the abundance of caspase-3 pathway. The antioxidant effects of pressurized WPI as shown by increased DC tissue FRAP activity and greater total antioxidant serum capacity could be due to increased GSH levels in the DC. Likewise, the GSH inducing effects of peptides derived from WPI hydrolysis in the Caco-2 cells could have provided additional GSH substrate for GSH peroxidise (GPx) which catalyzed the hydrogen peroxide (H_2O_2) to water (Netto *et al.*, 1996). Polyphenols derived from the digestion of potato extracts also exerted antioxidant

and anti-inflammatory effects in Caco-2 non-stimulated or stimulated with H_2O_2. Polyphenols may have prevented oxidative stress (OS)-induced by increasing abundance of B-cell lymphoma 2 (Bcl-2), and decreasing abundance Bcl-2-associated X protein (Bax), Bcl-2- associated death promoter (Bad) proteins (Youdim and Mandel, 2004), and reducing the abundance of TNF-related apoptosis-inducing ligand (TRAIL) and FasL/Fas resulting in decreased IL-8 release (Levites *et al.*, 2003). Also, polyphenols have a strong affinity for transition minerals such as ferrous ions which can catalyze processes leading to the generation of ROS (Iwahashi *et al.*, 1990; Ahn and Chen, 1998; Kono *et al.*, 1998).

6.2 STUDY LIMITATIONS

There are potential limitations in each one of the studies presented in this thesis. DSS was used to induce colitis in this piglet model of colitis despite the fact that in humans the etiology of colitis is still under investigation and the mechanistic effects of DSS is not clear. In addition, groups of well-nourished control piglets SM- and WPI-fed could have been added to have a better comparison for the analysed outcomes. The short duration of the study did not affect plasma protein synthesis in this moderate protein deficiency piglet model. Thus, a longer duration would be more appropriate to observe the impact of moderate protein restriction on plasma protein synthesis. The results from the Caco-2 studies are difficult to extrapolate to *in vivo* conditions because a cell culture system cannot be compared to the whole body complexity of metabolism. A limitation was that the specific bioactive peptides and amino acids derived from the digestion of native and pressurized WPI that could be involved in the antioxidant and anti-inflammatory effects were not characterized. Likewise, the specific bioactive antioxidant and anti-inflammatory components were not identified in the FW obtained from potato extract digestion, which could include not only polyphenols and their metabolites but also other by-products of gut metabolism such as organic acids and short chain fatty acids. In that regard, it is also not clear how the altered polyphenolic profiles produced via human simulated gut digestive and

microbial processes had altered the polyphenolic antioxidant and anti-inflammatory bioactivities as compared to the original polyphenols present in the potato extract before digestion. In addition, how the polyphenolic compounds could have affected the microflora of the dynamic simulated human gut system was not identified, which could have altered the microbial by-products to affect the measured outcomes.

6.3 FUTURE STUDIES

More studies are needed to further investigate the mechanisms involved in the antioxidant and anti-inflammatory effects of WPI, especially pressure-treated WPI, which can act as a potential nutraceutical agent for the treatment of IBD. Furthermore, tissue GSH and GSSG concentrations should be measured considering the antioxidant effects shown with WPI and polyphenolics (Alsaikhan *et al.*, 1995; Vilela *et al.*, 2006a). The anti-inflammatory effects of pressure-treated WPI were shown in the DC by reduced levels of TNF-α, lL-8, and IL-18 but another study could also analyse these cytokines systemically to further investigate the applicability of pressure-treated WPI as a potent anti-inflammatory agent. In addition, tryptophan and arginine could be added to the WPI feeding to avoid growth failure and low bone density, respectively, as compared to SM. Anti-apoptotic effects of WPI were found downstream in the caspase-3 pathway but the study could not show the proteins, which are up-regulating this apoptotic pathway. In that regard, caspase-10 and caspase-12 could be investigated because they have been shown to directly activate caspase-3 (Wang *et al.*, 2001; Kerbiriou *et al.*, 2009). In addition, the NF-κB pathway could also be investigated as another apoptotic pathway as it has been demonstrated in other models of gut inflammation (Okamoto *et al.*, 1993; Togawa *et al.*, 2002). The WPI hydrolysates resulted in the formation of several unidentified peptides. In that regard, *in vitro* and *in vivo* studies should identify and fractionate these peptides in order to identify those with antioxidant and anti-inflammatory activities. Further studies involving the Dynamic Human Gastrointestinal Model could examine the release of polyphenols in different phases of digestion such as at the gastric and small

intestinal levels to determine the polyphenolic release from the potato extract at the various digestion compartments. This approach would thus assess the impact of various digestive conditions on polyphenol release and bioactivity at each of the digestion compartments. The identification of the new peaks found in the FW + Phenolics samples could be done by gas chromatography-mass spectrometry (GC-MS) to examine for polyphenolic metabolites generated from the simulated human gut system at the different digestion compartments. Also, it would be important to study the capacity of the Onaway polyphenolic rich potato extract to decrease oxidative stress and inflammation in an *in vivo* experimental model of IBD to further verify the benefits of the potato extract for the treatment of IBD. An *in vivo* study using an experimental model of IBD could be designed to investigate the synergistic or additive effects of both the pressurized WPI and polyphenol-rich potato extracts. The antioxidant and anti-inflammatory effects of WPI likely involves a predominant systemic action to provide protection at the intestinal level whereas polyphenols are more likely to exert direct effects at the colonic level when they traverse into the colonic compartment.

6.4 KEY FINDINGS

The present thesis findings has demonstrated that pressurized WPI exerted anabolic, antioxidant and anti-inflammatory effects and decreased disease severity both systemically and in the DC in a piglet model of pediatric colitis. In addition, this study partly unveiled the mechanism by which WPI suppressed the inflammatory response mediated via the caspase-3 apoptotic pathway in the DC. In the cell culture system, WPI hydrolysates showed antioxidant and anti-inflammatory effects exhibited by decreasing IL-8 secretion and reduced intracellular ROS generation in H_2O_2-treated Caco-2 cells. However, pressurized WPI hydrolysates showed a greater extent of antioxidant and anti-inflammatory effects as compared to native WPI. Polyphenol-rich potato cultivar extracts exposed to digestive enzyme and bacterial metabolism via the simulated human gut model showed antioxidant and anti-inflammatory effects in H_2O_2-stimulated Caco-2 cells.

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Appendices

Appendix 1. Vitamin content of each study diet.

Compounds	Control (mg/L of diet)	SM[1] (mg/L of diet)	nWPI[2] (mg/L of diet)	pWPI[3] (mg/L of diet)
Vitamin A palmitate	1.5	1.5	1.5	1.5
Vitamin D3	0.7	0.7	0.7	0.7
Vitamin E acetate	11.5	11.4	11.2	11.2
Menadione sodium bisulfite	-	0.1	0.2	0.2
Cyanocobalamin	-	0.4	0.1	0.1
Folic acid	-	0.1	0.1	0.1
Nicotinic acid	4.8	5.5	6.1	6.1
Calcium pantothenate	-	0.0	3.8	3.8
Pyridoxine-HCl	-	0.3	0.6	0.6
Thiamin HCl	-	0.0	0.4	0.4
Riboflavin	-	0.0	1.3	1.3
Benefiber (filler)	110.0	110.0	110.0	110.0
Total (mg/L)	128.5	129.9	136.0	136.0
Total (g/L)	0.13	0.13	0.14	0.14

[1]SM = skim milk. [2]nWPI = native whey protein isolate. [3]pWPI = pressurized whey protein isolate.

Appendix 2. Mineral content of each study diet.

Compounds	Control (g/L of diet)	SM[1] (g/L of diet)	nWPI [2] (g/L of diet)	pWPI [3] (g/L of diet)
Calcium phosphate, dibasic (29.5% Ca, 22.8% P)	4.21	7.28	9.87	9.87
Magnesium oxide (60.3% Mg)	-	0.10	0.20	0.20
Sodium chloride (39.4% Na, 60.6%Cl)	-	0.99	1.77	1.77
Potassium acetate (38% K)	-	-	2.16	2.16
Cupric carbonate (57.5% Cu)	<0.01	<0.01	<0.01	<0.01
Ferrous sulphate (20.1% Fe)	0.18	0.18	0.18	0.18
Manganese sulfate (36.4% Mn)	<0.01	<0.01	<0.01	<0.01
Sodium selenite (45.7% Se)	<0.01	<0.01	<0.01	<0.01
Zinc sulphate (22.4% Zn)	0.24	0.25	0.24	0.24
Total (g/L)	4.64	8.81	14.42	14.42

[1]SM = skim milk. [2] nWPI = native whey protein isolate. [3] pWPI = pressurized whey protein isolate.