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The Investigation of Microbial-Intestinal Epithelial Cell Interactions in the Gut and Their Effects on Inhibitor of Apoptotic Proteins (IAPs)

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DEDICATION

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ABSTRACT

Probiotic bacteria such as *Lactobacillus plantarum* strain 299v (Lp299v) have been shown to upregulate facets of innate immunity. This study assessed whether Lp299v prevents apoptosis in intestinal epithelial cells (IECs) when faced with cytokine challenge. Both *in vitro* and *in vivo* systems were exposed for a pre-determined time to Lp299v or strain Lpadh- a non-adherent derivative of Lp299v. HT-29 cells were then challenged with cytokine mixture (TNF-α, IFN-γ, and IL-1a) to imitate conditions of inflammation. To assess for apoptosis, we evaluated both TUNEL analysis and caspase activity assays. Results of the assays indicate a marked decrease in apoptosis in samples treated with Lp299v. The adherent negative Lpadh- strain showed similar but less pronounced prevention of cytokine-induced apoptosis. IAPs were assessed both *in vitro* and *in vivo* and HIAP1 and HIAP2 were determined to be involved in this process. This is consistent with NF-kB activation by Lp299v. Probiotics may confer exogenous effects on IECs to elaborate protective mechanisms and alter cell homeostasis through alteration of caspase-dependent apoptosis.

TABLE OF CONTENTS

Acknowledgements i
Dedication ii
Abstract iii
Table of Contents iv
List of Abbreviations vii
List of Figures ix
List of Tables xi

CHAPTER 1: INTRODUCTION

1.1 OVERVIEW OF GASTROINTESTINAL PHYSIOLOGY
1.1.1 Anatomy of the Intestinal Tract 1
1.1.2 The Enteric Nervous System (ENS) and Mucosal Immune System 2
1.1.3 Cells of the Intestinal Tract 3
1.1.4 Naturally-Occurring Bacteria in the Gut 3
1.1.5 Defense Mechanisms of the Intestine 5
1.2 APOPTOSIS
1.2.1 Apoptosis in the Intestinal Tract and Clinical Progression 6
1.2.2 Therapeutic Relevance 8
1.2.3 Cell Signalling in Response to Microorganisms 10
1.3 INHIBITOR OF APOPTOSIS PROTEINS (IAPS)
1.3.1 Characterization of IAP Family Members 12
1.3.2 IAP-Mediated Signalling and Programmed Cell Death 13
1.3.3 Neuronal Apoptosis Inhibitor Protein (NAIP) and Inflammation 14
1.4 EXPERIMENTAL MODELS OF INFLAMMATORY BOWEL DISEASE (IBD)
1.4.1 Intestinal Epithelial Cells (IECs) 15
1.4.2 Cytokines in IBD 16
1.4.3 The Role of Macrophages 17
1.5 PROBIOTICS
1.5.1 Definition and Strains of Interest 18
1.5.2 Transcriptional and Translational Mucin Upregulation 18
1.5.3 Use in Conditions of IBD 19
1.5.4 Colonization and Related Signalling 20
1.6 HYPOTHESIS AND PRINCIPAL OBJECTIVES 21

CHAPTER II: MATERIALS AND METHODS 22
2.1 ANIMAL PROTOCOL
2.1.1 C57/Bl6 Mice 22
2.1.2 Sprague-Dawley Rats 22
2.2 BACTERIA
2.2.1 Probiotic Strains Used for Study 23
2.2.2 Bacterial Preparation and Quantification 23

2.3 RNA AND PROTEIN SOURCES AND EXTRACTION
2.3.1 Dissection of Distinct Intestinal Segments 24
2.3.2 In-vitro Cell Model 25
2.3.3 RNA Extraction 26
2.3.4 Protein Extraction 27
2.4 POLYMERASE CHAIN REACTION ANALYSIS OF GENE EXPRESSION
2.4.1 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction 28
2.4.2 Data Analysis 30
2.5 IN-VITRO ASSESSMENT OF CYTOKINE-INDUCED APOPTOSIS
2.5.1 Cell Model 30
2.5.2 Cytokine-Mediated Induction of Apoptosis 31
2.5.3 TUNEL Assay 31
2.5.4 Caspase Activity Assay 32
2.5.5 Analysis 33
2.6 IN-VITRO IAP EXPRESSION WITH MACROPHAGE CO-CULTURE
2.6.1 Cell Model 33
2.6.2 RNA and Protein Expression Analysis 34
2.6.3 Immunocytochemical Analysis of Macrophage Activation 34
2.7 IMMUNOBLOT ANALYSIS OF IAPS SHOWING INDUCTION
2.7.1 Western Blot Analysis of Genes Showing Induced Expression 35
2.8 ELUCIDATION OF CELL SIGNALLING
2.8.1 NF-kB Reporter Activity Assay 37
2.9 STATISTICAL ANALYSIS 38

CHAPTER III: RESULTS 41

3.1 IN-VITRO ANALYSIS OF PROBIOTIC EFFECT IN CYTOKINE-INDUCED APOPTOSIS
3.1.1 In-situ Peroxidase TUNEL Analysis: Probiotic-Induced Anti-apoptotic Effect 41
3.1.2 Caspase Activity Assay Results Confirm Prevention of Apoptosis 46
3.1.3 Analysis of Select IAP Gene Expression using QRT RT-PCR 55
3.1.4 Analysis of IAP Protein Expression using Western Blotting 65
3.2 ANALYSIS OF IAP ACTIVITY IN MACROPHAGE/HT-29 CELL CO-CULTURE
3.2.1 *Hiap1, hiap2,* and *naip* mRNA levels detected by QRT RT-PCR 69
3.2.2 IAP Protein Expression Following Co-incubation 72
3.2.3 Immunocytochemical Analysis of Macrophage Activation 75
3.3 EVALUATION OF PROBIOTIC ADMINISTRATION IN VIVO
3.3.1 Daily Water Intake of C57/Bl6 Mice 80
3.3.2 Bacterial Quantification 82
3.4 IN-VIVO ANALYSIS OF IAP EXPRESSION FOLLOWING TREATMENT
3.4.1 RNA Expression of Select IAPs 85
3.4.2 Analysis of Modulated IAPs at the Translational Level 96
3.5 ELUCIDATION OF POTENTIAL SIGNALLING PATHWAY
3.5.1 Luciferase Reporter Activity Assay 101

CHAPTER IV: DISCUSSION 104

4.1 PROBIOTIC-MEDIATED ANTI-APOPTOTIC EFFECT IN-VITRO
4.1.1 TUNEL+ Counts Indicate Lp299v and Lpadh- Protect Against Apoptosis 104
4.1.2 Evaluation of Caspase Activity Assay Results Confirms Protection 104
4.1.3 IAP RNA and Protein Expression 107
4.2 MACROPHAGE CO-CULTURE
4.2.1 HIAP RNA and Protein Expression Analysis 109
4.2.2 Macrophage Activation Following Co-culture and Endotoxin Exposure 111
4.3 PROBIOTIC-MEDIATED ANTI-APOPTOTIC EFFECT IN-VIVO
4.3.1 mRNA and Protein Expression following Probiotic Incubation 112
4.4 CELL SIGNALLING
4.4.1 Probiotic Protection Appears to be NF-kB Mediated 114
4.5 CONCLUSIONS AND FUTURE DIRECTIONS 115

REFERENCES 118

APPENDIX A: Sequence and strand location of *IAP* specific primers used in the analysis of QRT RT-PCR results. 125

APPENDIX B: Raw densitometric data for Western blots. 127

APPENDIX C1: Summary table of HT-29 *in vitro* results.
C2: Summary of macrophage studies.
C3: *In vivo* IAP expression in response to Lp299v. 129

APPENDIX D: Proposed Schematic. 130
CURRICULUM VITAE 131

LIST OF ABBREVIATIONS

Area Under the Curve (AUC)
Baculoviral IAP Repeat (BIR)
Colony Forming Units (CFU)
Crohn's Disease (CD)
Carboxyfluoroscein-labeled Fluoromethyl Ketone (FAM-DEVD-FMK)
Caspase Activation Recruitment Domain (CARD)
Dendritic Cell (DC)
Diaminobenzidene (DAB)
Enteric Nervous System (ENS)
Enteropathogenic *E. coli* (EPEC)
Fetal Bovine Serum (FBS)
Fluorochrome Inhibitors of Caspases (FLICA)
Glyceraldehyde Phosopho-Diesterase (GAPDH)
HT-29 galactose-dependent cells (HT-29 Gal)
HT-29 glucose-dependent cells (HT-29 Glu)
Human Inhibitor of Apoptosis Protein (HIAP)
Inflammatory Bowel Disease (IBD)
Inhibitor of Apoptosis Protein (IAP)
Interferon (IFN)
Interferon Response Factor (IRF)
Interleukin (IL)
Interleukin-Converting Enzyme (ICE)
Intestinal Epithelial Cell (IEC)
Lactobacillus plantarum 299v (Lp299v)
Lactobacillus plantarum adh- (Lpadh-)
Leucine Rich Repeat (LRR)
Lipopolysaccharide (LPS)
Luciferase Assay Substrate (LAR)
Meso-Diaminopimelic Acid (DAP)
Muramyl Dipeptide (MDP)
Macrophage Migration Inhibitory Factor (MIF)
Neonatal Necrotizing Enterocolitis (NEC)
Neuronal Apoptosis Inhibiting Protein (NAIP)
Nod-Like Receptor (NLR)
No Template Control (NTC)
Nucleotide Oligomerization Domain (NOD)
Optical Density (OD)
Passive Lysis Buffer (PLB)
Pathogen-Associated Molecular Patterns (PAMPS)
Phosphate-Buffered Saline (PBS)
Programmed Cell Death I (PCD I)
Programmed Cell Death II (PCD II)
Propridium Iodide (PI)
Reverse Transcription Polymerase Chain Reaction (RT-PCR)
Sodium Doecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Terminal Deoxynucleotidyl Transferase (TdT) Nick-End Labeling (TUNEL)
Toll-Like Receptor (TLR)
Tris-Phosphate Buffered Saline with Tween (TBST)
Tumour Necrosis Factor (TNF)
Ulcerative Colitis (UC)
X-linked Inhibitor of Apoptosis (XIAP)

LIST OF FIGURES

Figure 1: Peroxidase in-situ TUNEL staining of HT-29 Gal cells *in vitro* following cytokine exposure alone (a), incubation with Lpadh- for 1 hr prior to cytokine exposure (b), and incubation with Lp299v for 1 hr prior to cytokine exposure (c).

Figure 2: TUNEL results following enumeration of apoptotic cells per total cells counted in randomly selected microscopic fields.

Figure 3: Fluorescent caspase activity assay showing caspase$^+$ cells following conditions of cytokine exposure (a), in the presence of Lpadh- for 1 hr prior to cytokine exposure (b), and in the presence of Lp299v for 1 hr prior to cytokine exposure (c).

Figure 4: Caspase activity assay results following enumeration of apoptotic cells per total cells counted in randomly selected microscopic fields.

Figure 5: Fluorescent caspase-3,7 activity assay images showing caspase-3,7$^+$ cells following conditions of cytokine exposure (a), in the presence of Lpadh- for 1 hr prior to cytokine exposure (b), and in the presence of Lp299v for 1 hr prior to cytokine exposure (c).

Figure 6: Caspase-3,7 activity assay results following enumeration of apoptotic cells per total cells counted in randomly selected microscopic fields.

Figure 7: *Hiap2* mRNA induction following *in vitro* cell experiments where cells were incubated with probiotics in the presence (b) or absence (a) of cytokine exposure.

Figure 8: *Hiap1* mRNA induction following *in vitro* cell experiments where cells were incubated with probiotics in the presence (b) or absence (a) of cytokine exposure.

Figure 9: *Naip* mRNA induction following *in vitro* cell experiments where cells were incubated with probiotics in HT-29 Gal cells.

Figure 10: IAP mRNA induction following HT-29 Glu cell exposure to either Lp299v or Lpadh- for 1hr.

Figure 11: HIAP1 and HIAP2 protein levels *in vitro* detected using Western blotting.

Figure 12: NAIP protein levels *in vitro* detected using Western blotting.

Figure 13: *Hiap1* (a) and *hiap2* (b) induction following co-incubation of HT-29 Gal cells with activated RAW264.7 macrophages with or without select probiotics.

Figure 14: HIAP1, HIAP2 and GAPDH Western blots of RAW264.7/HT-29 cell co-culture protein extracts.

Figure 15: Immunocytochemical analysis of macrophage activation in HT-29 Glu cells co-incubated with macrophages subjected to probiotic exposure.

Figure 16: Quantification of activated macrophages in cell populations treated with endotoxin following incubation with either Lp299v, Lpadh-, or in the absence of probiotic treatment.

Figure 17: Probiotic-specific bacterial growth curves used to quantitate Lp299v (a) and Lpadh- (b) for samples used in the experiments.

Figure 18: *Hiap2* mRNA induction *in vivo* following gavage administration of either Lp299v or Lpadh- to C57/Bl6 mice for 2 days, n=31.

Figure 19: *Hiap1* mRNA induction *in vivo* following gavage administration of either Lp299v or Lpadh- to C57/Bl6 mice for 2 days, n=30.

Figure 20: *Naip1* mRNA induction *in vivo* following gavage administration of Lp299v to C57/Bl6 mice for 2 days, n=38.

Figure 21: *Xiap* mRNA induction *in vivo* following gavage administration of Lp299v to C57/Bl6 mice for 2 days, n=25.

Figure 22: *Naip* mRNA induction *in vivo* following administration of either high or low dose Lp299v to Sprague-Dawley rat drinking water for 2 days, n=16.

Figure 23: 2 day Lp299v ingestion dramatically raises mouse jejunal HIAP levels (a) with a moderate increase in NAIP (b).

Figure 24: Western blotting of jejunal and ileal rat protein extracts with RIAP1 antibody shows slight increase in protein level following treatment.

Figure 25: NF-κB reporter activity in cells following probiotic incubation for 1 hr.

LIST OF TABLES

Table 1: (a) Primary antibodies used in the experiments 39
(b) Secondary antibodies used in the experiments 40
(c) Stains used in experiments 40

Table 2: Rat study groups and daily intake of water 81

1. Introduction

1.1 Overview of Gastrointestinal Physiology

1.1.1 Anatomy of the Intestinal Tract

The gut, or gastrointestinal tract, is a long hollow tube of interconnected regions that runs as long as 10 m and is primarily responsible for obtaining nutrients from food, protecting against potentially noxious substances or deleterious pathogens and eliminating waste [1]. The different regions of the tract include the esophagus, stomach, small intestine, colon and rectum [1]. The muscle in the walls of the gut include an outer longitudinal layer and an inner circular layer to move its contents along the tract [1]. The submucosa contains various cellular immune and non-immune elements, nervous tissue, blood vessels and is lined by epithelial cells [1]. These intestinal epithelial cells not only act as a physical barrier but are able to produce a number of substances to aid in mechanical protection and enzymes to aid in digestion on nutrients such as carbohydrates. The small intestine is composed of the duodenum (for mineral absorption and where bile secretion, digestive enzymes and bicarbonate from the pancreas are secreted into), jejunum (where break down and absorption of main components of fat, protein and starches occurs) and the ileum (for further absorption of nutrients, water, and site-specific absorption of B_{12} and bile salts) [1]. Undigested compounds and substances are transported to the colon where a significant amount of water is absorbed, some further absorption of fermented material, and waste (including retired intestinal cells from the epithelial lining) is then excreted [1]. The intestine is supplied blood via the celiac, superior and inferior mesenteric arteries, and lymphatics are responsible for shuttling away absorbed fats, fat-soluble elements and fluid into the circulation [2].

1.1.2 The Enteric Nervous System (ENS) and Mucosal Immune System

The enteric nervous system (ENS) is responsible for governing the hormones, nerves, and neurotransmitters in relation to gut physiology including digestion [1]. An intricate nerve complex resides within the intestinal wall which relays information to and from the brain via the spinal cord and vagus nerve in the parasympathetic nervous system [1]. These nerves operate much like the spinal cord in giving and receiving information from the intestinal wall [1]. This *gut brain* is a complex system responsible for not only gathering physiological information, but also conveying signals back to the intestine [1].

The mucosal immune system operates at least superficially in a paradoxical manner. While requiring anti-inflammatory mechanisms for food, airborne allergen and commensal tolerance, it must also provide a means of rapid local defense against invading organisms [3]. This is achieved through the optimization of innate defenses (such as mucin and antimicrobial peptide production), a strong mucosal barrier, acting and available lymphocytes, and IgA secretion [3, 4]. The importance of resident bacteria in this system was determined through the use of germ-free mice. These animals are unable to induce mucosal B and T cells that constitute an immune barrier against microbial infection [5]. Moreover, commensals are tolerated by the mucosal immune system, and when in excess, their separation from dendritic cells (DCs) contained in mesenteric lymph nodes sequesters them from systemic immune responses [5]. Not only do these bacteria offer a means of regulating mucosal immunity due to their presence, in doing so they offer the possibility of disease treatment [3].

1.1.3 Cells of the Intestinal Tract

From the tall villi that protrude into the lumen of the intestinal tract, there is a thin single-cell lining of IECs each with microvilli projecting from their surface to increase overall surface area [2]. These cells are involved in water and nutrient absoption [2]. Scattered among the IECs are goblet cells which are responsible for mucus secretion and Paneth cells which secrete antimicrobial peptides as another means of innate defense [4, 6]. Inside each of the villi are small capillaries and a lacteal which is a specialized lymph vessel involved in immune system coordination [6].

Below the cells which comprise the epithelium resides the mucosa layer which contains macrophages, plasma cells, mast cells and lymphocytes involved in both immunity and chemical reactions to injury [2]. The mucosa itself is pleated to maximize fluid absorption as it lines the intestinal tract [2]. The mucosa is separated from the submucosa by a thin layer of muscle termed *muscularis mucosae* [2]. The layers of muscle, both the submucosal nerve plexus and the myenteric plexus, and the serosal layers of the gut underlie the submucosal layer [2]. It is in the immune cells of the mucosal and submucosal layers (infused with blood and lymph capillaries) where the sensing of foreign material takes place. Sensing occurs between the muscle layers in the submucosa in the enteric nerves and is assisted by DCs extending between IEC cells in the lumen. These reactions primarily orchestrate immune cell-mediated signalling and are highlighted in disease pathology [2, 7].

1.1.4 Naturally-Occurring Bacteria in the Gut

There are up to 10^{12} naturally-occurring bacteria or *commensals* per gram of lumenal content in the gastrointestinal tract, with the greatest concentrations in the colon. These strains in aggregate form a tolerated rapport with immune cells early in life [2, 5]. The

majority of these are Gram-negative anaerobes located in the colon [3]. Born with a sterile intestinal tract, infants first acquire their microbial flora from their environment chiefly from their mother upon vaginal delivery [8, 9].

Bacteria can break down and ferment dietary foodstuffs, as well as produce vitamins such as vitamin K and riboflavin that we cannot produce ourselves [8]. Alteration of one's microbiota may occur in certain disease conditions, as well as following courses of antibiotics. The alteration of a stable intestinal microbiota may lead to complications such as antibiotic-associated diarrhea whereby the most commonly associated pathogen is *Clostridium difficile* [8, 10]. Commensals are therefore vital to the healthy functioning of the intestinal tract. These microbes are tolerated well by immune cells as long as they remain in the lumen.

The principle activity of enteric bacteria is the fermentation of non-digested nutrients in waste, a metabolism that results in the production of short-chain fatty acids such as butyrate, propionate and acetate that provide further nutrients to IECs, and to maintain a balance of innate and adaptive immunity through their presence [1, 3]. Probiotics have been shown to increase mucin secretion, aid in intestinal host defense, strengthen intestinal tight junctions, enhance motility, produce metabolites that are beneficial to the intestinal tract, promote fermentation processes, produce antimicrobial bacteriocidins and help balance the microbial flora which can protect against pathogens [11]. Their outer glycocalyx layer, or capsule (typically composed of polysaccharide or polypeptide) helps evade immune detection as host cell receptors do not necessarily recognize them as disease-causing entities in the luminal compartment [12]. Thus, there is an effective compartmentalization of the microbiota. Short, hair-like projections called fibriae help certain bacteria adhere to mucosal surfaces [12]. This can be advantageous in terms of host-assisted metabolism or detrimental should

the bacteria be pathogenic in nature as adherence is the first step required for subsequent colonization, invasion and toxin delivery. It is in part the presence of probiotic (indigenous nonpathogenic) commensal bacteria that prevent the establishment and colonization of potentially harmful microbes [10]. However, the presence of bacteria and their interaction with the gut immune system is thought to be important in such diseases as Inflammatory Bowel Disease (IBD) given for example that various knockout mouse models of immune alterations in germ-free conditions do not develop colitis but in normal pathogen-free conditions develop chronic colitis [2]. An increased understanding of the role of probiotics in the healthy intestinal tract would lead to a better understanding of gastrointestinal physiology.

1.1.5 Defense Mechanisms of the Intestine

Inducible innate immune defense in the intestine includes mucin secretion by IECs and antimicrobial peptide secretion by Paneth cells [4, 13]. Beyond this are chemicals secreted into the intestinal lumen (including bile salts, gastric acid and pancreatic enzymes) and immune cells which are contained in gut mucosa and are called upon for cell-mediated defense against foreign invaders [2]. There is thought to be a low-level constitutive amount of controlled inflammation in the gut of normal individuals believed to keep the system in check or develop tolerance [2, 4]. Unchecked, chronic inflammation may develop characteristic of IBD, which includes both Crohn's Disease (CD) and Ulcerative Colitis (UC). This is no surprise considering approximately 80% of all immunologically active cells reside in gut-associated immune tissue [14].

Should immune cells be called upon, a variety of mononuclear cells can transform to perform a variety of requisite functions [2]. These include killer lymphocytes, T and B cells

and macrophages (which engulf and consume invading bacteria), to name a few [2]. Strong immune reactions are what lead to inflammation induced by the chemical secretions (including histamine and a myriad assortment of pro-inflammatory cytokines) from nearby mast cells [2].

In conditions of IBD the consequences of these responses can be seen in sample histology. Physiologically, prostaglandins are formed from arachidonic acid and can contribute to IBD, actions that are counteracted by the use of steriods for these conditions [2]. Blood vessels attribute to tissue damage via dilation, hemorrhage and fluid edema, whereas white blood cells including polymorphonuclear cells gather at the site of inflammation to compose the acute inflammatory response [2]. Following activation, macrophages secrete interleukin-1 (IL1) which signals the hypothalamus to raise the core temperature of the body (what we know as fever) in order to hinder the pathogen [10]. Accumulating evidence suggest there may be any number of triggering events required for chronic inflammatory bowel conditions in the genetically predisposed and suggest the IBD-related immune response is due to barrier deficit or a heightened immunological microbial response, or a combination of both [2]. Thus, preservation or enhancement of the barrier function of the intestinal tract may be an important consideration in preventing the initiation or moderating the chronicity of chronic bowel disease.

1.2 Apoptosis

1.2.1 Apoptosis in the Intestinal Tract and Clinical Progression

Epithelial cell homeostasis is maintained in the gastrointestinal tract mainly via apoptosis, a form of programmed cell death, distinct from necrosis in that it is held tightly under genetic control by the cell [15]. Apoptosis progresses via the activation of specific

cysteine proteases termed *caspases* [16]. Once activated, these enzymes proceed in executing the stages which proceed to programmed cell death. As discussed, IECs constitute the epithelial layer of the small intestine which due to their position is critical to the overall health of the host, as they constitute the cellular barrier between luminal contents and the host. Accordingly, IECs which are lost naturally through a physiologic process are replenished at an equally rapid rate in villi crypts, on average every 3-5 days [2, 17]. Due to this rapid cell turnover and the physiological requirement for apoptotic cell death, it follows that dysregulated apoptosis may be linked to the pathogenesis of a variety of gastrointestinal disorders [13, 17-20]. In previous studies, IEC cell apoptosis has been shown to be detrimental to survival of the host following bacterial threat [17]. There is an increasing amount of research which indicates that prevention of IEC apoptosis enhances survival in mouse models of sepsis and non-infectious inflammation [17].

In UC, increased IEC apoptosis is observed in cells of the crypt-villus axis leading to the tips of villi [21]. This increase in apoptosis is hypothesized to lead to functional loss of absorptive capacity, secretion of intestinal fluids, loss of barrier function and eventual progression to recognizable clinical effects [22].

In CD patients, not only is there a loss in IEC function, but all layers of the intestine are typically involved; interestingly the T-cells of the lamina propria seem to have an increased resistance to apoptosis [1, 2, 23]. This imbalance in cellular death results in T-lymphocytes which are resistant to subsequent Tumour Necrosis Factor (TNF)-mediated apoptotic death, characteristic of the disease [18, 22]. CD, characterized as chronic inflammation generally at the junction of the small intestine and colon, is generally treated using anti-inflammatory drugs to control the inflammation [21]. In advanced stages of the disease, immunosuppressants, corticosteroids, antibiotics and anti-TNF antibody infusion may be

required; if severe, the affected tissue itself may be removed [21]. A better understanding of IEC-mediated apoptosis could potentially lead to alternate forms of therapy to relieve the chronicity or development of IBD due to its critical function in cell and barrier function.

1.2.2 Therapeutic Relevance

It has been argued that IEC apoptosis occurring in villi will not lead to a significant clinical phenotype. However, increased cell apoptosis along the crypt-villus axis has been correlated to villus atrophy implicated in barrier disruption [24-26]. Villi atrophy results from a loss of proliferative capacity and failure to replace maturing IECs from villus crypts due to substantial or persistent apoptosis of single cells. This results in a shortening of IEC crypt-villus length, and increased progression of cell apoptosis [24]. Factors involved include increased expression of Fas ligand (FasL) on IECs from acting lymphocytes and a concurrent increase in pro-inflammatory cytokines such as IFN-γ (also acting through the Fas receptor) [27]. While anti-TNF therapies are somewhat successful in decreasing the inflammation associated with IBD, probiotic bacteria may also provide benefit as these have been shown to act in an anti-apoptotic manner in the presence of cytokines [28].

Probiotics have also been recently shown to modulate Toll-Like Receptor 2 (TLR-2), found to be critical in maintaining barrier function when cells are faced with inflammatory stress [29]. This barrier function is maintained via PI3K/Akt-mediated cell survival via MyD88 [29]. It follows that mice lacking TLR-2, MyD88 or with cells permissive to apoptosis are prone to barrier breach (controlled via TLR-2-mediated tight junctions) in inflammatory conditions [29]. It is barrier breach which predisposes the host to intestinal inflammation and injury [29]. Therefore, probiotics if acting in an anti-apoptotic fashion

while upregulating TLR-2, seem to be suited for use in conditions of inflammation and for the prevention of barrier breach.

Barrier breach has been implicated in other diseases of the intestinal tract such as neonatal necrotizing enterocolitis (NEC) where it is hypothesized that mediators of apoptosis act on IECs altering tight junction integrity leading to a breach in the intestinal barrier [30]. This permeability leads to bacterial translocation which activates the inflammatory cascade leading to widespread necrosis and NEC [30]. It has also been determined that apoptosis of a small subset of cells is what progresses to widespread necrosis and altered barrier integrity in NEC disease pathology [31]. Probiotics (specifically *Lactobacillus* and *Bifidobacterium* species) have been indicated in NEC improvement, hypothesized to be due to an increase in barrier function [30, 32]. It is the increased susceptibility to potentially pathogenic organisms together with a decrease in normal flora constituents which predispose neonates to NEC, again stressing the potential for probiotics in proper intestinal function [33].

1.2.3 Cell Signalling in Response to Microorganisms

To understand the role of apoptosis in IECs it is imperative to consider their complex nature. IECs exist in a milieu of heterogeneous microbes containing both commensal and sometimes potentially pathogenic species, and must actively use receptors to sense microbial Pathogen-Associated Molecular Patterns (PAMPS) absent from host systems [34]. TLRs sense the extracellular presence of LPS of Gram-negative bacteria, and lipoteichoic acid (LTA) of Gram-positive bacteria, leading to a variety of signalling cascades dependent upon the specific receptor [34-37]. TLR5 in particular, expressed on the basolateral surface of IECs is responsible for the pro-inflammatory response following detection of flagellin found on microbes such as enteropathogenic *E. coli* (EPEC) [38]. IECs sense different pathogenic

molecular species via TLRs responding with various cytokines if warranted. They must also sense pepidoglycan, bacterial DNA, viral double-stranded RNA, inter-species cross bacterial communication, protein products secreted by microbial populations, and pathogenic products that these species attempt to inject into cells via secretory pathways to invade cells and establish infection [34]. Therefore, when attempting to elucidate the role of apoptosis in IEC regulation, it is important to consider the effects of both pathogenic and probiotic species on these cells, since they may employ a vast array of signalling pathways under normal conditions [39].

In addition to TLRs located in the IEC membrane, there are an abundance of intracellular bacterial detectors known as Nod-Like Receptors (NLRs) [40]. This family of more than 20 members (including NOD1-5 and NAIP) work in tandem with TLRs in order to provide IEC defense against potential bacterial invasion [40]. NLRs are characterized by a C-terminal leucine-rich repeat (LRR), central nucleotide-binding NACHT domain and N-terminal Caspase Activation Recruitment Domain (CARD) [40]. NOD2 (Card15) has been shown to sense the intracellular presence of MDP common to both Gram-negative and Gram-positive bacteria, whereas NOD1 (Card4) senses meso-diaminopimelic acid (DAP) from Gram-negative bacteria [40].

Gram-negative probiotic organisms, which express lipopolysaccharide (LPS) are recognized by IEC TLRs in conjunction with NOD2 [40]. Frameshift mutations of NOD2 lead to failed sensing of bacterial presence and the subsequent establishment of infection [40]. Clinically, mutations in NOD1 as well as NOD2 have been linked to the establishment of inflammatory mucosal barrier disease [40]. NOD2 once activated by either Gram-negative or Gram-positive bacteria, activates a signalling cascade leading to the activation of transcription factor NF-kB. IKK is activated by NOD2 via RIP2 activity which then

phosphorylates the inhibitory IkB on NF-kB [40]. This leads to the migration of NF-kB into cell nuclei and the transcription of proteins involved in both cell survival and inflammation to protect against bacterial invasion [24]. This transient increase in protective inflammation could strengthen the intestinal barrier through recruitment of cell populations required for bacterial clearance [40]. We propose this to also be beneficial in the intestine devoid of previous chronic inflammation imposed by a disease state.

However, while bacterial recognition by TLRs can be protective in this manner, an increase in NOD2 also has negative effects. Elevated NOD2 activity can lead to an increase in Fas receptor sensitivity to FasL, which then in the presence of proinflammatory cytokines observed in conditions such as IBD (TNF, IFNγ) will increase caspase-8 mediated caspase-3 and -7 activation and subsequent apoptosis [27, 41].

There is evidence that microbes have differing effects on apoptosis: *Salmonella*, for example, a Gram-negative facultative intracellular bacteria, activates apoptosis in IECs and once contained in apoptotic bodies, activates proinflammatory cytokines in macrophages which aid in dendrite maturation to carry the bacteria to lymph nodes for spread [42]. Various other microbes stimulate apoptosis chiefly through LPS [43]. This is a rather surprising finding given that previous reports suggest LPS to be anti-apoptotic. In addition to attenuating chronic diarrhea and inducing remission in patients with UC [9], there is exciting evidence that certain probiotic strains are able to prevent cytokine-induced apoptosis in IECs via unknown gene regulation, and enhance epithelial barrier function [28, 44-49]

The therapeutic benefit of probiotics observed in conditions of acute inflammation, suggests that cell signalling in response to these microbes may evoke a protective pathway. There exist indications that IBD may be caused by an imbalance of enteric microflora, either due to the genetic predisposition or environment of the host. This, in conjunction with the

efficacy of antibiotics in the treatment of CD, lead us to believe that an introduction of these naturally-occurring bacteria into the intestinal milieu may act in such a way as to balance the microbial population to reduce excessive NOD2 signalling, potentially prevent caspase-3 and -7 mediated apoptosis and concurrent inflammation.

1.3 Inhibitor of Apoptosis Proteins (IAPs)

1.3.1 Characterization of IAP Family Members

One of the most interesting families of proteins to consider in apoptosis dysregulation in conditions of the gastrointestinal tract is the inhibitor of apoptosis proteins (IAPs). IAPs inhibit apoptosis by means of inhibiting key cysteine aspartyl specific proteases (caspases) involved in the propagation of programmed cell death. IAPs are being investigated as therapeutic targets for a variety of diseases as they show promising ability to regulate the activity of both initiator and effector caspases (notably caspase-3 and -7), grouped according to their function in either the initiation or execution of the events leading to cell death [16, 42, 50].

IAPs are characterized by one or more baculoviral IAP repeat (BIR) domains which are responsible for the hallmark caspase inhibition at their N-termini in addition to a C-terminal end unique to each IAP [50]. Distinct BIR domains are involved in binding active caspases; BIR domains 2 and 3 are able to bind caspases-7 and -9, for example [51]. X-linked inhibitor of apoptosis protein (XIAP), the most extensively studied IAP and a potent inhibitor of caspase activity [52] contains three BIR domains, in addition to a RING domain [50]. XIAP is most effective in caspase-3, -7 and -9 inhibition, which are key effector caspases [53]. Cellular IAP-1 (cIAP1/HIAP2) and cellular IAP-2 (cIAP2/HIAP1) contain the same BIR domains and RING domain but also an additional Caspase Recruitment

Domain (CARD) [50]. This CARD domain has been recently shown to have diverse functions beyond caspase inhibition including anti-viral type I interferon (IFN) induction [54]. Survivin is a potent IAP involved in cancer progression [55]. Should an induction of IAPs occur following probiotic exposure this would add merit to their apparent anti-apoptotic ability under conditions of cytokine stress.

1.3.2 IAP Mediated Signalling and Programmed Cell Death

IAPs, via inhibition of key caspase family members, impinge upon a distinct apoptotic pathway known as caspase-dependent programmed cell death (or Programmed Cell Death I, PCDI). This form of programmed cell death is also known as the *Classical Apoptotic Pathway* as it is the first known form of apoptosis [16]. This form of cell death involves two distinct pathways: the extrinsic (mediated by receptor-initiated death signals, TNF for example) and intrinsic pathway (mediated by cytochrome c release from mitochondria in response to a perceived intracellular death signal) [16]. Caspase-independent Programmed Cell Death (PCDII) or *autophagy* also exists [56]. PCDII conserves the limited energy of the cell via organelle recycling into autophagosomes under conditions of starvation or low energy [56]. There is evidence of autophagic cell death in response to Bcl-2 protein inhibition [56]. Due to an absence of methodology capable of detecting PCDII, the extent of autophagy is often deduced by measuring total cell death and PCDI occurrence.

In the intestine especially, anoikis occurs, or detachment-induced apoptosis [57]. Anoikis, while difficult to detect, has been shown to be enhanced in bacterially infected cells with human milk supplementation [57]. Overall however, cellular response to cytokines in terms of distinct PCD mechanisms remains to be elucidated.

Cellular death due to harsh conditions or lack the energy to undergo apoptosis, leads to necrosis, a process of cell lysis whereby none of the cellular constituents are conserved [16]. Both necrotic and apoptotic cell death result in DNA degradation, however both forms of PCD result in characteristic 180 bp DNA fragments, whereas necrosis is characterized by random DNA degradation [16]. The specific DNA fragments produced via PCD allow for its specific detection. Conversely, necrosis is detected by the presence of propridium iodide (PI) which indicates a break down in the cell membrane in dead or dying cells, as this dye is otherwise membrane impermeable [16]. Importantly both forms of PCD, involve distinct molecular pathways opening the possibility of a therapeutic interception. However, due to the requirements of specific caspases for cell differentiation and development, it is beneficial to specifically inhibit implicated executioner caspases alone, caspases-3 and -7 in particular [19, 58]. The prevention of cell death is a theoretical possibility in these pathways, yet necrosis once initiated cannot be stopped.

1.3.3 Neuronal Apoptosis Inhibitor Protein (NAIP) and Inflammation

Relevant to this study is the Neuronal Apoptosis Inhibitory Protein (NAIP), comprised of N-terminal BIR domains, C-terminal LRR and a Nucleotide-Binding Oligomerization Domain (NOD) [59]. The linkage of CD to NOD2 mutations in the LRR region, suggests the possibility of a role for NAIP (another LRR-containing protein) in this condition [40]. NOD domain-containing proteins such as NAIP and NALP3 have been implicated in *inflammasome* formation mediating inflammation upon bacterial recognition [40, 54]. This complex is composed of many proteins that ultimately lead to IL-1β converting enzyme (ICE; caspase-1) activation and inflammatory progression [60, 61]. This process prevents the death that would result from proinflammatory cytokine-conferred caspase-induced cell

death in response to microbes [59]. Due to its role, IL-1β is characteristic among intestinal disorders [60, 62].

It is hypothesized that since most NOD proteins (i.e. NOD2) activate NF-kB upon LRR-mediated LPS sensing, they are involved somehow in host-bacterial intestinal response to infection [40, 50]. Interestingly, mouse *naip5* determines *Legionella* susceptibility as mice with anomolous versions of this gene were shown to be susceptible to *Legionella* infection reflecting *naip*'s role as a critical bacterial sensor. This again highlights the potential role of NAIP in intracellular bacterial detection [63].

As previously stated, NAIP has been classified along with proteins such as NOD1 and NOD2, Ipaf, and Nalp1, 2-14 as NBS-LRR proteins for their NOD domain and LRR C-terminus [59]. Mutations in these proteins have been shown to lead to impaired bacterial sensing, bacterial-independent apoptosis, and constitutive NF-kB inflammation. While this highlights their role in IEC-microbial interactions, the role of these proteins in innate immune defense remains unknown [59, 63, 64]. There is evidence that NAIP acts as an intracellular flagellin sensor which acts independently from TLR-5 [40]. Of the IAPs mentioned here, XIAP, HIAP1, HIAP2 and NAIP are all found in both the epithelium of the small intestine and colon. Interestingly NAIP expression was found to increase as cells migrate towards the villus tip and coexists with p21 expression thought to play a protective role throughout this phase of differentiation [65-69].

1.4 Experimental Models of Inflammatory Bowel Disease (IBD)

1.4.1 Intestinal Epithelial Cells (IECs)

HT-29 and Caco-2 cells have been well established as *in vitro* models for gut physiology and immune function with respect to IEC behavior [25, 70]. It is of note that cytokine

activity of HT-29 cells in response to pathogenic bacteria or TNF is similar to that examined in freshly isolated IECs [71]. The similarity between IECs and HT-29 cells and the failure of fully differentiated intestinal cells to remain viable, make HT-29 cells a valuable line upon which to model IEC response to cytokines to those seen in IBD. These cells can also be differentiated along a small intestinal or colonic cell lineage dependent on their glucose exposure, which makes them ideal for our study. In addition to assessing the extent of IEC apoptosis, the effect of a probiotic strain on these cells when faced with cytokine challenge also remains to be studied. We postulate that the effects of toxic inflammatory responses may be moderated by probiotics to enhance survival of mucosal cells and may in turn provide enhanced mucosal barrier function.

1.4.2 Cytokines in IBD

Cytokines produced by activated macrophages are central to the pathology in CD and although anti-TNF therapies are available, a suppression of macrophage cytokine production is desirable as it may be more effective than suppressing TNF once already secreted [72]. Cytokine dysregulation is also prevalent in IBD. In patients with active UC, IL-1, IFNγ, and TNFα were listed among cytokines most prevalent in affected patient profiles [73]. While initially thought to be a Th-2 response and in CD a Th-1 response, newer studies and clinical response to anti-TNF therapies have cast doubt on this simple differentiation. TNFα is also implicated in NEC as the effects of anti-TNFα antibody has been shown to ameliorate this disease state likely by IEC anti-apoptosis [74, 75]. These cytokines are also found elevated in CD (IL-1) and lends support to the profiling of these three cytokines in our studies involving IEC apoptosis during cytokine stress [73]. TNFα itself is an inflammatory cytokine implicated in general IBD pathology [76]. It acts via the NF-kB classical pathway

to induce gene expression of various pro-inflammatory responses as well as those involved in tissue repair. HT-29 cells exposed to exogenous TNF have been shown to upregulate endogenous TNF in response in an auto-inducing manner [76]. In terms of clinical relevance, the exogenous TNF from activated macrophages and T cells observed in IBD is consistent with the efficacy of TNF-neutralizing antibodies in patients [76]. TNF has been shown to activate transcription factor IRF1 (interferon response factor 1) which regulates both inflammation and apoptosis by means of caspase-1 and -7 activation [77]. It would therefore be of use to not only screen cells for active caspase-3 and -7, but also to use a method capable of detecting multiple caspases, for the detection of caspase-1 (as indicated by the induction of IRF1) and caspase-8 (indicated in cytokine-dependent apoptosis consistent with inflammation) [26].

1.4.3 The Role of Macrophages

Macrophages are central to CD pathology, in large measure because of the potent cytokine response elicited when activated [78]. There is also evidence that macrophage migration inhibitory factor (MIF) and DCs are upregulated in UC, consistent with a central role for inflammatory progression in disease pathology [79]. In CD, not only is there is a constitutive activation of TNF secretion by macrophages, the cells also enhance apoptotic resistance due in part to an up-regulation of Bcl-2 [50]. In order to suppress this cell population, there exists a current reliance on anti-TNF neutralizing antibodies targeted towards overactive macrophage TNF secretion [80]. Prevention of macrophage activation in IBD would be of significant benefit, hence our interest in understanding the nature of this cellular activation.

Macrophage activation can occur in the presence of a broken cellular barrier such as that observed in IBD-conferred lesions or in response to a sensed pathogen [7]. Genetically-modified mice containing the LRR mutation associated with CD demonstrate an increased IL-1β release by macrophages when exposed to bacterial compounds such as MDP [40]. The assessment of probiotics for IBD should be informed at the level of IECs but also focussing on the peripheral effects of these microbes on surrounding cells.

1.5 Probiotics

1.5.1 Definition and Strains of Interest

Probiotics, "pro" meaning promoting and "biotic" derived from the Greek word for life, are non-pathogenic micro-organisms that confer health benefits to the host upon ingestion [9]. There is emerging evidence that probiotics can be of use for both acute and chronic conditions, including: digestive disorders, allergies, eczema, asthma, autoimmune disease, obesity and cancer [9, 81, 82].

The probiotic strain chosen for this study include *Lactobacillus plantarum* 299v (Lp299v), known to modulate other aspects of host innate immunity, such as mucin upregulation [83]. *Lactobacillus plantarum* are non-pathogenic, naturally-occurring bacteria that are typically found in grain products. Lp299v specifically was found adherent to human intestinal tissue obtained from endoscopic biopsies, attributing to its use in physiologic study [9]. It was also useful to assess the role of *Lactobacillus plantarum* Lpadh- (Lpadh-) as a control. Lpadh- is a non-adherent and non-protective derivative of Lp299v which lacks adherin protein and thus the ability to bind to IECs [84]. The failure of this microbe to induce either mucin transcript or protein suggests a role for microbial adherence in the beneficial probiotic effect.

1.5.2 Transcriptional and Translational Mucin Upregulation

Our previous research was based on both *in vitro* and *in vivo* studies focused on modulation of innate mucin expression in Sprague-Dawley rats following the administration of Lp299v to drinking water [83, 85-87]. Mucin is the main organic component of intestinal mucus, protecting IECs by limiting access of noxious substances and enteropathogens as it is secreted onto their luminal surface [86, 88-93]. Mucin gene predominance is site-specific; rMuc2 is chiefly expressed in the rat colon, whereas rMuc3 is found primarily in the rat small intestine [85]. Rats were used in this experimentation due to their rMuc3 and rMuc2 gene homology to human Muc3 and Muc2, respectively [94-96].

Probiotic strains Lp299v, *Lactobacillus rhamnosus* R0011, and *Bifidobacterium bifidum* R0071 increase mucin in a site-specific manner at both mRNA and protein levels [97]. This effect was noted following both short-term oral probiotic administration and rectal administration (unpublished, personal observation). The adherent-negative mutant Lpadh- or heat-killed Lp299v were unable to modulate mucin expression in a similar fashion. In our previous studies we also noticed that probiotic-induced mucin mRNA expression decreased over time, an effect that perhaps was possibly a consequence of a developing tolerance to these microbes. This observation is consistent with transient induction of cytokines specific to various commensals, dependent upon their microbial identity [98-100]. The success of probiotic Lp299v in modulating a key component of innate immunity, and evidence that the strain *Lactobacillus rhamnosus* GG protects cells against cytokine-induced apoptosis, prompted further investigation [28].

1.5.3 Use in Conditions of IBD

Probiotics have been shown to moderate pediatric antibiotic-associated diarrhea [9, 46, 101, 102]. They have also been shown to limit morbidity associated with acute infectious viral diarrhea in infants and children and decrease incidence of NEC in neonates [14, 102, 103]. There has also been success in validating *Lactobacillus rhamnosus* GG use, as this strain has been shown to prevent the recurrence of colitis in antibiotic-treated mice [104]. Despite the success of their implementation, a complete understanding of the mechanism of probiotic action remains to be determined [29]. In addition to boosting innate immunity, possible factors include evidence that probiotics mediate prevention of a T cell imbalance associated with CD (when T1 cells outnumber T2) and the stimulation of polymeric IgA secretion coating and protecting intestinal mucosa against bacterial invasion [105].

When incubated with a pathogenic challenge, Lp299v prevented EPEC adherence to intestinal epithelial cells *in vitro* [84, 106, 107]. We hope our investigation of IEC interaction in the context of programmed cell death can shed further light on the biochemical interplay of this strain and the cells with which it interacts.

1.5.4 Colonization and Related Signalling

When microbes are introduced into the lumen of the intestine there are usually detrimental effects should they fail to be effectively recognized by IECs and subsequently establish infection. Bacteria generally require cellular attachment and invasion prior to replication and infection [108]. Should the bacteria be commensal or non-pathogenic in nature, adherence may occur to IECs but without invasion or subsequent replication [108, 109]. Conversely, bacteria may simply bind to overlying mucus. Probiotics are thought to function in this manner. Many naturally-occurring microbes in the intestinal milieu are

sensed via TLRs, however since the molecular patterns are deemed to be non-threatening seldom do they induce a significant inflammatory response. IECs are also considered hypo-sensitive to these species, as they have a lower concentration of pattern recognition receptors than do cells of the mucosal immune system. There is however, some response induced by these microbes. Some inducible effects may be time-limited such as mucin overexpression induced by Lp299v [110] however other responses are suspected to be longer-lived; for example clinical studies have suggested probiotic ingestion in infancy prevents against later development of atopic dermatitis in childhood [111].

It is postulated that to benefit the host as a whole, IECs will undergo physiological cell death to prevent pathogenic replication and the opportunity for infection. Should probiotics be found to be anti-apoptotic under conditions of pro-inflammatory stress as typical in IBD, this may be a means of strengthening the IEC layer and further limiting barrier breaks, possibly moderating inflammation in the host.

1.6 Hypothesis and Principle Objectives

The goal of this research project is to further understand the complex relationship that exists between probiotics and IECs by assessing for and then elucidating the molecular nature of any anti-apoptotic effect. Specific aims include evaluating the extent of Lp299v-mediated anti-apoptosis in a model of cytokine-induced stress, evaluating relative IAP involvement, and assessing the role of adherence with any anti-apoptotic effect noted both *in vitro* and *in vivo*.

It is hypothesized that intimate probiotic interaction requiring adherence with IECs enhances cell survival through IAP-mediated alteration of programmed cell death (PCD) mechanisms.

2. Materials and Methods

2.1 Animal Protocol

2.1.1 C57/Bl6 Mice

Adult C57/Bl6 mice (Charles River Laboratories Inc., Montreal, Canada) were chosen for use in this study. There is significant homology in the IAP genes in both humans and rodents which allows us to monitor mouse IAP gene induction as a predictor for gene induction in humans [112]. Mice were transferred to the University of Ottawa Animal Care and Veterinary Services Facility and housed and bred in accordance with the Institutional Animal Care and Use Committee guidelines. Mice were housed in pairs according to assigned treatment group and had *ad libitum* access to food pellets and sterile filtered water. During treatment period, 100µl of 1 x 10^9 Colony-Forming Units (CFU) probiotic diluted in sterilized drinking water was administered via gavage to treatment group mice. Control mice were given 100µl sterilized water via the same method to serve as a negative control.

2.1.2 Sprague-Dawley Rats

Specific pathogen-free male Sprague-Dawley rats were also used to determine *in vivo* IAP response to oral probiotics as we could obtain larger tissue specimens using this animal model and assess correlation with mouse data (Charles River Laboratories Inc., Montreal, Canada). IAP gene homologues to those found in humans have been described [112]. 250g rats were acclimatized to vivarium conditions at the University of Ottawa for 5 days under standard conditions prior to probiotic administration. All experimentation was performed with the approval of the Animal Care Committee at the University of Ottawa. The reference body weight was taken after the acclimitization period and designated time 0 during the

experimental period. As was performed in the mouse experiments, rats were provided a standard pellet and sterile drinking water diet *ad libitum*. Daily water intake (recorded as ml per day) was monitored to ensure it was consistent between groups. Rats were divided into experimental groups based on the bacterial strain which would be administered. These experimental groups were given designated probiotics at an ingested concentration of 1×10^9 CFU/ml for a period of 2 to 5 days. Preparations were made fresh daily to ensure accurate quantification and viable bacteria. Final bacterial concentration was calculated based on daily drinking water consumption and was recorded to ensure this was similar between groups.

2.2 Bacteria

2.2.1 Probiotic Strain Used for Study

The bacterial strain chosen for experimentation was *Lactobacillus plantarum* 299v (Lp299v; Dr. S. Ahrne, University of Lund) originally obtained through jejunal patient biopsies. This strain has been shown by our laboratory to modulate intestinal mucin expression both *in vitro* and *in vivo*. *Lactobacillus plantarum* Lpadh- (Lpadh-; Dr. S. Ahrne) was used to determine the role of adherence in probiotic effect. Lpadh- is an adherent negative mutant of Lp299v which lacks adherin and thus the ability to bind to intestinal epithelial cells. Lpadh- was previously confirmed as a mutant strain lacking the ability to agglutinate *Saccharomyces cerevisiae* in a mannose-sensitive manner (Dr. S. Ahrne, personal communication). Experiments performed in our laboratory demonstrate that unlike the adherent strain Lp299v, administration of probiotic Lpadh- both *in vivo* and *in vitro* fails to upregulate mucin genes.

2.2.2 Bacterial Preparation and Quantification

Stock cultures of Lp299v and Lpadh- were maintained at -80 °C in a Revco Ultima II freezer (Ashville, NC). Bacteria were resuspended in Lactobacilli MRS broth and grown overnight at 37 °C in a ThermoForma Series II Water Jacketed CO_2 Incubator. Bacteria were harvested by spinning tubes in a Beckman GPR Centrifuge (Beckman Coulter Inc., Fullerton, CA) at 3000 rpm for 15 min. Expired media was removed and bacteria were suspended in ice-cold Phosphate-Buffered Saline (PBS) containing 8 g NaCl (Fisher Scientific, Fair Lawn, NJ), 20 mg KCl (Fisher Scientific, Fair Lawn, NJ), 1.44 g Na2HPO4 * 7H2O (Sigma-Aldrich Canada Ltd., Oakville, ON), and 24 mg KH2PO4 (Fisher Scientific, Fair Lawn, NJ) in 1 L ddH_2O (Sigma-Aldrich Canada Ltd., Oakville, ON), pH 7.4. This was repeated three times. Quantification of harvested bacteria was done through use of spectrophotometry readings at an Optical Density (OD) of 600 nm (Milton Roy Spectronic Genesys 5, Fisher Scientific, Fair Lawn, NJ). Absorbance readings were then plotted against bacterial CFU. To construct bacterial growth curves, serial dilutions of both Lp299v and Lpadh- were prepared and absorbance at 600 nm was recorded. 100µl dilutions were then individually spread plated in triplicate onto agar plates for quantification by enumeration of CFU. Growth curves were used in conjunction with absorbance readings from bacterial samples to enumerate bacteria in subsequent experimentation. Bacteria were added to rat drinking water so that they consumed 1 x 10^9 CFU/ml daily for the appropriate trial period. Water consumption was recorded to determine total number of bacteria ingested to ensure consistency between groups.

2.3 *RNA and Protein Sources and Extraction*

2.3.1 Dissection of Distinct Intestinal Segments

Following treatment mice were sacrificed via i.p. sodium pentabarbitol injection and intestines were excised. Tissue from jejunum, ileum, proximal colon and distal colon segments were cut lengthwise and mucin samples were obtained by scraping the mucosal surface using a microscope coverslip edge. Mucosal scrapings were placed in RNAlater for preservation and stored at -20°C for transcript level measurement. These were used to obtain IAP transcript expression levels. Additional 2 cm segments of the jejunum, ileum, small intestine and large intestine were excised and stored in a CMR/CMC Series Portable LN_2 Storage Container (Merlette, Ohio) for later protein isolation.

For studies involving rats, following the trial period animals were sacrificed via CO_2 euthanasia and 5 cm sections of the jejunum, ileum, small intestine and large intestine were collected in a similar matter to mouse sections. Samples were stored as described for mice for further analysis of both protein and RNA expression levels.

2.3.2 In-vitro Cell Model

Human Intestinal HT-29 cells (American Type Culture Collection, Manassas, VA) were chosen for use in our studies as it has been previously shown that they can be differentiated from a colonic phenotype to a small intestinal phenotype depending on growth conditions [113]. Furthermore, these cells may be induced to modulate innate gene expression levels using Lp299v [85]. Cells were grown in glucose-containing McCoy's 5A culture medium (Invitrogen Canada Inc., Burlington, ON) for colonic expression studies. To assess protein and RNA expression representative of the small intestine, HT-29 cells were

progressively transferred to a glucose-free, 5mM galactose-containing McCoy's 5A culture medium as previously described [85]. We have previously demonstrated that in the generation of HT-29 galactose-dependent cells (HT-29 Gal cells) mucin gene expression is altered from *MUC2* predominant in the large intestine, to *MUC3* characteristic of small intestinal cells. Culture medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 0.25% (Invitrogen Canada Inc.). An antibiotic/antimycotic mixture containing 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate, and 0.25 mg/ml amphotericin B (Invitrogen Canada Inc.) was also added to tissue culture media in addition to FBS. Cells were washed twice with PBS and incubated in antibiotic-free media prior to exposure to bacterial strains. Cells were cultured at 37 °C in 75 cm^2 culture flasks (Corning Inc., NY) in a ThermoForma Series II Water Jacketed CO_2 incubator (ThermoForma, Mariette, OH) with 5% CO_2 and were routinely passaged after washing with PBS using Trypsin-EDTA (Invitrogen Canada Inc.).

HT-29 Gal cells were used as an *in vitro* model to the test experimental hypotheses. Once cells had grown to approximately 85% confluence in culture flasks, cells were rinsed twice with 1xPBS. Media was replaced with an antibiotic-free media to which 1 x 10^9 CFU/ml of either Lp299v or Lpadh- were added for an incubation time of 1 hr at 37 °C. Cells were then washed twice with 1xPBS and challenged via the addition of a mixture of recombinant murine cytokines (100 ng/ml TNF-α, 100 ng/ml IFN-γ, and 10 ng/ml IL-1α) (PeProTech Inc., Rocky Hill, NJ) into antibiotic-free media for 15 min. Cells were then washed again with 1xPBS and harvested for later RNA and protein extraction.

2.3.3 RNA Extraction

RNA was isolated from rodent samples using an RNeasy kit (Qiagen Inc., Mississauga, ON) according to manufacturer's instructions. Mucosal scrapings preserved in RNAlater at -20 °C were placed in RLT lysis buffer and homogenized in 30 mg amounts using a QIAshredder spin column (Qiagen Inc., Mississauga, ON) for 2 min. Following this, RNA was eluted from spin column membranes in 30µl RNase-free water which was collected after its first column passage and used to re-elute remaining RNA. RNA concentration was calculated for samples diluted 1:100 in 10mM Tris HCl buffer, pH 8.5 using a spectrophotometer at an OD reading at 260 nm (Eppendorf Biophotometer, Hamburg, Germany) and stored at -80 °C until use.

RNA was isolated from HT-29 cells by adding 600 µl RLT lysis buffer (Qiagen Inc., Mississauga, ON) to cell culture flasks for 2 min. Cell lysates were transferred to eppendorf tubes and were homogenized using a 1 ml syringe and 20 gauge 1½ needle aspirated 4 times (Becton Dickinson and Company, Franklin Lakes, NJ). 70% ethanol was then added to samples and loaded into RNeasy spin columns (Qiagen Inc., Mississauga, ON) in 700 µl increments for RNA extraction as per manufacturer's instructions.

2.3.4 Protein Extraction

For protein extraction from tissue, excised intestinal segments stored in liquid nitrogen were placed in 15 ml tubes containing 2 ml RIPA buffer (200 µl 10x PBS, 20 µl IGEPAL (Sigma-Aldrich Canada Ltd., Oakville, ON), 20 mg taurocholic acid (Sigma-Aldrich Canada Ltd., Oakville, ON), 20 µl SDS 10% solution (Gibco Invitrogen, Grand Island, NY), 1.74 ml ddH_2O (Sigma-Aldrich Canada Ltd., Oakville, ON), 20 µl pepstatin (Sigma-Aldrich Canada

Ltd., Oakville, ON), 20 µl PMSF (Sigma-Aldrich Canada Ltd., Oakville, ON), 20 µl leupeptin (Sigma-Aldrich Canada Ltd., Oakville, ON), 4 µl aprotinin (Roche, Laval, QC), and 4 µl EDTA (Sigma-Aldrich Canada Ltd., Oakville, ON). Samples were homogenized using a handheld homogenizer (Model Pro 200 homogenizer, PRO Scientific Inc., Monroe, CT). Following this, 15 ml tubes were placed in a Beckman Centrifuge using a JA25.12 rotor and centrifuged for 10 min at 17500 rpm. Supernatant was removed and stored at -80 °C until assayed.

Protein extraction from HT-29 cells was similar to the methodology using tissue. Cells were detached from culture flasks using a disposable cell scraper into 1 ml of ice cold PBS. Lysates were then transferred into 1.5 ml eppendorf tubes and spun using a desktop centrifuge (Microfuge Beckman 22R Centrifuge, Beckman Coulter, Fullerton, CA) at 3000 rpm for 5 min at 4 °C. Cell supernatants were removed and pellets were stored at -80 °C until required. To analyze proteins, pellets were thawed on ice for 5 min and reconstituted in 100 µl RIPA buffer containing protease inhibitors. After mixing pellets with RIPA buffer, samples were incubated on ice for 1 hr. They were then centrifuged at 13000 rpm for 15 min at 4 °C. Supernatant was removed and stored at -70 °C until used for analysis of protein concentration using a standard Bradford Assay.

A modified protocol was required to lyse HT-29 cell nuclei and obtain protein for optimization of Western blot IAP detection and analysis. Since a large percentage of the HT-29 cell is composed of nucleus, these can be resistant to standard RIPA buffer lysis. When required for use specifically in Western blotting, 100 µl 3xdye containing 0.1M DTT (MP Biomedicals, Irvine, CA) was added to pellets. This reducing agent was used to disrupt protein disulfide bonds thus facilitating their movement through the SDS gel. Reconstituted pellets were then sonicated using a Transsonic 460 sonicator (DiaMed Lab Supplies Inc.,

Mississauga, ON) for 30 min to further help break down HT-29 cell nuclei. Protein extracts were then boiled for 10 min and quick chilled on ice prior to gel loading.

2.4 *Polymerase Chain Reaction Analysis of Gene Expression*

2.4.1 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

For analysis of IAP gene modulation by probiotics, Taqman© real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) was employed with reference to endogenous GAPDH gene expression. Real-time RT-PCR was chosen over traditional PCR due to the requirement for amplicon quantification, low sensitivity, and numerical expression of relative gene quantification, all of which are not possible with traditional PCR [114]. Mastermix was prepared using EZ RT-PCR core reagents (Applied Biosystems, Foster City, CA) and master cocktail reagent prepared according to manufacturer's instructions, modified for both probe and primer optimization. The master cocktail reagent contained the following: 1xEZ-Buffer, 300 μM dNTPs, 3 mM manganese, 200 nM each of forward and reverse IAP-specific primers, 100 nM IAP-specific probe, 200 nM each of forward and reverse GAPDH primers, 100 nM GAPDH probe, 0.1 U/μl RTth DNA Polymerase, 0.1 U/μl AmpErase UNG, and RNase-free water. For each reaction, 20 μl of master cocktail reagent was combined with 5 μl RNA (20 ng/ml) for a final reaction volume of 25 μl. *See Appendix A for specific probe and primer sequences.* Samples were analyzed in triplicate in a MicroAmp Optical 96-well Reaction Plate (Perkin Elmer, Norwalk, CT) with No Template Control (NTC) wells to check for contamination. Plates were analyzed for IAP-specific gene modulation with reference to *gapdh* internal control using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). *Gapdh* was chosen due to its

ubiquitous abundance irrespective of experimental conditions. RT-PCR chemistry allowed for the detection of fluorescence emitted from the reporter dye attached to specifically designed probes for this purpose. In the absence of target amplification, probes remained undetected due to reporter dye quenching from the opposing end of the probe. The emission of the reporter fluorescence allowed for real-time quantification of target amplification [114].

HT-29 cells grown in regular glucose-containing media (HT-29 Glu) or in galactose-containing media (HT-29 Gal) to enhance either MUC2 or MUC3 expression according to cell phenotype [85] were used to evaluate modulation of IAP expression in cells previously exposed to probiotics followed by cytokine challenge. For each group administered a probiotic strain there was a corresponding sample containing cells without added microbes which served as a negative control.

2.4.2 Data Analysis

Real-time RT-PCR data were imported from the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) into Excel (Microsoft) for calculation of induction using standardized formulae. In this process, a value reflecting cDNA amplification (resonance energy transfer, ΔRn) was measured at the end of each of each cycle. When this value exceeded the threshold of fluorescence detection (C_T) a calculation of mRNA abundance was calculated as these values are in inverse proportion. Data were compared using one way analysis of variance, and were standardized to *gapdh* mRNA values using the following formula: $(2^{-(C_T\ \text{test} - C_T\ \text{GAPDH})}) \times 100\%$. Following this, data were analyzed using the Tukey post-test for inter-condition comparison. Statistics were generated using Prism 4.0 (GraphPad Software, San Diego, CA) and are presented as mean +/- SEM.

2.5 *In-vitro Assessment of Cytokine-Induced Apoptosis*

2.5.1 Cell Model

For the *in vitro* assessment of cytokine-induced apoptosis, HT-29 cells were used to investigate whether probiotic strain Lp299v conferred IEC protection in an *in vitro* simulation of cytokine-induced cell death. As previously mentioned, these cells were experimentally manipulated to mimic small intestinal cell physiology by sequentially transferring cells from glucose-containing to galactose-containing media over a course of several days using a pre-established protocol [85]. HT-29 Gal cells have a number of features of small intestinal cell lineage including glycosyltransferase expression typical of small intestinal cells morphologically appear similar to cells of the small intestine when inspected using electron microscopy and overexpress Muc3 mucin as their predominant mucin [113]. Following growth to approximately 80% confluence on sterilized microscope slide coverslips, cells were incubated with fresh bacteria-free media, 1 x 10^9 CFU/ml Lp299v, or 1 x 10^9 CFU/ml Lpadh- for 1 hour. Following this, cells were washed twice with PBS and exposed to a mix of pro-inflammatory cytokines commonly associated with IBD (TNFα, IL-1α, and IFNγ) for a period of 15 minutes. After two subsequent washes, cells were assessed for apoptosis using Terminal Deoxynucleotidyl Transferase (TdT) Nick-End Labeling (TUNEL) and assessed for caspase activation using caspase activity assays.

2.5.2 Cytokine-Mediated Induction of Apoptosis

Cytokine profiles in the bowel with active inflammation include IL-1, IFNγ, and TNFα [60]. This mixture has also been previously found to induce IEC apoptosis [28]. We therefore chose a mixture of these three cytokines for an *in-vitro* analysis of the extent of apoptosis that they induce in our *in vitro* model. Following probiotic exposure for 1 hr, 100

ng/ml of IFNγ, 100 ng/ml of TNFα, and 10 ng/ml IL-1α were administered to HT-29 cells for a period of 15 min, as these concentrations were used in previous studies. Cells were then washed twice in PBS before further analysis of the extent of apoptosis using two distinct methods.

2.5.3 TUNEL Assay

Cells were assessed for the relative extent of total apoptosis using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA). This kit is a version of the TUNEL Assay and on the detection of 180 bp DNA fragments which are the hallmark of apoptotic cell death. Cleaved DNA fragments were detected by dNTP TdT enzyme labelling to 3' OH ends. An anti-digoxigenin antibody was then used which in the presence of peroxidase substrate such as diaminobenzidene (DAB) produced a brown colour indicative of apoptosis. Cells were then counterstained with hematoxylin for 10 seconds and viewed with a light microscope to distinguish stained cells. An ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) as a fluorescent TUNEL assay was also employed as a means of verification of $TUNEL^+$ cells due to the increased sensitivity of this method. This assay uses a fluorescent-conjugated primary antibody for detection of TdT enzyme activity instead of the peroxidase system.

2.5.4 Caspase Activity Assay

This assay can detect cells in the early stages of apoptosis that would otherwise be missed in the TUNEL assay, and also accounts for the degree of $TUNEL^+$ cells that were in fact necrotic and not truly apoptotic. Experiments were conducted using a multi-caspase activity assay measuring the activity of caspases 1-9. Additional experiments were

conducted using a caspase-3 and -7 activity assay to determine more specifically if these two main effector caspases are responsible for the noted effect [50]. Both the CaspaTag Multi-Caspase *In Situ* Assay Kit, and the CaspaTag Caspase-3/7 *In Situ* Assay Kit (Chemicon International, Temecula, CA) use Fluorochrome Inhibitors of Caspases (FLICA) reagent to detect and bind active caspases. The Caspase-3/7 kit uses a carboxyfluoroscein-labeled fluoromethyl ketone peptide inhibitor of caspase-3 (FAM-DEVD-FMK) to produce a green fluorescence. For both assays, Hoechst stain was used to label the nuclei of dividing cells and as a means of enumerating a *total cell* count. Coverslips were inverted onto a clean glass microscope slide on top of a drop of 1 x wash buffer before being analyzed via fluorescent microscopy. Green fluorescence indicative of apoptotic cell death was viewed using a bandpass filter (excitation 490 nm, emission 520 nm) and cell nuclei were viewed using a filter set to excitation 365 nm, emission 480 nm.

2.5.5 Analysis

Upon completion of the TUNEL assays, samples were inverted onto microscope slides and analyzed by counting the number of $TUNEL^+$ cells per 200 total cells detected via light microscopy (for peroxidase TUNEL) and fluorescent microscopy (fluorescent TUNEL) and displayed as a percentage $TUNEL^+$ cell death. Similarly, $Caspase^+$ cells were counted per 200 cells and graphically represented as a percentage of caspase-dependent cell death, or PCDI.

2.6 *In-Vitro IAP Expression with Macrophage Co-Culture*

2.6.1 Cell Model

In the human submucosa, macrophages are an integral part of the cellular milieu present and provide an important response to mucosal challenge. To evaluate whether there were synergistic effects of IECs and macrophages to both insults and protective intervention, we performed experimentation using a co-culture system. In these co-culture experiments, a murine macrophage cell line RAW264.7 (American Type Culture Collection, Manassas, VA) was used. We were then able to determine the extent of macrophage activation when in co-culture with HT-29 Glu cells both with and without probiotic pre-incubation. 5×10^4 cells/ml HT-29 Glu cells and 1×10^4 cells/ml macrophages were plated on sterilized coverslips in 6-well plates either together or in separate wells to serve as controls. After culturing cells for a period of 2 days, cells were experimentally treated as previously outlined in the cell model system used for *in vitro* testing of enhanced IAP expression.

2.6.2 RNA and Protein Expression Analysis

Following 1 hr incubation with either the adherent (Lp299v) or non-adherent probiotic (Lpadh-), cells (both alone or co-incubated with the macrophage cell line) were challenged with cytokines for 15 min. Cells were then washed twice with PBS, and harvested for both RNA and protein samples per condition. As endotoxin is a known activator of macrophages, an additional experiment was conducted wherein cells were incubated with 1μg/μl endotoxin (LPS, Sigma-Aldrich Canada Ltd., Oakville, ON) to detect both RNA and protein IAP expression following macrophage activation in the absence of external cytokines. RNA and protein samples were then collected as per the procedure outlined for the *in vitro* HT-29 cell model for analysis. RNA was analyzed by quantitative RT-PCR for IAP-specific modulation in reference to an internal *gapdh* control. Taqman conditions were similar to those used in the *in vitro* cell model for HT-29 Glu and HT-29 Gal cells.

Following protein isolation from cells, Western blots were completed to assess relative levels of NAIP, HIAP1 and HIAP2 protein. Antibodies used are outlined in Table 1.A. Protein expression was then visualized as described in the immunoblotting procedure.

2.6.3 Immunocytochemical Analysis of Macrophage Activation

In order to determine the extent of macrophage activation in co-culture with HT-29 cells, immunocytochemical techniques were employed. Cells were cultured as per the outlined conditions for the cell model system for 2 days. After this time, cells were exposed for 1 hr to either Lp299v or Lpadh-. Control cell populations included those not in co-culture (macrophages alone for confirmation of activation), cells in co-culture activated with endotoxin without prior exposure to probiotics, and those exposed to probiotics alone. Following probiotic exposure, cells were washed twice with PBS to remove bacteria and 2 ml fresh antibiotic-free glucose media was added per well. To activate macrophages, 1 μg/ml endotoxin (LPS, Sigma-Aldrich Canada Ltd., Oakville, ON) was added to cells and incubated for 24 hours. Following this, cells were washed twice in PBS and stained for immunofluorescence. iNOS/NOS Type II conjugated to FITC was then used as a stain for nitric oxide synthesis from activated macrophages. This was used in conjunction with an Actin Cytoskeleton and Focal Adhesion Staining Kit (Chemicon International, Temecula, CA) to detect macrophage morphology. Polymerized actin filaments were detected using rhodamine-phalloidin conjugated to FITC, and focal adhesion sites were detected using an anti-vinculin stain (Table 1.C). An anti-mouse goat secondary antibody was used for primary antibody detection as outlined in Table 1.B. Cell samples were inverted on clear glass microscope slides and examined for staining indicative of macrophage activation using fluorescent microscopy.

2.7 *Immunoblot Analysis of IAP Expression*

2.7.1 Western Blot Analysis

Protein was extracted as previously described from both *in vivo* and *in vitro* models to test our hypothesis. Proteins were first analyzed using the NuPAGE® Novex Pre-Cast Gel System optimized for large proteins due to the size of most IAPs (Invitrogen Canada Inc., Burlington, ON). In this system, crude protein extracts were diluted to 1 μg/μl in deionized water and 20 μl of protein was then added to 4 μl of NuPAGE® LDS Sample Buffer (4x) and 1 μl NuPAGE® Reducing Agent (10x) to equal a total volume of 25 μl per sample. Protein samples were also analyzed using the Mini-PROTEAN III gel system (BioRad Laboratories, Hercules, CA) due to increased efficacy of high molecular weight protein detection. In this process, 20 μl of prepared protein samples were diluted to 1 μg/μl with 1xRIPA buffer and 3xdye containing reducing agent DTT. In the case of both systems, prepared protein aliquots were heated to 70 °C for 10 minutes and were then quick chilled on ice prior to loading.

In accordance with the NuPAGE® system protocol, 20 μl of each protein sample was loaded into wells of a 10-well Novex Bis-Tris gel which was run at 150 V for 1 hour in 1xMES SDS running buffer. The type of gel used was optimized for IAP protein detection and separation. Using the mini-PROTEAN III system, standard 7.5% bis-acrylamide gels were prepared including a 4% stacking gel for use in manual Sodium Doecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The same amount of protein sample was run on each of the gel types used.

Following transfer and 5% milk blocking of the blots, PVDF membranes were washed for 1 hour in Tris-Phosphate Buffered Saline with Tween (TBST), incubated with primary antibody overnight (as indicated in Table 1.A) followed by subsequent washes and

incubation with species-specific secondary antibody (ECL Anti-mouse or Anti-Rabbit IGg, Amersham Biosciences, Piscataway, NJ) for 1 hour. The ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ) was used for detection of secondary antibody, and membranes were exposed to ECL Plus Hyperfilm (Amersham Biosciences, Piscataway, NJ) for optimized exposure time. Equal protein loading was confirmed by means of Western blotting for Glyceraldehyde Phosopho-Diesterase (GAPDH).

Western blots were scanned at a resolution of 100 dpi (dots per inch) on a standard computer scanner and saved as TIFF files. Files were analyzed using ImageJ, an image processing software program offered by the National Institute of Health (NIH) [115]. For analysis of protein expression, single boxes were drawn around protein bands so that densitometric data could be obtained. Gel lanes were then automatically plotted and peaks were obtained for each of the protein bands detected. Densitometric data were generated through a calculation of total area under the curve (AUC), and values are expressed in pixel density (*Appendix B*).

2.8 Elucidation of Cell Signalling

2.8.1 NF-kB Reporter Activity Assay

NF-kB activity was assessed in HT-29 Gal cells by use of a NF-kB luciferase activity assay according to manufacturer's instructions. Cells were cultured over a 2 day period until roughly 80% confluent and transfected as per the Cell Line Nucleofector Kit R instructions (Amaxa, Gaithersburg, MD). In this assay, 1 x 10^6 cells were counted following trypsinization and centrifuged to obtain media-free cell pellets. Cells were re-suspended in Nucleofector Solution R to a final concentration of 1 x 10^6 cells/100 µl. Cells were then

mixed with 4 µg NF-kB construct and 1 µg Renilla luciferase construct and transferred into Amaxa cuvettes for nucleofection. Cells were then cultured in 6-well plates at 37°C/5% CO2 and analyzed 24 hrs following the procedure using fluorescent microscopy to confirm transfection in positive control cells. Cell samples included groups experimentally treated with Lp299v or Lpadh- for one hour. Preceding analysis, cells were detached using 1 x Passive Lysis Buffer (PLB) for 15 min. To perform the Dual-Luciferase Reporter Assay, 20 µl of each of the PLB-treated lysates were added to a 96 well plate. A luminometer was used with both LAR II (Luciferase Assay Substrate) and Stop-n-Glo Reagent to measure relative firefly luciferase activity of the samples. Samples were each plated in triplicate and read twice to ensure accuracy.

2.9 Statistical Analysis

Data from all groups were analyzed using the Tukey post-test for inter-condition comparison. Statistics were generated using Prism 4.0 (GraphPad Software, San Diego, CA) and are presented as mean +/- SEM.

Table 1.A. Antibodies Used in the Experiments

Antibody	Supplier	Host	Species Reactivity	Dilution
Anti-RIAP1	Gift from Aegera	Rabbit	Human, mouse, rat	1:2000
HIAP1/BIR3	Epitomics	Rabbit	Human	1:1000
hNAIP	Gift from Aegera, Manufactured by Upstate Cell Signalling Technology	Rabbit	Human, mouse, rat	1:2000
Anti-GAPDH	Advanced Immunochemicals	Rabbit	Human, mouse, rat	1:500
iNOS/NOS TypeII: FITC	BD Biosciences	Mouse	Human, mouse, rat	1:500
Anti-Digoxigenin Peroxidase Conjugate	Chemicon International	Mouse	Human, rat	Ready-to-use
Anti-Digoxigenin Fluorescein Conjugate	Chemicon International	Mouse	Human, rat	47% v:v

Table 1.B. Secondary Antibodies Used in the Experiments.

Antibody	Supplier	Host	Species Reactivity	Dilution
ECL Anti-Rabbit IgG Horseradish Peroxidase Linked Whole Antibody	Amersham Biosciences	Donkey	Rabbit	1:2000
ECL Anti-Mouse IgG Horseradish Peroxidase Linked Whole Antibody	Amersham Biosciences	Sheep	Mouse	1:2000
Goat Anti-Mouse IgG (H+L) FITC Conjugated	Chemicon International	Goat	Mouse	1:500

Table 1.C. Stains Used in the Experiments.

Stain	Supplier	Specificity	Dilution
Rhodamine Phalloidin: FITC	Chemicon International	F-actin	1:500
Anti-Vinculin	Chemicon International	Focal Adhesion Sites	1:500
DAPI	Chemicon International	Nuclei	1:1000
Diaminobenzidine (DAB)	Chemicon International	Peroxidase	0.05% in 0.05M Tris
Hematoxylin	Invitrogen	Nuclei	Ready-to-use
Hoechst	Chemicon International	Nuclei	0.5% v:v
Fluorochrome Inhibitors of Caspase (FLICA)	Chemicon International	Active Caspase Heterodimer (FAM-DEVD-FMK)	1:5

3. Results

3.1 In Vitro Analysis of Probiotic Effect in Cytokine-Induced Apoptosis

3.1.1 In-situ Peroxidase TUNEL Analysis: Probiotic-Induced Anti-apoptotic Effect

HT-29 Gal cells were used to investigate whether probiotic strain Lp299v conferred IEC protection in an *in vitro* simulation of cytokine-induced cell death. Cells were assessed for apoptosis using an established TUNEL protocol. The number of $TUNEL^+$ cells (represented by peroxidase positive cells) per 200 total cells was counted and expressed as a percentage **(Figure 1)**. A significant number of apoptotic cells were noted following cytokine treatment using the TUNEL assay (43.6% +/- 6.2%) **(Figure 1a, Figure 2)**. Lp299v incubation prior to cytokine exposure resulted in a decrease in $TUNEL^+$ cells (18.7% +/- 4.1%, $p<0.01$) **(Figure 1c, Figure 2)**. Interestingly, Lpadh- incubation mimicked this effect (16.6% +/- 3.2%, $p<0.01$) **(Figure 1b, Figure 2)**. These results indicate that both Lp299v and Lpadh- pre-incubation on IECs similarly confer protection against cytokine-induced cell death involving DNA cleavage.

Figure 1: Peroxidase in-situ TUNEL staining of HT-29 Gal cells *in vitro* following cytokine exposure alone (a), incubation with Lpadh- for 1 hr prior to cytokine exposure (b), and incubation with Lp299v for 1 hr prior to cytokine exposure (c).

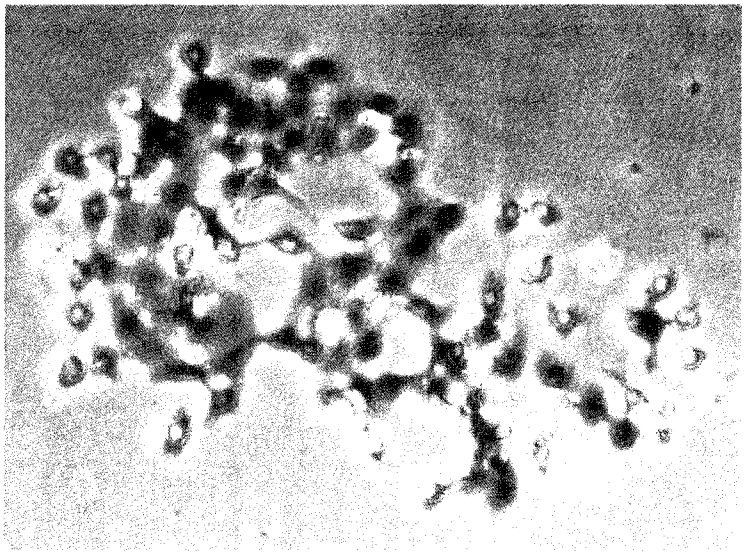
Shown are visual representations of randomly selected fields for each condition, using light microscopy. $TUNEL^{+}$ cells are indicated by the presence of brown colouration on a blue hematoxylin counterstain. Images were obtained using an Axioskop microscope and Northern Eclipse software for analysis.

(a) HT-29 Gal cells were exposed to a mixture of pro-inflammatory cytokines for 15 min with no probiotic pre-incubation.

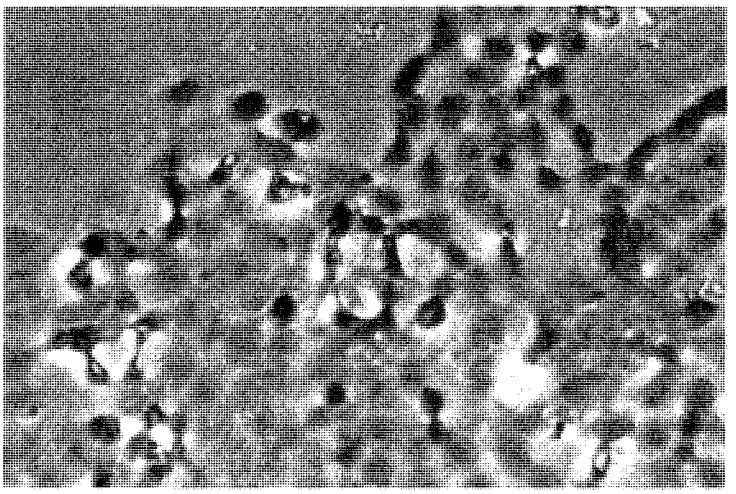
(b) HT-29 Gal cells were exposed to pro-inflammatory cytokines for 15 min proceeding 1 hr Lpadh- incubation.

(c) HT-29 Gal cells were pre-incubated with Lp299v for 1 hr prior to cytokine exposure.

Figure 1a.



b.



c.

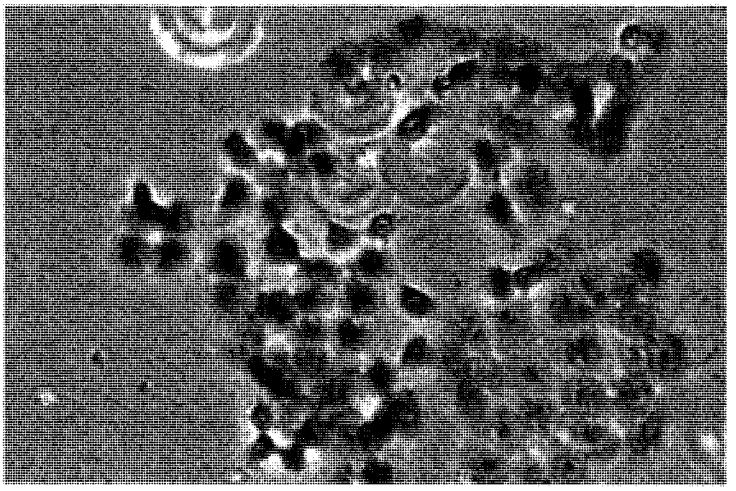
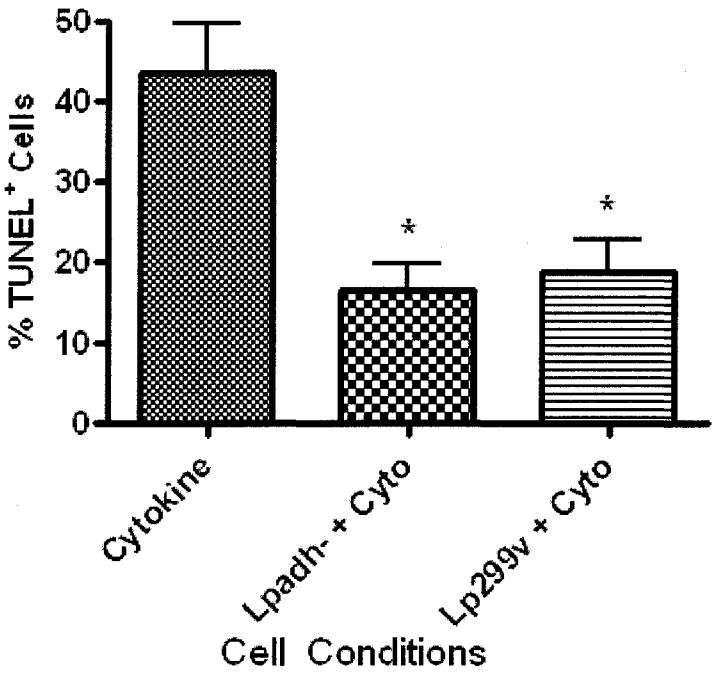


Figure 2: TUNEL results following enumeration of apoptotic cells per total cells counted in randomly selected microscopic fields.

Values shown represent mean +/- S.E. and are the result of 9 fields counted in 4 independent experiments. *=$p<0.01$ versus control.

Figure 2.


50
40
30
20
10
0
% TUNEL+ Cells
*
*
Cytokine
Lpadh- + Cyto
Lp299v + Cyto
Cell Conditions

3.1.2 Caspase Activity Assay Results Confirm Prevention of Apoptosis

To differentiate caspase-dependent cell death (PCDI) from necrosis and caspase-independent cell death (PCDII), HT-29 Gal cells were analyzed using the caspase activity assay as a means of caspase$^+$ cell detection. Caspase$^+$ cells were noted as green fluorescent cells with Hoescht blue nuclear stain to delineate total cell nuclei. When HT-29 Gal cells were incubated for 15 min with pro-inflammatory cytokines (in the absence of probiotics) there was an increase in caspase$^+$ cells shown by the significant increase in green cells compared to untreated cells (68.9% +/- 5.1%) (control data not shown) **(Figure 3a, Figure 4)**. There was a decrease in caspase$^+$ cells observed in the Lp299v pre-treated samples (3.6% +/- 0.6%, $p<0.01$) as opposed to both bacteria-free control slides and Lpadh- treated slides (46.8% +/- 17.5%) prior to 15 min cytokine exposure as indicated by the decrease in green fluorescent cells observed **(Figures 3b, 3c; Figure 4)**.

To distinguish between the number of caspase+ cells and caspase-3,7$^+$ cells, HT-29 Gal cells were subjected to experimental conditions similar to the caspase activity assay except with a fluorescent dye specific to activated caspase-3 and -7. This was to determine whether executioner caspases-3 and -7 were exclusively induced or all of the caspase family members. When cells were exposed to cytokines for 15 min there was an increase in caspase-3,7$^+$ cells (47.8% control +/- 25.3%) (control data not shown) **(Figure 5a, Figure 6)**. When cells were exposed to Lpadh- prior to cytokine exposure the percentage of caspase-3,7$^+$ cells increased (79.3% control +/- 20.8%) **(Figure 5b, Figure 6)**. When cells were incubated with Lp299v for 1 hr prior to cytokine exposure, the percentage of caspase-3,7$^+$ cells decreased (3.9% control +/- 0.3%), once again indicating protective benefit **(Figure 5c, Figure 6)**.

Figure 3: Fluorescent caspase activity assay showing caspase$^+$ cells following cytokine exposure (a), in the presence of Lpadh- for 1 hr prior to cytokine exposure (b), and in the presence of Lp299v for 1 hr prior to cytokine exposure (c).

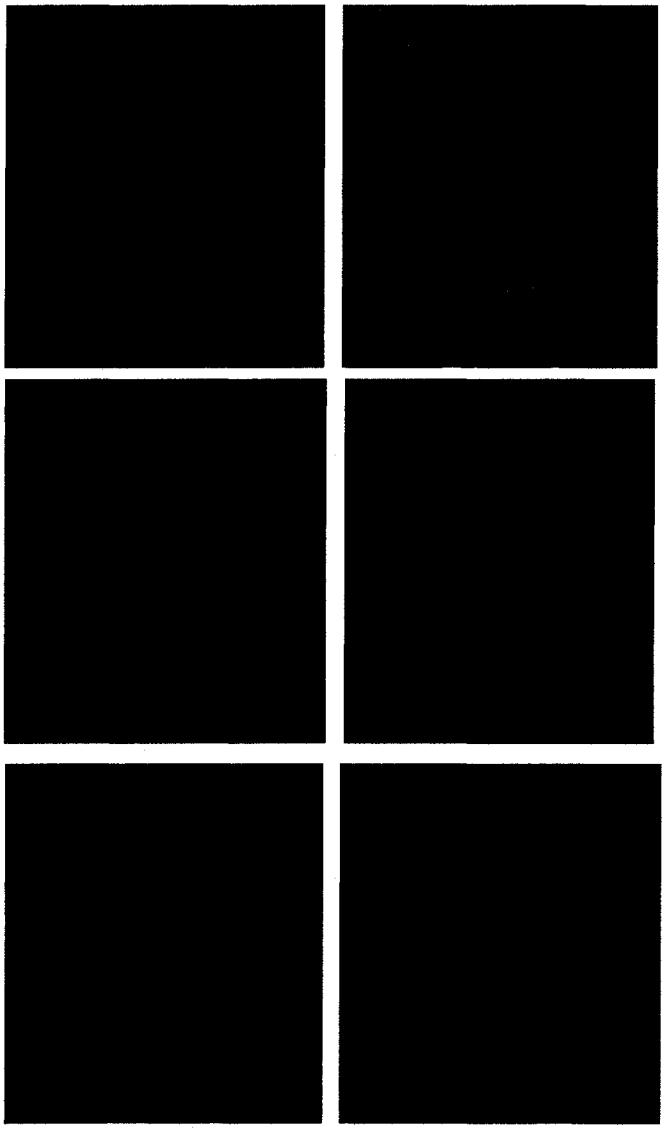
Caspase$^+$ cells are indicated by the presence of green fluorescence on a blue Hoescht counterstain which indicate cell nuclei. Images were obtained using an Axioskop microscope and Northern Eclipse software for analysis.

(a) Fluorescent caspase activity assay images showing HT-29 Gal cells exposed to cytokines for 15 min in the absence of probiotic pre-incubation.

(b) HT-29 Gal cells following Lpadh- incubation prior to cytokine exposure.

(c) HT-29 Gal cells following 1 hour Lp299v incubation prior to cytokine exposure.

Figure 3a.



b.

c.

Figure 4: Caspase activity assay results following enumeration of apoptotic cells per total cells counted in randomly selected microscopic fields.

Values represent mean +/- S.E. and are the result of 3 independent experiments. *=$p<0.01$ versus control.

Figure 4.

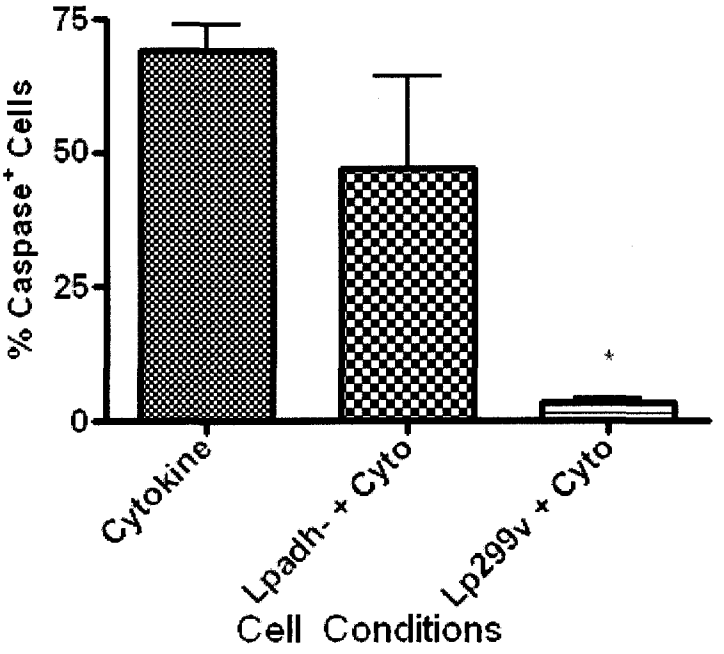

75
50
25
0
% Caspase+ Cells
*
Cytokine
Lpadh- + Cyto
Lp299v + Cyto
Cell Conditions

Figure 5. Fluorescent caspase-3,7 activity assay images showing caspase-3,7^{+} cells following cytokine exposure (a), in the presence of Lpadh- for 1 hr prior to cytokine exposure (b), and in the presence of Lp299v for 1 hr prior to cytokine exposure (c).

Assay specific to active caspases-3 and -7, as indicated by the presence of green fluorescent cells. Images obtained using an Axioskop fluorescent microscope and Northern Eclipse software for analysis.

(a) Fluorescent caspase-3,7 activity assay images showing cells exposed to cytokines for 15 min in the absence of probiotics.

(b) HT-29 Gal cells pre-incubated with Lpadh- incubation prior to cytokine exposure.

(c) HT-29 Gal cells pre-incubated with Lp299v for 1hr prior to cytokine exposure.

Figure 5.

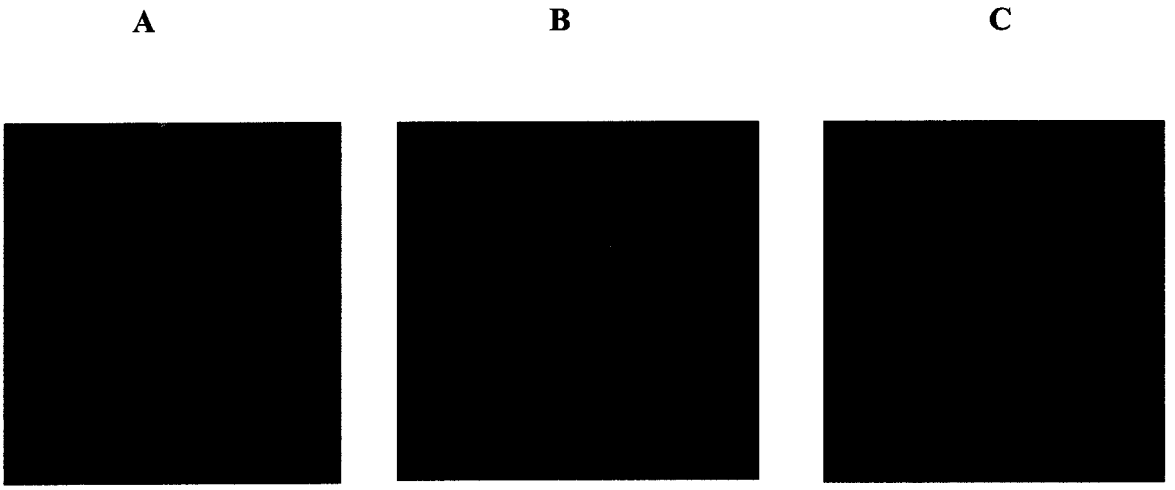
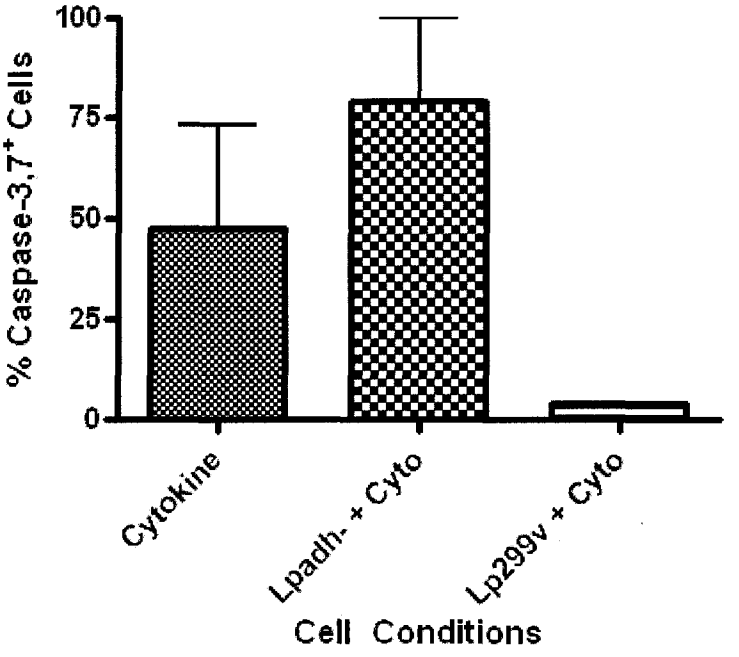


Figure 6: Caspase-3,7 activity assay results. Proportion of apoptotic cells per total cells counted in randomly selected microscopic fields.

Values represent mean +/- S.E. and are the result of a single independent experiment.

Figure 6.


100
75
50
25
0
% Caspase-3,7+ Cells
Cytokine
Lpadh- + Cyto
Lp299v + Cyto
Cell Conditions

3.1.3 Analysis of Select IAP Gene Expression using QRT RT-PCR

Taqman real-time RT-PCR was employed to determine mRNA expression levels of select anti-apoptotic genes in HT-29 Gal cells under conditions of probiotic incubation and pro-inflammatory cytokine stress. Of the IAPs assessed, (*hiap1, hiap2, naip, survivin,* and *xiap*), Lp299v induced a trend towards upregulation of *hiap2* mRNA expression (148% control +/- 2.3%, $p>0.05$) in the absence of cytokines **(Figure 7a)**. Although there was no statistical significance, changes were seen in multiple tests and trends were detected. Incubation with Lpadh- was similar to the control (109% control +/- 0.8% control, $p>0.05$).

Compared to control levels of *hiap2* transcript, pro-inflammatory cytokine exposure tended to upregulate transcription (156% control +/- 2.0% control, $p>0.05$) **(Figure 7b)**. When cells were co-incubated with Lp299v for 1 hr prior to cytokine exposure for 15 min, *hiap2* mRNA showed a tendency to increase (184% control +/- 5.1% control, $p>0.05$) compared to the untreated control. When incubated with the non-adherent strain Lpadh- prior to cytokines, *hiap2* induction in these cells showed only a modest trend (124% control +/- 2.0% control, $p>0.05$).

When cells were exposed to Lp299v in vitro in the absence of cytokines, *hiap1* transcript was similar to control untreated cells, although there was a slight trend of increase (148% control +/- 4.5% control) **(Figure 8a)**. Exposure to Lpadh- for the same time had no impact on *hiap1* mRNA expression (118% control +/- 2.3% control, $p>0.05$) as well as in the presence of cytokines alone which showed a trend to decrease (82% control +/- 1.0% control, $p>0.05$) **(Figure 8b)**. Lp299v and Lpadh- co-incubation prior to cytokine challenge failed to significantly induce *hiap1* (115% control +/- 0.9% control, 133% control +/- 1.7% control, $p>0.05$, respectively).

mRNA expression levels for all other IAPs tested in HT-29 Gal cells were not impacted to any significant degree (*survivin* and *xiap* data not shown). When examining *naip* levels in HT-29 Gal cells under conditions of Lp299v and Lpadh- exposure, levels tended to decrease (85% control +/- 4.1% and 58% control +/- 1.2% control, $p>0.05$, respectively) **(Figure 9)**.

IAP expression levels were also examined with HT-29 Glu cells that have a colonic phenotype, as previously described. Following Lp299v exposure, *naip* (89% control +/- 2.5% control, $p>0.05$), *survivin* (115% control +/- 6.0% control, $p>0.05$), and *xiap* (98% control +/- 6.3% control, $p>0.05$) failed to demonstrate any significant change **(Figure 10)**. Exposure to Lpadh- in these cells failed to induce *naip* (88% control +/- 0.9% control, $p>0.05$) and *survivin* (95% control +/- 2.6% control, $p>0.05$), however *xiap* mRNA showed a trend towards increase in response (175% control +/- 2.4% control, $p>0.05$) **(Figure 10)**. *hiap1* and *hiap2* are not shown as neither of these genes was significantly modified.

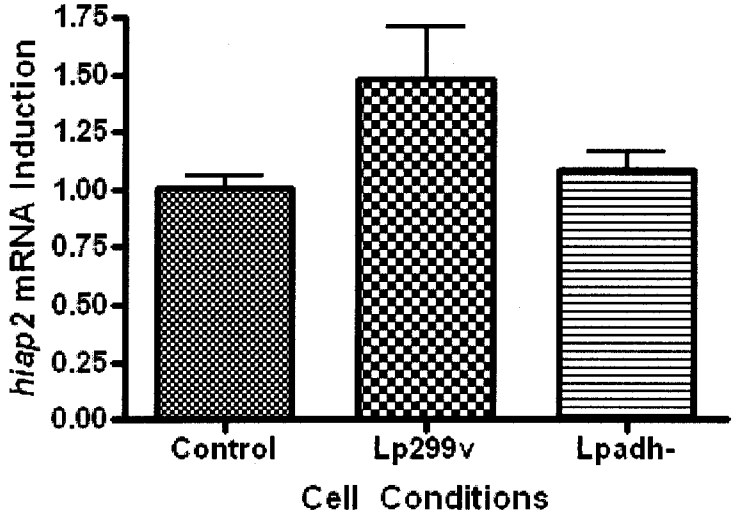
Figure 7: *Hiap2* mRNA induction following *in vitro* cell experiments where cells were incubated with probiotics in the presence (b) or absence (a) of cytokine exposure.

HT-29 Gal cells were cultured to 80% confluence over 2 days before incubation with either Lp299v or Lpadh- for 1 hr. mRNA modulation was detected using Taqman quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).

(a) *Hiap2* mRNA induction following incubation of HT-29 Gal cells in the presence or absence of select probiotics. Values represent mean+/-S.E. for samples run in triplicate, and are the result of four individual experiments.

(b) Samples were assessed for *hiap2* abundance following challenge with a mixture of proinflammatory cytokines (TNF-α, IL-1a, and IFN-γ) for 15 min in either the presence or absence of probiotic pre-incubation. Values represent mean+/-S.E. for samples run in triplicate, and are the result of four individual experiments.

Figure 7a.


1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
hiap2 mRNA Induction
Control
Lp299v
Lpadh-
Cell Conditions

b.

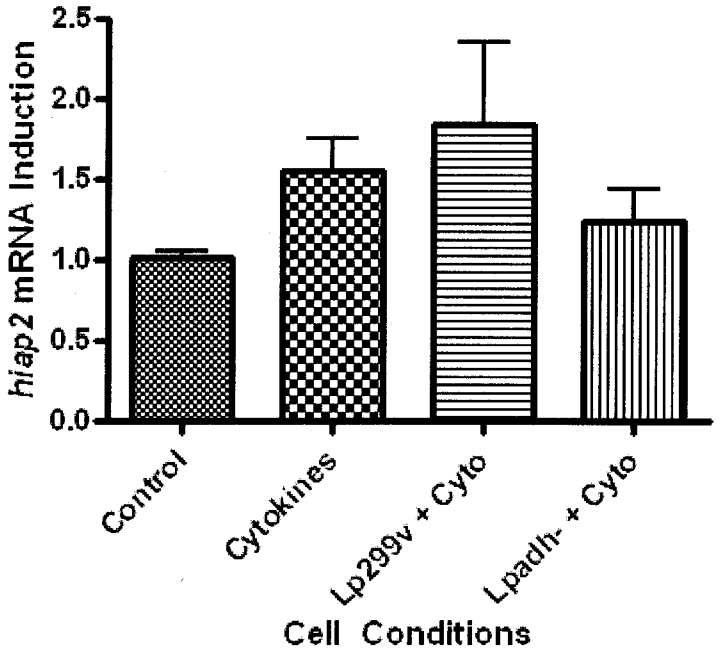

2.5
2.0
1.5
1.0
0.5
0.0
hiap2 mRNA Induction
Control
Cytokines
Lp299v + Cyto
Lpadh- + Cyto
Cell Conditions

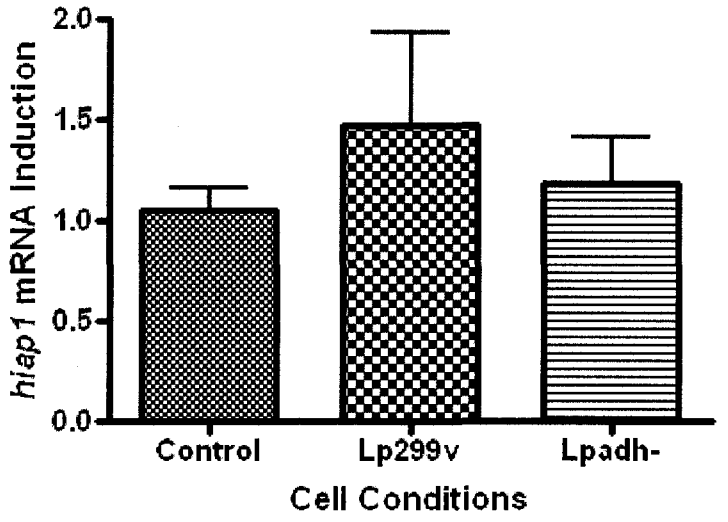
Figure 8: *Hiap1* mRNA induction following *in vitro* cell experiments where cells were incubated with probiotics in the presence (b) or absence (a) of cytokine exposure.

Cells were cultured to 80% confluence over 2 days before probiotic incubation for 1 hr. mRNA modulation was detected using Taqman quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).

(a) *Hiap1* mRNA induction following incubation of HT-29 Gal cells in the presence or absence of select probiotics. Values represent mean+/-S.E. for samples run in triplicate, and are the result of three individual experiments. Values were similar between experimental groups and control.

(b) *Hiap1* modulation is shown for samples that were challenged with a mix of proinflammatory cytokines including TNF-α, IL-1a, and IFN-γ for 15 min in either the presence or absence of probiotic pre-incubation. Values represent mean+/-S.E. for samples run in triplicate, and are the result of three individual experiments.

Figure 8a.


2.0
1.5
1.0
0.5
0.0
hiap1 mRNA Induction
Control
Lp299v
Lpadh-
Cell Conditions

b.

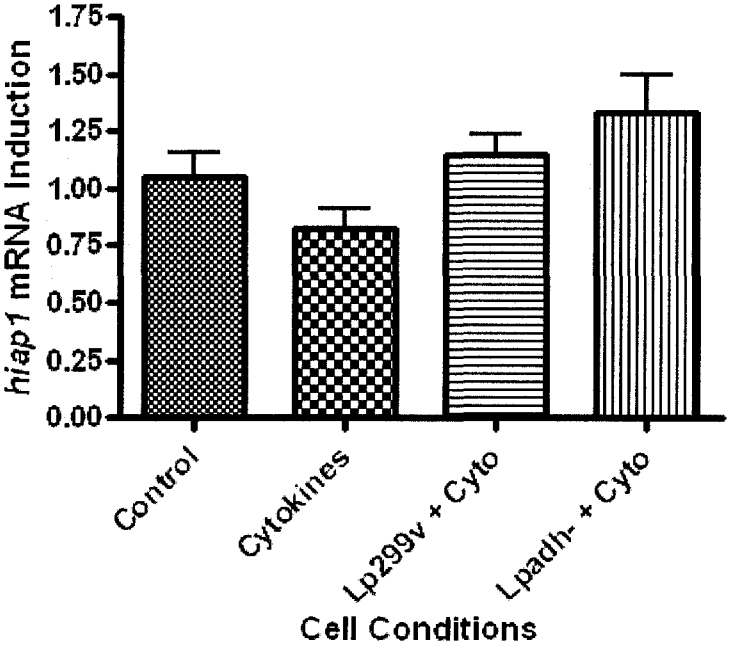

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
hiap1 mRNA Induction
Control
Cytokines
Lp299v + Cyto
Lpadh- + Cyto
Cell Conditions

Figure 9: *Naip* mRNA induction following *in vitro* cell experiments where cells were incubated with probiotics in HT-29 Gal cells.

Cells were cultured to 80% confluence over 2 days before probiotic incubation for 1 hr. mRNA levels were measured using Taqman quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Shown is *Naip* mRNA levels following incubation of HT-29 Gal cells in the presence or absence of select probiotics. Values shown are the result of four independent experiments.

Figure 9.

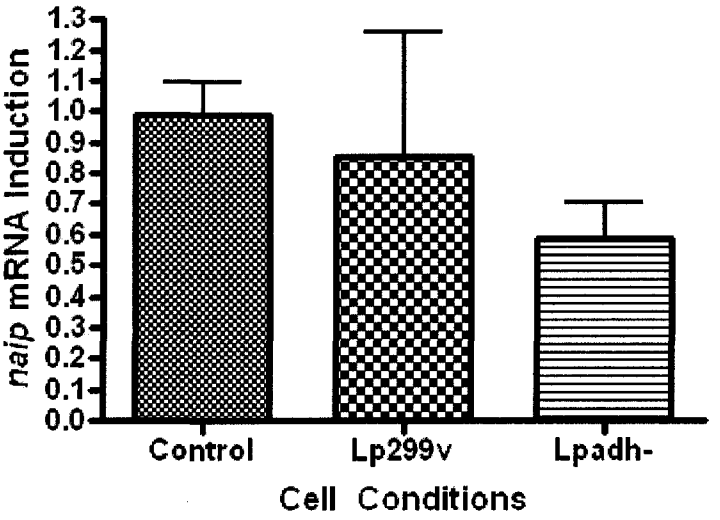

1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
naip mRNA Induction
Control
Lp299v
Lpadh-
Cell Conditions

Figure 10: IAP mRNA induction following HT-29 Glu cell exposure to either Lp299v or Lpadh- for 1hr.

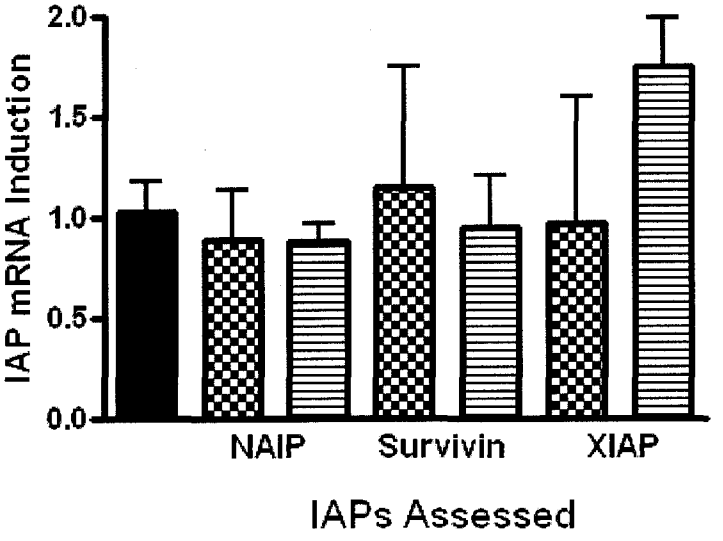
Cells were cultured to 80% confluence over 2 days before probiotic incubation for 1 hr. mRNA modulation was detected using Taqman quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Naip, *survivin* and *xiap* mRNA induction are shown following incubation of HT-29 Glu cells in the presence or absence of select probiotics.

Values represent mean+/-S.E. for each experimental group for samples run in triplicate and each are the result of 2 independent experiments.

Glu cells not exposed to either probiotic are shown with a solid bar, exposure to Lp299v is indicated with squared boxes, and exposure to Lpadh- is indicated with horizontal lines.

Figure 10.


IAP mRNA Induction
2.0
1.5
1.0
0.5
0.0
NAIP
Survivin
XIAP
IAPs Assessed

3.1.4 Analysis of IAP Protein Expression using Western Blotting

Western blots were performed using a range of polyacrylamide gels, systems, and buffers in order to optimize results for HIAP1 and HIAP2 band separation, as there is only a two kDa difference between the two proteins (HIAP1, 70 kDa; HIAP2, 68 kDa). Both HIAP1 and HIAP2 can be detected using the RIAP1 antibody (Table 1A). In figure 11, samples from HT-29 Gal cells incubated with either Lp299v or Lpadh- showed approximately equal expression of both HIAP1 and HIAP2 **(Figure 11, lanes 1 and 2)**. Interestingly, when cells were exposed to cytokines, HIAP1 increased approximately 2 fold when compared to probiotic exposure induction **(Figure 11 lane 3)**. There was no change in HIAP2 expression when the appropriate band was compared to its respective GAPDH value. Protein levels measured by Western blotting produced interesting results compared to mRNA levels detected using RT-PCR. Similar to insignificant *hiap2* mRNA levels found *in vitro*, Lpadh- followed by cytokines also failed to increase HIAP2 protein levels (Lpadh- exposure results were 4 times higher than when cells were exposed to both Lpadh- and cytokines combined) **(Figure 11, lanes 2 and 5)**. Similarly, Lp299v exposure in the presence of cytokines failed to further induce HIAP2 expression beyond those induced by Lp299v, with expression decreased by half **(Figure 11, lanes 1 and 4)**.

In figure 12, samples from HT-29 Gal cells were evaluated using Western blotting and a human anti-NAIP antibody in order to assess NAIP protein expression. NAIP protein expression was consistent between cell groups exposed to cytokines, Lp299v and Lpadh- **(Figure 12, lanes 3-5)**. This value was approximately double that noted for NAIP expression in control HT-29 Gal cells **(Figure 12, lanes 2 versus lanes 3-5)**. NAIP expression was three times higher in control cells compared to cells exposed to Lp299v prior to cytokine exposure **(Figure 12, lanes 1 and 2)**. There was no difference in expression

noted after incubating cells with both Lpadh- and cytokines versus control cells **(Figure 12, lanes 2 and 6)**. Taken together with mRNA data, probiotics protect against apoptosis but not through detectable modulation of IAP expression as assessed *in vitro*.

Figure 11: HIAP1 and HIAP2 protein levels *in vitro* detected using Western blotting.

Protein was isolated from HT-29 Gal cells incubated in the presence or absence of select probiotics for 1 hr prior to 15 min exposure to a mixture of pro-inflammatory cytokines. Refer to Appendix B for raw densitometric values.

Lanes from left to right (1-6):

1. Lp299v 1 hr
2. Lpadh- 1hr
3. Cytokine Treated Control
4. Lp299v 1hr/cytokine 15 min
5. Lpadh- 1 hr/cytokine 15 min

Figure 12: NAIP protein levels *in vitro* detected using Western blotting.

Protein isolates were obtained from HT-29 Gal cells incubated in the presence or absence of select probiotics for 1 hr prior to 15 min exposure to a mix of pro-inflammatory cytokines. Refer to Appendix B for raw densitometric values.

Lanes from left to right (1-6):

1. Lp299v 1 hr/cytokine 15 min
2. Untreated Gal Control
3. Cytokine Treated Control
4. Lpadh- 1 hr
5. Lp299v 1 hr
6. Lpadh- 1 hr/cytokine 15 min

Figure 11.

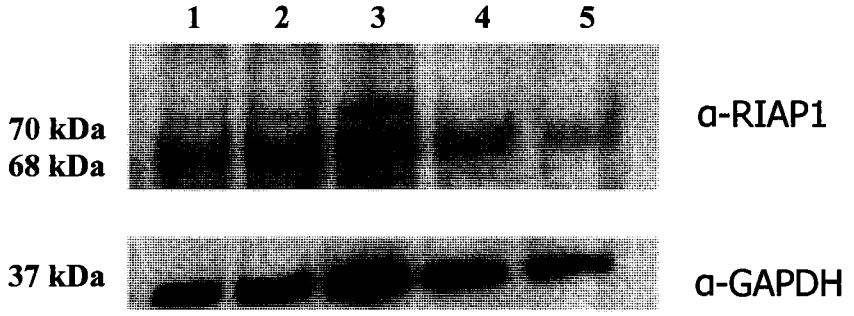
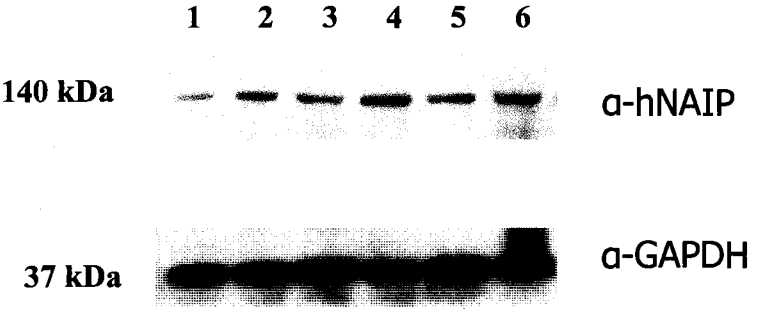


Figure 12.



3.2 Analysis of IAP Activity in Macrophage/HT-29 Co-Culture

3.2.1 *Hiap1*, *hiap2,* and *naip* mRNA levels detected by QRT RT-PCR

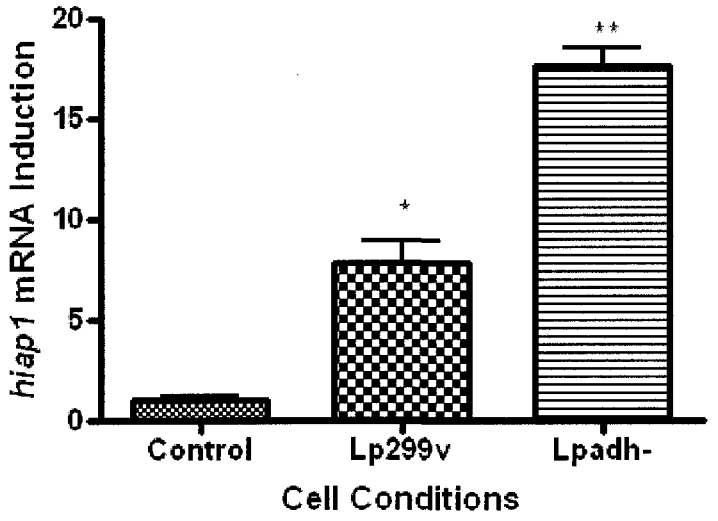
To detect the amount of *hiap1, hiap2,* and *naip* mRNA in cells in the presence and absence of cytokines, HT-29 Glu cells were co-incubated with RAW264.7 macrophages for 2 days. Following macrophage activation with endotoxin, HT-29 cells treated with Lp299v showed significant increase in *hiap1* levels and a trend of *hiap2* upregulation (780% control +/- 11% control, $p<0.01$ and 2300% control +/- 46% control, respectively) **(Figure 13)**. When incubated with Lpadh- for 1 hour, both *hiap1* and *hiap2* increased beyond levels obtained after Lp299v incubation (1760% control +/- 9.0% control, $p<0.001$ and 3430% control +/- 146% control, $p<0.05$ respectively) **(Figure 13)**.

Figure 13: *Hiap1* (a) and *hiap2* (b) induction following co-incubation of HT-29 Gal cells with activated RAW264.7 macrophages with or without select probiotics.

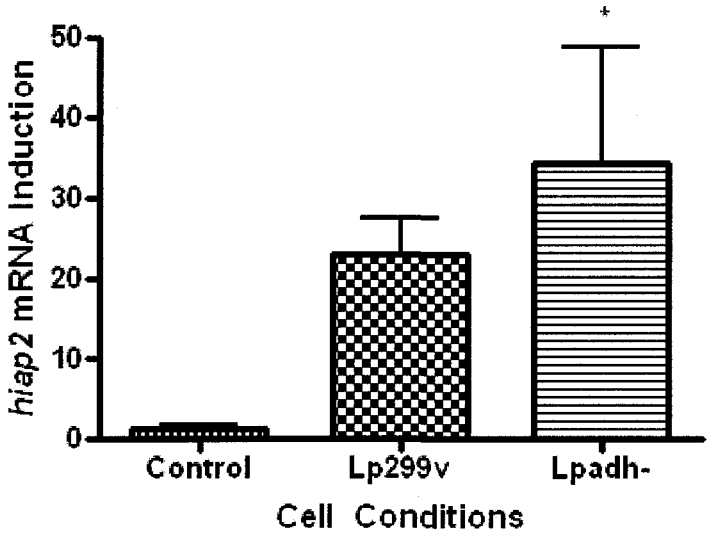
(a) *Hiap1* mRNA induction following co-incubation of HT-29 Gal cells with activated RAW264.7 macrophages in the presence of either Lp299v or Lpadh- for 1 hr. Values shown are mean +/- S.E. and are the result of a single experiment, run in triplicate; *=$p<0.01$, **=$p<0.001$.

(b) *Hiap2* mRNA induction following co-incubation of HT-29 Gal cells with activated RAW264.7 macrophages in the presence of either Lp299v or Lpadh-. Values shown are mean +/- S.E. and are the result of two independent experiments, run in triplicate; *=$p<0.05$.

Figure 13a.


20
15
10
5
0
hiap1 mRNA Induction
*
**
Control
Lp299v
Lpadh-
Cell Conditions

b.


50
40
30
20
10
0
hiap2 mRNA Induction
*
Control
Lp299v
Lpadh-
Cell Conditions

3.2.2 IAP Protein Expression Following Co-incubation

To correlate mRNA expression with protein expression, Western blotting was used with co-cultured samples. Macrophages were activated with endotoxin A (with the exception of lane 4) in order to assess protein expression in the presence of activated immune cells. All other lanes are the result of macrophage and HT-29 Glu cell co-incubation. Co-incubated cells in the absence of probiotics (**Figure 14, lane 5**) were used as a negative control. In lane 2 there was an absence of protein due to difficulties encountered obtaining protein extracts from an insufficient amount of macrophages.

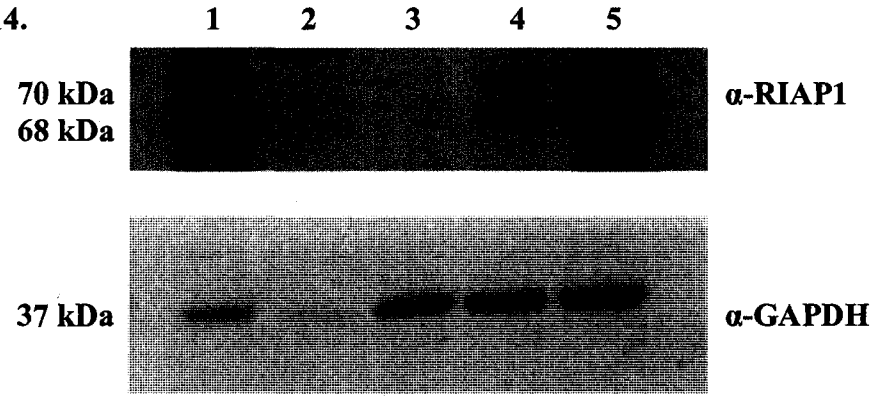
In response to activated macrophages co-incubated with Lpadh-, both HIAP1 and HIAP2 were dramatically increased to the point of pixel saturation (as determined using the RIAP1 antibody) **(Figure 14, lane 1)**. Alternately, when co-incubated cells were exposed to Lp299v alone, there was no detectable HIAP2 expression with only trace amounts of HIAP1 **(Figure 14, lane 3)**. There did seem to be an induction in both HIAP1 and HIAP2 protein expression following co-cultured cell exposure to Lp299v when endotoxin was excluded **(Figure 14, lane 4)** however the expression of HIAP1 in these cells was three times control cell expression, and five times less for HIAP2 (**Figure 14, lanes 4 and 5**).

Figure 14: HIAP1, HIAP2 and GAPDH Western blots of RAW264.7/HT-29 cell co-culture protein extracts.

Lanes are labelled as follows (1-5):

1. Co-incubated RAW264.7/HT-29 cells exposed to Lpadh-
2. Macrophages exposed to Lp299v
3. Co-incubated RAW264.7/HT-29 cells exposed to Lp299v
4. Non-activated RAW264.7 macrophages & HT-29 cells exposed to Lp299v
5. Co-incubated RAW264.7/HT-29 cells with no probiotic exposure.

Figure 14.



3.2.3 Immunocytochemical Analysis of Macrophage Activation

To provide insight into the role of macrophages in the IEC-microbe interaction in the presence of probiotics, co-cultured cells were stained to indicate macrophage activation. It was confirmed that 1μg/1μl endotoxin was required for macrophage activation as seen in **Figures 15c** in the absence of probiotics (86%) **(Figure 16)**. It is noted that pre-incubation of cells with Lp299v for 1 hour induces dramatic macrophage activation (100%) **(Figure 15a, Figure 16)**. Incubation with the non-adherent strain Lpadh- also leads to full activation of macrophages similar to co-culture in the presence of endotoxin though not to the extent of incubation with Lp299v (64.3%) **(Figure 15b, Figure 16)**. Thus in these conditions, both adherent and non-adherent Lp299v activate macrophage cells when in co-culture.

Figure 15: Immunocytochemical analysis of macrophage activation in HT-29 Glu cells co-incubated with macrophages subjected to probiotic exposure.

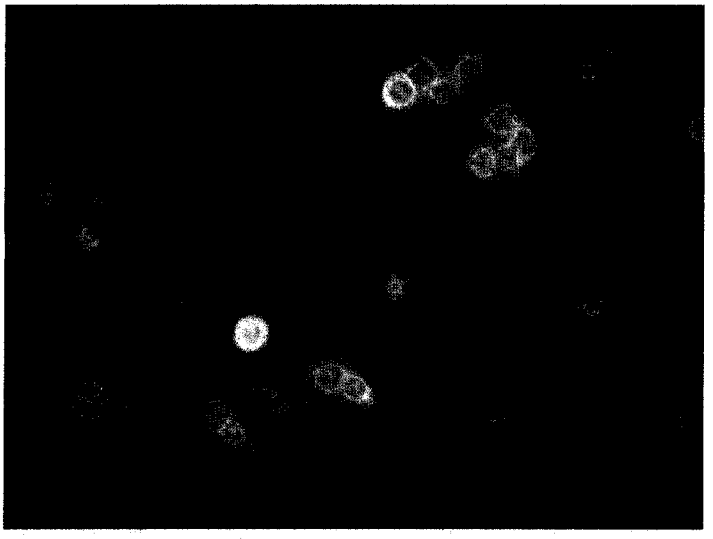
Cells were stained for activation following probiotic exposure using iNOS and phalloidin-TRITC staining and imaged using an Axioskop fluorescent microscope, analyzed using Northern Eclipse imaging software. Activated macrophages are indicated by red staining, green staining indicates phalloidin-TRITC focal adhesion contacts. Magnification 40x.

(a) Immunocytochemical analysis of macrophage activation in HT-29 Glu cells co-incubated with both macrophages and Lp299v for 1 hour.

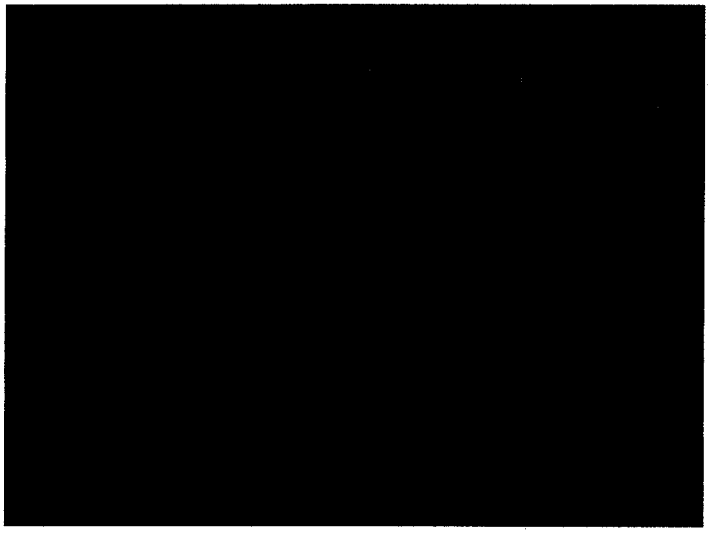
(b) Immunocytochemical analysis of macrophage activation in HT-29 Glu cells co-incubated with both macrophages and Lpadh- for 1 hour.

(c) Immunocytochemical analysis of macrophage activation in HT-29 Glu cells co-incubated with macrophages in the absence of probiotics.

Figure 15a.



b.



c.

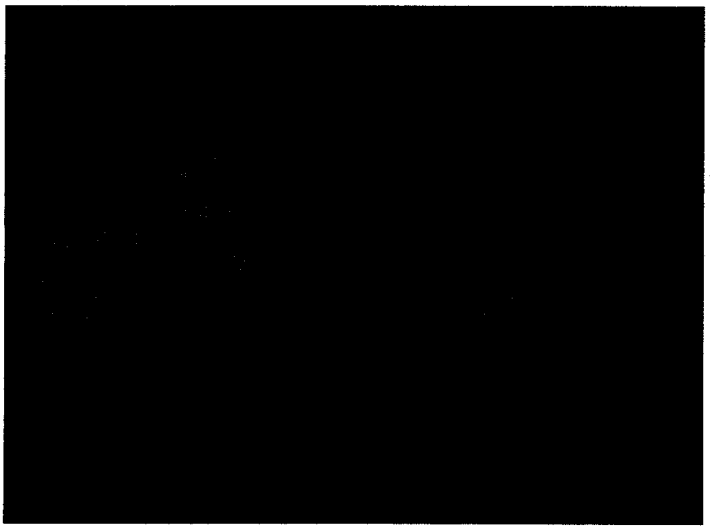
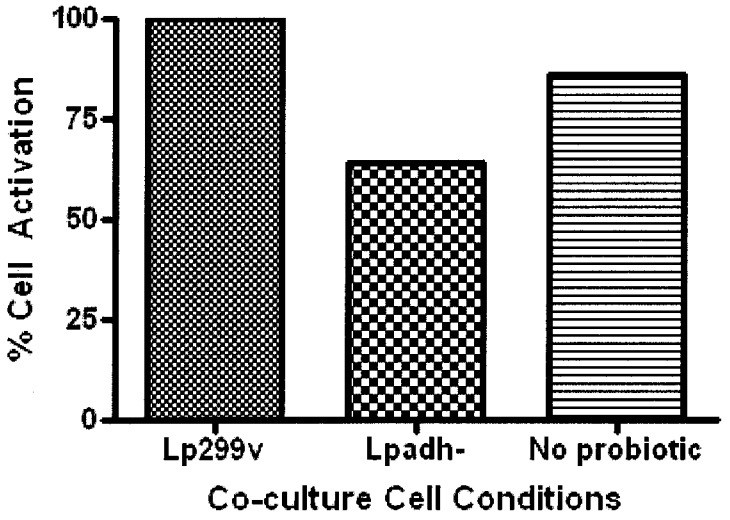


Figure 16: Quantification of activated macrophages in cell populations treated with endotoxin following incubation with either Lp299v, Lpadh-, or in the absence of probiotic treatment.

Random fields were selected for counting and are expressed as a percentage of activated cells per 200 cells counted. Percentages are compared to 0% activation achieved when cells are co-cultured in the absence of endotoxin exposure (data not shown). Values shown are the result of a single independent experiment.

Figure 16.


100
75
50
25
0
% Cell Activation
Lp299v
Lpadh-
No probiotic
Co-culture Cell Conditions

3.3 Evaluation of Probiotic Administration In Vivo

3.3.1 Daily Water Intake of Animals

Without significant IAP expression in our HT-29 cell model as a consequence of using a cancer cell line, we wanted to evaluate whether probiotics would influence IAP expression in an *in vivo* model. Probiotics were quantified and added to the drinking water of Sprague-dawley rats. Daily water intake was recorded to confirm that rodents were ingesting similar quantities of probiotic-containing water per day **(Table 2)**. For C57/Bl6 mice, water intake was too low (approx. 1cc/day) to have confidence in the number of ingested microbes. The provision of equal ingestion amounts of bacteria in small water volumes would require water to be turbid. Thus, to ensure that mice were ingesting 1 x 10^9 CFU of probiotics per day between experimental groups, probiotics were actively administered to mice via gavage on a daily basis rather than allowed to be passively ingested by mice in drinking water. Therefore, the amount of bacteria ingested by mice on a daily basis was consistent with those ingested by rats.

Table 2: Rat study groups and daily intake of water.

Group	Rats	Rat ID	Bacterial strain	Duration (days)	Organism count (per 200ml water)	Av. Water intake (ml/day)
6	2	211,212	None (Control)	2	-	39.0
28	2	1112,1113	Control	2	-	48.5
25	3	140-142	Control	2	-	30.0
8	2	216,217	L. plantarum 299v	2	10ml of 10^7 CFU/ml	41.7
29	3	1114-1116	L. plantarum 299v	2	10ml of 10^9 CFU/ml	23.7
26	4	144-147	L. plantarum 299v	2	10ml of 10^9 CFU/ml	35.0

Rats were separated into groups on a basis of administered strain (Lp299v or Lpadh-) and a concentration of either 10 ml of 10^7 CFU/ml or 10^9 CFU/ml of probiotic in a total of 200 ml drinking water. Note untreated control animals in each trial. Daily water intake values are the average daily intake as recorded for each rat within the specific trial group, note its relative consistency between study groups.

3.3.2 Bacterial Quantification

In order to quantitate probiotic strains to enable addition of 10 ml 10^9 CFU/ml of probiotic in a total of 200 ml drinking water, bacterial strain-specific absorbance versus CFU curves were developed. Absorbance readings (OD=600 nm) of serial dilutions were plotted against quantified CFU values to construct graphs. It may be noted that the growth curves for both the adherent and non-adherent probiotic are distinct, as might be expected since there were differences in outer membranes (see background) **(Figure 17a and 17b)**. Subsequent experiments utilized these curves through absorbance readings to quantify bacteria.

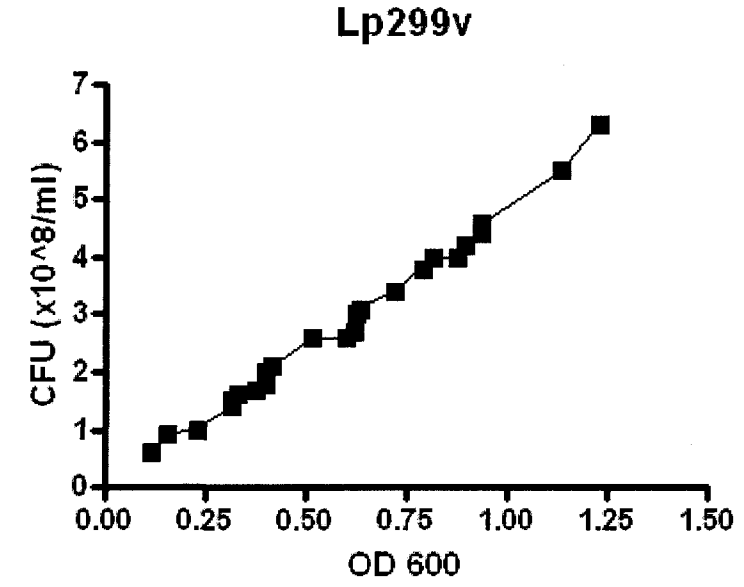
Figure 17: Probiotic-specific bacterial growth curves used to quantitate Lp299v (a) and Lpadh- (b) for samples used in the experiments.

Data were used to determine the bacterial quantification of each strain following spectrophotometric reading of randomly selected samples. Following sample OD_{600} nm readings, corresponding bacterial concentration could be read in 10^8 units.

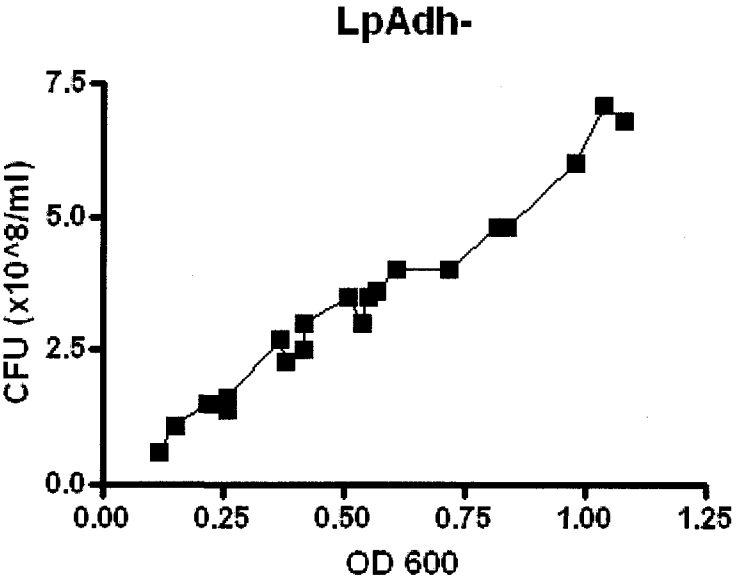
(a) Growth curve for *Lactobacillus plantarum* 299v.

(b) Growth curve for Lpadh-.

Figure 17a.


Lp299v
CFU (x10^8/ml)
0
1
2
3
4
5
6
7
0.00
0.25
0.50
0.75
1.00
1.25
1.50
OD 600

b.


LpAdh-
CFU (x10^8/ml)
0.0
2.5
5.0
7.5
0.00
0.25
0.50
0.75
1.00
1.25
OD 600

3.4 *In Vivo Analysis of IAP Expression* Following *Probiotic Treatment*

3.4.1 RNA Expression of Select IAPs

Taqman real-time RT-PCR semi-quantitative mRNA analysis revealed that *hiap2* mRNA expression was significantly increased in the mouse jejunum after Lp299v ingestion for 2 days (160% control +/- 10% control, $p<0.001$). *Hiap2* mRNA expression following Lpadh- ingestion was similar to controls (100% control +/- 10% control, $p<0.001$) **(Figure 18)**. IAP modulation was standardized to rodent *gapdh* internal control expression in all experiments.

Hiap1 mRNA was not found to be significantly modulated in response to treatment with either Lp299v or Lpadh- for 2 days (110% control +/- 4.0% control, 80% control +/- 4.0% control, respectively) **(Figure 19)**.

Naip1 levels were determined in mice in order to correlate this data to both rats and humans. *Naip1* mRNA levels following the administration of Lp299v for 2 days were significantly increased (160% control +/- 7.0% control, $p<0.0001$) **(Figure 20)**. There was no detectable change in *xiap* mRNA transcript expression following ingestion of Lp299v **(Figure 21)**. *In vivo naip1* and *hiap2* were the most responsive genes assayed following 2 day administration of the adherent probiotic Lp299v to C57/Bl6 mice.

Naip mRNA modulation was also assessed in a dose response manner using jejunum samples from Sprague-Dawley rats administered probiotics for a period of 2 days in their drinking water. Ingestion of 1×10^7 CFU per day only resulted in moderate *naip* increase (110% control +/- 5.0% control, $p<0.05$), while ingestion of 1×10^9 CFU per day increased this level substantially (170% control +/- 1.0% control, $p<0.001$) **(Figure 22)**. All other IAPs assessed in rats lead to transcriptional responses similar to mouse results and experiments were therefore carried out with the higher dosage to maximize gene response (data not shown).

Figure 18: *Hiap2* mRNA induction *in vivo* following gavage administration of either Lp299v or Lpadh- to C57/Bl6 mice for 2 days, n=31.

Mice were administered probiotics (1 x 10^9 CFU per day) or plain water (control) over 2 days before sacrifice and jejunal RNA isolation. mRNA modulation was detected using Taqman quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Values shown represent mean +/- S.E., and are the result from 4 independent experiments, run in triplicate. **=$p<0.001$, compared to controls.

Figure 18.

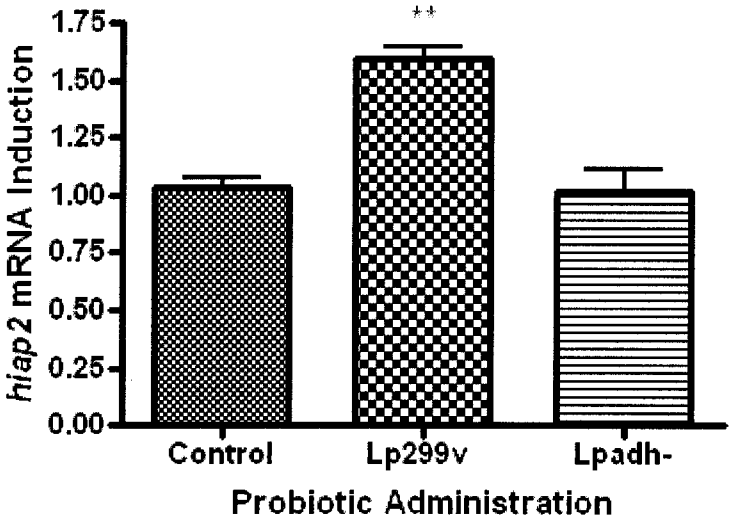

**
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
hiap2 mRNA Induction
Control
Lp299v
Lpadh-
Probiotic Administration

Figure 19: *Hiap1* mRNA induction *in vivo* following gavage administration of either Lp299v or Lpadh- to C57/Bl6 mice for 2 days, n=30.

Mice were administered probiotics or plain water control over 2 days before sacrifice and jejunal RNA isolation. mRNA modulation was detected using Taqman quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Values shown represent mean +/- S.E., and are the result from 4 independent experiments, run in triplicate.

Figure 19.

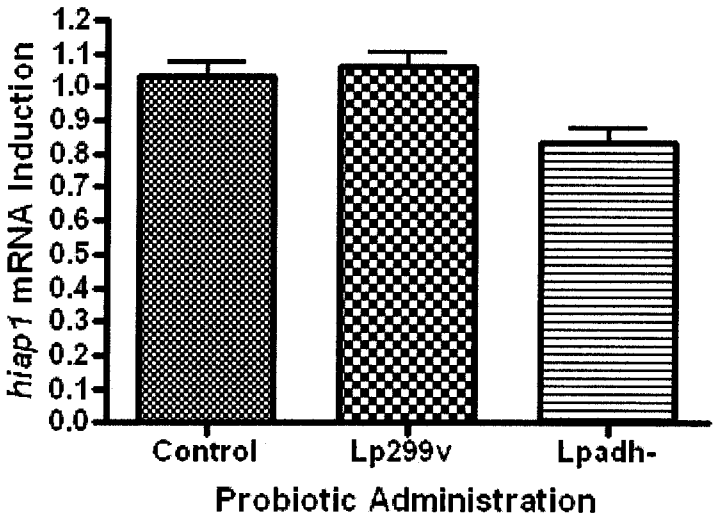

hiap1 mRNA Induction
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
Lp299v
Lpadh-
Probiotic Administration

Figure 20: *Naip1* mRNA induction *in vivo* following gavage administration of Lp299v to C57/Bl6 mice for 2 days, n=38.

Mice were administered probiotics or plain water control over 2 days before sacrifice and jejunal RNA isolation. mRNA modulation was detected using Taqman quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Values shown represent mean +/- S.E., and are the result from 2 independent experiments, run in triplicate. ***=$p<0.0001$ versus control.

Figure 20.

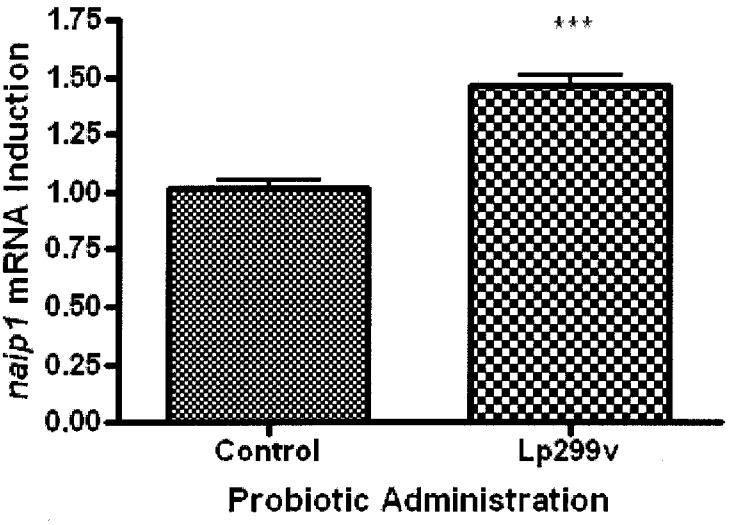

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
naip1 mRNA Induction
***
Control
Lp299v
Probiotic Administration

Figure 21: *Xiap* mRNA induction *in vivo* following gavage administration of Lp299v to C57/Bl6 mice for 2 days, n=25.

Mice were administered probiotics or plain water control over 2 days before sacrifice and jejunal RNA isolation. mRNA modulation was detected using Taqman quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Values shown represent mean +/- S.E., and are the result from 3 independent experiments, run in triplicate.

Figure 21.

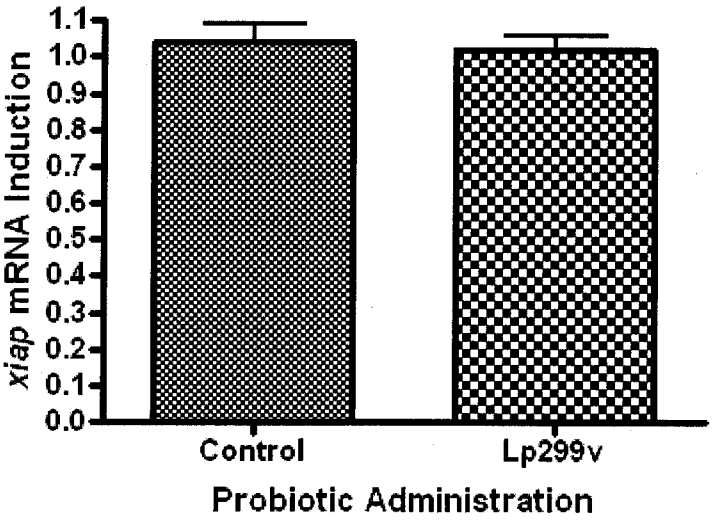
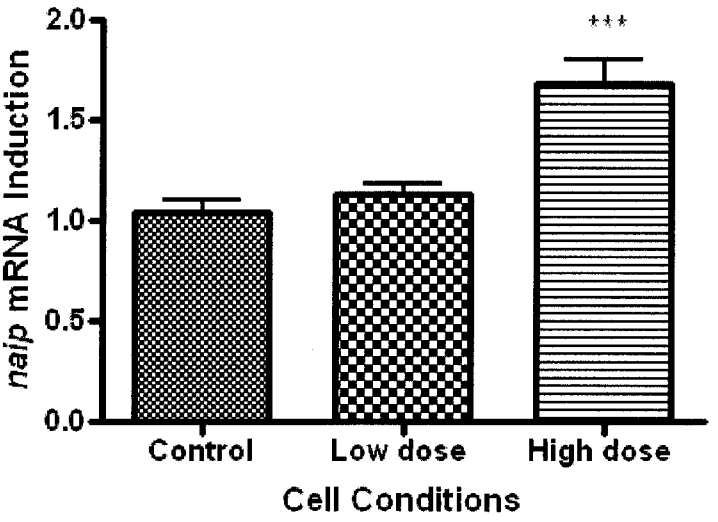

1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
xiap mRNA Induction
Control
Lp299v
Probiotic Administration

Figure 22: *Naip* mRNA induction *in vivo* following administration of either high or low dose Lp299v to Sprague-Dawley rat drinking water for 2 days, n=16.

Rats were administered probiotics or plain water control over 2 days before sacrifice and jejunal RNA isolation. mRNA modulation was detected using Taqman quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Values shown represent mean +/- S.E., and are the results of a single experiment, run in triplicate. ***=$p<0.0001$ versus control.

Figure 22.


2.0
1.5
1.0
0.5
0.0
naip mRNA Induction
***
Control
Low dose
High dose
Cell Conditions

3.4.2 Analysis of Modulated IAPs at the Translational Level

HIAP1, HIAP2, and NAIP expression were assessed in protein samples obtained from isolated mouse jejunal segments following probiotic treatment for 2 days. RIAP1 antibody was used to detect both HIAP1 and HIAP2 expression and a human anti-NAIP antibody was used to detect rodent NAIP. Westerns were scanned as TIFF files and densitometry was performed using appropriate software.

HIAP1 protein expression was only moderately higher in Lpadh- and Lp299v treated samples compared to control **(Figure 23a)**. GAPDH expression was similar in all lanes. There was no difference observed in HIAP2 for any of the experimental conditions **(Figure 23a)**. In *in vitro* HT-29 Gal cell studies mRNA data were inconsistent with protein findings. *In vivo* NAIP experiments revealed this as well as there was no modulation protein expression as levels remained unchanged **(Figure 23b)**.

Sprague-Dawley rat segments were also evaluated. Protein isolates were obtained from both rat ileum and jejunum samples and analyzed by Western blot for HIAP1 and HIAP2. NAIP was not assessed as protein had not been significantly altered in mouse intestine. HIAP2 levels in rat jejunum increased approximately two-fold following a 2 day course of Lp299v **(Figure 24 lane 2 versus lane 4)**. In contrast, no significant protein expression was noted for HIAP2 in ileal samples **(Figure 24 lanes 1 & 3)**. HIAP1 was not detected in either jejunal or ileal samples **(Figure 24)**.

Figure 23: 2 day Lp299v ingestion dramatically raises mouse jejunal HIAP1 levels (a) with a moderate increase in NAIP (b).

Western blot analysis was conducted by loading 20 μg of protein extracted from the jejunum of C57/Bl6 mice on 7.5% Acry./Bis. gels using anti-rodent RIAP1 primary antibody (a) and anti-human NAIP antibody (b). Following subsequent species-appropriate secondary incubation membranes were analyzed via IVIS (In Vitro Imaging System) imaging equipment. GAPDH was used as a means of loading control.

Lanes labelled as follows (1-9):

A:

1,2,7,8: Untreated control
3,4: Lpadh- treated 2 days
5,6,9: Lp299v treated 2 days

B:

1: Molecular Weight Ladder (MWL)
2,3,8,9: Untreated control
4,5: Lpadh- treated 2 days
6,7: Lp299v treated 2 days

Figure 23.

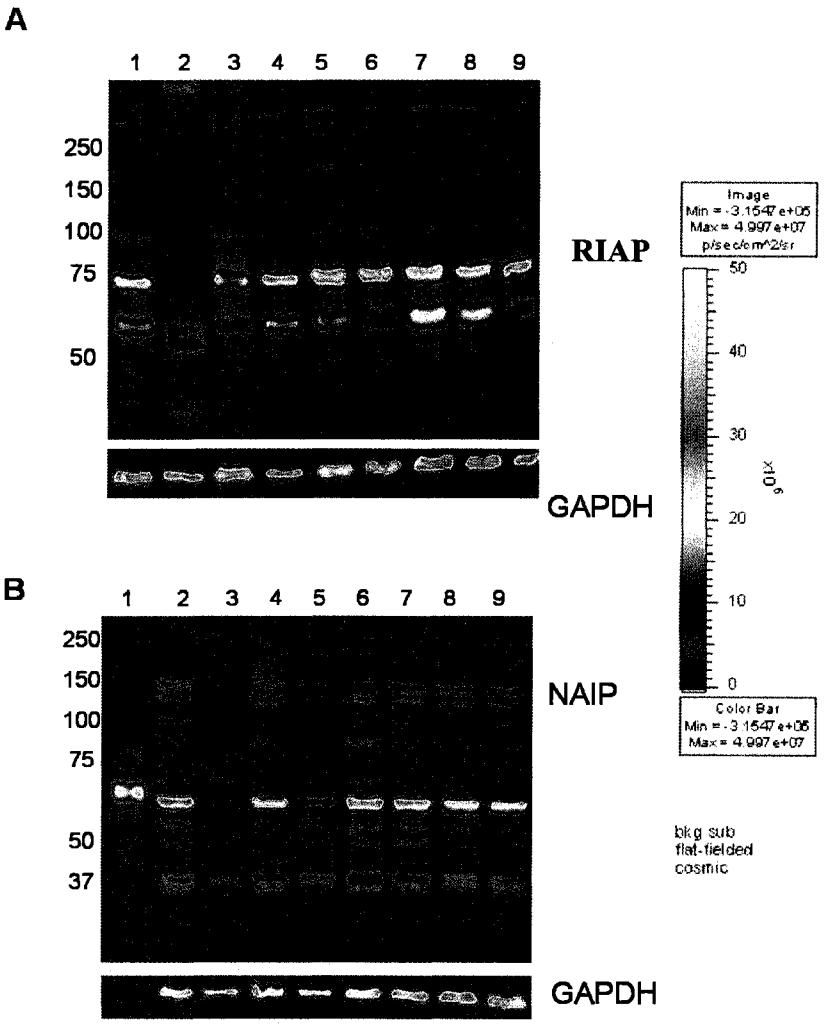
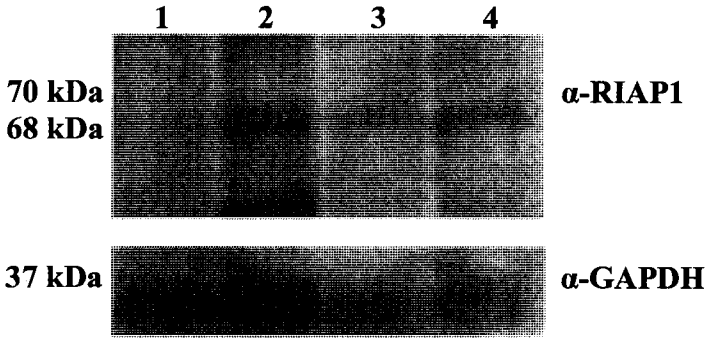

A
1 2 3 4 5 6 7 8 9
250
150
100
75
50
RIAP
GAPDH
B
1 2 3 4 5 6 7 8 9
250
150
100
75
50
37
NAIP
GAPDH
Image
Min = -3.1547e+05
Max = 4.997e+07
p/sec/cm^2/sr
50
40
30
20
10
0
x10^6
Color Bar
Min = -3.1547e+05
Max = 4.997e+07
bkg sub
flat-fielded
cosmic

Figure 24: Western blotting of jejunal and ileal rat protein extracts with RIAP1 antibody shows slight increase in jejunal HIAP2 level following treatment.

Lanes labelled as follows (1-4):

1. Ileum (Lp299v treated)
2. Jejunum (Lp299v treated)
3. Ileum (untreated)
4. Jejunum (untreated).

Figure 24.



3.5 Elucidation of Potential Signalling Pathway

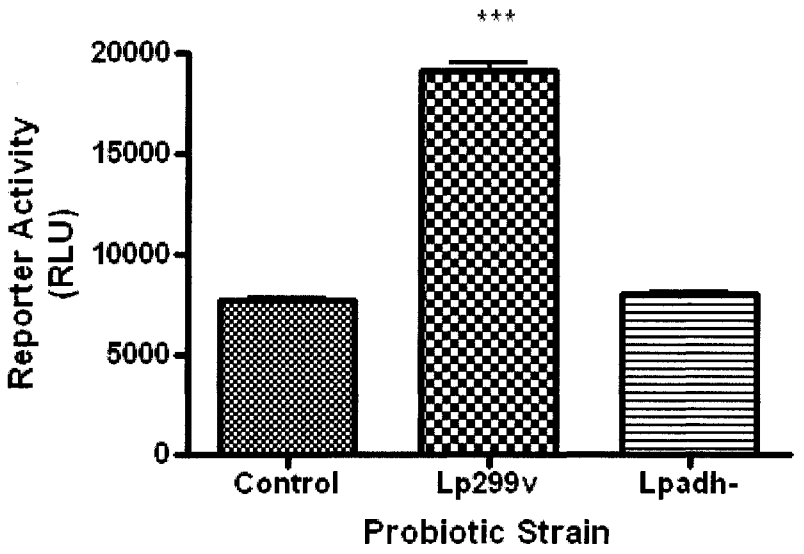
3.5.1 Luciferase Reporter Activity Assay

NF-kB activity was assessed in HT-29 Gal cells using a NF-kB luciferase activity assay. Following 1 hr Lp299v or Lpadh- incubation, samples were plated in triplicate and read twice to ensure accuracy. Lp299v incubation increased NF-kB activity significantly (19137 +/- 350.3 RLU versus 7705 +/- 107.6 RLU, $p<0.001$) in contrast to Lpadh- for 1 hr (7969 +/- 156.4 RLU, $p>0.05$) **(Figure 25)**.

Figure 25: NF-κB reporter activity in HT-29 Gal cells following probiotic incubation for 1 hr.

HT-29 Gal cells were transfected with 4 μg NF-kB construct and 1 μg Renilla luciferase construct and visualized via fluorescent microscopy to ensure control cell transfection. Cells were then incubated with either Lp299v or Lpadh- for one hour, and in the absence of probiotics. A Dual-Luciferase Reporter Assay was performed on cells plated in a 96-well plate, and read by a luminometer. Analysis was performed using values for luciferase assay substrate versus firefly luciferase control values. Samples were each plated in triplicate and read twice to ensure accuracy. Values are expressed in relative luminescence units, with ***=$p<0.0001$ statistically significant.

Figure 25.


***
20000
15000
10000
5000
0
Reporter Activity (RLU)
Control
Lp299v
Lpadh-
Probiotic Strain

4. Discussion

4.1 Probiotic-Mediated Anti-Apoptotic Effect In Vitro

4.1.1 TUNEL + Counts Indicate Lp299v and Lpadh- Protect Against Apoptosis

In vitro TUNEL assays measured the extent of cell death involving DNA cleavage characteristic of apoptosis. HT-29 Gal cells exposed to cytokines for 15 min showed $TUNEL^+$ (43.6% +/- 6.2%). This is in keeping with IEC apoptosis in response to pro-inflammatory cytokines [116]. Although there are distinct trends where Lp299v and Lpadh- elicit unique responses from HT-29 cells, interestingly the two microbes had seemingly similar effects on apoptosis as detected via TUNEL. Using this method, Lp299v decreased the amount of $TUNEL^+$ cells (18.7% +/- 4.1%, $p<0.01$) similar to Lpadh- (16.6% +/- 3.2%, $p<0.01$). Using the TUNEL assay, it seems as though both Lp299v and Lpadh- similarly prevent apoptotic cell death.

4.1.2 Evaluation of Caspase Activity Assay Results Confirms Protection

Consistent with TUNEL assay results, an increase in apoptotic HT-29 cell death was observed following cytokine exposure in the absence of probiotics (68.9% +/- 5.1%). However, adherent and non-adherent probiotic incubation prior to cytokine exposure elicited divergent levels of cytoprotection; Lpadh- decreased caspase-dependent cell death moderately (46.8% +/- 17.5%) while Lp299v was significantly more potent in reducing $caspase^+$ cells (3.6% +/- 0.6%, $p<0.01$). We are thus confronted with a striking disconnect; roughly similar reduction of TUNEL positivity by adherent and non-adherent probiotic strains while the adherent strain imparts a dramatic, almost complete abolition of caspase reactivity. Possible explanations include a soluble cytoprotective agent secreted by Lpadh- which inhibits PCDII; although reconciling this with the genetic differences between the two

microbes is somewhat problematic. It follows that Lp299v seems to protect cells from caspase-dependent PCDI. We therefore find grounds to investigate the possible involvement of IAPs in this phenomenon. Again, the failure of Lpadh- to protect against PCDI suggests that this strain may act on caspase-independent PCDII, necrosis, or perhaps lends credibility to the specificity of the caspase activity assay versus the TUNEL method in specifically detecting apoptosis. This assay can detect cells in the early stages of apoptosis that would otherwise be missed in the TUNEL assay, and also accounts for the degree of $TUNEL^+$ cells that were in fact necrotic and not truly apoptotic.

Earlier experiments were conducted using a multi-caspase activity assay to measure the activity of caspases 1-9. This was of benefit while assessing the relative degree of whole caspase involvement measuring IRF1-mediated caspase-1 involvement and caspase-8 activity consistent with inflammation. However, further experiments were conducted using a caspase-3 and -7 activity assay to determine more specifically whether these key executioner caspases were being inhibited in this phenomenon. Should these caspases be involved to a large extent, this would mechanistically exclude the pathway of FAS-mediated apoptosis involving cytokine-c release and subsequent caspase-9 involvement [27].

HT-29 Gal cells were subjected to a caspase activity assay with a fluorescent dye specific to activated caspases -3 and -7 alone. With cytokine exposure alone, there was an increase in $caspase^+$ cell death (47.8% +/- 25.3%). Surprisingly, when cells were exposed to Lpadh- prior to cytokine exposure the percentage of caspase-$3,7^+$ cells increased substantially (79.3% +/- 20.8%). Due to the absence of adherence factor on its microbial surface, it is possible that IECs do not correctly identify Lpadh- as a commensal organism as in the case of Lp299v. In the event of a cytokine response to this bacteria, an increase in caspase-dependent cell death, which would appear as an increase in caspase-$3,7^+$ cells would be

predicted. This could be due in part to increased IFN-γ-induced FAS expression which is known to sensitize cultured IECs to cytokine-mediated apoptosis [27]. An increase in FAS expression would lead to an increase in pro-caspase-8 cleavage into active caspase-8, which would increase caspase-3 and -7 activity as a result [27]. Should apoptotic cell death be blocked by probiotic action against caspase-3 and -7, this would again elude to the role of IAP involvement.

As with the multi-caspase activity assay, when cells were incubated with Lp299v for 1 hr prior to cytokine exposure, the percentage of caspase-3,7^{+} cells markedly decreased (3.9% +/- 0.3%), suggesting that the strain's reduction of caspase-dependent programmed cell death may be mediated by caspase-3 and -7 inhibition. The lack of protection conferred by the non-adherent Lpadh- points both to the suspected role of adherence in preventing cytokine-induced caspase activation, as well the importance of adherence for bacterial recognition.

Successful disease interference would be aided by knowledge of the form of cell death indicated for the specific disease [56]. While the predominant form of cell death in IBD is unknown, the large degree of necrosis and IEC death associated with a similar disease, NEC, is instructive [11, 117]. Since the comparatively circumscribed level of apoptosis is thought to trigger opportunistic necrotic cell death, PCDI inhibition could potentially block the subsequent necrosis. This would be likely more successful than attempting to block necrotic cell death. Since Lp299v demonstrates an anti-apoptotic phenotype associated with caspase-3 and -7 inhibition, therapeutic interference of PCDI could potentially improve conditions of IBD from progressing to full-blown chronic inflammation (attributed to in part by necrosis). If probiotics impinge selectively on caspase-3 and -7, thus mainly affecting the end stage of apoptosis, this may permit the activity of other caspases involved in differentiation and cell development (contraindicated in other apoptotic therapies) [19].

4.1.3 IAP RNA and Protein Expression

For the most part, IAP analysis following probiotic pre-incubation on HT-29 Gal cells reveals insignificant results. Exposure of cells to Lp299v alone in the absence of cytokines only tended to modulate *hiap2* transcript (148% control +/- 45% control, Figure 7a) and protein (Figure 11). In a similar fashion, *hiap1* transcript and protein was found to be only moderately raised in these cells (148% control +/- 23% control, Figure 8a, Figure 11). Although not achieved using this model, perhaps in using a non-cancer cell line the role of both HIAP1 and HIAP2 could be better determined. Upon the initial detection of microbes, IECs may upregulate both HIAP1 and HIAP2 to counter the apoptotic stress of a potential invasion. It would therefore also be interesting to follow IAP expression over time to see if expression tapers back to baseline following tolerance.

No change of NAIP was detected in either mRNA or protein analysis *in vitro*. This may be consistent with NAIP's role as a sensor for intracellular bacteria. Consistent with the pattern of p21 expression lining intestinal villi, NAIP expression increases in abundance at villi tips [118]. In addition to pathogen recognition, it has been suspected to play a role in the protection of terminally differentiated cells, due to this geographic location [118]. As a suggested bacterial sensor, there does not seem to be a direct purpose in NAIP modulation in the face of microbial exposure as baseline expression could suffice. Similar to *naip,* neither *survivin* nor *xiap* mRNA responded to Lp299v incubation for 1 hr.

Using the non-adherent probiotic, we found that Lpadh- did not significantly modify either *hiap1* or *hiap2*. *Hiap2* mRNA levels remained unchanged, and *hiap1* levels were only moderately altered (118% control +/- 2.3% control). There was a slight decrease in *naip* expression in response to Lp299v, however this was not significant (58% control +/- 12% control).

Expression was assessed in an *in vitro* model of large intestinal cells as well. HT-29 Glu cells were exposed to Lpadh- and assessed for IAP gene induction. Although insignificant, there was a moderate decrease in both *naip* (88% control +/- 9.0% control) and *survivin* (95% control +/- 26% control) observed, however *xiap* mRNA tended to increase in response (175% control +/- 24% control). This is perhaps due to *xiap* being the most potent anti-apoptotic IAP [52]. Due to its cap-independent mechanism of translation in conditions of inflammatory stress, it would be expected that XIAP protein would be maintained or increased in the presence of cytokines [119]. XIAP, functioning effectively under inflammatory stress, is perhaps most suited to handle cell protection and thus an induction of the other IAPs may be redundant [119].

As previously mentioned, the fluctuation in IAP expression following probiotic exposure may be a form of microbial pattern recognition. It would also be beneficial to assess the microbial response of these genes in conditions of pro-inflammatory stress, as observed in IBD. Positive findings would provide a therapeutic basis for the use of probiotics as a means of cell protection in the face of inflammatory stress. *In vitro* experiments were therefore conducted using a probiotic pre-incubation period followed by exposure to a mixture of pro-inflammatory cytokines. Selective upregulation of cytoprotective IAPs in response to cytokine-conferred stress may be potentiated by Lp299v.

It is important to note that there were distinct gene and protein changes in response to either Lp299v or Lpadh-. This again could be due to the central role of adherence in the potentiation of cell survival signals, whereas the effects of Lpadh- could be due a secreted factor unique to *L. plantarum*. HT-29 Gal cell results indicate that exposure to cytokines only moderately increased both *hiap2* transcript levels (156% control +/- 20% control) and HIAP2 protein expression (Figure 11). Conversely, *hiap1* expression tended to be lower

(82% control +/- 10% control). While insignificant, this is consistent with decreased HIAP1 protein expression seen in Figure 11 which is reassuring. There was no alteration observed in either NAIP protein or gene expression *in vitro*.

Lp299v-conferred IAP modulation in the presence of cytokines was assessed. When cells were co-incubated with Lp299v for 1 hr prior to cytokine exposure for 15 min, *hiap2* mRNA tended to increase (184% control +/- 51% control), however HIAP2 protein did not reflect this trend.

Following Lp299v exposure, NAIP protein levels were approximately doubled compared to the control (Figure 12). Perhaps the slight increase in *naip* mRNA which was observed following Lp299v exposure subsequently provided ample transcript for later translation incurred by cytokine exposure. However moderate, elevated *hiap2* and NAIP expression would be consistent with a protective benefit from Lp299v exposure under conditions of simulated chronic inflammation, especially given that *hiap1* and *hiap2* are actively transcribed in cell stress situations [51].

Lpadh- incubation prior to cytokine exposure did not elicit similar results. *hiap2* induction tended to fall (124% control +/- 20% control). The lack of adherence by Lpadh- may lack immune detection by evasion of IECs consistent with the absence of *hiap1* modulation. As *hiap1* transcript tended to decrease in the presence of cytokines it would not be expected to change in the presence of both cytokines and Lpadh-. Accordingly there was only a slight modulation of *hiap1* noted (133% control +/- 17% control, $p<0.05$). A secreted factor that transiently up-regulates *hiap1* transcription under cytokine-mediated stress is a possible explanation. Evidence of such a secreted factor which modulates other facets of innate immunity (such as mucin upregulation and prevention of apoptosis) is gaining interest.

Recently a soluble factor was implicated in enhanced IEC survival raising the role of putative IAP involvement [120].

4.2 Macrophage Co-Culture

4.2.1 HIAP RNA and Protein Expression Analysis

We realized that using a single cell line in order to predict IAP gene response in a complicated environment such as the gut was rather simplistic. In order to increase the complexity of the *in vitro* model of inflammatory stress, HT-29 Glu cells were co-incubated with endotoxin-activated RAW264.7 macrophages for 2 days [121]. It was hoped that the presence of macrophages would more closely mimic the biochemical activity which would occur *in vivo* in response to microbes. Probiotics would encounter these activated cells under circumstances such as a break in the epithelial barrier due to IBD-conferred lesions, as macrophages are key in the compartmentalization of the mucosal immune response [121].

RNA was isolated for real-time RT-PCR analysis where it was found that *hiap1* and *hiap2* mRNA levels increased following incubation with Lp299v in co-culture (780% control +/- 11% control, $p<0.01$ and 2300% control +/- 46% control, respectively). *Hiap1* (1760% control +/- 9.0% control, $p<0.001$) and *hiap2* (3430% control +/- 146% control, $p<0.05$) were significantly altered with exposure to Lpadh- as well. In this system, there was no change in *naip* levels. Binding to mucins *in vivo* could reduce bacterial exposure to activated macrophages, making Lp299v less stimulatory than Lpadh-; when these cells come into direct contact with Lp299v however, it's possible that they activate macrophages directly. It is also possible that macrophages were able to recognize the adherent probiotic as non-pathogenic, whereas Lpadh- lead to a subsequent protective boost in *hiap* levels due to lack

of a detectable adherence factor. Attempting to make our *in vitro* system more complex in this case, lead to results that are not necessarily representative of a living system.

The absence of HIAP1 and HIAP2 protein when cells are exposed to Lp299v could be a direct result of their ability to bind and downregulate these proteins due in part to the abundance of HIAP mRNA. Since HIAP1 and HIAP2 are induced to approximately equal levels following incubation with Lpadh- or in the absence of probiotics, it is possible that Lpadh- is unable to similarly regulate HIAP1 and HIAP2, or again is seen as more of a potential pathogen. This could also be representative of the endogenous levels of these proteins in HT-29 cells as modulated by activated macrophages. Once activated, these cells may induce IAP induction in IECs in order to boost protection in the face of such a perceived threat. Upon bacterial recognition by immunological cells such as activated macrophages, appropriate cell signalling could either taper or exacerbate IAP levels in IECs [121].

4.2.2 Macrophage Activation Following Co-culture and Endotoxin Exposure

Using stains for the detection of activated macrophages, it was noted that both Lp299v and Lpadh- lead to macrophage activation *in vitro*, although not to the same extent. Lp299v activated macrophages entirely, whereas incubation with Lpadh- lead to activation of only 64.3% of macrophages. As expected, there was a high amount of macrophage activation noted in the presence of endotoxin. This strengthens the concept of relative immune evasion by Lpadh-, as it is not sensed by macrophages as effectively as Lp299v, which possesses the adherence factor. As previously mentioned, Lp299v is probably not as prone to macrophage detection due to its adherence factor and subsequent ability to bind to IECs. In the case of this experiment where probiotics came into direct contact with macrophages, it would be expected that Lp299v would cause a greater amount of activation for the same reason. This

again leads us to the importance of *in vivo* studies in order to properly assess this hypothesis in a more relevant context.

What these results do imply however, is the ability of these probiotics to activate macrophages in a context of epithelial barrier breach *in vivo*. This would lend caution towards their therapeutic use when IBD-conferred ulcers or lesions are present. As mentioned, it is difficult to assess the functioning of both cell populations when they are largely being used out of the scope of their physiological context. This experiment would be worth pursuing further in terms of macrophage-dependent apoptosis when cells are exposed to both probiotic pre-incubation and further cytokine challenge [122]. Either the implementation of an experimental IEC-barrier inclusive of tight junctions or *in situ* staining for activated macrophages in mice exposed to Lp299v would produce more relevant results.

4.3 Probiotic-Mediated Anti-Apoptotic Effect In Vivo

4.3.1 mRNA and Protein Expression following Probiotic Incubation

In order to test our insignificant yet perceived trends *in vitro* in an *in vivo* model, probiotics were administered to mouse groups over a 2 day period and the jejunum of test mice was collected for RNA and protein extraction. It was hoped that this model would produce significant results. Analysis of mRNA from these samples found that in contrast to *in vitro* studies, *hiap2* mRNA expression was significantly increased (160% +/- 1.0% control, $p<0.001$). Also in accordance with *in vitro* work, there was no alteration in *hiap2* observed when mice were treated with Lpadh- (100% control +/- 1.0% control, $p<0.001$). Further convincing are the results from rat groups in which HIAP2 protein levels are increased approximately 2-fold after Lp299v administration in jejunal samples.

Hiap1 mRNA following treatment with either Lp299v or Lpadh- failed to increase significantly (110% control +/- 4.0% control, 80% control +/- 4.0% control, respectively), although there was slight induction in mouse HIAP1 protein levels following Lp299v administration. While this is not in agreement with *in vitro* results, this is perhaps one of the discrepancies between using a cancer cell line versus a living system.

HIAP1 is the most abundant IAP present in the small intestine [67]. Due to this fact, it is hard to assess the magnitude of its presence versus a slight modification in the other two IAPs assessed (HIAP2 and NAIP). Though HIAP2 is suspect, it may also be HIAP1 that is the regulator involved in the anti-apoptotic effect of Lp299v. A specific knock-out cell model or knock-out animal model with induced colitis would facilitate the assessment of whether this protein is induced to protect IECs. It would also be worth exploring the expression of both HIAP1 and HIAP2 *in situ* and to conduct *in vivo* apoptosis assays to determine which of these proteins is responsible for the apoptotic-prevention observed via both TUNEL and caspase activity assays.

Naip mRNA levels were significantly increased following 2 day Lp299v treatment in mice (160% control +/- 7.0% control, $p<0.0001$) and in rats (170% control +/- 1.0% control, $p<0.001$). It is possible that Lp299v successfully upregulates intracellular *naip* due to its correct physiological location in villi tips in this system, as predicted following the negative results in *in vitro* experiments.

Lp299v upregulation of *naip* was found to be dose-dependent in rats. In addition, when looking at protein expression the effect was site-specific with upregulation in jejunum and not in the ileum. We found from the majority of our preliminary results that this could be due to a question of accessibility. Due to the site-specific action of the administered probiotics, it seemed that a larger dose delivered orally was effective in modulating small

intestinal gene function. However, there was little to no effect observed in the colonic cells. Conversely, when the same dose of probiotics was administered rectally in rats, there was a significant modulation of genes in the colonic cells. Possible explanations are related to effective mucosal concentration in ileum or altered jejunal adherence of the Lp299v. Lp299v was initially isolated from jejunal biopsy but it is unknown if it binds to ileal intestinal cells. The effective concentration may be more plausible as mucin gene expression work did not yield colonic upregulation with oral probiotic administraion, but when these were administered rectally mucin was significantly upregulated (unpublished, personal observation).

It follows that the larger dose administered to mice would optimize the effect we noted when examining the jejunum. As mentioned, HIAP2 was increased in the rat jejunum approximately 2-fold when rats were treated for two days with Lp299v. This effect was not noted in the ileum. Therefore, similar to what was observed for mucin site-specificity, it seems that Lp299v most effectively alters gene expression in the jejunum.

4.4 Cell Signalling

4.4.1 Probiotic Protection Appears to be NF-kB Mediated

Using a NF-kB-specific luciferase activity, it was noted that Lp299v significantly activates the NF-kB gene construct (19137 +/- 350.3 RLU). This indicates a strong NF-kB pathway activation which may be involved in the protection elicited by this probiotic. This again lends support to HIAP1 or HIAP2 being responsible for apoptotic protection, as these IAPs are regulated in part by NF-kB. Another aspect of cellular protection conferred by Lp299v could be the induction of maintenance levels of inflammation via NF-kB. This is suspected to be through signalling via the adherence factor, as Lpadh- failed to induce the

NF-kB gene construct (7969 +/- 156.4 RLU, $p<0.001$ versus Lp299v), levels similar to those of untreated control cells. However, in the presence of cytokines indicative of pre-established inflammation, Lp299v could switch cell signalling towards an anti-apoptotic IAP response. This action could also act via NF-kB due to the plethora of pathways which impinge upon this pathway. It would be of value to therefore assess NF-kB activity in the presence of both Lp299v and cytokines.

Due to the actions of Lp299v examined via the TUNEL and caspase activity assay, there seems to be a role for adherence in both NF-kB activation and the associated apoptotic-prevention observed. It is therefore through an alteration of IAPs that we suspect this is achieved. However, NF-kB has been shown to have organ-specific responses to various stimuli [123]. This transcription factor can activate numerous genes which can potentiate either a pro- or anti-apoptotic response highly contextually-dependent [123].

Due to potential induction of HIAP via the NF-kB pathway, this outlines a potential mechanism of action whereby Lp299v is recognized via NAIP or an intracellular NOD protein leading to the activation of NF-kB and the further induction of HIAP in order to boost cellular defense in the presence of cytokines. Since HIAP1 and HIAP2 seem to function in a self-regulating feedback loop [55], there probably exists a protein to cease the functioning of this pathway following the identification of Lp299v as commensal and for the tolerance that follows (*Appendix D*).

4.5 Conclusions and Future Directions

There is increasing evidence that certain probiotic strains have the ability to prevent cell apoptosis in conditions of inflammatory stress [28, 116]. While there is evidence of this phenomenon using *Lactobacillus plantarum* GG, through experimentation we have shown

that an adherent form of *Lactobacillus plantarum* 299v similarly exhibits an anti-apoptotic effect when assessed using an *in vitro* system. This strain was able to prevent caspase-dependent PCDI, and more specifically caspase-3,-7-dependent death. Using a non-adherent probiotic Lpadh- to assess the role of adherence, we demonstrated a decrease in the amount of total cell death as indicated by both TUNEL and multi-caspase activity assay, however PCDI was not significantly altered to the extent it was by Lp299v. It follows that Lpadh- effectively prevents either necrosis or caspase-independent PCDII, and we conclude that the prevention of cytokine-induced PCDI is dependent on microbial adherence.

Interestingly, when analyzing IAP activity *in vitro* following both probiotic incubation and cytokine exposure, although there was a tendency for *hiap2* to increase with Lp299v, this was not significant. However, noting distinct trends in IAP mRNA expression, we investigated protein expression. Using the *in vitro* system, protein results were rather inconclusive. We then attempted to make the system more complex by the addition of activated macrophages. When HT-29 cells were co-incubated with activated macrophages, both *hiap1* and *hiap2* mRNA levels increased in the presence of either probiotic. When IAP protein levels were examined it was found that Lp299v-treated cells had lower HIAP1 and HIAP2 levels than Lpadh-. This could be due to the ability of Lp299v to bind and moderate protein expression, however due to direct probiotic access to macrophages in these experiments, it is not known as to how relevant these results are to the complex *in vivo* intestinal environment.

It would be beneficial to use a non-cancer cell model in order to assess IAP function by means of knock-outs to assess putative IAP involvement more effectively. HT-29 cells are beneficial for use *in vitro* due to their viability, however their resistance from cell death makes them a less desirable cell model for recapitulating what occurs *in vivo*. Cells remain

sensitized to cytokine-mediated apoptosis through the use of IFN-γ, however it is possible that the signalling pathway deviates from ideal conditions.

In vivo experiments were conducted in order to assess IAP activity trends more accurately than in previous HT-29 cell work. HIAP2 was found to be moderately up-regulated although HIAP1 was not. This could be due to the baseline abundance of *hiap1* transcript in these samples as the predominant IAP of the small intestine. As IAP expression is found normally in the small intestine, the implications of IAP modification by Lp299v may be to allow cells better survival. With previous studies showing mucin increases this would be plausible. Alternatively, they may induce differentiation as NAIP expression mirrors p21 expression along villi tips.

Upon analysis of mRNA, the increase in *hiap2* suggested by *in vitro* work was confirmed *in vivo*. *hiap2* mRNA was most notably increased in mouse jejuneal samples of all IAPs assessed, and similarly, HIAP2 expression was upregulated in the rat jejunum and absent from comparable samples from the ileum. It would be useful to correlate this specific IAP activity with the anti-apoptotic effect visualized in the *in vitro* system to determine if either HIAP1 or HIAP2 are responsible for this protective effect.

As Lpadh- exposure prior to cytokine incubation fails to protect cells to the extent of Lp299v, we conclude that adherence plays a role in apoptotic cell protection. Adherence also seems to be necessary for related IAP induction, as it causes both IAP-specific modulation and demonstrates dose-dependent effects.

It was determined that Lp299v significantly activates NF-kB, whereas Lpadh- does not. We propose that as adherent microbes are sensed by an intracellular detector (NOD2 or NAIP), NF-kB is activated and moves into the nucleus to modify the expression of HIAP1 and/or HIAP2 in order to protect IECs against apoptosis (Appendix D). This cellular

protection is beneficial in the presence of inflammatory cytokines, as Lp299v is able to confer apoptotic-resistance to cells by means of IAP-mediated PCDI inhibition.

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APPENDIX A: Sequence and strand location of *IAP* specific primers used in the analysis of QRT RT-PCR results.

IAP	*Sequence*	
Human NAIP	**Probe**	5'-(FAM)CTG TAC CGT GTC CTB TTT ACC TGT AAA GAC AAA GC(TAMRA)-3'
	F-Primer (4936)	5'-GCC ATT TTA TGT CCA AGG GAT ATC-3'
	R-Primer (4935)	5'-CTT CCC AAT TTC CTA AAC ATC CA-3'
Mouse Naip1	**Probe**	5'-(FAM)AGC ATG CCA AGT GGT TCC CCA AAT G(TAMRA)-3'
	F-Primer (4987)	5'-TTC CTG TGG GGG AAG CTT-3'
	R-Primer (4988)	5'-TGG GCA ATT TCC TCT GAA GAT T-3'
Rat Naip	**Probe**	5'-(FAM)TGC CAA GTG GTT CCC CAA ATG TGA AT(TAMRA)-3'
	F-Primer (RNAIP-101)	5'-AGG AGA TGA TCC CTG GAA GGA-3'
	R-Primer (RNAIP-102)	5'-TCC TCT GTG GAT TTC TTA CTT TGA AG-3'
Human XIAP	**Probe**	5'-(FAM)CCA CGC AGT CTA CAA ATT CTG GTA TCC AGA ATG(TAMRA)-3'
	F-Primer	5'-AAT TGC AGA TTT ATC AAC GGC TTT-3'
	R- Primer	5'-CAG ATG GCC TGT CTA AGG CAA A-3'
Human HIAP1	**Probe**	5'-(FAM)CTC ACA CCT TGG AAA CCA CTT GGC ATG(TAMRA)-3'
	F-Primer (4925)	5'-TGG AGA TGA TCC ATG GGT TCA-3'
	R-Primer (4924)	5'-GAA CTC CTG TCC TTT AAT TCT TAT CAA GT-3'
Human HIAP2	**Probe**	5'-(FAM)CTC ACA CCT TGG AAA CCA CTT GGC ATG(TAMRA)-3'
	F-Primer (4927)	5'-TCT GGA GAT GAT CCA TGG GTA GA-3'
	R-Primer (4926)	5'-TGG CCT TTC ATT CGT ATC AAG A-3'

Mouse HIAP1	**Probe**	5'-(FAM)TTG AGC AGC TAT TAT CTA CGT CAG ACT CCC CA(TAMRA)-3'
	F-Primer (4991)	5'-AAG TTC AAG CTG GCT ATC CTC ATC-3'
	R-Primer (4992)	5'-ACG ATT GCT GCG TCT GCA T-3'
Mouse HIAP2	**Probe**	5'-(FAM)TTC TTG AGC AGC TGT TGT CCA CTT CAG ACA(TAMRA)-3'
	F-Primer (4990)	5'-TAC GGA TGA AGG GTC AGG AGT TT-3'
	R-Primer (4989)	5'-TCT GTA GGG TCA GCA TTT TCT TCT T-3'

APPENDIX B: Raw densitometric data for Western blots.

Figure	Protein	Lane	AUC*
11	HIAP1	1	768
		2	1146
		3	3028
		4	0
		5	0
	HIAP2	1	5889
		2	7578
		3	8504
		4	5074
		5	1784
	GAPDH	1	5862
		2	6586
		3	8757
		4	7174
		5	5656
12	NAIP	1	2985
		2	5345
		3	9642
		4	9661
		5	8218
		6	5074
	GAPDH	1-6	Equivalent
14	HIAP1	1	Saturated
		2	0
		3	1070
		4	2399
		5	1425
	HIAP2	1	15519
		2	0
		3	0
		4	2137
		5	13105
	GAPDH	1	936
		2	476
		3	2435
		4	3263
		5	4132
23A	HIAP1	1	6822
		3	7446
		4	7342
		5	7626
		6	8653
	HIAP2	1	6104

		3	6765
		4	6019
		5	5243
		6	6273
	GAPDH	1	8043
		3	8489
		4	9125
		5	8356
		6	8699
23B	NAIP	1	10036
		4	10405
		5	11876
		6	9524
		7	9840
	GAPDH	1	7306
		4	7052
		5	7686
		6	7169
		7	7388
24	HIAP1	1	0
		2	0
		3	0
		4	0
	HIAP2	1	0
		2	2839
		3	1415
		4	1099
	GAPDH	1	5400
		2	5710
		3	4056
		4	3501

**AUC: Area Under the Curve as calculated in pixel density.*

APPENDIX C1: Summary table of HT-29 *in vitro* results.

Test		Lp299v
mRNA	*hiap1*	Not significant
	hiap2	Not significant
	Naip	Not significant
Protein	HIAP1	Same as Lpadh-
	HIAP2	Same as Lpadh-
	NAIP	Same as Lpadh-
Apoptosis	TUNEL	Significant decrease
	Caspase	Significant decrease
	Caspase-3, -7	Significant decrease
NF-kB		Significant activation

APPENDIX C2: Summary of macrophage studies.

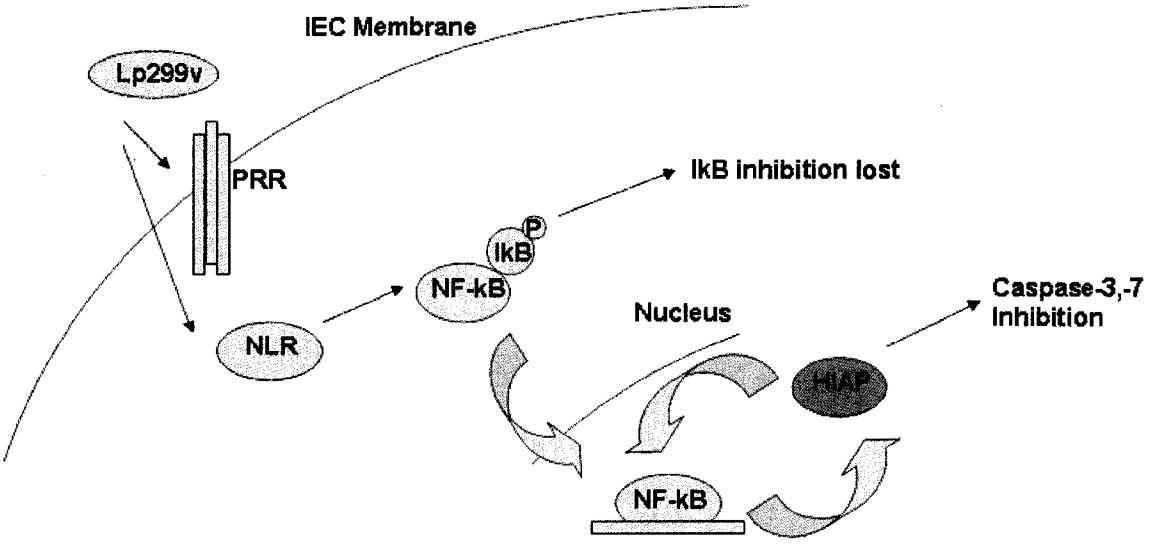
Test		Lp299v
mRNA	*hiap1*	Significant increase
	hiap2	Not significant
	Naip	Not assessed
Protein	HIAP1	Trace amounts
	HIAP2	None
	NAIP	Not assessed
Activation		Significant increase

APPENDIX C3: *In vivo* IAP expression in response to Lp299v.

	Test		Lp299v
Mouse	mRNA	*hiap1*	Not significant
		hiap2	Significant increase
		Naip	Significant increase
	Protein	HIAP1	Moderate increase
		HIAP2	No change
		NAIP	No change

	Test		Lp299v
Rat	mRNA	*hiap1*	Not assessed
		hiap2	Not assessed
		Naip	Significant increase
	Protein	HIAP1	None
		HIAP2	2-fold increase
		NAIP	Not assessed

APPENDIX D: Proposed Schematic.



PRR: Pattern Recognition Receptor (ie. TLRs)
NLR: Nod-Like Receptor (ie. NOD2, NAIP)

Natalie S. Dykstra (Née Godwin)

EDUCATION

Master of Science in Biochemistry
Thesis Submission: September 20, 2007
University of Ottawa, Ontario

- **Research Project:** The Investigation of Microbial-Intestinal Epithelial Cell Interactions in the Gut and their Effects on Inhibitor of Apoptosis Proteins (IAPs)
- **Project Supervisors:**

Dr. Alex MacKenzie, MD PhD, Children's Hospital of Eastern Ontario
Dr. David Mack, MD, Children's Hospital of Eastern Ontario

Bachelor of Science, Honours Biopharmaceutical Science, Major in Genomics
2005
University of Ottawa, Ontario

- **Research Project:** Intestinal Mucin Gene Modulation in Response to Probiotic Bacteria
- **Project Supervisors:**

Dr. Alex MacKenzie, MD PhD, Children's Hospital of Eastern Ontario
Dr. David Mack, MD, Children's Hospital of Eastern Ontario

PROFESSIONAL DEVELOPMENT

Publications:

1. **Godwin, N.S., Hyde, L., Mack, D.R. and A.E. MacKenzie.** *Intestinal Mucin Gene Over-Expression Induced by Probiotics is Associated with a Reduction of Dextran Sodium Sulphate Induced Colitis in Sprague-Dawley Rats.* (Manuscript, to submit).
2. **Godwin, N.S., Hyde, L., Mack, D.R. and A.E. MacKenzie.** *Probiotic Pulse Therapy Imparts Ongoing Modulation of Intestinal rMuc3 Mucosal Mucin Expression in Rats.* (Manuscript, in preparation).
3. **Godwin, N.S., Hyde, L., and D.R. Mack.** *Probiotic-Mediated Inhibition of Apoptosis.* Abstract. The Canadian Journal of Gastroenterology, 2007:(21) Suppl.A:1A-176A.
4. **Godwin, N.S., Hyde, L., and D.R. Mack.** *Intestinal Mucin Gene Modulation in vivo using Orally Administered Probiotic Bacteria.* Abstract. The Journal of Pediatric Gastroenterology and Nutrition, 2005.

Conferences:

February 16-20th, 2007: I presented "Probiotic-Mediated Inhibition of Apoptosis" in the first oral plenary session of the Canadian Digestive Disease Week (CDDW) Conference of the Canadian Association of Gastroenterology (CAG).

October 2005 : I was a primary speaker at the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN) conference held in Salt Lake City, Utah. I presented my research results as "Intestinal mucin gene modulation in vivo using orally administered probiotic bacteria."

Awards & Honours:

Crohn's and Colitis Foundation of Canada Student Research Prize: Awarded for my submitted research to the 2007 CDDW conference. CCFC student research prize was formally awarded at the annual awards ceremony held during the conference and applauded my work using probiotics for experimental treatment for IBS.

CHEO Research Institute Dr. J. Wiley Scholarship Fund: $1500 CDN awarded on the basis of merit in working with a researcher at the CHEO Research Institute on a project related to the field of pediatric research.

Grants & Proposals:

Rosell Proposal, 2006: Personally prepared and submitted a research proposal and budget workup for the next year of natural health research in our lab for submission.

Summer Studentship, 2004: Recipient of a research grant from Abbott Laboratories.

DISTINCTIONS

- Melotte Installatietechniek Award of Excellence, 200 Dutch Guilders
- Hermes Groep Award of Excellence, 300 Dutch Guilders
- Reids Company Limited Prize in Biology, $100 CDN
- Lieutenant Governor's Community Volunteer Award for Students
- Induction into the National Honour Society
- University of Ottawa Admissions Scholarship
- Aiming for the Top Tuition Scholarship, $100 CDN
- University of Toronto Book Award
- Canada Day Youth Award, trophy
- Rotary Club Scholarship, $200 CDN
- Student Leadership Excellence Award, $100 CDN

EMPLOYMENT & VOLUNTEER EXPERIENCE

Present

Medical Researcher

Apoptosis Research Center, CHEO Research Institute Phase II

CHEO, 4

- Currently completing an M.Sc research project using probiotics both *in vivo* and *in vitro* to determine both mechanism of action and efficacy of probiotic supplementation to counter conditions of inflammatory bowel syndrome (IBS)
- Train and supervise summer student research projects

Present
Science Outreach Partnership Program Volunteer
Let's Talk Science
Lady Evelyn Alternative School,

- I am partnered with the National Coordinator of *Let's Talk Science* and together we work to create innovative programs for students in grades 1-6, working with their teachers to explore and encourage science in the classroom. We are currently partnered with eight teachers and have already worked hard this year to explore the concepts of magnetism, photosynthesis, and Rube Goldberg machines as they apply to physics

2005 & 2006
Teaching Assistant
Faculty of Science, Biochemistry, University of Ottawa

- Conducted laboratory sessions for undergraduate biochemistry and molecular biology classes, graded laboratory reports and assessed student performance

2004-2005
High School Science & Math Tutor
Ottawa-Carleton School Board Tutors in the Classsroom Program
OCSB 133

- Tutor at Lisgar Collegiate Institute, Grades 9-12 advanced science and math courses

2003
Activity Program Coordinator
Rideau Gardens Retirement Residence

- Volunteer on the assisted care floor of the home, working with residents with Alzheimer's Disease and developing activity program

2001
Volunteer, Maternity Ward
Belleville General Hospital Maternity Ward

- Worked as a volunteer throughout the year with duties including: Preparation of pediatric medical supplies such as facial masks and dressings, post-operative and post-delivery patient interaction and instruction

Summer 2000
Dental Assistant/Laboratory Volunteer
Geilenkirchen American Dental Clinic
Bldg 198, NATO Airbase, Geilenkirchen, Germany.

- Aided in keeping medical records, working the front desk, chair-side dental assistance, inventory, laboratory work, and cleaning/sterilizing of treatment rooms

1999-2000
Elementary School Teacher's Assistant
AFCENT International Elementary School
APO AE 09703, Brunssum, The Netherlands.

- Position in the pre-teacher co-op program, with the following duties: Lesson planning, teaching, and the evaluation of students