An investigation of the relationship between dietary fiber, fecal bacterial composition, and colon cancer

by

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B. Sc. Honours-Carleton University, 2010

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE AND POSTDOCTORAL AFFAIRS IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE WITH SPECIALIZATION IN CHEMICAL AND ENVIRONMENTAL TOXICOLOGY.

MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY

CARLETON UNIVERSITY
OTTAWA, ONTARIO, CANADA

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-93547-7

Our file Notre référence
ISBN: 978-0-494-93547-7

ABSTRACT

Colon cancer (CC) is the second leading cause of all cancer-related deaths in North America. Dietary fiber (DF) may be an important risk factor in the aetiology and pathogenesis of CC. The anticancer effects of dietary fiber were investigated with a focus on fecal bacterial diversity and toxicity of bacterial metabolites in the aqueous phase of feces (fecal water: FW) that contains bile acids, short chain fatty acid, lactate, succinate, etc. Briefly, male Fischer-344 rats were randomized to one of 3 diets: alphacel (control), fructooligosaccharides (FOS) or wheat bran (WB) with a total fermentability level of 3% (wt/wt). Rats were injected with saline or azoxymethane (AOM) to induce tumors. FW toxicity was tested on HCT-116 cells. Rats fed alphacel and FOS diets had significantly more colon tumors than those fed WB. FW from both FOS and alphacel significantly increased apoptosis and DNA damage, and induced cell cycle arrest in HCT-116 cells after a 48 hr treatment whereas FW of WB had no effect on those cell parameters. Lower pH of FW was associated with more tumors incidence and higher cell toxicity. FOS diet was significantly associated with more *Allobaculum sp.* whereas *Lactobacillus sp.* and *Clostridium XI sp.* were associated with WB diet. These results suggest that dietary fiber can be an influence in CC development. This seems to be related to changes in bacterial population and bacterial metabolic activities.

ACKNOWLEDGEMENTS

First of all, I would like to remember Almighty Allah (God) for giving me the strength in the entire period of my great success. I would like to take this opportunity to formally thank both my supervisors Dr. Stephen Brooks and Dr. Kenneth Storey for the knowledge, patience, time, and unlimited supports they have provided over the last two years. I am so grateful for the opportunities to conduct this research, explore my passions, and grow as a little scientist and individual.

I would like to emphasis my great appreciation for Jan Storey, Judy Green and Fernado Matias's enduring patience and assistance in the preparation of this thesis, edits of my works, discussions and interchange of ideas. Both Judy and Fernado also helped me in following experimental protocol.

I would like to thank Dr Jayadev Raju and his lab members to allow me to work in their cell culture lab and for providing me with tumor data. Thanks to Emily Chomyshyn for her help with flow cytometer data analysis. I would like to thank all of my teachers and professors for their massive contributions in my study life.

I am grateful to my family for their love, encouragement, financial and moral support.

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LIST OF ABBREVIATIONS

AOM	azoxymethane
ATP	adenosine triphosphate
bp	base pair
Br-dUTP	bromolated deoxyuridine triphosphates
BSA	bovine serum albumin
CC	colon cancer
DMSO	dimethyl sulfoxide
DF	dietary fiber
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
FITC	fluorescein isothiocyanate
FOS	fructo-oligosaccharide
Fo	feruloyl oligosaccharide
FW	fecal water
GIT	gastrointestinal tract
HCT-116	human colorectall carcinoma cell line
Hg	mercury
hr	hours
min	multiple intestinal neoplasia
NaBut	sodium butyrate
OTU	operational taxonomic unit
ODC	ornithine decarboxylase

PA	phytic acid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PS	phospholipid phosphatidylserine
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDG	secoisolariciresinol diglycoside
SCFA	short chain fatty acid
SFP	Sulforaphane
SOC	super optimal catabolite
TAE	tris-acetate buffer
TdT	terminal deoxynucleotidyl transferase enzyme
UV	Ultraviolet
WB	wheat bran

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CHAPTER 1

General Introduction

Colon cancer (CC) is the second leading cause of all cancer-related deaths in both men and women in North-America (Canadian Cancer Society: Canadian Cancer Statistics 2011; Jemal *et al.* 2011). According to the American Cancer Society, there were about 104,950 new cases of colon cancer and 40,340 new cases of rectal cancer in 2005 in the United States. These will cause about 56,290 deaths. The risk of colon cancer increases after the age of 40, and in fact, more than 9 out of 10 people found to have colon cancer are older people (Mager 2006). Several factors play a role in the initiation and development of CC, including genetic and environmental factors as well as lifestyle components including smoking, obesity, non exercise, alcohol consumption, and sedentary lifestyle (Giovannucci 2002; Bird 1986). It has been estimated that approximately 30% of cancers are related to lifestyle and dietary factors (Donaldson 2004).Environmental factors include persistent pollutants, certain dietary constituents, and nutritional factors such as excessive energy intake.

Dietary fiber (DF) may be protective against colon cancer development. DF is the cell wall components of plants that consists of complex carbohydrate polymers that reach the colon without being digested (Young *et al.* 2005). Intake of dietary fiber is extremely variable across individuals and throughout the world. Different kind of foods contains different types of fiber and these fibers ferment differently in various regions of the gut that alters the environments of the gut (McIntyre *et al.* 1991; Folino *et al.* 1995). DF is divided into two different classes: soluble fiber (such as fruits, oats, etc.) and insoluble fiber (e.g. wheat bran, cellulose, lignins, and hemicelluloses). Studies have shown that insoluble fiber is more protective than soluble fiber in animal models and humans because insoluble fiber trends to ferment in the distal colon (McIntyre *et al.* 1991)

whereas soluble fiber is more proximally and more rapidly fermented in the cecum and proximal region of the gut. The insoluble fiber had greater impact on reducing secondary bile acid (Reddy *et al.* 2000). Recently, it has been shown that total dietary fiber intake was not associated with colon cancer development whereas consumption of whole grain had an association with reduction of colon cancer (Schatzkin *et al.* 2007). In addition, it was found that the highest intake of dietary fiber decreased the risk of colon cancer compared with the lowest intake in both men and women (Wakai *et al.* 2007). On the other hand, studies found that consumption of a diet low in fat and high in fiber, fruits, and vegetables did not reduce the risk of colon adenomas in both men and women (Schatzkin *et al.* 2010).

The human gut harbours 15,000-36,000 bacterial species, amounting to more than 100 trillion bacteria (Frank *et al.* 2007; Neish 2009) and their composition in the human gastrointestinal tract is highly diverse and variable across individuals (Gosalbes *et al.* 2011). Bacteria play significant roles in various biological process including metabolic capacity, maturation and development of the mucosal immune system, and cell proliferation and differentiation (Turnbaugh *et al.* 2007). Fermentation of dietary materials (e.g., carbohydrate and protein) produces metabolic end products that may interact with the host (Zhu *et al.* 2011) and provides substrate for bacterial growth and reproduction. The gut bacteria also interact with the gut associated lymphatic tissue (GALT), which has been suggested to heighten immunity and provide protection against the growth of pathogens as well as reduce susceptibility to chronic disease (Davis and Milner 2009; Wexler 2007). The colonic microflora influence inflammatory bowel disease (IBD) pathogenesis (e.g. Crohn's disease and ulcerative colitis) (Frank *et al.*

2007) and may play an important role in the development of colorectal cancer through the production of phenolic compounds, amines, N-nitroso compounds, indoles, and inositol phosphates (Davis and Milner 2009) and many others (Cummings and Bingham 1987). A significant risk associated with IBD is the development of colorectal cancer (Uronis and Jobin 2009). Uronis and Jobin (2009) proposed that bacteria use Toll-like receptor (TLR) mediated signalling components such as myeloid differentiation factor 88 ($_{MYD}88$) to promote the development of colon cancer. Furthermore, innate bacterial sensing receptors such as Toll-like-receptor (TLR) and Nod-like receptor alert the host to the presence of bacteria (Beutler 2009). Bacteria may produce toxins (e.g. procarcinogens, carcinogens, or cocarcinogens) and bioactivate fecal carcinogens that have been shown to induce DNA damage and apoptosis in cultured colon epithelial cells. It is also possible that colonic bacteria participate in the development of inflammation of the colon and inflammation-associated colon carcinogenesis (Terzix *et al.* 2010; Davis and Milner 2009; Mager 2006; Moore and Moore 1995; Zoetendal *et al.* 2008).

Diet plays an important role in physiological processes since it provides substrate in the form of dietary fiber (DF) and will, therefore, dictate the type of metabolites that are produced in the lumen and will influence the relative abundance of the resident bacterial species and strains. In addition, DF may have some physical properties (such as the ability to bind bile acids or minerals) that can also influence the availability of these metabolites throughout the colon. DF has been defined as: "carbohydrate polymers with ten or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans" (Codex Alimentarius Commission 2008). The CODEX Alimentarius Commission is an international organization that develops food standards,

guidelines and codes of practice to protect consumer health and promote fair trade practices. The Commission, established in 1963 by the FAO and WHO, also promotes the coordination of food standards work undertaken by international governmental and non-governmental organizations (CODEX Alimentarius Commission 2012). The current DF CODEX definition is written in such a fashion to give individual jurisdictions the power to include or exclude oligomers. In Canada, all carbohydrate oligomers of degree of polymerization (dp) ≥ 3 that reach the large intestine are considered as DF.

DF has long been believed to have antitumorigenic properties (Kim and Milner 2007) related to its processing by the colonic bacteria community and its physical effect on physiological processes. In the colon, DF is fermented to short chain fatty acids (SCFA such as acetate, propionate, and butyrate), lactate, succinate, and metabolic products that include vitamins but also many other substances (Cummings and Bingham 1987). The SCFA decrease fecal pH, which has, by itself, been proposed as a marker for improved colonic health (Salminen *et al.* 1998). Particulate dietary fiber and material that is more slowly fermented in the colon has been linked to a regularisation of the colonic transit time and subsequent increased laxation (Cummings 1984) and increased stool output has also been inversely correlated with colon cancer rates around the world (Bingham *et al.* 1992). There is epidemiological evidence to suggest that DF reduces the risk of colon cancer in humans (Mager *et al.* 2006) but trials with wheat bran supplemented subjects have not confirmed this data (Macrae 1999; Schatzkin *et al.* 2000; Cottet *et al.* 2005).

DF from various sources (e.g. high amylose maize starch, fructooligosaccharides, etc.) was effective in reducing the number of aberrant crypt foci (ACF) in the colon of

rats (Nakanishi *et al.* 2003; Buecher *et al.* 2003), induced apoptosis in rat colon (Pool-Zobel *et al.* 1996), reduced colon tumor occurrence in *Min* mice (Pierre *et al.* 1997), and might lower the risk of coronary heart disease, stroke, hypertension, diabetes, and obesity (Saura-Calixto 2011).

The original *Min* mouse has a truncation mutation at codon 850 of the APC gene. This causes the spontaneous appearance of up to 100 polyps in the small intestine as well as colon tumors. Newer APC knock out models (truncating mutation at codon 716) can develop up to 300 polyps. A combination of the 716 mutation with a mutation in the CdX2 gene has shifted polyp formation to the colon. The *Min* mouse colon cancer model has been used to evaluate the effect of diets and chemopreventive agents, as well as understanding of carciogenesis.

Cancer initiation is followed through the appearance of aberrant crypt foci (ACF), which are precursor lesions of the adenoma-carcinoma sequence in both humans and experimental animals. They are larger and have a thicker layer of epithelial cells than normal cells and can be identified though microscopy of stained tissue (Takayama et al. 2005; Pretlow and Pretlow 2005). ACF forms before colorectal polyps and represents the earliest changes in the colon that may lead to cancer. A diagram of ACF is shown in Figure 1A. ACF were first identified as lesions with a microscope by staining surgical specimens with methylene blue agent (Takayama et al. 2005; Pretlow and Pretlow 2005).

Figure 1.01: Figure illustrating aberrant crypt foci (ACF) in the colon of rats treated with (DMH) after staining with methylene blue. Adapted from Kumar et al. (2010). Arrow indicates location of ACF.

Butyrate is thought to be the most important metabolite among the SCFA and is believed to play a protective role in colon cancer initiation. Recently, it has been suggested that butyrate might have protective effects on the colon because it induces apoptosis, gene expression, cell differentiation, and decreases the conversion of primary bile acids to secondary bile acids (Heetdt et al. 1994; Wang et al. 2006; Chapkin et al. 2000; Wong et al. 2010). DF is fermented in the colon by microflora to produce SCFA, including butyric acid. Butyric acid is the primary fuel used by normal colonocytes. It appears to play an important role in gene expression, cell cycle and apoptosis in colon epithelial cells (Wang et al. 2006; Chen et al. 2010). It's protective role in human colon cancer is thought to be through an inhibition of histone deacetylase and a promotion of apoptosis (Lupton 2004). In addition, butyrate has an anti-proliferation activity on many human colon cancer cell lines (e.g. CaCo-2, HT-29, and HCT-116) and suppresses adenoma development (Wong et al. 2006; Hijova and Chmelarova 2007). However, there are data that suggest that butyrate also promotes tumour initiation and growth. For example, administration of butyrate in drinking water actually enhanced tumor development in rats compared with control (Freeman 1986), although it is unclear whether the butyrate actually reached the large intestine or not. Lupton (2004) suggested that the chemo-preventive effect of butyrate depends on the amount, exposure time with respect to the stage of tumor, and type of fat in diets. Caderni et al. (1998) showed that there was no protective effect of butyrate on aberrant crypt formation (ACF) (a precursor of colon tumours) after butyrate was supplied in the form of slow release pellets to the colon of rats. Based on these findings the term "the butyrate controversy" was coined."

Intake of diets high in fat (varying in fatty acid composition) has been shown to increase the concentration of surfactants (e.g. bile acids and fatty acids) in the feces, which may damage colon epithelial cells and may lead to an increased risk of developing colon tumors (Bernstein *et al.* 1999; Lapre *et al.* 1993; Ten Bruggencate *et al.* 2006). This highlights the fact that several dietary components can impact colon health.

Azoxymethane (AOM) is an orally active chemical compound. AOM is not only a carcinogenic compound but also a genotoxic compound that induces tumors in the colon of rats, mice, and rodents (Takahashi and Wakabayashi, 2004; Sohn *et al.* 2001). AOM can be metabolically activated by the enzyme cytochrome P450 2E1 (CYP2E1). The hydroxylation of AOM by CYP2E1 induces DNA guanine alkylation in various organs (e.g. colon, liver, kidney, and lung), and promotes the formation of colonic aberrant crypt foci (precursors of tumor formation; ACF) *in vitro* and *in vivo*. Methylazoxymethanol is a proximate metabolite of AOM, an unstable compound with a half-life of approximately 12 hr under physiological conditions (Nagasawa *et al.* 1972; Feinberg and Zedeck 1980). Methylazoxymethanol spontaneously decomposes to formaldehyde and a highly reactive alkylating agent (e.g. methyldiazonium ion) (Nagasawa *et al.* 1972) that is responsible for DNA alkylation in the colon of rats and mice. Methylazoxymethanol can be readily transported to the colon via the bloodstream after conjugation with glucuronic acid (Sohn *et al.* 2001, Fiala *et al.* 1991). The glucuronide conjugate is deconjugated by microbial β-glucuronidase, and then released methylazoxymethanol can attack colonic mucosal DNA (Pozharisski *et al.* 1975, Fiala 1977). It was noticed that *E. coli* and *Clostridium* have the highest level of β-glucuronidase whereas *Lactobacillus* and *Bifidobacterium* have lower levels (Hawkswotith *et al.* 1971). In support of this, Lee *et al.* (1999) demonstrated that

an intake of *Bifidobacterium longum* HY8001 significantly lowered the activities of fecal bacterial enzymes (e.g. β-glucuronidase and nitroreductase) in human volunteers. Furthermore, consumption of *Bifidobacterium longum*, inulin or both as dietary supplements decreases β-glucuronidase activity and ammonia concentration in the fecal contents of rats (Rowland *et al.* 1998). Alcohol dehydrogenase plays a significant role in the activation of methylazoxymethanol in the colon mucosa of rats (a tissue with a deficiency of cytochromes P450) (Sohn *et al.* 1991). However, AOM and methylazoxymethanol could be human carcinogens, but not commonly consumed. However, methylazoxymethanol occurs naturally as a product of palm *Cycas circinalis* (Laqueur and Matsumoto 1981). Carcinogenic compounds associated with diets are primarily metabolized by different enzymes including cytochrome P450 which is found in the liver. Cytochrome P450 is a family of powerful detoxifying enzymes. There are many subclasses of CYP450 present in humans and bacteria. Over 60 forms of CYP450 are known with hundreds of genetic variations possible (Spatzenegger et al. 2001). The bacterial enzymes might also play an important role in inducing toxicity. Diets are metabolized by host enzymes and produce different types of metabolites (e.g. nitrosocompounds, fecal bile acids, hetero cyclic amines, etc.) that may induce cellular damage in tissues. The metabolites of carcinogenic compounds have been shown to induce toxic effects in different organelles, but primary compounds such as AOM are usually not toxic.

The P450 metabolites are transported to different parts of body with the help of different transport proteins (Multi-drug resistance protein, P-glycoprotein, albumin, etc.). Once in the cell, these metabolites mostly target DNA and induce changes in gene

expression. Different compounds or metabolites have different physiochemical properties that determine the solubility of those compounds in different organs because different organelles have different chemical compositions. For example, lipid soluble drugs or compounds are metabolized in the liver whereas water soluble drug are metabolized in the blood.

The glutathione-S-transferase (GST) is an enzyme that detoxifies metabolites such as N-acetyl-para-benzoquinone imine (NAPQI) via a conjugation reaction. However, if glutathione stores are depleted, the toxic metabolites (e.g. NAPQI) covalently bind to cellular proteins and DNA and disrupt the function of cellular proteins (Lee 2004). For example, butyrate induces GST in cultured CaCo-2 cells to enhance the detoxification capacity of intestinal cells against carcinogens (Stein et al. 1996; Treptow-van Lishaut et al. 1999), and inhibit the genotoxic activity of nitrosamides and H_2O_2 in human colon cells (Wollowski et al. 2001).

OBJECTIVES

The aim of my research is to identify whether anticancer effects are associated with dietary fiber (DF) and perhaps begin to show the relationship of dietary fiber to cancer aetiology. The information gathered will be used by Canadian Food Regulators to evaluate the safety and efficacy of dietary fiber.

The aim of phase I (chapter 2) is to determine the effects of different fiber sources on the species diversity of gut resident bacteria in rats.

The aim of phase II (chapter 3) is to investigate whether fecal water derived from different dietary fiber types modulates the growth of human colon cancer cells either *in vitro* or in an *ex-vivo* system.

CHAPTER 2

Fiber source effects on the populations of resident bacteria in the feces of rats

INTRODUCTION

The origin and development of colon cancer is a multistage process (e.g. initiation, promotion, and progression) and is complex (Foulds 1958). The ultimate cause is DNA damage in the proto-oncogenes and tumor suppressor gene that leads to cancer initiation (Fearon and Vogelstein 1990). However, several factors can play a role in cancer initiation and the growth of tumors. Colon cancer may arise from the combined actions of environmental factors such as genotoxic agents, dietary factors, and endogenous formation of tumorigenic substances (Rao *et al.* 1991). Prevention has been associated with daily intake of fermented vegetables and fermented-milk products which can decrease the bacterial enzymatic activities such as β-glucuronidase, and nitroreductase enzymes (Wollowski *et al.* 2001). A high intake of protein-rich and fat-rich diets increases the glucuronidase activity which leads to formation of a higher amount of toxic compounds (e.g. bile acids, heterocyclic amines, etc.) in the colon of rats (Wollowski *et al.* 2001).

The gut processes and digests food and regulates the rate and extent of macronutrient breakdown and absorption through the epithelial cells (Salminen *et al.* 1998). The absorption rate of nutrients (e.g. calcium) and metabolites (e.g. bile acids, SCFA, etc.) is varied across the gut (Mitra and Flynn 2007). While the upper GI tract provides the host with the majority of metabolic fuels or energy-rich nutrients and mineral/vitamins derived from food, important events also occur in the lower gut. Dietary material such as resistant starch and non-starch polysaccharides (e.g. cellulose, hemicelluloses, pectin and gum) that escape digestion in the upper gastrointestinal tract can enter into the lower gut and undergo fermentation. This material can include proteins

(e.g. elastin, collagen and albumin) which can be used as growth factors for colonic microflora (Salminen *et al.* 1998). Foods are fermented and digested in the lower gut by the combined action of intestinal microflora and host enzymes (e.g. primary bile acid and pancreatic juice). The gut typically contains about 10^{11}–10^{12} bacteria per gram feces and their activities are significantly different in the different regions of the colon. The bacterial number and composition varies though the lower gastrointestinal tract because of changes in luminal pH and content (Salminen *et al.* 1998). The right (proximal) colon has high substrate availability due to dietary input with low pH and rapid transit whereas the distal colon has lower concentrations of substrates with neutral pH and bacteria grow more slowly. The proximal region tends to be a more saccharolytic (hydrolysis of sugars) environment than the distal gut, the latter having higher bacterial proteolysis (Salminen *et al.* 1998).

Fermentation plays an important role in food processing. Specifically, fermentation of dietary fiber takes place in the large intestine with the combination action of fecal bacteria under anaerobic conditions. DF has been defined differently through the years, but can be thought of as carbohydrate polymers that are not hydrolyzed by the endogenous enzymes in the small intestine of human (Trowell *et al.* 1972). The fermentation process produces short chain fatty acids (e.g. butyrate, acetate and propionate) that can be used as an energy source by the host and are specifically used by the epithelial cells lining the lower gut (Topping and Clifton 2001; Salminen *et al.* 1998). It has been suggested that butyrate acts as controlling substrate for the suitable ratio of beneficial bacteria (e.g. *Roseburia, Lactobacilli*, etc.) in the colon of rats and humans (Salminen *et al.* 1998; Barcenilla *et al.* 2000). The rate of fermentation and production of

metabolites depend on type of dietary fiber and may vary across individuals, and includes species to species variability that may play significant roles in colon cancer development. The relationship between fermentation and tumor development is not simple and depends on several factors.

Diets can modulate the composition of microflora (Salminen *et al.* 1998). The bacterial cells secrete hydrolyase enzymes that help to digest a wide range of carbohydrates. Topping and Clifton (2001) proposed that the principal products of fermentative reactions are SCFA (acetate, propionate, and butyrate), succinate, lactate, CO_2, CH_4, H_2, and heat in humans, as described in equation 1 below. In addition, substrate specific differences in fermentation are apparent. Diets and the use of drugs (e.g. antibiotics) can significantly alter the microbial composition which is associated with inflammatory diseases including Crohn's disease and ulcerative colitis (Zoetendal *et al.* 2008). Fructo-oligosaccharide (FOS) and galacto-oligosaccharide (TOS) diets significantly increased H_2 and CH_4 excretion whereas a gluco-oligosaccharide (GOS) diet only increased CH_4. All three oligosaccharide diets significantly increased total SCFA and decreased fecal pH in humans (Djouzi and Andrieux 1997). Andrieux *et al.* (1991) previously showed that a FOS diet modified the excretion ratio of H_2 and CH_4 in gnotobiotic rats. The CH_4-producing flora was frequently found in the low risk of colon cancer populations (Segal *et al.* 1988).

$$\mathbf{59\ C_6H_{12}O_6 + 38\ H_2O \rightarrow 60\ CH_3COOH + 22\ CH_3CH_2COOH + 18\ CH_3CH_2CH_2COOH + 96\ CO_2 + 268\ H^+ + heat + additional\ bacteria}$$

(Equation 1)

Bacteria play an important role in fermentation and developing a barrier that prevents pathogenic bacteria from invading the gastrointestinal tract (Salminen *et al.* 1998). Tuohy *et al.* (2005) suggested that colonic microflora may mediate the effects of diet, reducing the risk of colon cancer development. Bacteria also synthesize vitamins; metabolize xenobiotics, and ferment carbohydrates (and amino acids) that escape digestion in the small intestine.

Bacteria have also been postulated to play a direct role in colon cancer through their effects on cells. For example, Moore and Moore (1995) found that some Lactobacilli species may decrease the risk of colon cancer development. An increased intake of *Lactobacilli* and *Bifidobacteria* were effective in protecting rats from colonic DNA damage as measured by the Comet assay (Pool-Zobel *et al.* 1996; Tuohy *et al.* 2005). It was also found that providing *Lactobacilli* and *Bifidobacteria* strains in diet supplements decreased mutagenic and carcinogenic metabolites (they are also known as enzymes) (e.g. urease, nitroreductase, etc. and microbial enzymes (e.g. β-glucuronidase, β-glucosidase, etc.) in healthy humans (Roberfroid, 2000). These bacteria also significantly inhibited 2-amino-3-methylimidazo[4,5-] quinoline (IQ)-induced colon and liver tumors, as well as reduced a multiplicity of colon, liver, and small intestinal tumors in male rats (Reddy and Rivenson 1993). Microbial enzymes, specifically β-glucuronidase, may play a role in the activation of carcinogens (AOM). In addition, some *Bacteroides* species can induce oxidative DNA damage and alter signaling pathways by converting fat soluble materials to secondary bile acids and fecapentaenes which are related to colon carcinogenesis (Povey *et al.* 1991; Sghir *et al.* 2000).

Although fermentation products are generally thought to be beneficial, they can be detrimental to colonic cells. For example, the final fermentation products of protein are phenolic compounds, amines, N-nitoso compounds, and indoles that are known as toxic or carcinogenic compounds to the host (Davis and Milner, 2009). In addition, research found that several bacteria themselves produce toxins that may alter cell cycle and apoptotic pathways, which may initiate tumor formation (directly causing mutations) or promoters (facilitating mutations) (Mager, 2006).

The *Lactobacilli* and *Bifidobacteria* species are most widely used as probiotics. The current definition for probiotics is: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [meeting of the FAO/WHO (2008) Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, American Córdoba Hotel, Córdoba, Argentina, October 1-4, 2001]. In the past, they have been thought to act on the indigenous microbioal population, rather than the host. Parker (1974) was the first to define probiotics as "organisms and substances that contribute to intestinal microbial balance". They are available to consumers as powders, tablets, drinks and fermented dairy products as well as a supplement with traditional fermented foods (e.g. yoghurt, sauerkraut, and kefir) (Holzapfel and Schillinger 2002)..

Reddy *et al.* (1997) found that feeding of oligofructose and inulin, used to stimulate the growth of *Bifidobacteria,* significantly inhibited ACF formation in AOM-treated F344 rats. However, the FOS diets did not significantly increase the growth of *Bifidobacteria* so the association with beneficial bacteria is questionable in this study. Some countries (e.g. Brazil, Singapore, and Malaysia) consider inulin and oligofructose

as prebiotics, defined by Gibson and Roberfroid (2009) as non-viable food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon.Gibson and Roberfroid (2009) originally thought of *Bifidobacteria* and *Lactobacilli* as benficial bacteria, although that idea is changing.

Both bacteria and diet have been shown to independently affect colon cancer development, however it is possible that their effects are additive for protecting against colon cancer. This bacteria and dietary component combination has been studied using a mixture of probiotic bacteria and prebiotics referred to as a symbiotic. It has been shown that these mixtures exert synergistic effects that inhibit ACF formation in rats (Liong, 2008). Studies found that rats fed a high-fat and low-fiber diet supplemented daily with the probiotic *B. polyfermenticus* (3×10^8 cfu/1.3g) had a 50% reduction in ACF formation in rats compared to controls (Rowland *et al.* 1998). They also found that the combination of inulin and *B. longum* decreased ACF formation by 80%, whereas inulin alone decreased ACF formation by 41% and *B. longum* alone decreased ACF by 26%.

Bacterial 16S ribosomal RNA Genes:

The 16S rRNA gene is a component of the small subunit of prokaryotic ribosomes that provides significant molecular information of genetic relatedness or phylogenetic (evolutionary) relationships (Chanama 1999). It plays a pivotal role in identification of bacterial species and aids in the discovery of novel bacteria (Woo *et al.* 2008). Analysis of 16S rRNA gene sequences helps clinical researchers and microbiologists to gain new insights into the process of infectious bacteria-related diseases and even discover

relationships that may affect chronic disease (Clarridge 2004). The length of the 16S rRNA gene is about 1550 bp and it contains nine variable and nine conserved regions, as documented by Petrosino *et al.* (2009). The structure of the 16S gene is shown in **Figure 2.01.** A single bacterium can have multiple copies of 16S rRNA genes and between 2 and 14 copies have been noted (Case *et al.* 2007; Clarridge 2004).

The 16S rRNA gene sequence is well conserved so that organisms having greater than 97% similarity in their DNA have been classified as the same species. The difference of 3% (approximately 45 nucleotides) between genes is not evenly distributed along the primary structure of the molecule (Stackebrandt and Goebel 1994). The 16S rRNA gene sequence can differentiate organisms at genus and species levels (Woo *et al.* 2008), classifying strains at multiple levels (e.g. species and subspecies level) (Clarridge 2004), to describe and compare the diversity of a bacterial community, and 16S rRNA genes evolve at different rates in different organisms (Woese, 1987). The 16S rRNA analysis helps researchers to identify strains or species of bacteria from a large collection of bacterial clones (Woo *et al.* 2008). The 16S rRNA gene is relatively short (1.5 kb) so it is faster and cheaper to sequence than many other unique bacterial genes.

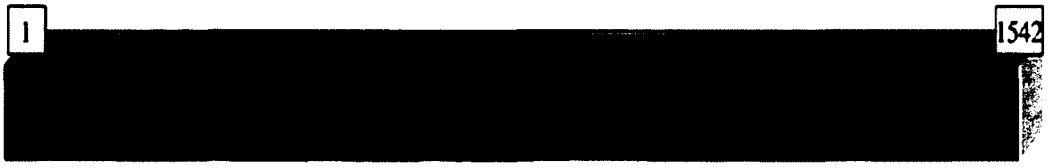

Figure 2.01: The modified structure of the bacterial 16S rRNA gene from Petrosino *et al.* (2009) showing the 9 interspersed conserved regions (C1-C9, highlighted in gray color) and 9 hypervariable regions (V1–V9, highlighted in different colors).

MATERIALS AND METHODS

Animals and Diet:

This research was approved by the Health Canada Ottawa Animal Care Committee. All animals were housed in polyethylene cages lined with wood chips according to the Guidelines of the Canadian Council for Animal Care. Animals were monitored for their overall health. The 6 week-old male F344 rats were procured from Charles River Laboratories (Montreal, QC). They were weaned onto the AIN-93G basal diet and had access to drinking water and food *ad libitum.*

At 7 weeks of age, rats were randomized into three groups to receive either a control (alphacel) or an experimental diet with fructooligosaccharides (FOS) or wheat bran (WB) with a total fermentable level of 3% (wt/wt). There were 24 AOM rats and 12 saline rats assigned for each diet. Composition of the three diets is shown in **Table 2.01** and physiological parameters of the diets are given in **Table 2.02.**

After 2 weeks, all AOM rats received two subcutaneous doses of azoxymethane (AOM; 15 mg/Kg body weight) in saline a week apart to induce colon tumors. Saline alone was injected in the controls. All rats remained on the experimental diets for 24 weeks (post-injection). Fresh feces were collected 29 weeks after injection and stored at -80°C to isolate bacterial species and to prepare fecal water.

Table 2.01: The composition of the experimental diets that were fed to rats.

Diet ingredient (g/Kg)	**FOS**	**WB**	**Control (Alphacel)**
Casein	200.0	178.4	200.0
Native waxy-maize starch	390.1	377.0	390.8
Maltodextrins	124.6	111.5	125.3
Sucrose	92.6	79.5	93.3
Soybean oil	70.0	65.4	70.0
AIN-93mineral mix	35.0	35.0	35.0
AIN-93 vitamin mix	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5
L-Cystine	3.0	3.0	3.0
Alphacel	40.0	3.3	70.0
Extra Fiber	32.2	134.3	0.0
Total of all dietary Fiber sources (Alphacel + Extra)	72.2	137.6	70.0
Total dietary Fiber %	7.0	7.0	7.0
Total fermentable material %	3	3	0
Total Protein %	20.0	20.0	20.0
Total Carbohydrate %	60.9	59.0	60.9
Total Fat %	7.0	7.0	7.0
Estimated Energy content (kcal/kg)	3931.9	3788.9	3874.9

Table 2.02: Physiological properties of experimental diets that were fed to rats.

Degree and rate of fermentation	**Fiber source**	**Abbreviation**
Completely and rapidly fermented	Fructooligosaccharide	FOS
Incompletely and slowly fermented	Wheat bran	WB
Poorly fermented	Alphacel	Control

At the end of the animal study, all rats were sacrificed and the colons were dissected and laid flat on a cold plate to record tumors and their coordinates. The cross-sections of colons were stained with hematoxylin and eosin and the incidence of colon tumors were recorded. The average tumor size (mm^2) per group was calculated using the following formula: total size (mm^2) of tumors in the group was divided by the number of tumors in the group. The tumor multiplicity was calculated using the following formula: total tumors were divided by total tumor bearing rats. This histological analysis of colon tumors was conducted by Raju *et al.* (unpublished data) at Health Canada.

Bacterial genomic DNA Isolation:

The isolation of bacterial DNA from rat feces was performed using the QIAamp DNA Stoolmini Kit (Qiagen Inc., Mississaugua, ON) as described by the manufacturer (and outlined below) and using the included premade buffers and reagents. Rat feces were weighed to within 180-220 mg and placed into a 2 mL micro-centrifuge tube containing ASL buffer that lysed stool samples. The tubes were instantly placed on ice and bead beating followed using a Precellys24 homogenizer (ESBE Scientific, Canada) for 15 sec at 6800 rpm to disperse the samples. Samples were placed in liquid N_2 for few seconds. This process was repeated for a total of 3 times to break down the cell wall of bacteria, specifically Gram positive that have thick cell walls.

The suspensions were then heated for 10 min at 95°C in a digital heat block (VWR®, Canada) to lyse bacterial cells in the stool. Samples were vortexed for 15 sec and then centrifuged for 1 min at 17800 x g to pellet stool particles. The supernatant was pipetted into a new 2 mL microcentrifuge tube and the pellet was discarded. One

InhibitEX tablet was added to each tube and the tubes were vortexed immediately and continuously for 1 min or until the tablet was completely suspended. Stool samples typically contain many compounds that can degrade DNA and inhibit downstream enzymatic reactions. The inhibitEX tablet can efficiently adsorb these substances early in the purification process so that they can easily be removed by a quick centrifugation. The suspension was incubated for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix. The suspension was centrifuged at 17800 x g for 3 min to pellet inhibitors bound to the InhibitEX matrix. The supernatant was pipetted into a new 1.5 mL microcentrifuge tube and the pellet was discarded. The sample was then centrifuged at full speed for 3 min. An aliquot of 15 µL of proteinase K was pipetted into a new 1.5 mL microcentrifuge tube and 200 µL of supernatant was then added. Then 200 µL of AL buffer was added and the sample was vortexed for 15 sec and then incubated for 10 min at 70°C. Finally, 200 µL of ethanol (96%) was added and tubes were vortexed to mix.

The complete lysate was carefully transferred to a QIAamp spin column. The tube was centrifuged for 1 min at 17800 x g to adsorb DNA onto the QIAamp silica-gel membrane. The QIAamp spin column was placed in a new 2 ml collection tube and the tube containing filtrate was discarded.

The DNA bound to the QIAamp membrane spin column was washed with 500 µL AW1 Buffer by centrifuging at 17800 x g for 1 min. The DNA bound QIAamp membrane spin column was placed in a new 2 mL collection tube and the collection tube containing the filtrate was discarded. A 500 µL aliquot of buffer AW2 was then added to the tube followed by centrifugation at 17800 x g for 3 min to ensure complete removal of any residual impurities without affecting DNA binding. The collection tube containing

the filtrate was discarded. The QIAamp spin column was placed in new 2 mL collection tube. The cap was closed and centrifuged at 17800 x g for 1 min. The QIAamp spin column was then transferred into a new labeled 1.5 mL micro-centrifuge tube and 200 µL of AE Buffer was added directly onto the QIAamp membrane. The spin column was incubated for 1 min at room temperature, and then centrifuged at 17800 x g for 1 min to elute the DNA.

Assessing the quality of bacterial genomic DNA:

DNA quality was assessed by gel electrophoresis. TAE buffer (50 mL) and agarose (0.4g) were mixed and heated for 3 min in microwave. A 1 uL aliquot of ethidium bromide (10 mg/mL) was then added and mixed and the gel was poured into the gel rack. A comb was placed at the edge and the gel was allowed to solidify for 15 min. A genomic DNA sample with loading dye (1:1 v/v) was loaded into gel and the sample was run for 30 min at 150 volts (Bio-RAD). A picture of the gel was then taken with a bio-imaging system (PerkinElmer, USA).

Determining the Concentration of DNA and Pooled DNA Sample:

Genomic DNA concentration was determined using a Nanodrop Spectrophotometer (Applied Biosystem, HITACHI, Canada). AE buffer was run as a blank because AE buffer was used in DNA isolation. A 2 µL DNA sample was loaded into the channel and read a total of 3 times.

A pooled DNA sample was used to determine bacterial composition. Pooled samples eliminate variability among rats. The pooled DNA sample was added into a PCR

tube that contained a white PCR bead consisting of stabilizers, bovine serum albumin (BSA), dATP, dCTP, dGTP, dTTP, ~2.5 units of puReTaq DNA polymerase and reaction buffer. When a bead is reconstituted to a 25 µL final volume, the concentration of each dNTP is 200 µM in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM $MgCl_2$. A total of 25 µL pooled DNA sample was prepared to be used in PCR reaction (Biometra®, Montreal Biotech Inc., Canada). The total sample contained universal primers including 2 µL of F44 and R1543, 2 µL of PCR products, and 21 µL of PCR water. GC content of the R1543 primer was 36.84% and its total length was 19 bp; GC content of the F44 primer was 38.89% and its total length was 18bp. The sequences of F44 and R1543 primers were 5'-RGTTYGATYMTGGCTCAG-3' and 5'-GGNTACCTTKTTACGACTT-3' respectively. The PCR reaction was run for 12 cycles. The annealing temperature was 72°C.

Purification of PCR DNA fragments:

The minElute PCR Purification Kit was used to purify double stranded DNA fragments from PCR reactions (following manufacturer's instructions), resulting in high concentrations of DNA and DNA fragments ranging from 70 bp to 4 kbp. A 125 µL aliquot (5X volume of the PCR sample) of PB buffer was added to a PCR reaction tube followed by a quick centrifugation (Fisher Scientific, Canada) for a few seconds. The sample was transferred to the minElute and the tube was centrifuged (17800 x g) for 1 min. The filtrate was discarded and the minElute column was placed back into the same tube. Buffer PE (750 µL) was then added to the minElute column to wash the DNA sample followed by centrifugation at 17800 x g for 1min. The filtrate was discarded and

the minElute column was placed back in the same tube and the column was centrifuged for additional 1 min. The minElute column was placed in a clean 1.5 mL micro-centrifuge tube and 12 µL of pure PCR ddH_2O (pH 8.5) was added to the center of the membrane. The column was incubated for 1 min at room temperature and then centrifuged for 1 min at 17800 x g. The fresh PCR products were used for cloning.

TOPO® Cloning Reaction:

The chemically competent *E. coli* cloning method was used. A total of 4 µL fresh PCR product was mixed with 1 µL of pCR2.1-TOPO vector (Invitrogen, Carlesbad, Canada) and 1 µL salt solution (1.2 M NaCl, 0.06 M $MgCl_2$). The sample was gently mixed and incubated for 1 h at room temperature (22-23°C). It was found that a longer time incubation provided higher efficiency of cloning.

A 4 µL aliquot of the TOPO cloning reaction sample was gently mixed with One Shot Chemically Competent *E. coli* TOP10 (Invitrogen, Carlesbad, CA) and then the tube was incubated on ice for 10 min. The cells were heat-shocked for 30 sec at 42°C without shaking and then the tube was immediately transferred to ice. SOC medium (250 µL at room temperature) was then added to the vial and it was attached to a horizontal shaker (200 rpm) for 1 hr at 37°C. The components of the SOC medium are 0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose. Fifty and seventy five micro-litres of the transformed cells were spread on two pre-warmed agar plates and incubated overnight at 37°C.

Analysing Transformants and Harvesting Bacterial Colonies:

The overnight incubated plates were taken out of the incubator and colonies were analyzed. One millilitre of LB (Luria Broth) growth medium containing ampicillin (25 mg/tablet) was pipetted into each well of a 96 well growth plate to harvest bacterial colonies. Well-isolated single white bacterial colonies were picked with a sterile toothpick and placed into a well of the growth plate. The growth plate was covered securely with the supplied gas-permeable foil. The plate containing the bacterial culture was shaken at 250 rpm in the incubator at 37°C for 16 hr. The overnight recombinant *E. coli* culture was centrifuged (Kendro® SORVALL®, Kendro Laboratory Products Inc, Asheville, USA) at 2100 x g for 10 min and the supernatant was discarded without disturbing the bacterial pellets.

Bacterial Plasmid DNA Isolation:

This procedure was performed with the use of a Biomek NX^P robot (Beckman Coulter, USA) by following the PureLink™ Pro Quick96 Plasmid Purification Kit, as discussed briefly below. A 250 µL aliquot of buffer with RNase A was added to each well of a growth plate to completely resuspend the bacterial cells. Then, 250 µL of lysis buffer was added to each well and gently vortexed to lyse the cells. The lysis reaction was allowed to run only for 5 min to avoid denaturing supercoiled plasmid DNA. Then 350 µL of neutralization or binding solution was added and gently vortexed to stop the lysis reaction and precipitate the cell debris.

The lysate solution was transferred into the clarification plate. The PureLink™ 96 Well Plasmid filter plate was placed inside the vacuum manifold base. The PureLink™

96 Plasmid clarification plate was placed on the top of the filter plate. The vacuum pressure was set at –12 to –15 Hg for the purification steps. The lysate was completely passed through the clarification plate into the filter plate by applying vacuum for 1 min. The vacuum was then released and the clarification plate was discarded. All the lysate was passed through the filter plate by applying vacuum for 1-2 min. The filter plate was placed in the manifold base and 900 µL Wash Buffer II with ethanol was added into each well of the filter plate. The filter plate was washed by applying vacuum for 1 min. The washing step with Wash Buffer II was repeated once more. The filter plate was vacuumed for 10 min to remove residual ethanol. The PureLink™ Pro 96 elution plate was then placed under the filter plate and 100 µL Elution Buffer was added into each well of the filter plate. The plates were incubated at room temperature for 1 min and then centrifuged (Kenodro® SORVALL, Kendro Laboratory Products Inc, USA) at 12000 x g for 1 min. The elution plate was sealed with foil tape and plasmid DNA was stored at -20°C.

Sequencing Reaction and Cleanup:

DNA sequence reactions were carried out using the BigDye Terminator v3.1 cycle sequencing kit and were run on an ABI 3130xl genetic analyzer (Applied Biosystems, HITACHI, USA) using a 36 cm capillary column and POP7 polymer. An aliquot of 10 µL of plasmid DNA sample was transferred from the elution plate into each well of the PCR_{96} reaction plate and 10 µL of Master Mix was then transferred into each well. The master mix solution contained primers, BDT mix, buffer, and water. The sequences of primers such as M13-forward, M13-reverse, and 16S-internal were 5'-

CACGACGTTGTAAAACGAC-3', 5'-GGATAACAATTTCACACAGG-3', and 5'-TCACRRCACGAGCTGACGA-3', respectively. GC content of the M13-F primer was 47.37% and its total length was 19 bp, GC content of M13-R primer was 40.00% and its total length was 20 bp, and the 16S-I primer had 52.63% GC and its total length was 19 bp. The PCR_{96} well reaction plate was quickly centrifuged (Kenodro® SORVALL, Kendro Laboratory Products Inc, USA). The plasmid DNA sample on the reaction plate was amplified through the BIO-RAD DNAEngine® machine (Bio-Rad Laboratoris Ltd, Canada). The BDSEQ1 condition was selected with a plate containing 20 μL samples. This procedure took 3 hr to amplify DNA sample.

Subsequently, 10 μL of Injection Solution was added into each well of the Millipore Seq96 plate. Then all amplified DNA samples from the PCR_{96} reaction plate were transferred to a Millipore Seq96 plate and the PCR_{96} reaction plate was discarded. The Millipore Seq96 plate was placed in the manifold and vacuumed at approximately 23 in Hg pressure until the wells dried. The vacuum was continued for another 20 sec after the last well dried (approximately 2 min total). This is an important step to make sure the solutions have completely gone through the filter in order to minimize dye blobs. The bottom of Millipore Seq96 plate was blotted on Kimwipes to dry. A 20 μL aliquot of injection solution was added to each well to clean up the amplified DNA sample and the vacuum was run until the wells were dry. The vacuum was continued for another 20 sec (approximately 3 min in total) and the plate was blotted on Kimwipes. A final volume of 25 μL of injection solution was added into each well of the Millipore Seq96 plate followed by shaking (SARSTEDT shaker) for 10 min at 600 rpm. Samples (20 μL) from each well of the Millipore Seq96 plate were transferred into a 96 well reaction plate

which is specific to be fitted into the genetic analyzer and the reaction plate was stored at -20°C. The plate was quickly centrifuged before being run in the genetic analyzer.

Data Quantification and Statistics:

The Sequencher 4.7 program (Gene Codes Corporation, USA) was used to align three sequencing fragments including M13-F, M13-R, and 16S intergenic region to produce consensus sequences. The base calling was performed using Sequencer 4.7 program.

Statistical Program and Tool:

Mothur is a name given to a collection of sub-programs that make analysis of bacterial communities and comparisons among communities possible. It takes raw DNA sequences and outputs α and β diversity using 16S rRNA gene sequences from diverse environments (Scholss *et al.* 2009). The program is composed of several tools: DOTUR (for separating sequences into operational taxonomic units), SONS (for calculating the richness of operational taxonomic units shared between communities), TreeClimber (screening sequences to generate phylogenetic tree), LibShuff (calculating pairwise distance to determine whether two or more communities have the same structure), ∫-LibShuff, and UniFrac to incorporate the following features: i) quantify the ecological parameters for measuring α and β diversity; ii) Venn diagrams, heat maps, and dendrograms tools to visualize the relative abundance of bacterial population within diverse samples; and iii) a pairwise sequence distance calculator to determine the coverage (Scholss and Handelsman 2006; Scholss *et al.* 2009).

The ∫-LibShuff component compares two bacterial libraries using 16S rRNA sequence abundance data. This analysis describes the libraries in terms of library coverage (C), which is calculated from the number of unique 16S rRNA sequences (Mx) and the total number of sequences in a single library (m). The LibShuff program calculates the coverage of a 16S rRNA library for values of evolutionary distance (D) ranging from 0.0 to 0.5 in increments. The evolutionary distance is the fractional cutoff for the definition of identical taxonomic units. For example, using $D \leq 0.03$, we consider that sequence differences ≤ 0.03 all belong to the same species (form a group). At D = 0.0 sequences forming a group must be identical (100% similarity) and at D = 0.5 an evolutionary distance that is close to the maximal evolutionary distance within the prokaryotic domain, all the sequences form 1 group. The coverage of a single library can be calculated using the formula: $C_x = 1-(M_x/m)$. The Cx value increases with increasing evolutionary distance because the number of unique sequences tends towards a value of 0.0 at large distances. In this equation, each sequence in the library is compared to the other sequences within the same library to generate a homologous coverage curve (internal comparison).

In order to compare two libraries, the LibShuff program also calculates an inter library coverage value (C_{XY}), termed heterologous coverage. This is calculated by comparing each sequences in library X to all sequences in Y to determine whether or not the X sequence is found in the Y library. This is described by the following equation, $C_{XY} = 1-(N_{XY}/m)$, where N_{XY} is the number of sequence of X that are found in library Y, and m is total number of sequences in library X. Both heterologous and homologous values are determined across evolutionary distances from 0.0 to 0.5 to obtain coverage curves.

The heterologous and homologous coverage curves are shown in **Figure 2.02**. Mutiple libraries comparisons are also shown in **Appendix Figure A.01.** Libraries derived from similar sources should have similar homologous and heterologous coverage curves. The difference between these curves is quantified using the Cramer-von Mises test statistic (ΔC). If two libraries are identical, then the coverage curves of the two libraries should be the same or close. Significance is determined by comparing the ΔC value for the two libraries against 1000 randomly separated libraries using the same sequences. Two separate C_X and C_{XY} values are calculated for a comparison of two libraries, we'll call A and B. In one case X is library A and B is library Y, in the other case the order is reversed. Differences are called significant if either of the two comparisons is significant.

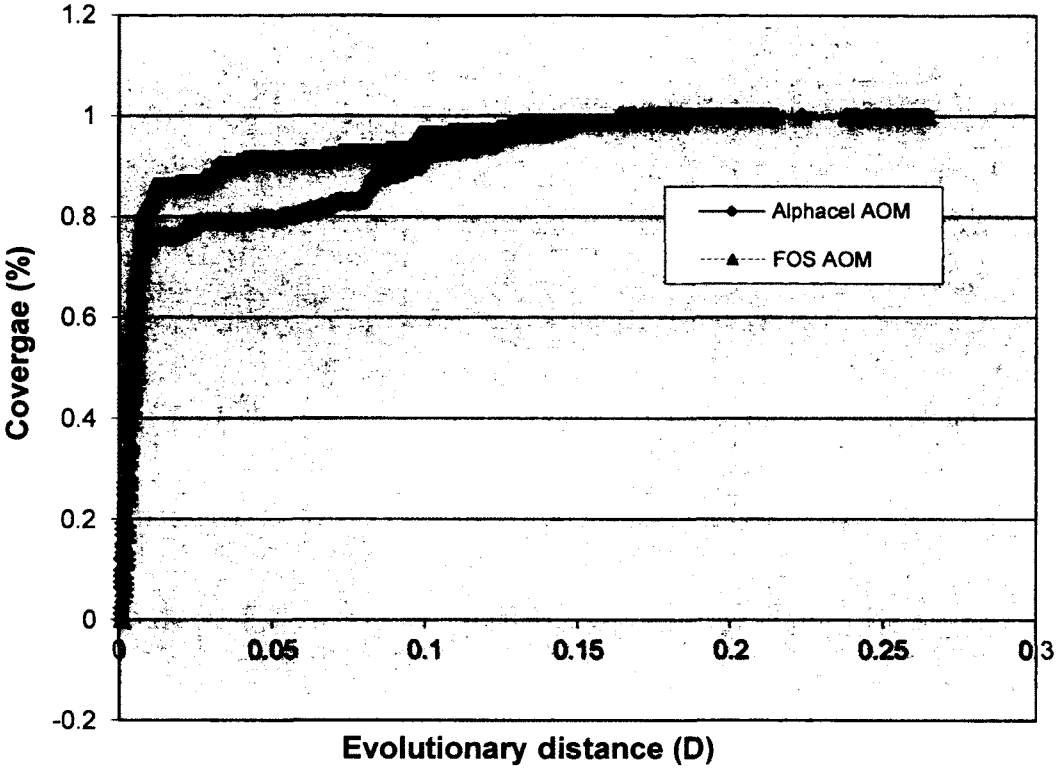

Figure 2.02: Shows the partial comparison of bacterial libraries based on 16S rRNA gene sequences.

The Library Compare Tool (RDP II website) was used to taxonomically classify the libraries of 16S rRNA sequences using the RDP naïve Bayesian classifier (*http://rdp.cme.msu.edu/comparison*). The library comparison tool requires that each library have at least 200 bases and the number of query sequences is limited to 40000. Each library of 16S rRNA sequences is assigned into hierarchical taxa from the phylum to genus rank along with a confidence value (95%) with p-value ≤ 0.05.

Mothur software was used to trim, screen, align sequences, and calculate distances between sequences. The sequences were screened before analysis in order to remove more than 4 ambiguous (N) bases and a string of homo-polymers longer than 8. The assigned DNA sequences of 16S rRNA gene fragments were converted into operational taxonomic units (OTUs) at a cutoff level of 3% using Mothur software. The filtered file from the Mothur program was exported as .meg file that was used to construct a neighbour-joining tree using the Mega5 program. The filter file is the OTUs of 16S rRNA sequence that have 97% sequence similarity and it was used to identify bacterial species. The tree was exported as Newick Standard file.

The LibShuff program (described above) uses pairwise distance matrix and coverage values to determine whether or not two libraries are significantly different. This test can also illustrate whether two samples are drawn from the same population or whether one is a subset of other. The Bonferroni correction was applied to analyze our LibShuff results. This is a multiple-comparison correction used when several dependent or independent statistical tests are being performed simultaneously (Bonferroni 1935) that provide new limits of critical value.

We treated six different samples so the number of comparisons = $N_{treatment}$ [$N_{treatment}$-1] = 6*(6-1) = 30. The total number of performed tests (n) = 30 and the probability = 0.05. The significances level for the entire series of our experimental data was = 0.05/30 = 0.0017, which was used the limit of probability (p value) for significance (original p = 0.05). That is, p values smaller than 0.0017 were compared to as statistically significant and p values higher than 0.0017 were not statistically significant.

The parsimony test specifies the distribution of species along the branches to see if they are random and equally distributed between two 16S rRNA gene libraries. This test generates random trees from a pool of species sequences and compares the number of changes in tree topology among communities against the randomized null (Scholss *et al.* 2009). This test constructed 10,000 randomly joining trees for each library from 16S rRNA gene sequences. The Bonferroni correction was applied to analyze our parsimony test due to the large number of comparisons. The analysis resulted in a new cutoff p-value of 0.003 to analyze the significance of libraries.

RESULTS AND DISCUSSION

Tumor incidence in rats:

Analysis of tumor incidence in the rat study conducted at Health Canada, which was used as a source of material for this study (Raju *et al.*, unpublished data; see **Appendix Table A.01**.) is summarized in **Table 2.03**. The control diet had the highest percentage of tumor incidence (62.50%) and tumor multiplicity (2.07) compared to the FOS and WB diets. The tumor multiplicity is the average number of tumors per tumor-bearing rat. The second highest tumor incidence was associated with the FOS diet which was 58.33% and its tumor multiplicity was 1.50. These data were statistically significant different from the control. In addition, FOS had the highest average tumor size (30.66 mm^2). On the other hand, WB had the lowest colon tumor incidence (13.4%) and lowest tumor size (9.73 mm^2). The WB group was significantly different from the other diets. The present data indicate that fermentation-associated events can play a significant role in cancer development in the AOM-treated rat model. However, the extent of fermentation does not appear to be the most important factor. This is supported by the fact that both experimental diets (WB and FOS) except alphacel (control) contained the same amount of fermentable material, but these diets had significantly different incidences of colon tumors. Thus, if the total amount of dietary fiber was the only factor in tumorigenesis, then all the diets should have the same tumor incidence and multiplicity.

The estimated energy density was higher in the FOS diet and that might enhance tumor growth and multiplicity. However, Pierre *et al.* (1997) showed that FOS also dramatically decreased or suppressed colon tumors and concomitantly developed gut-associated lymphoid tissues in *Min* mice whereas WB did not show any effect. It was

noticed that a significant number of macroscopically detectable lymphoid nodules were seen in the small intestine of *Min* mice that might play a role in inhibiting tumor formation with the FOS diet (Pierre *et al.* 1997). It was found that WB fed rats had a lower incidence of colon tumors compared to oat bran fed rats (Zoran *et al.* 1997). WB diet is a complex fiber that contains many morphologic components such as aleurone and the pericarp seed-coat and phytates (Cheng *et al.* 1987; Kirby and Nelson 1988). WB is feremented to produce butyrate and propionate within normal limits. It also can dilute luminal contents to reduce the exposure of colonic epithelial cells to carcinogens (Zoran *et al.* 1997)

Table 2.03: Evidence of colon tumors in rats after administration of azoxymethane (AOM) and feeding FOS, WB or Alphacel (Control) diets. These data were gathered by Raju *et al.* (unpublished data) at Health Canada for the rats in my test groups.

Characteristics	**FOS**	**WB**	**Alphacel (Control)**
Number of rats (n)	24	23	24
Number of tumor-bearing rats	14	3	15
Tumor incidence (%)	58.33	13.04	62.50
Tumor multiplicity	1.50 ± 0.29	1.00 ± 0	2.07 ± 0.27
Average tumor size (mm^2)/ tumor-bearing rat	30.66 ± 10.12	9.76 ± 2.43	16.39 ± 4.35

NB: Results were statistically analyzed by ANOVA, $p<0.05$ was considered significant and the ± values were SD.

Bacterial Diversity in the feces of rats:

A total of 971 16S rRNA genes were sequenced. These resolved into 204 OTUs distributed among the 6 treatments: AOM and saline injected rats fed alphacel (control), FOS and WB diets. The distribution of OTUs among the 6 treatments after AOM and saline injection in rats is shown in **Table 2.04.** OTUs occur in more than one condition such that the sum of OTUs across diets/injections does not equal 204. There was a lower number of OTUs compared to total number of 16S rRNA clones. This is due to repeat amplification of the most dominant bacterial genomic DNA through PCR reaction. The final OTU distribution is thought to accurately reflect the clone distribution in the DNA sample. However, many possible factors may affect this distribution including the quality of the DNA sample, differential PCR amplification (PCR bias), formation of PCR artifacts, contaminating DNA, and the quality of universal primers.

Table 2.04: The distribution of total clones and OTUs among the 6 treatments after AOM and saline injection in rats fed alphacel (control), WB, and FOS.

Features	Diets					
	Alphacel AOM	**Alphacel Saline**	**FOS AOM**	**FOS Saline**	**WB AOM**	**WB Saline**
Number of Clones	172	141	166	171	143	178
Number of OTUs	67	60	47	49	53	71

The cutoff value (phylogenetic distance = 0.03) used to define an OTU represents a 97% 16S rRNA sequence similarity and is used for species identification: each of the clones in one OTU is less than 3% divergent from each other. Larger distances have been used to define other taxonomic levels. For example, a distance of 0.05 represents the genus level, and a distance of 0.20 represents phylum level (Hughes *et al.* 2001; Sait *et al.* 2002; Ward 1998). In addition, a phylogenetic distance smaller than 0.03 represents the strain levels. The phylogenetic relationship in terms of OTUs among 6 treatments is shown in **Figure 2.03**. While we have used 3% and 5% as functional cutoffs for defining species and genus, microbiologists have used inconsistent arbitrary cutoff values such as 0.01-0.05 (1-5% 16S rRNA gene dissimilarities) to classify bacterial diversity at the species level which provides high or less closely related bacterial species (Hughes *et al.* 2001). If we draw a tangent line of the phylogenetic distance on **Figure 2.03**, then the cutoff value has not significantly changed within the region between 0.025 - 0.035. Therefore, we decided to use the middle point (0.03) as the cutoff value for OTU to classify bacterial species. The intervening sequence (IVS) of 16S rRNA gene is a potential marker to specify species or strains. The length of IVS is 120 to 131 base pair (Rainey *et al.* 1996). It has shown that there are 1-14 rRNA operons in bacterial genomes (Young and Cole 1993), and rRNA genes are mostly linked in the following order 5'-16S-23S-5S-3' as a transcription unit (Nomura *et al.* 1984). The difference of nucleotides between any pair of 16S rRNA genes within a genome is 0 to 19 Nts and their corresponding similarities is 100 to 98.74 % (Coenye and Vandamme 2003). Therefore, 16S rRNA gene was a good marker for identification and phylogenetic study of the gut microflora in rats.

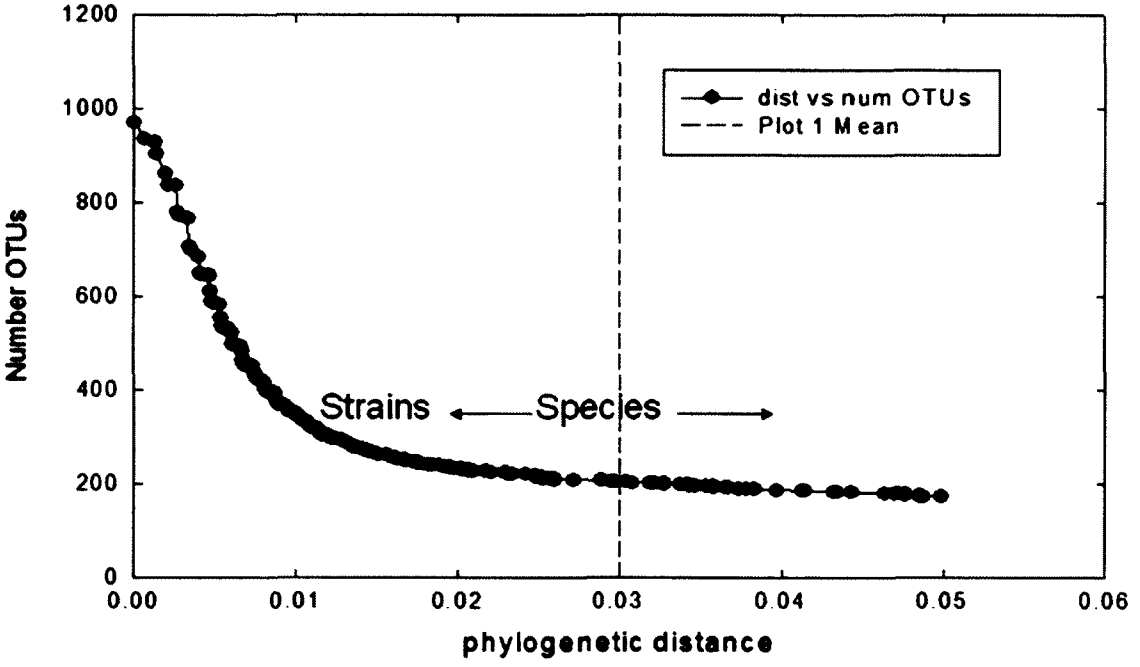

Figure 2.03: The phylogenetic relationship among isolated bacterial populations: operational taxonomic unit (OTUs) versus phylogenetic distance.

The phylogenetic tree shows the bacterial diversity in the feces of AOM- and saline-injected rats fed diets (e.g. alphacel, FOS and WB). The bacterial diversity in the feces of rats after AOM- and saline-injected is shown in **Figure 2.04**. The greatest number of bacterial species belonged to the family *Ruminococcaceae*. The second and third most abundant bacterial families were *Lachnospiraceae* and *Erysipelotrichaceae*, respectively. The other bacterial families were minimally present in the phylogenetic tree. However, this phylogenetic tree cannot differentiate the relative percentage of bacterial diversity as a function of diet and AMO treatment. The phylogenetic tree also provides the genetic relationship among bacteria.

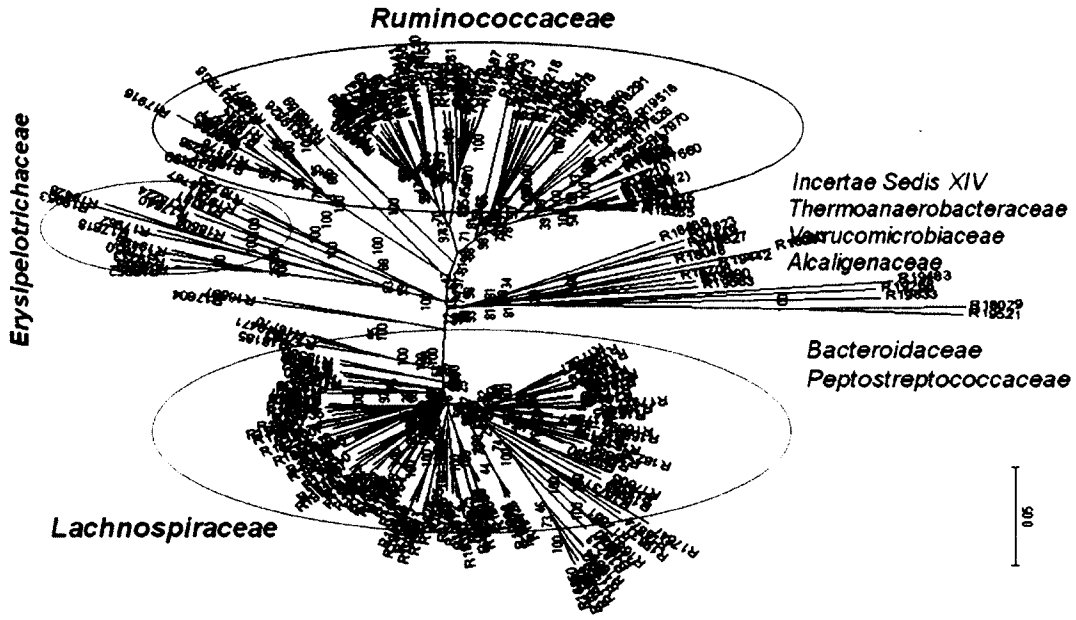

Figure 2.04: The phylogenetic tree of bacterial families where each R number represents a single 16S rRNA sequence or operational taxonomic unit (OTU). These sequences represent the diversity of bacterial species and the scale bar indicates 0.05 changes per nucleotide.

The relative distribution of bacterial species at the family level was determined among the six treatments from the 16S rRNA gene sequences using RDPII online software. The relative ratios of bacterial families are shown in **Figure 2.05**. The relative bacterial diveristis are also shown in **Appendix Table A.02.** The most abundant bacterial family in the feces of all treatment groups was *Ruminococcaceae* representing about 50% of the bacterial species identified in control groups (both with or without AOM treatment) and somewhat less in the WB and FOS groups. The second most abundant bacterial family was *Lachnospiraceae* in three treatment groups except for the WB-AOM, FOS-AOM and FOS-saline groups where *Erysipelotrichaceae* and *Incertae* were the second most abundant. The most abundant bacterial groups (e.g. *Lachnospiraceae sp* and *Ruminococcaceae sp.*) were those that ferment carbohydrate. However, the distribution of bacterial families in the pie chart does not provide an easy analysis of the relative abundance of bacterial species with respect to diets. The heatmap diagram in **Figure 2.06** reworks the data to show the relative abundance of bacterial species with respect to each diet group. The only difference between these two figures was that the abundance of bacterial populations is directly proportional to the intensity of the color in the Heatmap diagram whereas in the pie chart, the abundance of bacterial diversity depends on the area within the chart. It was observed in both figures that most of the bacterial species identified belong to the Firmicutes phylum which is known as Gram-positive bacteria. It is well-known that Gram-positive bacteria (e.g. *Lactobacillus* and *Bifidobacterium*) are more beneficial than Gram-negative type (e.g. *Bacteroides*); Gram-positive bacteria also have a thicker cell wall than Gram-negative bacteria. Some species of *Bacteroides* such as *B. fragilis* are pathogenic to human because these species causes

diseases including infections in the peritoneal cavity and gastrointestinal surgery (Ryan and Ray 2004), and become resistance to various antibiotics including β-lactams, aminoglycosides, erythromycin and tetracycline (Salyers *et al.* 2004; Löfmark *et al.* 2006). It was also noticed that the relative ratio of Gram-negative bacteria was lowered compared to Gram-positive bacteria in the six treatments. However, both figures cannot provide the exact percentage of bacterial species among treatments.

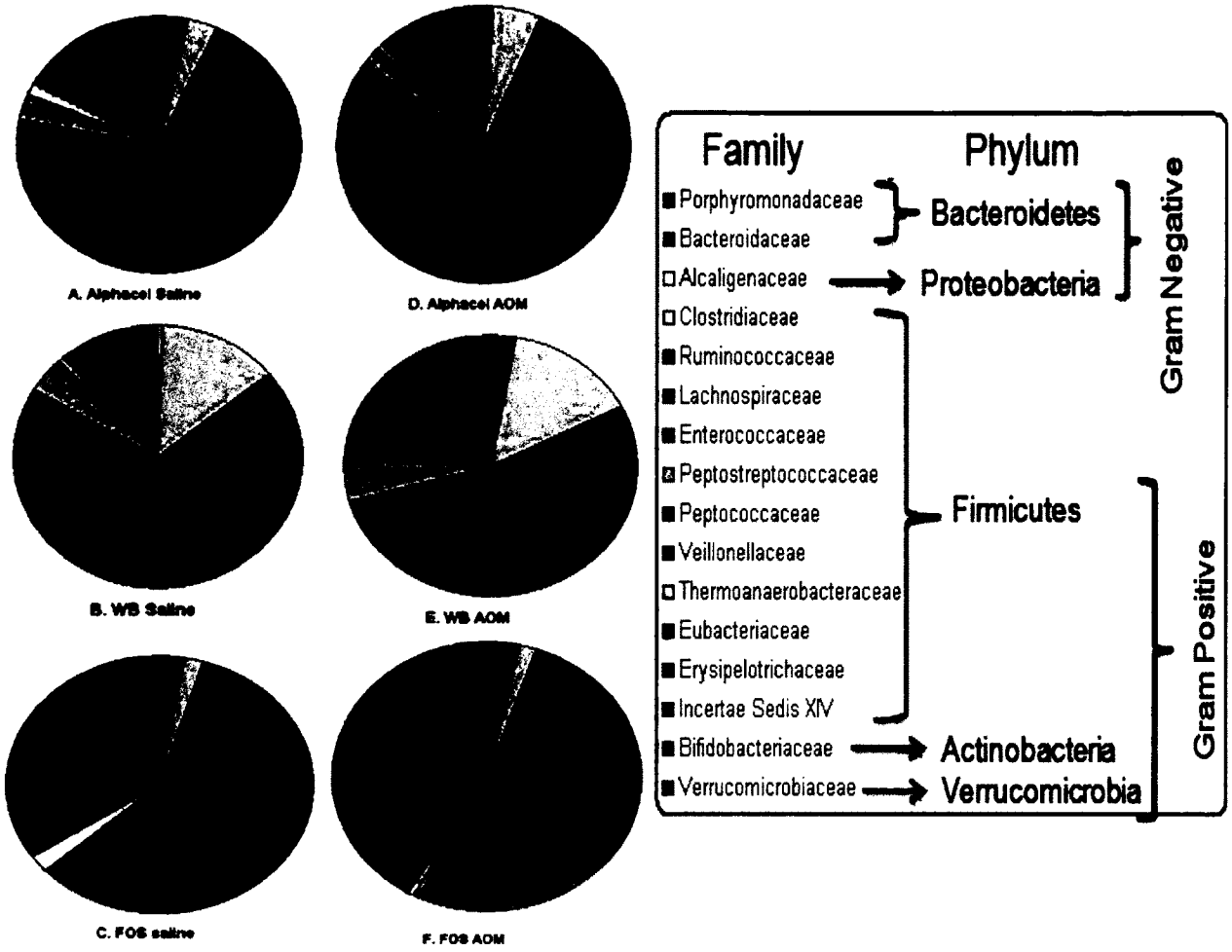

Figure 2.05: The relative ratio of different bacterial families among the six treatment groups. The results were analyzed using RDPII online software. Different colors represent different bacterial families.

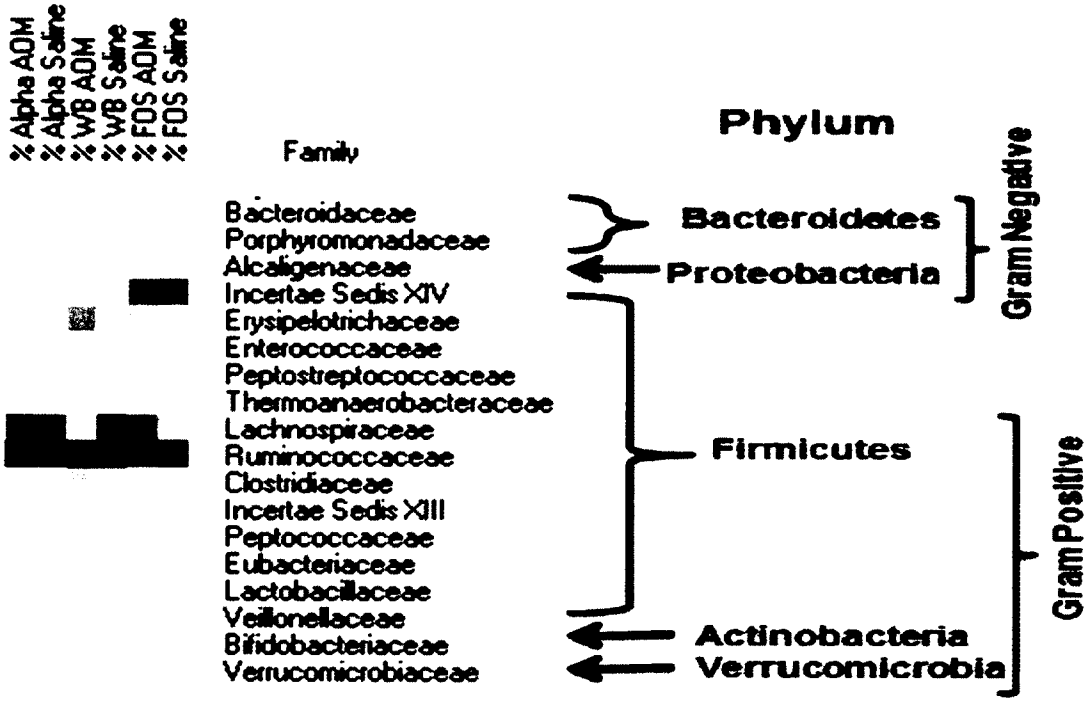

Figure 2.06: The heatmap image shows the relative abundance of bacterial families among each of the diet/treatment groups including Alphacel, WB, and FOS diets, each with (AOM) or without (saline) AOM-injection.

The classification was performed using RDPII Naïve Bayesian classifier and provided the relative percentage of bacterial diversity among the six different treatment conditions. The percentage of the most abundant bacterial family, the *Ruminococcaceae*, was 54.2%, 38.5%, and 31.3% in AOM-injected rats fed alphacel, WB and FOS respectively. The percentages of bacterial compositions among the 6 treatments are shown in **Table 2.05**. The *Ruminococcaceae* family was also the most abundant in the saline-administrated rats, representing 46.7%, 38.3%, and 44.5%, respectively, for the three diets. The second most abundant bacterial family found in the feces of rats were *Lachnospiraceae* at 25.1%, 14.7%, and 20.4% in AOM-injected rats fed alphacel, WB, and FOS diets; comparable numbers for the saline-injected rats were, respectively, 21.2%, 31.5%, and 14.1%. The AOM-injected rats had tumor incidence whereas saline-injected rats were healthier since they did not have tumors in the colon. The similar ratio was observed in the phylogenetic tree. The phylogenetic tree of 16S rRNA genes is shown in **Figure 2.04**.

The least abundant bacterial family was the *Alcaligenaceae* (0.6%) and was found only in WB fed rats with saline-injection. The family *Alcaligenaceae* belongs to the *Proteobacteria* and is Gram negative. There are several genera such as *Bordetella* and *Alcaligenes* and *Achromobacter*, *Pigmentiphaga* and *Kerstersia* that are also assigned to the *Alcaligenaceae* (Yabuuchi *et al.*, 1998; Blümel *et al.*, 2001). Most of genera were isolated from human and veterinary clinical samples including respiratory secretions of cystic fibrosis (CF) patients and some of them are environmental microorganisms that are not pathogenic to human or animals (Coenye *et al.* 2005).

The *Bifidobacteriaceae* family was present at 0.7% and 1.2% in the feces of only AOM-injected rats fed WB and FOS diets, respectively. Indeed, the results found that the FOS diet enhanced the growth of *Bifidobacteria* compared to other diets. Roberfroid *et al.* (1998) found that FOS is an efficient substrate for most strains of *Bifidobacteria* and only small portion (1.2%) of *Bifidobacteria* grew in the feces of AOM-treated rats. They also found that feeding FOS reduced the number of *Clostridium* bacteria, but increased the number of *Bifidobacteria* in rats. In addition, Gibson *et al.* (1995) found that consumption of FOS increased the number of *Bifidobacteria* in humans (Gibson *et al.* 1995). Fructooligosaccharides (FOS) are natural occurring oligosaccharides composed of several beta (1-2) or (1-6) linked fructosyl units that usually attach to a terminal glucose residue (Sharp *et al.* 2001). FOS can be found in many plants (e.g. onion, Jerusalem artichoke, chicory, garlic, and barley) that pass the upper gastrointestinal tract of humans without being hydrolysed (Gibson and Roberfroid, 1995), but FOS are partly or completely degraded in the ileum of pigs (Houdijk *et al.* 1999). Mikkelsen *et al.* (2003) hypothesised that dietary FOS are fermented in the proximal colon that has bifidogenic effects that increased beneficial bacteria (e.g. *Bifidobacteria* and *Lactobacilli*) in piglets, *Bifidobacteria* in mice (Howard *et al.* 1995), and significantly increased *Bifidobacteria* in humans (Probert *et al.* 2004; Gibson and Roberfroid 1995; Ito *et al.* 1993). FOS and galacto-oligosaccharide (TOS) also significantly increased the growth of *Bifidobacteria* in gnotobiotic rats (Rowland and Tanaka 1993), and humans (among others; Ito *et al.* 1993; Gibson *et al.* 1995).

The least abundant bacterial species may have the lowest number of bacterial species in the gut of rats among treatments; however, they may participate in tumor

development or play a significant role in the host. The least abundant bacterial species (genus level) were *Parasutterella sp* (0.6%) and *Peptococcus sp* which belong to the class of *Betaproteobacteria* and *Clostridia*; these were only present in saline-injected rats fed WB, respectively. Furthermore, it was also found that the lowest percentages of *Cryptanaerobacter sp.* (0.6%) and *Caloranaerobacter sp.* (0.6%) were present in the feces of AOM-injected rats fed the alphacel (control) diet whereas *Sporobacterium sp.* (0.7%) and *Desulfonispora sp.* (0.7%) were lowest in saline-injected control rats. The percentage composition of bacterial species is shown in **Table 2.05**.

Caloranaerobacter is a thermophilic, Gram-negative, and chemo-orgatrophic bacterium that optimally grows at 65°C and pH 7.0. This species was first isolated from a deep-sea hydrothermal chimney sample (Wery *et al.* 2001) that ferments proteinaceous substrates (e.g. heart/brain infusion and gluten) and carbohydrates (e.g. glucouse, xylan, and starch). The 16S rRNA sequence of *Caloranaerobacter azorensis* 92% is similar to *Thermohalobacter berrensis* and 91% is similar to *Clostridium purinilyticum* (Wery *et al.* 2001).

Cryptanerobacter (e.g. *Cryptanerobacter phenolicus*) is a Gram-positive, anaerobic, and phenol transforming bacteria that optimally grow at 30-37°C and pH 7.5-8.0 and was originally isolated from a mixture of swamp water, sewage sludge, swine waste, and soil (Bisaillon *et al.* 1993; Zang *et al.* 1990). The presence of 4-hydroxybenzoate (4-OHB) and phenols is essential for its growth; these are converted to benzoate that it uses as an energy source (Juteau *et al.* 2005). However, some phenolic compounds play an important role in colon cancer development. The bacterium has GC content 51% of its DNA sequences and its major membrane fatty acid is anteiso-$C_{15:0}$

(Juteau *et al.* 2005). *Desulfonispora* (e.g. *Desulfonispora thiosulfatigenes*) is a Gram-positive, motile, spore-forming bacterium that ferments organosulfonate compounds such as taurine (2-aminoethanesulfonate) and produces metabolites such as acetate, ammonia and thiosulfate (Denger *et al.* 1997; Cook *et al.* 1998).

Sporobacterium (e.g. *Sporobacterium olearium*) are strictly chemo-organotrophic, anaerobic, Gram-positive bacteria that use crotonate, methanol, and degrade many aromatic compounds including 3,4,5-trimethoxybenzoate (TMB), 3,4,5-trimethoxycinnamate (TMC), 3,4,5-trimethoxyphenylacetate (TMPA), 3,4,5-trimethoxyphenylpropionate (TMPP), syringate, ferulate, etc.) to produce methanethiol (MT), acetate and butyrate. They share 90% sequence similarity with *Clostridium aminovalericum* and *Eubacterium fissicatena* (Mechichi *et al.* 1999). The GC content of the *Sporobacterium* genomic DNA was 38% and optimum growth was at 37 and 40°C, pH 7.2 (Mechichi *et al.* 1999).

Enterococcus species belong to the class *Bacilli* which were only found in the feces of saline-injected alphacel fed rats. These species produce high amounts of extracellular superoxide (O_2^-), and reactive oxygen species such as H_2O_2 and hydroxyl radical (Huycke *et al.* 2002). Rats colonized with *E. faecalis* showed significantly increased DNA damage and increased chromosomal instability with sporadic adenomatous polyps and colorectal cancer in rats (Huycke *et al.* 2002). However, it is important to note that there was no incidence of colon tumors in saline-injected rats fed alphacel, FOS, and WB diets.

The *Roseburia* and *Lactobacillus* species were only found in the feces of WB fed rats with AOM- and saline-injections, but the percentage of both species were double in

saline injected rats compared with those injected with AOM. Therefore, we suspect that these bacterial species may be protective against colon cancer development. Both species produce butyrate in human large intestine (Barcenilla *et al.* 2000) and it is believed that butyrate producing bacteria play an important role against colon cancer development. The library comparison results suggested that *Lactobacillus* had a significant effect against colon cancer development. The library comparison results are shown in **Table 2.08.**

The genus *Roseburia* was only present at 0.7% and 1.1% in the feces of WB fed rats with AOM- and saline-injection, respectively. Four different species of *Roseburia* were isolated from human feces: *R. intestinalis* (Duncan *et al.* 2002), *R. faecis, R. hominis,* and *R. inulinivorans*. The species *R. cecicola* (Stanton and Savage 1983) was isolated from mouse intestine and shown to share many characteristics and a close relationship with *R. intestinalis*. These species are strictly anaerobic, Gram-positive, and non-spore forming rods (Duncan *et al.* 2002) that mostly produce butyrate via fermentation in the human large intestine (Barcenilla *et al.* 2000). Butyrate is used as a nutrient for growth of coloncyte cells and is a signalling molecule in cell proliferation and apoptosis (Scheppach *et al.* 1995; von Englhardt *et al.* 1998). The DNA GC content of *Roseburia* strains was low (29-31%) and shared less than 95% sequence identity with the novel strains. In addition, Collins *et al.* (1994) illustrated that the low GC content type bacteria mostly produces butyrate found in human feces.

Table 2.05: The percentage composition of bacterial species (genus level) as determined by 16S rRNA gene sequences in AOM-treated versus saline-treated rats fed on Alphacel, WB, or FOS diets.

Class	Genus	WB Saline (%)	WB AOM (%)	FOS Saline (%)	FOS AOM (%)	Alphacel Saline (%)	Alphacel AOM (%)
Bacteroidia	*Parabacteroides*	0	2.1	0	2.4	2.1	0
	Bacteroides	0	0.7	2.9	1.2	1.4	1.2
Betaproteobacteria	*Parasutterella*	0.6	0	0	0	0	0
Clostridia	*Clostridium*	13.5	14.7	1.8	1.8	3.5	4.1
	Robinsoniella	14.1	4.9	0.6	0.6	7.1	9.9
	Anaerotruncus	10.7	7.0	4.7	1.2	14.2	20.9
	Acetivibrio	5.7	4.2	2.4	0	3.5	3.5
	Ethanoligenens	0.6	0.7	3.5	1.8	2.1	0.6
	Blautia	2.8	0	26.9	21.7	2.1	1.2
	Coprococcus	6.2	2.8	5.9	7.8	6.4	7.6
	Oscillibacter	9.6	5.6	28.1	24.1	11.3	14.5
	Ruminococcus	4.5	11.2	4.7	4.2	12.1	10.5
	Marvinbryantia	1.7	0	5.3	8.4	2.9	5.2
	Cryptanaerobacter	0	0	0	0	0	0.6
	Subdoligranulum	0	0.7	0	0	0	1.2
	Syntrophococcus	1.1	0	0	0	2.1	0.6
	Sporacetigenium	3.4	4.9	0	0.6	2.8	1.2
	Mahella	0.6	0	2.3	0.6	1.4	0.6
	Lachnobacterium	0.6	0	0	0.6	0	0
	Anaerovorax	0.6	1.4	0.6	0	4.3	3.5
	Papillibacter	3.9	8.4	1.2	0	2.1	1.2
	Sporobacterium	0	0	0	0	0.7	0
	Desulfonispora	0	0	0	0	0.7	0
	Dorea	1.7	2.8	0	0.6	0.7	0.6
	Acetanaerobacterium	1.7	0	0	0	1.4	1.2
	Eubacterium	0.6	0.7	0	0.6	0.7	0
	Acetitomaculum	0.6	0	0	0	0.7	0.6
	Anaerostipes	0	0	1.8	2.4	0.7	0
	Anaerosporobacter	0.6	0.7	0.6	0	0	0.6
	Roseburia	1.1	0.7	0	0	0	0
	Sporobacter	1.7	0.7	0	0	0	0.6
	Caloranaerobacter	0	0	0	0	0	0.6
	Propionispira	0.7	0	0	0	0	0.6
	Peptococcus	0.6	0	0	0	0	0
Erysipelotrichi	*Coprobacillus*	0	1.4	0.6	1.2	5.0	3.5
	Holdemania	0	0.7	0	0	0	0
	Turicibacter	2.8	16.1	0	3.6	0	0.6
	Allobaculum	0	0	2.9	7.8	0	0
Bacilli	*Lactobacillus*	3.9	2.8	0	0	0	0
	Enterococcus	0	0	0	0	2.8	0
Verrucomicrobiae	*Akkermansia*	4.5	3.5	3.5	5.4	5.0	3.5
Actinobacteria	*Bifidobacterium*	0	0.7	0	1.2	0	0

Comparisons among pairs of libraries showed Alphacel-AOM versus FOS-AOM, Alphacel-AOM versus WB-AOM, and WB-AOM versus FOS-AOM were all significantly different ($p \leq 0.0017$). The significant ($p \leq 0.0017$) and non-significant ($p \geq 0.0017$) relationships between libraries is shown in **Table 2.06**. These libraries were significantly different and each of these libraries was not a subset of another. Small p value such as $p \leq 0.0007$ indicated that two libraries of 16S rRNA sequences were not sampled from the same library. These libraries such as Alphacel-AOM versus WB-AOM ($p < 0.0001$) and WB-AOM versus Alphacel-AOM ($p = 0.0052$) indicated whether two libraries of 16S rRNA sequences were sampled from the same library or whether one library was a subsample of other library. The same was found for the comparison of the libraries from WB-AOM versus WB-Saline, and FOS-AOM versus FOS-Saline; these pairs were not significantly different.

Table 2.06: The significant ($p<0.0017$) and non-significant ($p>0.0017$) relationship of bacterial populations between AOM-treated and Saline-treated rats fed on the three diets, as analyzed using the LibShuff program.

Comparison	$dC_{XYScore}$	p-value	Significance
Alphacel-AOM versus Alphacel-Saline	0.001041	0.0185	Not Significant
Alphacel-Saline versus Alphacel-AOM	0.000977	0.0068	Significant
Alphacel-AOM versus FOS-AOM	0.012600	<0.0001	Significant
FOS-AOM versus Alphacel-AOM	0.020400	<0.0001	Significant
Alphacel-AOM versus FOS-Saline	0.010884	<0.0001	Significant
FOS-Saline versus Alphacel-AOM	0.011761	<0.0001	Significant
Alphacel-AOM versus WB-AOM	0.003819	<0.0001	Significant
WB-AOM versus Alphacel-AOM	0.001095	0.0052	Significant
Alphacel-AOM versus WB-Saline	0.004731	<0.0001	Significant
WB-Saline versus Alphacel-AOM	0.004339	<0.0001	Significant
Alphacel-Saline versus FOS-AOM	0.009228	<0.0001	Significant
FOS-AOM versus Alphacel-Saline	0.017630	<0.0001	Significant
Alphacel-Saline versus FOS-Saline	0.009720	<0.0001	Significant
FOS-Saline versus Alphacel-Saline	0.009154	<0.0001	Significant
Alphacel-Saline versus WB-AOM	0.004099	<0.0001	Significant
WB-AOM versus Alphacel-Saline	0.004972	<0.0001	Significant
Alphacel-Saline versus WB-Saline	0.006889	<0.0001	Significant
WB-Saline versus Alphacel-Saline	0.004690	<0.0001	Significant
FOS-AOM versus FOS-Saline	0.001179	0.0007	Significant
FOS-Saline versus FOS-AOM	0.000141	0.2878	Not Significant
FOS-AOM versus WB-AOM	0.019172	<0.0001	Significant
WB-AOM versus FOS-AOM	0.003511	<0.0001	Significant
FOS-AOM versus WB-Saline	0.023828	<0.0001	Significant
WB-Saline versus FOS-AOM	0.013549	<0.0001	Significant
FOS-Saline versus WB-AOM	0.015613	<0.0001	Significant
WB-AOM versus FOS-Saline	0.015919	<0.0001	Significant
FOS-Saline versus WB-Saline	0.017079	<0.0001	Significant
WB-Saline versus FOS-Saline	0.013242	<0.0001	Significant
WB-AOM versus WB-Saline	0.000220	0.2934	Not Significant
WB-Saline versus WB-AOM	0.000474	0.0745	Not Significant

Small p values ($p \leq 0.003$) in the parsimony test indicate that bacterial species are evenly distributed along the phylogenetic tree in the two libraries being compared. The compared libraries are shown in **Table 2.07**. The comparison of multiple libraries is shown in **Appendix Figure A.01.** The idea is that differences in distribution between libraries highlights different species distributions related to changing diet or experimental conditions. This test showed that Alphacel-AOM versus Alphacel-Saline, WB-AOM versus WB-Saline, Alphacel-Saline versus WB-AOM, and FOS-AOM versus FOS-Saline libraries were significantly different. The parsimony test illustrated that diets with different treatments altered bacterial diversity in the gut. This result agrees with the results from the RDP II program. The difference between the two tests is that the parsimony test only can detect a difference at the species level whereas RDP II can detect a difference in bacterial diversity at all levels including the species level.

Table 2.07: The relationships among bacterial populations of AOM-treated and saline-treated rats after feeding the three diets (Alphacel, FOS and WB) were analyzed using a Statistical Parsimony Test.

Groups	UW_{Score}	p-value	Significance
Alphacel AOM-Alphacel Saline	0.626653	0.023	NS
Alphacel AOM-WB AOM	0.670984	0.006	NS
Alphacel AOM-WB Saline	0.709217	<0.0010	S
Alphacel AOM-FOS AOM	0.765449	<0.0010	S
Alphacel AOM-FOS Saline	0.725029	<0.0010	S
Alphacel Saline-WB AOM	0.660446	0.004	NS
Alphacel Saline-WB Saline	0.732104	<0.0010	S
Alphacel Saline-FOS AOM	0.733001	<0.0010	S
Alphacel Saline-FOS Saline	0.678029	<0.0010	S
WBA OM-WB Saline	0.673379	0.142	NS
WB AOM-FOS AOM	0.694505	<0.0010	S
WB AOM-FOS Saline	0.734141	<0.0010	S
WB Saline-FOS AOM	0.811363	<0.0010	S
WB Saline-FOS Saline	0.795148	<0.0010	S
FOS AOM-FOS Saline	0.575825	0.04	NS

NB: S and NS represent significant and non-significant.

Table 2.08: RDPII library comparison analysis of 16S rRNA gene libraries in the feces of AOM-treated and saline-treated rats after feeding with three diets (Alphacel, WB, and FOS).

Genus	WB-Saline	WB-AOM	FOS-Saline	FOS-AOM	Alphacel-Saline	Alphacel-AOM
Turicibacter§	6	23↑	0	6	0	0
*Clostridium XI***	6	0	0	0	0	0
Clostridium sensu stricto	24	11↓	3	3	7	6
Blautia¤	0	0	24	13↓	1	0
*Lactobacillus***	7	3↓	0	0	0	0
Clostridium IV¤	16	6↓	5	0	22	11↓
Allobaculum	0	0	5	13↑	0	0
*Bacteroides**	0	0	5	0	0	0

The data indicate that *Clostridium XI* and *Lactobacillus* bacteria might have protective activities against colon cancer because these bacterial species are significantly present in the feces of rats that did not show tumors incidence. The library comparison results examining the significant bactrial species that might have protective effects or an assiociation in the colon cancer development are shown in **Appendix Table A.03 to Table A.11**. **Table 2.08** summaries the bacterial species that is significantly different among libraries. It was found that *Lactobacillus* species were significantly reduced in WB-AOM treated rats compared to WB-Saline rats. There are many species of *Lactobacillus* found in the gut of most warm-blooded animals (Salminen *et al.* 1998). Our results are consistent with epidemiological studies that have suggested that these bacterial species play a significant role against colon cancer development. It was found that administration of cultured *Bifidobacterium longum* HY8001 and *Lactobacillus acidophilus* HY2104 significantly inhibited the formation and multiplicity of ACF in the colon of AOM-treated rats (Arimochi *et al.* 1997; Lee and Lee 2000). In addition, the *Lactobacillus casei* strain Shirota (LcS) has potent anti-tumor and anti-metastatic effects on transplantable tumor cells, and intrapleural administration of LcS into tumor-bearing mice inhibited tumor growth and increased survival rate (Matsuzaki 1998). The feeding of *Lactobacillus acidophilus* NCFM and N-2 strains significantly decreased the activity of three bacterial enzymes (nitroreductase, β-glucuronidase, and azoreductase) in humans (Goldin and Gorbach 1984), and feeding viable *Lactobacillus acidophilus* reduced colon cancer incidence in rats (Goldin and Gorbach 1980). It was also shown that lactic acid bacteria play important roles in the metabolism of carcinogens including increasing activities of colonic P450 reductase (Pool-Zobel *et al.* 1996) and glutathione-S-transferase (Challa *et al.* 1997), and

reducing hepatic uridine diphosphoglucuronyl transferase activity (Abdellai *et al.* 1995) in rats.

It was noted that *Allobaculum* species were a significant presence in the feces of FOS fed AOM-treated rats. The correlation between tumor incidence and *Allobaculum* suggest it might play a role in aiding tumor generation because the second highest incidence of colon tumors was found in FOS fed rats. However, this species was not found in other treated rats. There is not much information on the *Allobaculum* group. Greetham *et al.* (2004) first isolated a novel rod shape anaerobic organism from canine feces which was called *Allobaculum stercoricanis* and proved to be a Gram-positive, non-spore forming, and non-motile bacterium. The GC content of *Allobaculum stercoricanis* is 37.9% and the major end product of glucose metabolism is lactic and butyric acids (Greetham *et al.* 2004) that are known as promoters of colon cancer development in FOS feeding rats. It was also found that the DNA sequence identity of *Allobaculum stercoricanis* was similar to *Ruminococcus obeum* in DMH-treated rats (Wei *et al.* 2010).

The genus *Blautia* was significantly present in the feces of FOS feeding rats under both AOM and Saline treatments but not in the other diets. It is also very interesting that *Blautia* species were almost twice (as numerous) in the FOS-Saline treatment than in the FOS-AOM treatment. It could be possible that AOM and its metabolites might have negative effects and interactions with these bacterial species. There were several *Blautia* species found in the feces of humans and other mammals (Liu *et al.* 2008; Furuya *et al.* 2010; Park *et al.* 2012). The 16S rRNA sequence of *Blautia glucerasei* has similarity (95.3%) to the strain of *Ruminococcus obeum* (Furuya *et al.* 2010). The GC content of its genome DNA is 40.7% and optimum growth is at 37°C and pH 7.0. *Blautia* are Gram-positive, strictly anaerobic, oval spore

forming rods, and motile. They mainly ferment carbohydrates that produce acetate, formate, and lactate (Furuya *et al.* 2010; Liu *et al.* 2008). It is worth mentioning that the genus *Blautia* is newly proposed and formerly *Blautia* species belonged to the genus *Ruminococcus* and *Clostridium* (Liu *et al.* 2008).

Turicibacter species were significantly present in the feces of AOM-treated rats upon feeding with the WB diet. It is suspected that AOM and its metabolites might have positive effects on this bacterial species. It may be possible that AOM might not have an impact on *Clostridium sensu stricto* species because the number of this species remained unaffected for all three diets. An important observation was that *Clostridium IV* species were significantly decreased from saline treatment to AOM treatment with respect to diets (e.g. WB, FOS, and Alphacel). It may be possible that AOM might play a significant role in the growth of this bacterial species. It was found that infection with *Clostridium septicum* had a significant link to malignancy with high mortality in humans (Chew and Lubowski 2001; Mirza *et al.* 2009). Larson *et al.* (1995) showed that 50% of malignancy was association with *Clostridium septicum* sepsis compared with other clostridial infections in humans. The authors showed that among those malignancies 70, 24, and 6 percent were related to colorectal carcinoma, haematological malignancy, and gall bladder carcinoma, respectively.

CHAPTER 3

Toxic effects of fecal water on HCT-116 human colon cancer cells

INTRODUCTION

Fecal water (FW) is the aqueous phase of feces obtained after homogenizing feces and pelleting the particular matter. The fluid portion, therefore, contains the soluble metabolites that remained after absorption in the colon. Several compounds including short chain fatty acids (SCFA), lactate, succinate, bile acids, nitrate, sulphate, and vitamins were measured in FW (Cumming and Bingham 1987). Rafter *et al.* (1987) proposed that the components of fecal water may interact with colon epithelial cells to perform normal physiological functions or, on the other hand, cause pathogenesis (possibly leading to inflammation or colon carcinogenesis). FW composition is known to be altered in patients with colorectal adenomas in comparison to healthy individuals (Nordling *et al.* 2003).

It was observed by Marchesi *et al.* (2007) that a high concentration of glycerol was obtained from the feces of patients with inflammatory bowel disease (IBD) that was due to high metabolic activities. In addition, they identified reduced levels of butyrate, acetate, methylamine, and trimethylamine in the fecal extracts of IBD patients compared to normal individuals. These metabolites are a result of fermentation in the colon by resident microflora; and the depletion of these metabolites in feces was suggested to be involved in the disruption of the normal bacterial ecology in patients with IBD (Marchesi *et al.* 2007).

Studies identified potentially beneficial factors (e.g. polyphenols and SCFA) as well as potential risk-associated factors (e.g. N-nitroso compounds and heterocyclic amines) in FW (De Kok *et al.* 2000). Some of these can be related to colon cancer risk. For example, many studies identify bile acids and their derivatives as being important components. Cummings *et al.* (1976) reported the protective effects of dietary fiber against human colon cancer, principally through its effect on fecal

parameters such as the ability to modify fecal bile acid output. Lapre and Van der Meer (1992) demonstrated that steroid supplementation in the diet increased the lytic activity of FW on colon epithelial cell proliferation *in vivo,* illustrating the effect of diet in influencing the risk for colon cancer (inhibition of normal epithelial cell proliferation). The lytic activity of FW in human patients with adenomatous polyps in their colon has been shown to be decreased by supplementing the diet with calcium confirming that the protective effects of FW can be amended by nutritional manipulations and indirectly suggesting that bile acids may be involved since they form insoluble salts with calcium. Protective effects of bile acid derivatives have also been found. The effect of the soluble bile acid fraction in FW (e.g. deoxycholic acid (DCA) and muricholic acids) was shown to be protective against AOM-induced colon tumorigenesis in the rodent model; this effect was apparently related to the lowering of secondary bile acid (DCA) levels in the feces (Batta *et al.* 1998). The FW of adenoma patients also induced more DNA strand breaks in cultured cells than normal individuals, an effect that could be due to higher concentrations of lithocholic acid (LCA) in fecal water as well as fecapentaene-12 in feces (Nordling *et al.* 2003).

Bile acids may be one of the important carcinogenic metabolites present in FW that may play a significant role in colon cancer development (Reddy *et al.* 1992). It has been shown that primary and secondary bile acid administration to the small intestine of germfree and convectional rats induced tumors developing in the colon (Narisawa *et al.* 1971; Reddy *et al.* 1977; Corpet *et al.* 1997). In addition to primary bile acids, most of the secondary bile acids, such as deoxycholic acids (DCA) and lithocholic acid (LCA), are also carcinogenic. It was found that rats fed dietary DCA supplements showed enhanced proliferation of colonic tissues, which is thought to be one factor in tumor growth (Wargovich *et al.* 1983; Hamada *et al.* 1994). In a

systematic meta-analysis of research conducted in patients to address the relationship between fecal bile acids and colon cancer, it was concluded that bile acids indeed play a role in the development of colon cancer (Tong 2008). There are several publications linking increased fecal bile acid secretion to colon cancer incidence in animal models (Bajor *et al.*, 2010). However, bile acids are necessary since they play a role in normal physiological functions of the colon including colon motility and motor function (Bajor *et al.* 2010). Bile acids are produced in the liver and secreted into the small intestine through the biliary tree where they act to enhance the absorption of dietary lipids (Bajor *et al.* 2010). Although the majority of bile acids are reabsorbed before they reach the cecum, a minimal concentration reaches in the lower gut, where passive absorption to re-enter the hepatic circulation can occur. The re-absorption of bile acids is dependent on colonic pH and chemical modifications (such as deconjugation and/or dehydroxylation) by the resident microflora (Bajor *et al.* 2010). It was also noticed that the resident gut microflora also appear to play a role in homeostasis of the bile acids in the colon through the fermentation process (Hammer *et al.* 2011). Baijal *et al.* (1998) demonstrated that the tumorigenic effect of bile acids in the colon was dependent on the time of intervention in the carcinogenic process (i.e. initiation versus post-initiation/promotion stages). The carcinogenic effects of bile acids are thought to be exerted through several cellular mechanisms, including pathways associated with growth, proliferation and inflammation. For instance, bile acids are known to activate the epidermal growth factor receptor (EGFR), and thus disturb associated signaling molecules such as p38 and members of the mitogen activated protein kinase (MAPK) family (Rhodus *et al.* 2005; Jaiswal *et al.* 2006). It was shown through *in vitro* experiments that bile acid-induced cellular acidification

involved nitric oxide (NO)-mediated inhibition of Na^+/H^+ exchangers, which in turn leads to DNA damage (Goldman *et al.* 2010).

Rafter *et al.* (1987) found a significant weak correlation between bile acid concentration and cell lysis, which indicates that there might be other compounds in FW that may exert cytotoxicity. Studies have found that dietary calcium phosphate supplements decreased the cytolytic activity of FW from 47±9% to 27±8% ($n = 12$, $P<0.05$) in the intestinal lumen in rats, which decreased epithelial cell proliferation (Lapre *et al.* 1993). Lapre *et al.* also showed that dietary calcium supplements in humans decreased the molar ratio of hydrophobic bile acids to hydrophilic bile acids by precipitating the hydrophobic dihydroxy bile acids. As a consequence, the cytotoxicity of fecal water decreased. This synergistic effect increases hydrophobicity of the bile acids in FW (Lapre *et al.* 1993).

DF may reduce the concentration or carcinogenic activities of bile acids in FW. Diet plays an important role in controlling bile flow in the secretion and excretion of secondary bile acids such as DCA and LCA (Thompson *et al.* 1985; Villalon *et al.* 1992). It was shown that diets low in fiber increased LCA levels in FW, and similar higher levels of LCA have been shown to induce DNA strand breaks, form DNA adducts, and inhibit DNA repair enzymes *in vivo* (Chaplin 1998). Diets supplemented with fiber (from wheat bran or rye) significantly reduced the concentration of fecal secondary bile acids that lead to a lower level of potential fecal mutagens (e.g. fecapentaenes) in humans compared to those that did not receive dietary fiber supplements (Reddy *et al.* 1987). Additionally, Reddy *et al.* (1992) reported that the modifying effect of dietary fiber on secondary bile acids and bacterial enzymes depended on the type of dietary fiber consumed. Subsequently, a high intake of wheat bran dietary fiber was shown to be associated with a significantly

lower incidence of colon tumors in humans. There are several possible mechanisms that can explain the relationship between the DF and FW carcinogens such as bile acids. A direct mechanism proposes that undigested dietary fiber (insoluble or soluble dietary) binds to carcinogens and transports them out of the intestines in the feces. DF also increases fecal volume and decreases transit time (Harris and Ferguson 1993). This is thought to dilute mutagens in the lumen and decrease exposure to potentially carcinogenic compounds. The extent of binding depends on the nature of the carcinogens and DF. Studies found that dietary fiber, especially wheat bran, had 20 times more bulking capacity than pectin (Stephen and Cummings 1979; Hariss *et al.* 1993; Janicke *et al.* 2010).

FW may be cytotoxic and genotoxic. It was shown that the water fractions and not the lipid components of human feces induced apoptosis in colonic cells *in vitro* (Haza *et al.* 2000), giving rise to the concept that the aqueous phase of the feces exerts a direct cytotoxic effect rather than those components of the feces that are bound to food residues and bacterial mass. The importance of FW cytotoxicity and potential genotoxicity to normal physiological function in the colon, and any health consequences observed in the colon (such as colon cancer) have been inferred from these studies. The cytotoxic effects of FW on colon epithelial cells have also been shown predominantly through *in vitro* studies using human colon epithelial cell lines (Haza *et al.* 2000; Lee *et al.* 2005) but direct cytotoxic effects of FW have been shown *in vivo* by measuring total alkaline phosphatase activity in colon epithelial cells of rodents fed different diets (Lapre *et al.* 1993). The genotoxicity of FW has been assessed using classical methods such as the Comet assay, measurement of DNA adducts, and cytokinesis-block micronucleus (CBMN) assay, among others (Cross 2006; Fenech *et al.* 2006).

In preliminary studies on the relationship between bile acids and the colon epithelium, Rafter *et al.* (1986) reported that it was not the total concentration of bile acid in the colon that was responsible for the cellular damage occurring in the colon epithelium but it was the concentration in solution, suggesting that FW is the more important component. For example, the cytotoxic effects of bile salts on the colon epithelium were reduced by the increasing colonic calcium concentrations or by lowering the pH (Wargovich *et al.* 1983).

Intake of high fat diets counteracts the effect of dietary calcium because fat stimulates bile acid release that overwhelms the binding of calcium. As a result, high fat diets increased the cytotoxicity of FW, increased cell proliferation and damaged epithelial cells in colon (Lapre *et al.* 1993). Free fatty acids appear to be even more toxic: not only did they damage the colonic epithelium, but also they also induced hyperproliferation in cultured cells. Calcium supplements can decrease the solubility and concentration of surfactants in FW (Lapre *et al.*1993).

It is possible that intake of high amounts of animal fats, high protein diets, or red meat may increase the toxicity of FW and may be associated with tumorigenesis. It has been shown that high protein diets increase N-nitroso compounds in FW that induce more DNA damage. Those compounds have been associated with the initiation and development of colon tumorigenesis in humans and experimental animals (Bird 1986; Gratz *et al.* 2011). The typical Western diet has been shown to be associated with higher amounts of heterocyclic mutagenic amines (e.g. 2-amino-3,4-dimethylimidazo (4,5-f) quinoline and 2-amino-3-methylimidazo(4,5-f) quinoline) that exert damaging effects on the colonic epithelium of C57BL/6J mice *in vivo* (Bird 1986). They also have been shown to induce DNA strand breaks in CaCo-2 and HT-

29 cells *in vitro* (Gratz *et al.* 2011; Reiger *et al.* 1999). It was shown that dietary heme increased DNA damage in rats compared to the control diet (de Vogel *et al.* 2008).

The toxicity of FW may depend on various factors such as concentration, time, dietary composition, weight, and age. It was noticed that the concentrated FW (individuals on diets high in fat and meat but low in dietary fiber) induced more oxidative DNA damage on HT-29 cells (Rieger *et al.* 1999). Joosen *et al.* (2009) found that the high consumption of red meat stimulates the production of mutagenic nitroso compounds in humans. The authors also suggested that the processing of meat was associated with stimulation of nitrosation and DNA damage that might increase risk of colorectal cancer in humans (Joosen *et al.* 2009). It was also found that pyrolyzates are potent mutagenic and carcinogenic heterocyclic amines that are formed during the processing of foods (Morotomi and Mutal 1986). Recent studies have found that the FW of vegetarian (VEG) diets significantly induced more DNA strand breaks than the meat diets in CaCo-2 cells (Joosen *et al.* 2009). This is controversial and further studies need to be performed to clarify these findings.

Background on ViaLight Assay:

The ViaLight assay analyzes cell proliferation and cytotoxicity by measuring adenosine triphosphate (ATP) levels in living cells. This assay is more useful than other conventional methods (e.g. MTT) used in drug discovery and drug toxicity testing because it very fast and sensitive, as well as it does not require any radioisotopes (Slater 2001). The ATP bioluminescence method utilizes the luciferase enzyme, which catalyses the formation of light from ATP and luciferin to determine the ATP levels in metabolically active cells (Crouch *et al.* 1993). The luciferin-luciferase reaction is shown in the following equation 2.

$$ATP + Luciferin + O2 \xrightarrow[Mg^{2+}]{Luciferase} Oxyluciferin + AMP + PPi + CO_2 + LIGHT$$

Equation 2

The absorbance of light is directly proportional to the ATP concentration that is measured by a luminometer or beta counter at ambient temperature (18-22°C). This is the optimum temperature for the luciferase enzyme (Campbell, 1988).

Background on Apoptosis Assay:

The term apoptosis was first proposed by Kerr *et al.* in 1972 to describe a specific morphological pattern of cell death that occurs during embryonic development and during maintenance of tissue homeostasis in healthy adult tissue. Apoptosis is a controlled physiologic process that removes individual cells of an organism without destruction or damage to the organism (Kerr *et al.* 1972). It is also known as programmed cell death. Oxidative stress has been suggested to mediate apoptosis (Bergman *et al.* 1996). The morphologic features of the apoptosis process are characterised by loss of plasma membrane asymmetry and attachment, condensation of cytoplasm and nucleus, and DNA damage (i.e. injuring DNA that may result in a mutation or block of DNA replication). In the plasma membrane of apoptotic cells, a phospholipid (phosphatidylserine) is translocated from the inner to outer surface of the plasma membrane.

An inhibition of apoptosis contributes to tumor formation and is thought to be one cause of colon cancer progression. It is strongly regulated by diets (Bedi *et al.* 1995; Chapkin *et al.* 2000). DF has a critical role in maintaining the steady state balance between proliferation and apoptosis in the colon (Hijova and Chmelarova

2007; Chapkin *et al.* 2000). This is thought to occur through production of SCFA, especially butyrate, which is a well-known chemo-preventive agent due to its ability to stimulate apoptosis *in vitro*.

Annexin-V is a Ca^{2+} dependent binding protein that binds to phosphatidylserine (PS) (Meers *et al.* 1991; Moss *et al.* 1991). A conjugate between Annexin V and a fluorochrome compound retains its high affinity binding for PS and acts as a sensitive probe for monitoring the amount of external membrane PS. This is related to the number of apoptotic cells within a population that are actively undergoing apoptosis. Fluorescein isothiocyanate (FITC) is a derivative of fluorescein that is widely used in the flow cytometry. The combined use of FITC Annexin V with propidium iodide (PI) dye detects different stages of apoptosis based on their interaction and permeability to the membranes of cells. Neither FITC Annexin V nor PI dyes are permeable to viable cells (O'Brien and Bolton 1995). However, FITC Annexin V is permeable to intact cell membranes during early apoptosis whereas PI is only permeable to the membrane of late apoptotic and dead cells.

Hence in this assay, four classes of cells are possible: FITC-Annexin V and PI negative cells represent viable cells, FITC-Annexin V positive and PI negative cells indicate cells in early apoptosis, FITC-Annexin V positive and PI positive cells represent cells in late apoptosis, and FITC-Annexin V negative and PI positive cells represent dead cells. The dead cells only contain PI dye. This is represented in an idealized flow cytometer output **Figure 3.01**.

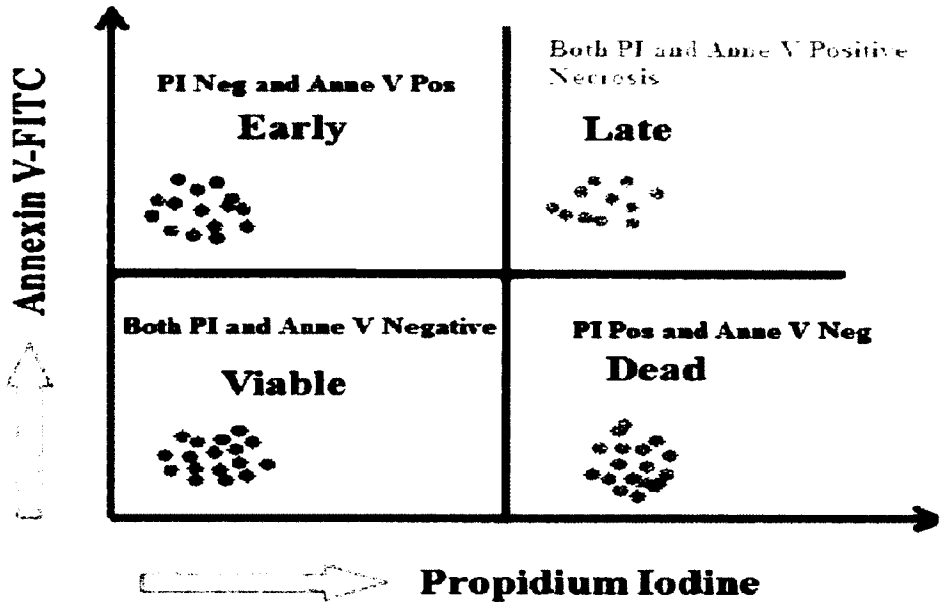

Figure 3.01: The schematic representation of apoptotic cells in different stages of apoptosis.

Background on BrdU Assay/DNA Damage

DNA damage refers to the disruption (break) in the nucleotide strands by the action of factors (e.g. UV light, carcinogens, xenobiotics, dietary components) (Parrish *et al.* 1982). The most important characteristic of late apoptosis is DNA fragmentation through the activation of endonucleases that occurs in 95% of cell types. These nucleases degrade the higher order chromatin structure of DNA into fragments of 50 to 300 kb and then further degrade then into smaller DNA pieces (approximately 50 bp in length) (Walker *et al.* 1999). In the apoptotic process, the genomic DNA strands are broken by cellular nucleases and the broken DNA pieces are labelled at the 3'-hydroxyl ends with bromolated deoxyuridine triphosphate nucleotides (BrdUTP), a fluorochrome, to identify apoptotic cells. A diagram showing the labelling of broken DNA with BrdUTP dye is shown in **Figure 3.02**. The BrdU assay is known as the gold standard for the detection of apoptosis. In this assay, a terminal deoxynucleotide transferase (TdT) enzyme directly attaches Br-dUTP to the 3'OH ends in the DNA breaks (Li and Darzynkiewicz 1995). This assay can detect two parameters such as apoptotic cells and total DNA content by adding an intercalating dye such as PI whereas conventional methods (e.g. Comet assay) can only detect DNA damage on gel electrophoresis (Walker *et al.* 1993) which cannot detect smaller fragments of DNA. It has been shown that this assay increases detection sensitivity by rapidly incorporating BrdUTP dye into apoptotic cells. It is an improvement on the deoxynucleotide triphosphate method that required binding to larger ligands (e.g. fluorescein, biotin or digoxigenin) (Lawry 2004).

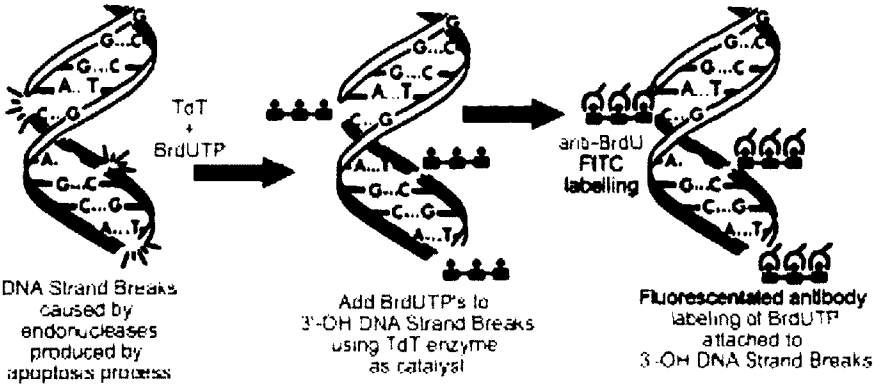

Figure 3.02: The adapted figure from the BrdU kit that represents the APO-BrdU labelling by a FITC-labelled anti-BrdU mAb.

Background on the Cell Cycle Assay:

Cell cycle arrest refers to a halt in the progression of cells through the phases (G_1/G_0, S, and G_2/M) of the cell cycle. The spindle-assembly checkpoint blocks chromosome segregation by inactivating CDC20 (e.g. Slp1 and Fizzy) and degrading proteins (e.g. ubiquitin ligase or cyclosome (APC/C) (Nilsson *et al.* 2006). The important cellular functions, including mitotic spindle pole formation, centrosome assembly, and centriole-centriole cohesion are tightly regulated in the cell cycle. The centrosomes are duplicated in the S phase of the cell cycle and they grow in size in the late G_2 phase. They migrate to the opposite poles of the nucleus and form bipolar mitotic spindle at the G_2/M transition phase of cell cycle (Mayor *et al.* 2002; Lutz *et al.* 2001).

Hydroxycinnamic acid, ferulic acid (FA) and p-coumaric acid (p-CA) are important components of dietary fiber that significantly affect the expression of several genes involved in the cell cycle that may play a significant role against colon cancer development (Janicke *et al.* 2010). These authors found that treatment of CaCo-2 cells with 150 μM of FA or p-CA significantly affected cell cycle regulating genes. In addition, FA treatment induced a delay in the S-phase and up-regulated genes such as RABGAP1, CEP2, and SMC1L1 whereas p-CA treatment induced a delay in G_2/M phase and up-regulated genes in the cell cycle regulating system including CCNA2, CCNB1, MYC, ODC1, CDKN1A (Janicke *et al.* 2010). In their previous study, the same authors showed that FA inhibited cell proliferation of CaCo-2, and inhibited the growth of T47D breast cancer cells and ECV304 endothelial cells (Kampa *et al.* 2004; Hou *et al.* 2004). Hariss *et al.* (1993) proposed two mechanisms (direct and indirect) for how DF protects from developing colon cancer. The effects of dietary FA and p-CA followed an indirect mechanism where the hydroxycinnamic

acids are bound with polysaccharide through ester bonds to the hemicellulose component of dietary fiber (associated with plant cell wall) and this entered into the colon. The colonic bacteria then released the hydroxycinnamic acid through fermentation (Chesson *et al.* 1999, Janicke *et al.* 2010; Plumb *et al.* 1999). The positive effects of FA and p-CA on the intestine have been shown in several animal models. FA has a blocking effect on colon carcinogens and reduced the number of aberrant crypt foci (ACF) in AOM-induced ACF rats after administration of FA (Kawabata *et al.* 2000). Janicke *et al.* (2005) found in their earlier treatment of CaCo-2 cells with 1500 μM FA and p-CA that this decreased the proportion of cells in G_1 phase and increased the proportion of cells in the S and G_2 phases whereas FA significantly increased the length of S phase.

In the assay adopted in this analysis, the fluorescence intensity of cells stained with PI dye remained stable for at least 48 hr, allowing a quantitative estimate of the fractions of cells in different phases of cell cycle (Fried *et al.* 1976). The distribution of cells in each phase is determined by the fluorescence intensity of cells after staining with PI where the intensity of G_2/M phase is equal to twice of G_1/G_0. The intensity of S phase is proportional to the DNA contents that are placed between G_1/G_0 and G_2/M phases (Fried *et al.* 1976). The distribution of DNA contents of cells in different phases significantly represents the stage of tumors (Armitage *et al.* 1987). The distribution of DNA content of HCT-116 cells after 48 hr treatemnet with dietary fecal water is shwown in **Figure 3.03.**

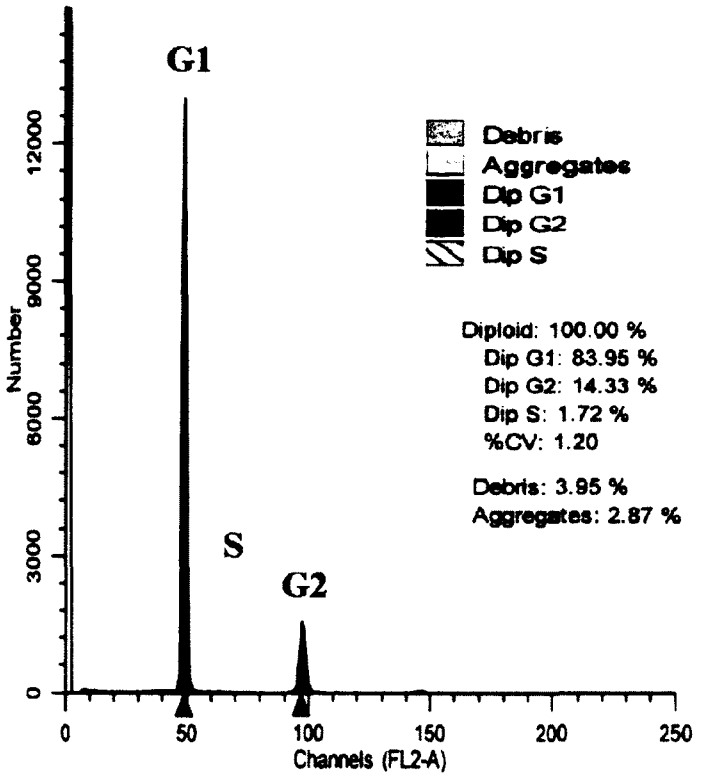

Figure 3.03: Cell cycle arrest of HCT-116 cells was assessed by staining DNA with propidium iodide (PI) dye and flow cytometry analysis. HCT-116 cells were treated with FW 1 of alphacel diet for 48 hr.

Background on Flow Cytometry:

Flow cytometry is a technological process that measures fluorescence signals from cells and quantifies them. In 1972, Herzenberg developed a cell sorter that separated cells stained with fluorescent antibodies, which is known as Fluorescence Activated Cell Sorter (FACS). It used laser lights to excite cells to emit fluorochrome signals from cells within the size of 0.5-40 μm in diameter. First, antibodies or dyes recognizing specific molecules on the surface of cells are attached followed by antibodies or dyes conjugated to fluorochromes. The prepared sample is first injected into a flow cytometer where cells are randomly distributed in three-dimensional space in solution. The sample must be ordered into a stream of single particles by the fluidics system. The overall systematic procedures of a flow cytometer are shown in **Figure 3.03**. The fluidics system consists of a central channel that contains faster flowing fluid that creates greatest velocity at its center and zero velocity at the wall to pass cells through the laser one cell at a time (Rahman 2005; Darzynkiewicz *et al.* 2008).

When the cells pass through laser beam and they scatter and absorb light, and fluorochromes are excited to higher energy state. The molecules relax to a lower state by releasing energy as a photon with specific spectral properties unique to different fluorochromes. The scattered and emitted light from cells is detected and converted into electrical pulses by optical detectors. The detector used in flow cytometer is the photomultiplier tube (PMT) that converts light into electrical pulses and then is processed by a series of linear and logarithmic amplifiers that are used to measure fluorescence in cells. The different pulses are processed by an Analog to Digital Converter (ADC) that can be plotted on a graphical scale (e.g. One Parameter, Two parameter Histograms).

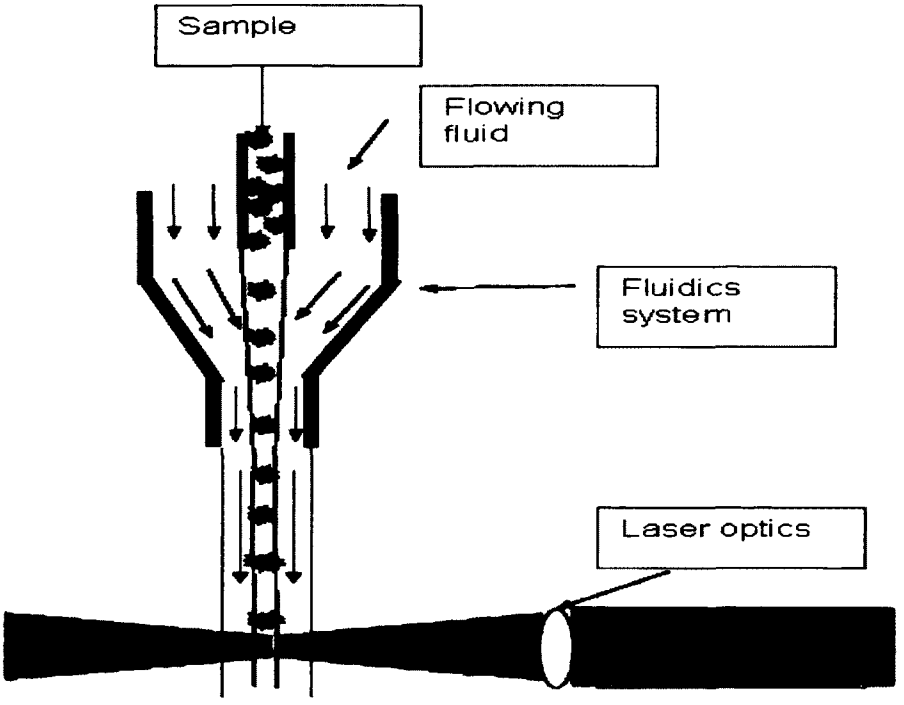

Figure 3.04: The adapted diagram that represents hydrodynamic focusing of a single stream of cells from the following source (http://biology.berkeley.edu/crl/flow_cytometry_basic.ht mL).

The electrical pulse is shown on a histogram graph as an output of cell measurement. A one-parameter histogram is a graph where the numbers of events (cells count) are expressed on the y-axis and the measurement parameter (relative fluorescence or light scatter) on the x-axis. The specific fluorescence light generates pulses or signal for each event. The intensity of fluorescence light is proportional to the height of the pulse displayed on the histogram. A two parameter histogram represents two measurement parameters as dot density gradient on the x-axis and cell count on y-axis.

An important principle of flow cytometery is the gating procedure that eliminates unwanted particles (e.g. dead cells, clumps, and debris) from the cells of interest. This is a rapid process that can provide information on individual sort or separate subpopulations of the cells. The important feature of a flow cytometer is that it measures fluorescence per cell or particle compared to spectrophotometry.

MATERIALS AND METHODS

Fecal Water Preparation:

Stool samples from the three diets were used to prepare FW at three different times. Stool samples were taken from a parallel rat study where rats were fed 5% fermentable material WB or FOS, and alphacel (control). First, feces was weighed and mixed with ice cold PBS buffer (1X PBS) (1.5 g stool sample was mixed with 4.5 mL of ice cold PBS) and homogenized in centrifuge tubes. The tubes were centrifuged at 21800 x rpm for 2 hr at 10°C in a Beckman Coulter Floor Model Ultracentrifuge (Beckman Coulter Canada, Inc. Canada) to dissolve the metabolites in feces (SCFA, bile acids, nitroso compounds, etc.). The supernatant was collected and filtered through a sterile 0.2 μm pore size membrane (MILLEX®GP, Carrighwohill Co. Cork, Ireland) to remove bacteria in order to avoid bacterial contamination when using these extracts with the HCT-116 human colon cancer cells. The FW was frozen at -20°C until use. The percentage of recovery of FW was calculated. The pH of FW was determined using pH meter (SevenEasy™ pH Meter S20, Mettler-Toledo AG, Switzerland).

Cell Culture and Treatments:

HCT-116 human colon cancer cells were cultured and treated with FW obtained as above to determine the effects of FW on parameters including cytotoxicity, apoptosis, cell cycle arrest and DNA damage. Cells were grown in McCoy's 5A medium (HyClone, Logan, UT) modified with 10% (v/v) fetal bovine serum (HyClone, Logan, UT), and 1% penicillin/streptomycin (HyClone, Logan, UT) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 mixture in BD Falcon T25 flasks.

Cell Density (Cells Counting) Measurement:

One hundred microlitres of the cell suspension and trypan blue were mixed 1:1 (v/v) in a sterilized centrifuge tube with pipetting up and down. A glass slide was always placed on top of the hemocytometer cell and then the cell mixture (approximately 50 μL) was added into the hemocytometer cell. Cell counting was performed using a microscope and the cell density was noted using the following formula.

Volume of Counter Plate:

= 1 mM (length) x 1 mM (wide) x 0.1 mM [where, 0.1 mM is the thickness of plate]

= 0.1 mM^3 = 0.1 μL = $0.1x10^{-3}$ mL = 10^{-4} mL

We knew that, 1 mM^3 = 1 μL and 1 mL= 1000 μL. So 1 μL = $1x10^{-3}$ mL

Cell density (cells/ mL) = {(Total Number of Cell) ÷8} x $2x10^4$ cells/ mL

The counter plate contains 4 quadrants in one field and it has a total of 2 fields. Therefore, two fields contain total 8 quadrants. The second 2 stands for the dilution factor because we have a total of 200 μL solution after the 100 μL cell suspensions were mixed with 100 μL trypan blue.

Cell Proliferation and Cytotoxicity Assay:

The ViaLight Plus Kit (Rockland, USA) was used for the direct detection of living HCT-116 cells that were treated with fecal water samples. The ViaLight Plus Kit measured living cells that contained adenosine triphosphate (ATP).

HCT-116 cells ($1.0 x 10^4$ cells/well) were seeded into a 96-well plate for 24 hr in the incubator at 37°C with a mixture of 95% air and 5% CO_2 and treated for 0.5, 24, 48, and 72 hr with various diluted FW preparations (e.g. FW1, FW2, FW3, FW4,

FW5, FW6 and FW7), 1 mM, 3 mM and 10 mM of sodium butyrate, 1X PBS buffer (negative control) and 9.79 M H_2O_2 (positive control) in order to determine the optimum time line for our toxicity research. The original FW was diluted with PBS buffer. In preliminary tests, the cytotoxicity assay had provided optimum results at 48 hr with FW1 and FW3 dilutions. Therefore, all others assays were performed at 48 hr.

HCT-116 cells were seeded into a 96-well plate for 24 hr in the incubator at 37°C with a 95% air and 5% CO_2 mixture. After 24 hr incubation, the cells were treated with 10 µL of FW. Cell medium was placed in the outer well of 96-well plate to control humidity. The plates were placed on a shaker for 10 min and then placed in the incubator for 48 hr for treatment. Following 48 hr of incubation, 50 µL of cell lysis reagent was added into each well to lyse the cells and the plate was placed on a shaker (speed 3) for 10 min. Finally, 100 µL of ATP monitoring reagent was added into each well which catalyzed the formation of light from ATP as ATP was released from living cells. The plate was placed in the shaker for 3 min. The fluorescence was measured using a luminometer (POLARstar OPTIMA and BMG LABTECH, USA). The data were recorded and analyzed with statistical software SigmaPlot (version 12.0).

Apoptosis Assay:

Cell Culture and Treatment:

HCT-116 cells (1.5 x10^6 cells per flask) were seeded into 25 cm^2 flasks and incubated for 24 hr at 37°C in a mix of 95% air and 5% CO_2. Each flask contained a total of 3 mL cell medium. The old medium was aspirated from each flask and fresh medium (2700 µL) was added in its place. Then the seeded cells were treated with 300 µL of FW, 1X PBS, 10 mM NaBut, or 1 mM NaBut for 48 hr in the incubator.

Washing and Staining Procedure:

Following treatment, 10 polypropylene falcon tubes (15 mL) and thirty polystyrene flow cytometer tubes (5 mL) were labelled. The cell confluency was checked under a microscope. All floating cells were collected into 15 mL falcon tubes and they were kept in an incubator. The treated attached cells on the surface of the flask were washed with 5 mL of cold PBS buffer and then 0.75 mL of 1X Trypsin was added into each flask. Flasks were then incubated for 4 min to detach the cells from the surface of the flask.

A 4.25 mL aliquot of cell medium was then added into a flask. It was rotated and then all of the supernatant was transferred to the tube containing the floating cells. The tubes were centrifuged for 5 min at 1000 x rpm and the supernatant was aspirated. Five millilitres of fresh cell medium was added to the pellet and the tubes were gently vortex to mix the solution, and a 100 µL aliquot was transferred into a 1.5 mL tube for cell counting. All tubes containing solution were kept in an incubator until the cell counting was completed. The tubes were centrifuged for 5 min at 1000 x rpm (Eppendorf Centrifuge 5810R, VWR Canlab, Canada) and the supernatant was aspirated. PBS treated cells were killed by boiling them in heater at 100°C for 10 min.

Cells were washed twice with 5 mL of ice-cold PBS and centrifuged for 5 min at 1000 x rpm. The supernatant was aspirated. The cell pellets were re-suspended with 1X Binding Buffer to a concentration of $1x10^6$ cells/mL and then a 100 µL solution was transferred ($1x10^5$ cells) into a 5 mL flow tube. A 5 µL aliquot of fluorescein isothiocyanate (FITC) Annexin V and 5 µL of propidium iodide (PI) were added into each tube and the cells were gently vortexed. Three extra 5 mL flow tubes were prepared as control tubes for the apoptosis assay. Tube 1 contained unstained cells

that contained both reagents/dyes (FITC Annexin V and PI). Tube 2 cells were stained with only FITC Annexin V. Tube 3 cells were stained with only PI.

The cells were incubated for 15 min at room temperature in the dark. At the end of incubation, 400 µL of 1X Binding Buffer was added into each tube. The samples were analyzed by flow cytometry (FACSCalibur, BD Bioscience, USA) within 1 hr of staining.

Cell Cycle Arrest Assay:

The CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson Canada Inc. Ontario, Canada) was used for identification of DNA distribution in the cell cycle phase for the treatment of HCT-116 cells with FW from alphacel, FOS and WB diet groups. The HCT-116 cells were also treated with three concentrations of sulforaphane (10 µM, 20 µM, 30 µM) and taxol (100 µM, 200 µM, 300 µM) in order to determine a positive control for the HCT-116 cell line (Singh *et al.* 2004; Groschel and Bushman 2005). We found that G_2 phase cell cycle was obtained most prevalently with the use of 30 µM sulforaphane.

HCT-116 cells Culture and Treatment:

HCT-116 cells (1.5 million cells per flask) were seeded into 25 cm^2 flasks for 24 hr in an incubator at 37°C with a mixture of 95% air and 5% CO_2. The old medium was aspirated from each flask before the treatment. The seeded cells were treated with 300 µL of FW from alphacel, FOS, and WB diet groups, 1X PBS, and 30 µM Sulforaphane (positive control) for 48 hr in the incubator.

Following treatment, eight 15 mL polypropylene falcon tubes, twenty four 5 mL polypropylene flow tubes, and twenty four 5 mL of strainer cap containing flow

tubes (Becton Dickinson Labware, Franklin, USA) were labelled. Cell density was checked under a microscope. The floating cells were collected into 15 mL falcon tubes and they were put back into the incubator.

The treated attached cells on the surface of the flask were washed with 5 mL of cold PBS buffer solution (1X PBS) and followed by the addition of 0.75 mL of 1X Trypsin into each flask and then incubation for 4 min to detach the cells from the surface of the flask.

Following incubation, 4.25 mL of cell medium was added into a flask. It was rotated back and forth and then all of the supernatant was transferred to the tube containing the floating cells. Tubes were centrifuged for 5 min at 1000 x rpm at room temperature and the supernatant was removed. The cell pellets were re-suspended into 5 mL of fresh cell medium. Tubes were centrifuged at 300 x g for 5 min at 25°C and the supernatant was aspirated but leaving approximately 50 µL of residual fluid.

Cells Washing and Staining:

The cells were washed twice with 2 mL of Buffer Solution and gently vortexed. The buffer solution contained sodium citrate, sucrose, and dimethyl sulfoxide (DMSO). Samples were centrifuged for 5 min at 300 x g at 25°C and the supernatant was aspirated. Two millilitres of Buffer Solution was added into each tube to re-suspended pellets. A 100 µL aliquot was transferred into a 1.5 mL tube for cell counting using a hemacytometer. The cell concentration was adjusted to obtain $1x10^6$ cells/mL by addition of Buffer Solution. The cells were centrifuged for 5 min at 400 x g at room temperature (Kendro® SORVAILL®, Kendro Laboratory Products Inc. Asheville, USA) and all of the supernatant was carefully decanted and a tissue was used to remove the remaining drop.

Solution A (250 μL) (Becton Dickinson Canada Inc. Ontario, Canada) was added into each tube and mixed gently. Solution A contains trypsin in a spermine tetrahydrochloride detergent buffer that disaggregates the solid tissue fragments and digests cell membranes and cytoskeletons. Tubes were incubated for 10 min at room temperature. Solution B (250 μL) (Becton Dickinson Canada Inc. Ontario, Canada) was then added and mixed gently. Solution B contains trypsin inhibitor and ribonuclease A in citrate stabilizing buffer with spermine tetrahydrochloride to inhibit the trypsin activity and to digest the RNA.Tubes were incubated for 10 min at room temperature. Solution C (250 μL) (Becton Dickinson Canada Inc. Ontario, Canada) was then added and mixed gently while sitting on ice for 10 min in the dark. Solution C contains PI and spermine tetrahydrochloride in citrate stabilizing buffer that binds to the clean, isolated nuclei at a final concentration of at least 125 μg/mL. The sample was then filtered through a 50 μM cell strainer cap into a 5 mL flow tube to prevent cell clumping. The sample was kept on ice and covered with foil paper, and then run on a flow cytometer (FACSCalibur, BD Bioscience, USA) within 3 hr. PI-stained nuclei emit fluorescent light primarily at wavelengths between 580 and 650 nm. The resulting fluorescence histograms were analyzed to detect the presence of an abnormal DNA stem line (DNA aneuploidy).

BrdU (DNA damage) Assay:

The assay takes approximately one hour to stain the cells. In the first step, the cells must be fixed with paraformaldehyde before staining to cross link the DNA so that it will not wash out during the staining process and stop apoptosis during staining. The APO-BRDU™ Kit is a two color staining method that was used to

determine DNA breaks and total cellular DNA of HCT-116 cells after treatment with FW of diets by flow cytometer (FACSCalibur, BD Bioscience, USA).

Cell Culture and Treatment:

HCT-116 cells (1.5 million) were seeded into a 25 cm^2 flask and incubated for 24 hr in an incubator at 37°C with a mixture of 95% air and 5% CO_2. Each flask contained a total of 3 mL cell medium. Fresh cell medium was added into each flask by removing old medium. Then the seeded cells were treated with a 1:3 dilution (v/v) of FW, 300 μL 1X PBS, 10 mM NaBut, and 1 mM NaBut for 48 hr in the incubator.

The confluency of treated cell was observed under a microscope. Floating cells were collected into 15 mL falcon tubes and they were put back in the incubator. The treated attached cells on the surface of the flask were washed with 5 mL of cold PBS buffer solution and at the end of washing, 0.75 mL 1X Trypsin was added into each flask and incubated for 4 min to detach the cells from the surface of the flask.

After completion of incubation, 4.25 mL of cell medium was added into each flask and rotated back and forth and all of the supernatant was transferred into tube containing the floating cells. Tubes were centrifuged for 5 min at 1000 x rpm at 5°C and the supernatant was aspirated. Fresh cell medium (5 μL) was then added into the tubes to re-suspended pellets. A 100 μL aliquot was transferred into a 1.5 mL tube for cell counting. Tubes were centrifuged for 5 min at 1000 x rpm at 5°C (Eppendorf Centrifuge 5810R, VWR Canlab, Canada) and the supernatant was aspirated.

Cell Fixation and Staining:

The cell pellets were re-suspended in 4% (w/v) Para-formaldehyde in PBS (pH 7.4) at a concentration of 1-2 x 10^6 cells/mL. The cells treated with PBS buffer

were killed by boiling them at 100°C on a heat block. The cell suspension was placed on ice for 1 hr or more. After completion of the incubation, the cells were centrifuged for 5 min at 300 x g at room temperature (Kendro Laboratory Products Inc. Asheville, USA) and the supernatant was discarded. The cells were washed twice with 5 mL of PBS. The cell pellet was re-suspended in the residual PBS in the tube by gently vortexing. The cell concentration was adjusted to 1-2 x 10^6 cells/ mL in 70% (v/v) ice cold ethanol. The cells were stored at -20°C until use.

The positive and negative control cells provided with the APO-BRDU™ Kit were already fixed. Treated cell tubes were taken out of the freezer (-20°C) and placed at room temperature and gently vortexed them before transferred into flow tubes. One millilitre aliquots of the cell suspensions (approximately 1x10^6 cells/mL) were transferred into 12x75 mM flow cytometer centrifuge tubes. The cell suspensions were centrifuged for 5 min at 300 x g at room temperature (Kendro Laboratory Products Inc. Asheville, USA). The 70% (v/v) ethanol was removed by aspiration without disturbing the cell pellet. The cell pellet was re-suspended by addition of 1.0 mL of wash buffer and gently vortexed. Samples were centrifuged as previously and the supernatant was removed by aspiration. This washing process was repeated. The cell pellets were re-suspended in 50 μL of the DNA labelling solution that contained reaction buffer (10 μL), TdT emzyme (0.75 μL), Br-dUTP (8 μL), and distilled water (32.25 μL).

The cells in the DNA labelling solution were incubated for 60 min at 37°C in a water bath with shaking every 15 min to re-suspend. At the end of the incubation time, 1.0 mL of rinse buffer was added to each tube and the tubes were centrifuged at 300 x g for 5 min at room temperature and the supernatant was removed by aspiration. The washing process was repeated. The cell pellet was re-suspended with 100 μL of

the FITC-labelled anti-BrdU antibody staining solution that contained FITC-Labeled Anti-BrdU (5 μL) and rinsing buffer (95 μL).

The cells with the FITC-labelled anti-BrdU antibody solution were incubated in the dark for 30 min at room temperature. At the end of incubation, 0.5 mL of the PI/RNase Staining Buffer was added into each tube. The cells were incubated in the dark for 30 min at room temperature. The cells were analyzed by flow cytometer (FACSCalibur, BD Bioscience, USA) within 3 hr of staining to obtain optimal results.

Data quantification and statistics:

The luminescence meter/beta counter was used to determine the viable cells by determining ATP levels in the living cells. The flow cytometer was used to detect apoptotic cells for apoptosis assay, cell cycle assay, and BrdU assay. SigmaPlot (version 12.0) statistical program/software was used to analyze data from cell viability, cell cycle arrest, apoptosis, and DNA damage. A paired t-test was used to analyze the fecal water data. SigmaPlot software was also used to create graphs.

RESULTS AND DISCUSSION

The recovery of fecal water (FW) was 73.2%, 66.4%, and 63.7% from the feces of rats that were fed FOS, alphacel, and WB diets, respectively. The fecal pH of WB, FOS, and alphacel diets were 8.1, 5.5, and 6.8 respectively. The recovery of FW and fecal pH are shown in **Table 3.01.**

The FW contains various metabolites that were left in the feces after absorption in the colon of rats that may induce toxic effect on epithelial cell lines. WB had the lowest recovery of FW compared to FOS/Alphacel because the feces of WB were drier and contained long chain polycarbohydrates and undigested cellulose that bound the liquid more tightly. It may also be possible that some of heavy toxic metabolites may not dissolve into PBS due to its polarization activity, or the nature of metabolites, or centrifugation time (2.5 hr) was not sufficient to dissolve the metabolites of feces with solvent (PBS). Diets fermented differently and produced different metabolites that may have distinct nature of dissociation with solvent.

Table 3.01: The fecal pH and fecal water recovery of WB, FOS, and Alphacel (Control) diets.

Parameters	**Diets**		
	WB	**FOS**	**Alphacel**
Fecal pH	8.1	5.5	6.8
Fecal water Recovery (%)	63.7	73.2	66.4

HCT-116 cells were treated with FW from the WB diet group for 0.5, 24, 48, or 72 hr to rationalize the FW concentration and incubation time before assessing the effects of FW on cell parameters. The preliminary results suggested that the inhibitory effect of FW was lowered on HCT-116 cells, but there was no consistency within three experiments after 0.5 and 24 hr incubation. The inhibitory effect of FW after 24 hr incubation is shown in **Figure 3.05.** It is possible that these times were not sufficient to assess the effect of FW on HCT-116 cells. The 72 hr experiments suggested that growth of treated HCT-116 cells was abnormal. The effect of FW after 72 hr incubation is shown in **Figure 3.07**. The cell growth was higher than the initial cell number. This effect is called recovery effect. The possible reason was that the concentration of FW might not be adequate to exert its toxic effect on HCT-116 cells for the full 72 hr. Other factors may also be involved such as the stability and half-life of fecal toxic compounds. It was discovered from the ViaLight assay that FW showed a better cytotoxic effect on HCT-116 cells at 48 hr compared to the other incubation times (0.5, 24, 72 hr). The cell growth rate with FW from WB is shown in **Figure 3.07**. The toxic effects of FW were concentration and time dependent. The cell viability data for 24, 48, and 72 hr treatments are shown in **Figures 3.05, 3.06, and 3.07** respectively. It was noted that the lowest dilution of FW had the highest inhibitory effect on cells growth and vice versa. The effect of FW was also dependent on the initial concentration of cultured cells in the assay. This is because the cells grow during the assay and must not become confluent during the assay as this will interfere with the final result. For example, the cell concentration of cell cycle assay was 1.11×10^6 cell/ mL and 0.82×10^6 cells/ mL with corresponding passage numbers of 7 and 12 respectively.

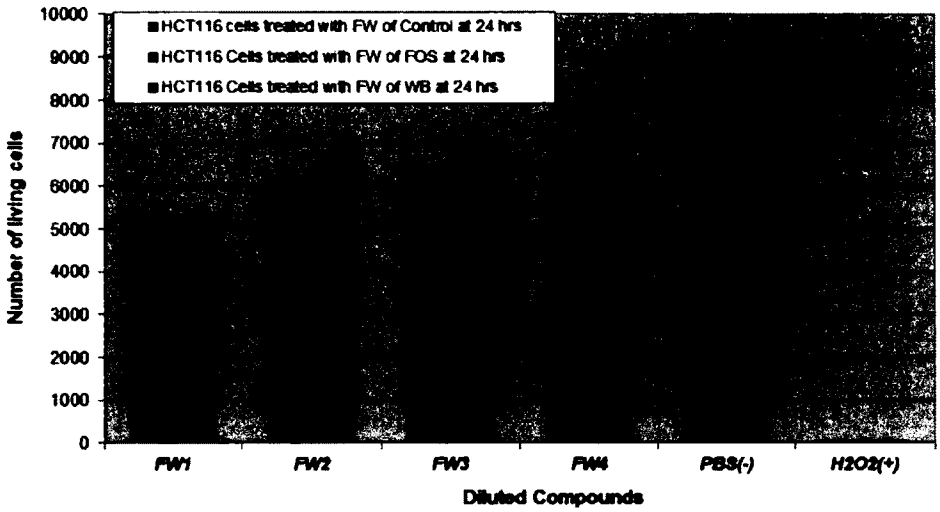

Figure 3.05: The number of HCT-116 living cells after 24 hr treatment with FW from the three diet groups (alphacel, FOS and WB), as measured by a luminescence spectrometer. FW 1-4 represent the diluted fecal water (1:1, 1:2, 1:3, and 1:4) with PBS (v/v), respectively. These data were analyzed by Excel program. PBS and H_2O_2 groups represented negative and positive controls. The column represent the mean (n=6) from 6 replicates and bars represent SD.

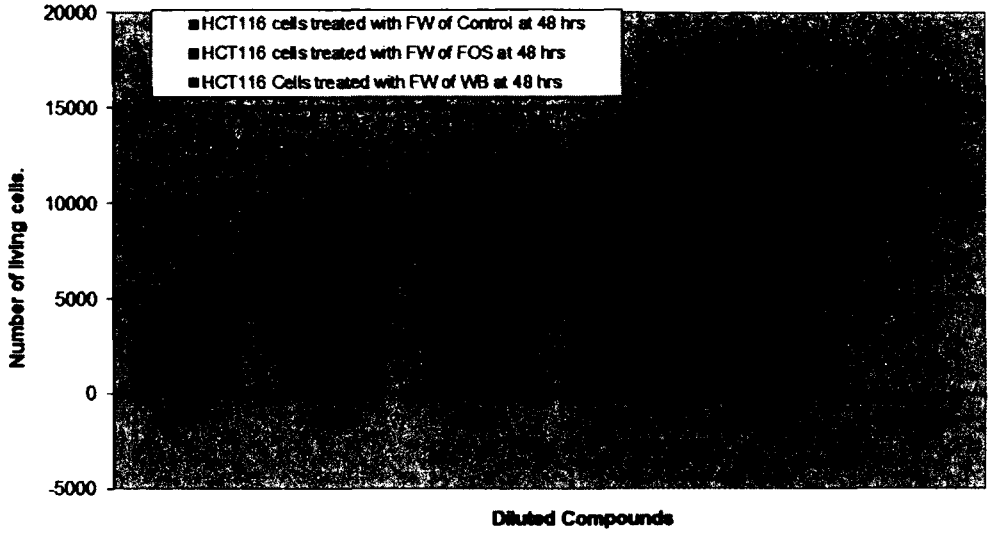

Figure 3.06: Effect of 48 hr treatment with FW from the three diet groups (alphacel, FOS, and WB) on HCT-116 cell viability; other information as in Figure 3.05.

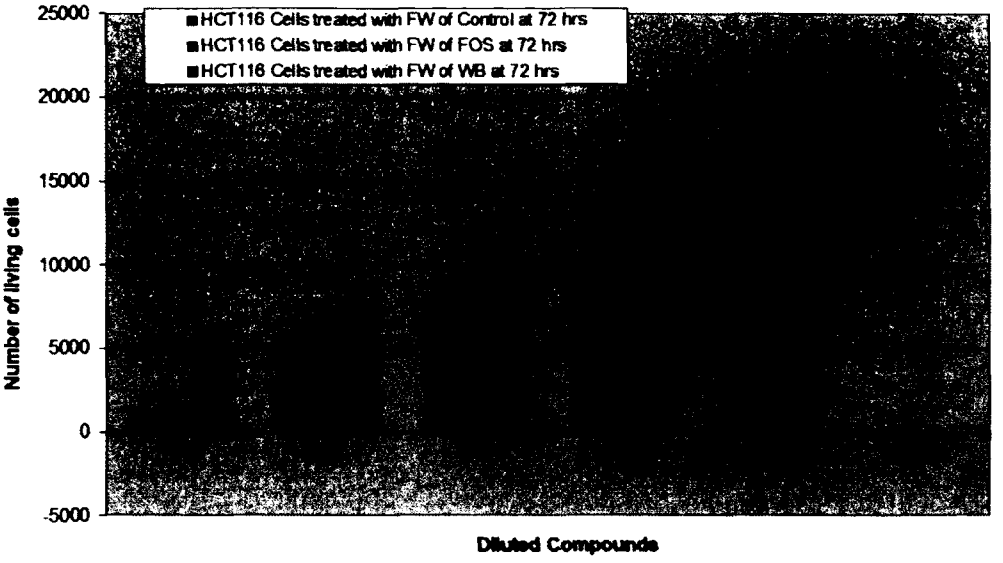

Figure 3.07: Effect of 72 hr treatment with FW from the three diet groups (alphacel, FOS, and WB) on HCT-116 cell viability; other information as in Figure 3.05.

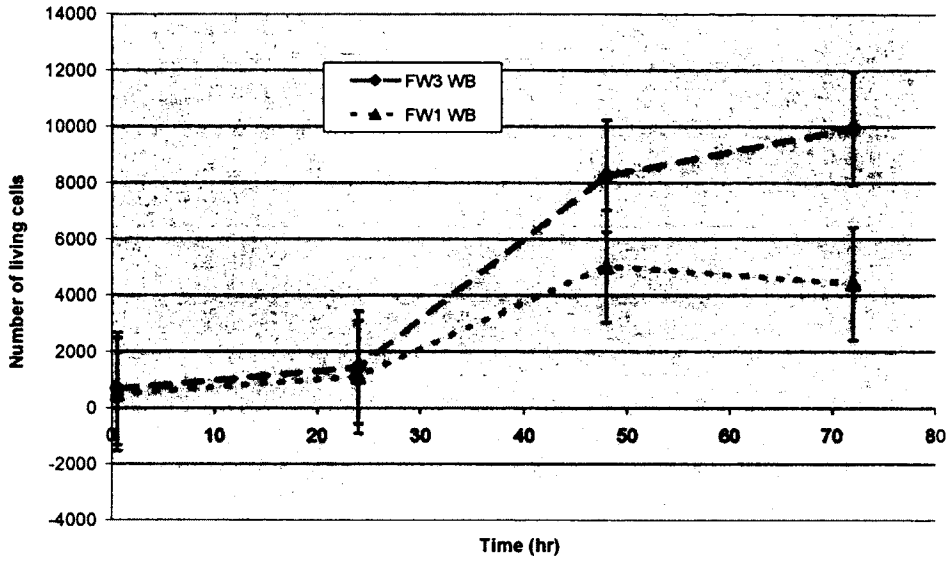

Figure 3.08: Growth curve of HCT-116 human colon cancer cells treated with FW1 (1:1 dilution) or FW3 (1:3 dilution) from the WB diet group at 24, 48, and 72 hr. HCT-116 cell growth increased with increasing time and the higher concentration of FW had a higher inhibition rate.

Effects of FW (FOS/WB/Alphacel) on cell viability

The raw data of cell viability is shown in **Appendix Table A.12 and Figure A.02 to Figure A.06**. The FW study found that WB had no significant effect on HCT-116 cell viability whereas FW derived from the FOS and alphacel diets both had significant effects on HCT-116 cell viability. The toxic effect of FW on cell growth is shown in **Figure 3.09**. The analyzed results of cell growth using paried t-test are shown **in Appendix Tables A.16 –A.19.**

Sang *et al.* (2006) found that the hydrophobic fraction of WB dietary material was more active than the water fraction against growth of human colon cancer cells (e.g. HCT-116 and HT-29) and a 2% WB oil extract significantly inhibited tumoregenesis by 35.7% in the $Apc^{min/+}$ mouse model. The oil fraction of WB consists of the nonpolar and phytochemicals (e.g. phytosterols, phytosterol ferulates, 5-alk-(en)-ylresorcinols, and unidentified constituents). The phytochemical fraction of WB oil had higher inhibitory effects on HCT-116 cells than the nonpolar fraction (Sang *et al.* 2006). Reddy *et al.* (2000) showed that removal of lipid and lipid soluble components prior to feeding WB had high numbers of colon tumors in AOM-induced rats compared to control and suggested that WB oil-soluble material is an important component of WB that inhibits the formation of tumors. Animal and human studies suggested that dietary WB altered the risk factors that are related to colon cancer development such as fecal mutagenicity and secondary bile acids. In addition, WB was more protective than corn bran and oat bran (Reddy *et al.* 1989; Reddy *et al.* 1992). Our data agree with the finding of several labs that feeding of WB diets or dietary supplements to male F344 rats lowered the incidence and multiplicity of colon tumors, as well as decreased the formation of aberrant crypt foci (ACF) compared to control rats after administration of AOM (Reddy and Mori 1981; Reddy *et al.* 1981;

Ferguson and Harris 1996). It was noticed that WB diet had no significant effect in distal cell proliferation in rats (Le Leu *et al.* 2002).

WB is a source of dietary fiber and phenolic compounds including hydroxycinnamic acids and hydroxybenzoic acids that have antioxidant activities. Ferulic acid is one of the phenolic acids found in WB that has important roles in antioxidant, anticancer, antimicrobial, anti-inflammatory, antithrombotic, lowering cholesterol in serum and liver, and increasing sperm cell viability (Ou *et al.* 2004). In addition, feeding of feruloyl oligosaccharides significantly protected pancreatic tissue from oxidative stress and damage in diabetic rats (Ou *et al.* 2007). Wang *et al.* (2008) found that the cytotoxicity (cell viability) of feruloyl oligosaccharides (FOs) were greater than 96% when FOs was incubated with human lymphocyte cells at 37°C for 30 min. However, no dose-response relationship was apparent: there was no significant difference in cell viability from 10 to 500 μmol/L FO (Wang *et al.* 2008). It was suggested that FOs contain non-ionic hydrophilic oligosaccharides that may pass through cell membranes with a high density of inner negative charges more easily than the negative free phenolic compound to exert beneficial effects in immune system (Ou *et al.* 2007). Water soluble FOs from insoluble dietary fiber including wheat bran had no cytotoxicity and genotoxicity when tested on normal human blood lymphocytes (Wang *et al.* 2008). However, FOs had significant cellular protective effects against DNA damage induced by H_2O_2 under *ex vivo* conditions (Wang *et al.* 2008). FW from our experimental WB diets had no significant effects on HCT-116 cells, but WB might have protective effects in normal epithelial cells against colon cancer development. Many factors could be important, including the concentration of WB in the diet. Boffa *et al.* (1992) postulated that diets containing moderate levels of fiber may have protective effect on cell proliferation, differentiation and

carcinogenesis whereas diets supplemented with high fiber contents might have potential effects to promote colon carcinogenesis. This idea was based on their finding that feeding a 5% WB diet decreased both colonic epithelial cell hyper-proliferation and hyperplasia compared to a control diet in rats whereas 10% and 20% WB diets increased hyperplasia and hyper-proliferation. These data agree with gene expression data from our lab showing that 2% WB did not affect gene expression in rats whereas the 5 and 10% WB diets had effects on the expression of 133 and 111 genes (Chen *et al.* 2011).

Fisher male F344 rats fed DCA diets and steroid diets with injection of AOM had significantly elevated cell proliferation compared to control (saline-treated rats) that may play an important role in the colon cancer aetiology (Hori *et al.* 1998). They also found that AOM treatment with a steroid diet had an additive effect on cell proliferation and colonic crypt mitotic activity (Hori *et al.* 1998). Ten Bruggencate *et al.* (2005) found that FOS increased the cytotoxicity of FW and intestinal permeability in rats. In their later research work on humans and rats, they found that intake of FOS increased flatulence and intestinal bloating. Thus, FOS only increased mucin excretion in humans, but the overall effects are more moderate than in rats (Ten Bruggencate *et al.* 2006). FOS diets had effects on gene expression such as 116, 1674, and 3364 genes in rats, respectively (Chen *et al.* 2011).

Butyrate is a short-chain fatty acid fermentation end product which is used as primary aerobic fuel by normal colonocytes (Wang *et al.* 2006). Butyrate appears to play a significant role in cell proliferation and differentiation, induces apoptosis, and inhibits angiogenesis (Davis and Milner, 2009). Rats fed WB diet produced higher concentration of butyrate than the rats fed oat bran diet (Zoran *et al.* 1997). Our data suggested that butyrate had no significant effect on HCT-116 cell viability. The effect

of butyrate on cell growth is shown in **Figure 3.09**. Kim *et al.* (1980) found that butyrate (5 mmol/L) had no effects on human colon adenocarcinoma cell lines (e.g. SW-620, SW-480) after incubation for 8 days. After doubling the incubation time (1.18 and 7.6-fold), cell viability remained unchanged. But, upon removal of butyrate from medium cells, cells were growing rapidly. Gamet *et al.* (1992) also demonstrated that butyrate (2-5 mmol/L) and propionate (2-10 mmol/L) inhibited growth of HT-29 cells. Furthermore, acetate (2-10 mmol/L) had no effect on cell proliferation. Milovic *et al.* (2000) found that butyrate has a potential inhibitory effect on cell proliferation in human colonic adenocarcinoma cell lines (such as HT-29, Colo-320, and SW-948). Therefore, the effect of butyrate on cell growth is controversial.

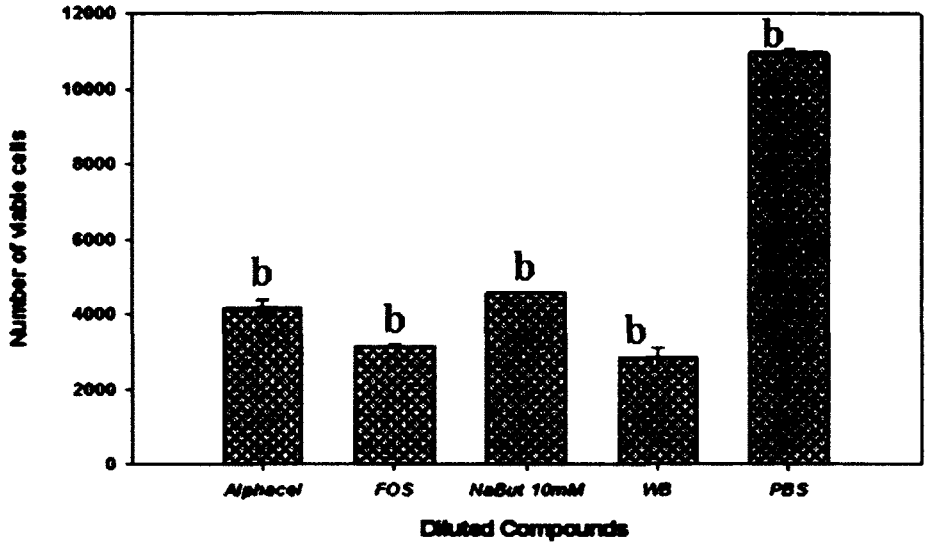

Figure 3.09: The measurement of viable HCT-116 cells after 48 hr treatment with FW from the three diet groups (Alphacel, FOS, and WB). Results were analyzed by a pair-T test (Sigma Plot software 12.0). The column represent the mean (n=4) from separate experiments and bars represent SD. Diet groups and NaBut were compared to PBS (control). Letters a and b represent significance and non-significance of the experiment respectively.

Effects of FW (FOS/WB/Alphacel) on apoptosis

The present study found that FW of WB had no significant apoptotic effect ($p \geq 0.05$) on HCT-116 cells after a 48 hr treatment (**Figure 3.10**) but found that FOS and alphacel diets significantly affected the early and late stages of apoptosis (**Figures 3.11 and 3.12**). In addition, only the FOS diet significantly increased the percentage of cells in the early stage of apoptosis compared to WB and alphacel (**Figure 3.10**). Three **Figures 3.10, 3.11**, and **3.12** represent viable, early, and late plus dead stages of apoptosis.The analyzed results of apoptosis using paried t-test are shown in **Appendix Tables A.20 –A.31**.

FW preparations have been shown to affect apoptosis. The primary data of apoptosis are shown in **Appendix Table A.13** and **Figures A.07-A.10** respectively. Haza *et al.* (2000) showed that the lipid component of human fecal water, possibly containing bile acids and butyrate, induced apoptosis in human cancer cells (e.g. HT-29 and FHC). The effect may be cell-dependent since they also found that FW had less apoptotic effect on human fetal colonic mucosa cells (FHC) than HT-29 (Haza *et al.* 2000). WB itself has been shown to influence apoptosis. However, Jenab and Thompson (2000) found that 25% WB, 25% dephytinized wheat bran (DWB), and 1% phytic acid (PA) diets significantly increased the rate of apoptosis and cell differentiation in the crypts of AOM-treated rats, as well as decreased the number of crypts in the colon compared to control diets. Le Leu *et al.* (2002) found that fed WB diet significantly enhanced apoptosis in the distal colon of rats, increased fecal bulk and butyrate levels, and lowered fecal pH compared to cellulose and NF (no fiber) diets. Compher *et al.* (1999) found that intake of WB significantly increased apoptosis and conserved normal proliferation during tumor initiation whereas WB did not show a significant effect in any stage of apoptosis when using an *in vitro* system. On the

other hand, WB diet had a significant inhibitory effect on tumors formation in the colon of rats.

WB diet has antineoplastic effects in early carcinogen exposure and produced significant amounts of fecal butyrate in the feces of rats (Compher *et al.* 1999). It was shown in our previous study that WB diet produced lower amount of butyrate and acetate in cecum of rats than FOS (Chen *et al.* 2010). The relative amount of butyrate in the cecum of rats is shown in **Table 4.02** of the following chapter. This is possibly due to the fact is that FOS diet rapidly fermented and produced higher amount of SCFA, especially butyrate, acetate, etc. in the cecum of rats compared to WB diet.

It is noted that butyrate significantly increased apoptosis on HCT-116 cells after 48 hr incubation. The significant effect of butyrate on apoptosis is shown in **Figure 3.12**. Recently, it was shown that butyrate significantly increased apoptosis in both cell lines HT-29 and AA/C1 cells, and it was also suggested that butyrate had beneficial effects against colon cancer development (McMillan *et al.* 2000). Therefore, butyrate enhanced apoptosis on HCT-116 cells.

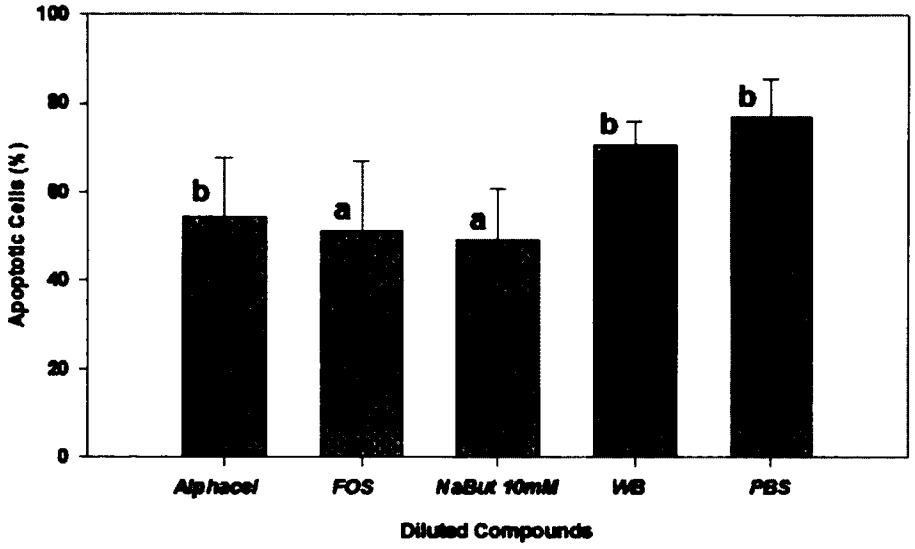

Figure 3.10: The percentage of apoptotic cells (HCT-116 cells) in a viable stage of apoptosis after 48 hr treatment with FW (Alphacel, FOS, and WB). The column represent the mean (n=4) from 4 separate experiments and bars represent SD. Diet groups and NaBut were compared to PBS (control). Letters a and b represent significance and non-significance of the experiment respectively.

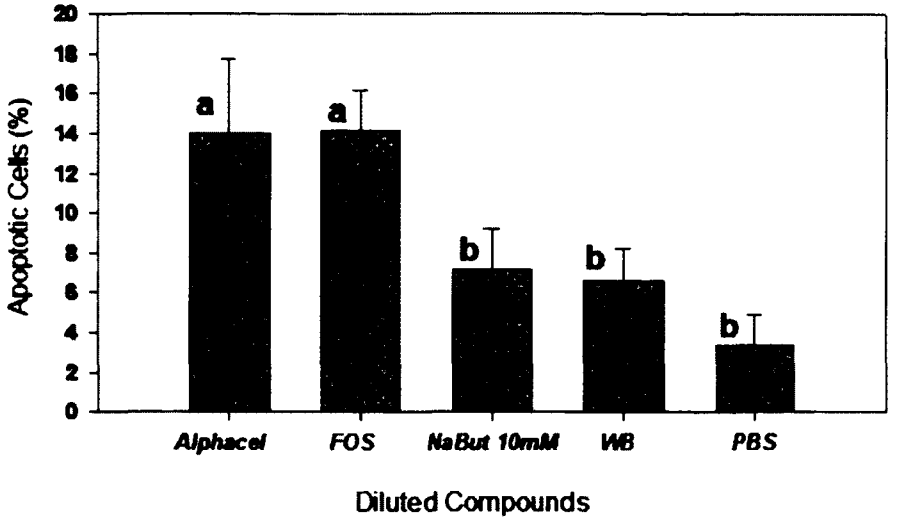

Figure 3.11: The percentage of apoptotic cells (HCT-116) in early apoptosis after 48 hr treatment with FW from the three diet groups (Alphacel, FOS, and WB) as detected by flow cytometry. The column represent the mean (n=4) from 4 separate experiments and bars represent SD. Diet groups and NaBut were compared to PBS (control). Letters a and b represent significance and non-significance of the experiment respectively.

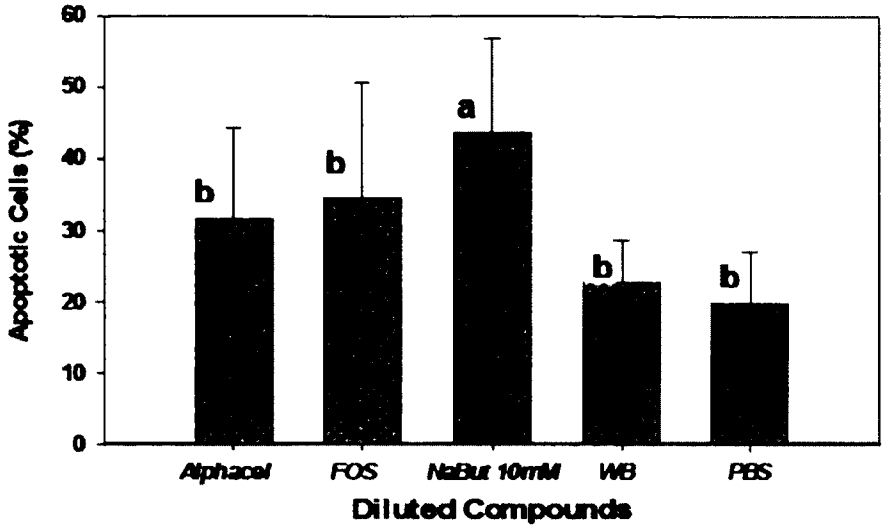

Figure 3.12: The percentage of apoptotic cells in late and dead stage of apoptosis after 48 hr treatment with FW from the three diet groups (Alphacel, FOS, and WB). The column represent the mean (n=4) from 4 separate experiments and bars represent SD. Diet groups and NaBut were compared to PBS (control). Letters a and b represent significance and non-significance of the experiment respectively.

Effects of FW (FOS/WB/Alphacel) on cell cycle arrest

The raw data of cell cycle assay are shown in **Appendix Table A.15** and **Figures A.11-A.13** respectively. Our studies found that only the FOS diet significantly reduced the number of apoptotic cells in the G_1/G_0 phase (n=3; p = 0.03) of the cell cycle compared to WB and alphacel (n=3; p = 0.71) diets but the effect was quite small. The toxic effects of FW in G_1 pagse of cell cycle are shown in **Figure 3.13 and Table 3.02**. Analyzed results of cell cycle arrest using paried t-test are shown in **Appendix Tables A.32–A.40**. Recently, it was shown that HT-29 cells treated with human fecal water (people are on normal diets) arrested most of the cells in G_1 phase and cancer disease progression is associated with cell cycle arrest (Zeng and Davis 2003). In addition, human FW significantly inhibited HT-29 cell cycle progression and cell growth. The down-regulation of PCNA and cyclin A genes by human fecal water is an important factor related to cell cycle arrest that depends on dose and dietary factors (Zeng and Davis 2003; Glinghammar *et al.* 1997). Furthermore, components of the FW including 3-phenylpropionic acid, 3-hydroxyphenylacetic acid, and 3-(4-hydroxyphenyl)-propionic acid decreased COX-2 protein levels and the PGE2 expression in HT-29 cells that is related to colon tumors development (Karlsson *et al.* 2005). In addition, alphacel and FOS diets significantly ($p<0.05$) arrested the percentage of HCT-116 cells that were arrested in the S- and G_2/M phases of cell cycle, compared to the WB ($p>0.05$) diet and the PBS control. The effects of FW in G_2 and S-phases of cell cycle are shown in **Figures 3.14 and 3.15 respectively.** Both diets had the highest incidence of colon tumors in rats. However, some studies have found that the components of dietary fiber had significant effects in different phases of cell cycle in an *in vitro* system whereas our WB fiber did not show a significant effect in any phase of the cell cycle. However, the

WB diet had the lowest colon tumor incidence in rats compared to FOS and alphacel diets. The WB diet contains many phytochemicals (e.g. lignans) that play significant roles in reducing colon cancer risk (among others: Jenab and Thompson 2000; Qu *et al.* 2004; Nilsson *et al.* 1997). Lignans are diphenolic compounds that present in the outer layer of bran and the major lignan in WB is secoisolariciresinol diglycoside (SDG) that is converted into enterolactone and enterodiol by microflora (among others: Mazur *et al.* 2000; Stitch *et al.* 1980; Setchell *et al.* 1981). The metabolites of lignan (e.g. enterolactone and enterodiol) act as antioxidants and free radical scavengers that may decrease the risk of colon cancer development (Bird 1995). Qu *et al.* (2004) found that metabolites enterolactone and enterodiol had effects on human colon cancer SW480 cells after 72 hr incubation with 0-40 μmol/L. The cell viability was time and dose dependent. Both enterolactone and enterodiol metabolites alone or in combination significantly increased apoptotic cells compared to control. SW480 cell growth was significantly inhibited and blocked cell cycle progression in S phase by both metabolites (Qu *et al.* 2004). The same authors suggest that cell cycle arrest may trigger the DNA repair machine that may lead to apoptosis (Qu *et al.* 2004). The suggestion is that the FW of our WB diet did not contain these metabolites or fewer amounts might be present in FW because only 3% of total dietary fiber was fermentable, or the incubation time (48 hr) was short too induce an effect on the cell cycle.

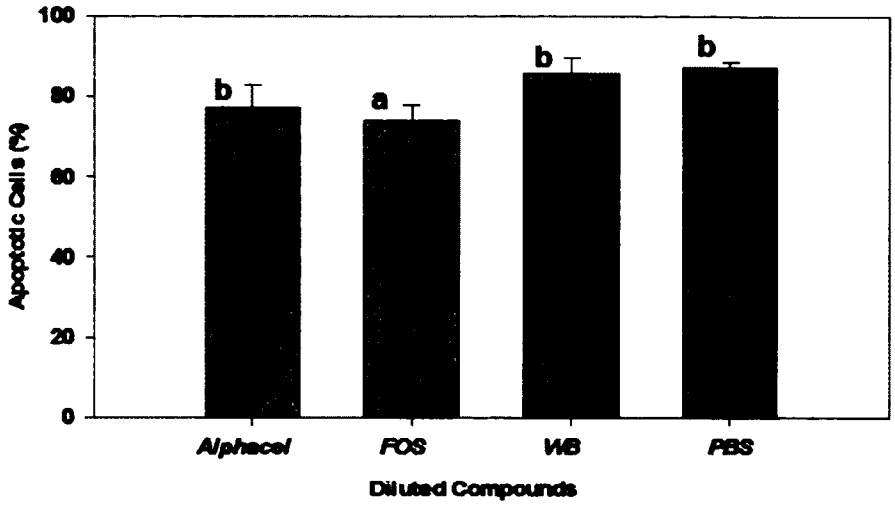

Figure 3.13: Distribution of apoptotic cells (%) in the G_1/G_0 phase of the cell cycle after 48 hr treatment with FW from the three diet groups (Alphacel, FOS, and WB). The column represent the mean (n=3) from 3 separate experiments and bars represent SD. Diet groups and NaBut were compared to PBS (control). Letters a and b represent significance and non-significance of the experiment respectively.

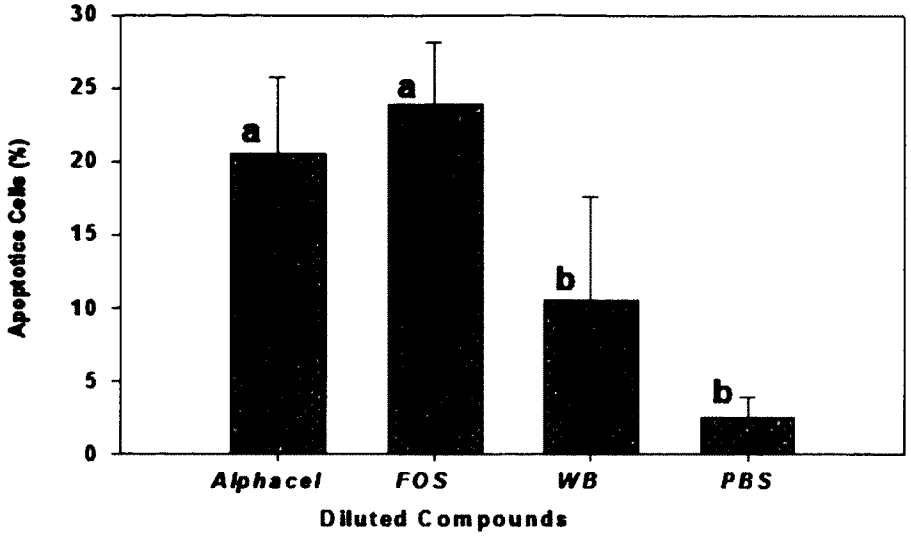

Figure 3.14: Distribution of apoptotic cells (%) in the G_2/M phase of cell cycle after 48 hr treatment with FW (Alphacel, FOS, and WB). The column represent the mean (n=3) from 3 separate experiments and bars represent SD. Diet groups and NaBut were compared to PBS (control). Letters a and b represent significance and non-significance of the experiment respectively.

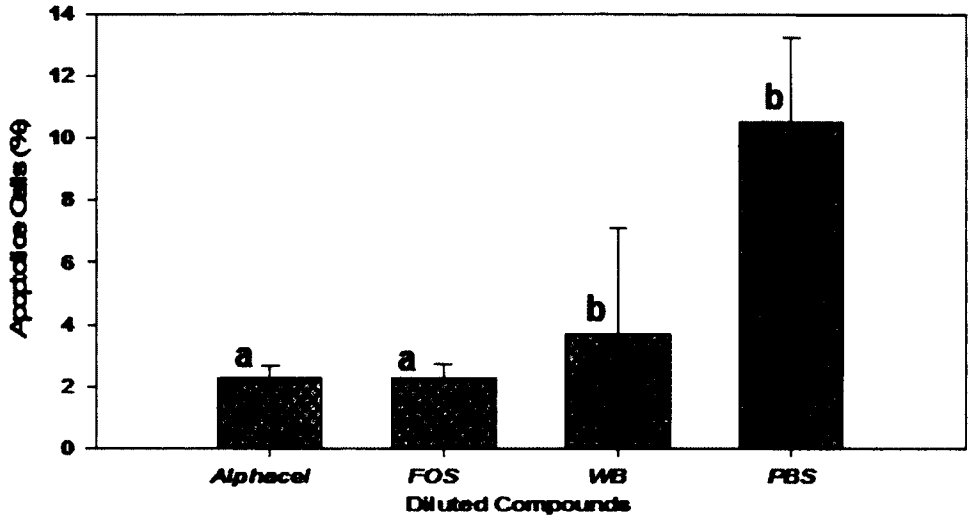

Figure 3.15: Distribution of apoptotic cells (%) in S phase of the cell cycle after 48 hr treatment with FW (Alphacel, FOS, and WB). The column represent the mean (n=3) from 3 separate experiments and bars represent SD. Diet groups and NaBut were compared to PBS (control). Letters a and b represent significance and non-significance of the experiment respectively.

Effects of FW (FOS/WB/Alphacel) on DNA damage

Our BrdU assay results suggested that FW of WB diet had no significant effect on DNA damage whereas both FOS and alphacel diets significantly ($p < 0.05$) induced DNA damage on HCT-116 cells after a 48 hr treatment (**Figure 3.16**). The primary data on DNA damage are shown in **Appendix Table A.15** and **Figures A.14-A.17** respectively. The analyzed results of DNA damage using paried t-test are shown in **Appendix Tables A.41–A.44.**

Intake of high protein diets increased N-nitroso compounds in FW and resulted in greater DNA damage. These compounds are related to the initiation and development of colon tumorigenesis in humans and experimental animals (Bird 1986; Gratz *et al.* 2011). The typical Western diet has been shown to be associated with colonic heterocyclic mutagenic amines (e.g. 2-amino-3,4-dimethylimidazo (4,5-f) quinoline and 2-amino-3-methylimidazo(4,5-f) quinoline) that exert their nuclear damaging effect on colonic epithelium of C57BL/6J mice *in vivo* (Bird 1986). These colonic heterocyclic mutagenic amines also induced DNA strand breaks in CaCo-2 and HT-29 cells, (Gratz *et al.* 2011; Reiger *et al.* 1999). High consumption of dietary heme has also been shown to increase DNA damage 10-fold and increase crypt depth compared to control diet in rats (de Vogel *et al.* 2008). However, Joosen *et al.* (2009) found that the high consumption of red meat only stimulates the production of mutagenic nitroso compounds in humans. The authors suggested that the processing of meat was associated with stimulation of nitrosation and DNA damage that might increase the risk of colorectal cancer in humans. It was also found that FW of vegetarian (VEG) diets significantly induced more DNA strand breaks than meat diets in CaCo-2 cells (Joosen *et al.* 2009). This is controversial. However, all three of our diets had the same percentage of protein (20%). Therefore, these diets should produce

the same types and same amounts of metabolites specifically nitroso compounds in FW. FW from three diets should have similar effects on DNA damage. But, FW of three diets differently affected on DNA damage (**Figure 3.16**). There could be other factors or compounds such as bile acids or pH in FW that may affect DNA damage. It was shown that primary and secondary bile acids administration in the small intestine of germfree and conventional rats induced tumors developing in the colon (Narisawa *et al.* 1971; Reddy *et al.* 1977; Corpet *et al.* 1997). Reddy *et al.* (1992) reported that the effect of dietary fiber on secondary bile acids depended on the type of dietary fiber consumed. Subsequently, a high intake of wheat bran fiber was shown to be associated with significantly lower colon tumors incidence (Chen *et al.* 2010). The authors postulated that this was due to lower secondary bile acid concentration in FW. The pH of our FW was different for the three diets. WB diet had the highest pH (8.1) compared with FOS (5.5) which may play a role in DNA damage as well as in colon cancer development.

It was noted that butyrate significantly induced more DNA damage than FOS, WB, and alphacel diets. Wollowski *et al.* (2001) illustrated that butyrate is a protective agent that may lower the risk of colon cancer and may inhibit the genotoxic activity of nitrosamides and hydrogen peroxide in human colon cells. It was shown that butyrate increased the glutathione-S-transferase π enzyme in cultured CaCo-2 cells. The glutathione-S-transferase π is a detoxifying enzyme that enhanced the detoxification capacity of cells against carcinogens (Stein *et al.* 1996). But our three diets had a different fermentation rate and produced different amount of butyrate in the different regions of colon. We suspected that a metabolite of WB, specifically butyrate, may be absorbed by normal epithelial cells in protecting against colon cancer development.

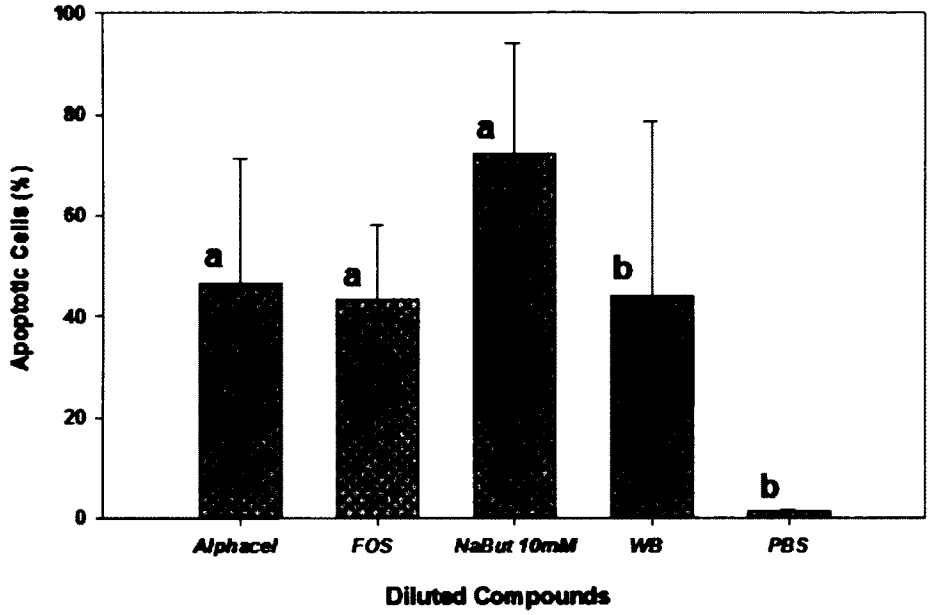

Figure 3.16: Distribution of apoptotic cells (%) after 48 hr treatment with FW from the three diet groups (Alphacel, FOS, and WB). The columns represent the mean (n=4) from 4 separate experiments and bars represent SD. Diet groups and NaBut were compared to PBS (control). Letters a and b represent significance and non-significance of the experiment respectively.

Table 3.02: All results were analyzed to assess significant differences among the dietary fecal water groups and their toxic effects (e.g. viability, apoptosis, cell cycle arrests and DNA damage) on HCT-116 cell lines using paired T-test (SigmaPlot 12.0 software).

Treatments	ViaLight Assay	Annexin-V Assay			BrdU Assay	Cell Cycle Assay		
	Cell Growth	Viable	Early apoptosis	Late apoptosis + Dead	DNA damage	G_0/G_1	G_2/M	S
FOS Vs PBS	0.28 (0.11)	0.67 (0.03*)	4.21 (0.005*)	1.75 (0.09)	6.89 (0.004*)	0.85 (0.03*)	9.57 (0.02*)	0.22 (0.05*)
WB Vs PBS	0.22 (0.13)	0.92 (0.08)	1.96 (0.084)	1.15 (0.13)	5.51 (0.24)	0.99 (0.71)	4.23 (0.16)	0.35 (0.09)
Alphacel Vs PBS	0.38 (0.09)	0.71 (0.08)	4.15 (0.03*)	1.69 (0.18)	7.36 (0.03*)	0.89 (0.12)	8.20 (0.03*)	0.22 (0.04*)
10 mM NaBut Vs PBS	0.42 (0.09)	0.12 (0.006*)	2.13 (0.10)	2.21 (0.02*)	9.26 (0.04*)	ND	ND	ND

NB: statistically significant p-values are highlighted with star symbol inside the brackets ($p<0.05$), and non-significant p-values are highlighted without the star ($p>0.05$); ND stands not detected with those methods. The values outside of the bracket represent the ratio of median FOS/PBS, WB/PBS, Alphacel/PBS, and NaBut/PBS.

CHAPTER 4

General Discussion

DF is an important factor that may have significant control or influence on bacterial diversity in the gut and contribute in producing various metabolites in FW that may be associated with colon cancer development. Burkitt (1971) postulated that a high incidence of colon cancer in Western countries could be attributed to carcinogens produced by the action of bile acids in the colon via the resident gut bacteria; typical Western diets alter not only the bacterial diversity but also the chemical components of FW. The components of diet have been shown to change the fecal and FW composition and maintain normal functioning of the colon epithelium and have been thought to confer protection against the risk of developing colon cancer (Jensen *et al.* 1982; Cummings *et al.* 1976). For example, WB diets have been associated with the lowest incidence and multiplicity of colon tumors in AOM-treated rats (Watanabe *et al.* 1979; Reddy *et al.* 1981; Raju *et al.* (unpublished data)). WB has a fecal dilution effect and lowers transition time so that fecal material passes through the colon quicker and the colon epithelial cells are exposed to lower amounts of potential carcinogens (Harris and Ferguson 1993). Gazzaniga and Lupton (1987) also found that WB had better dilution potential in the three colonic regions including cecum, proximal colon and distal colon of Sprague-Dawley rats compared to pectin, guar gum, oat bran, and cellulose. The same authors implanted radiopaque markers along the colon of Sprague-Dawley rats and found that fiber free diets, cellulose, guar gum, pectin, oat bran, and wheat bran had the longest to the shortest transition times, respectively. The most important factors that might be associated with colon cancer development are shown in **Table 4.01.**

Table 4.01: The most important factors for or against colon cancer development.

Factors	Association
Fecal Microflora	Results suggested that fecal microflora help to ferment various dietary fibers and produce various metabolites that might have an association with colon cancer development.
Fecal pH	Diets and microflora modulate the colonic pH. Therefore, pH might have a role in colon cancer development.
Bile acids	There was no association of bile acids with colon cancer development because all three experimental diets had similar protein component (20%) in diets.
Nitroso compounds	Nitroso compounds may not be an important factor since three diets had the same protein and fat components. The production of nitroso compounds in the FW should be same.
SCFA	SCFA may be an important factor because different types of fiber ferment differently and produce different amount of SCFA in the different regions of the gut.
DF	DF had significant effects on bacterial diversity in the gut (e.g. WB diet) and helps to maintain a good ratio of beneficial fecal microflora that might protect against colon cancer development.

One of the factors in colon cancer development appears to be the cytotoxicity and genotoxicity of FW (and FW components). It has been shown that dietary components, transit time, weight and age are all significant factors related to this (Cross *et al.* 2006). However, it is virtually impossible to isolate a single important factor because FW contains upwards of 160 compounds (such as phenols, aromatic compounds, etc.)(Jenner *et al.* 2005). In recognition of this, our experimental design was intended to investigate the relationships between diet, FW toxicity and cytotoxicity and the relationship to bacterial changes in general without specifically identifying individual components.

It is difficult to test toxicity *in vivo* and we consequently used cultured cells to indirectly test the effects. It was reasoned that if diet altered the chemical composition of FW then differences in FW composition might induce significant toxic effects on colon cancer cells: HCT-116 cells. We suspected that fecal pH may be related to colon cancer development because the FW of diets had significantly different effect on HCT-116 cells after 48 hr treatment. The pH is related to the presence of fermentable materials since the microbial fermentation of carbohydrates produces volatile fatty acids and lowers luminal pH in the colon (Roberfroid *et al.* 2010). But the total percentage of fermentable materials (3%) such as DF was same in WB and FOS except alphacel (0%) and therefore it was predicted that these diets should produce same amount of volatile fatty acids and change in pH. However, it was found that the pH of FW was significantly different for the different diets. For example, dimethylhydrazine (DMH)-injected rats that consumed lactulose and/or sodium sulphate (non-fermentable) had lower fecal pH and had significantly fewer colon tumors than controls (Samelson *et al.* 1985). Our fecal pH data showed that the WB diet had a higher fecal pH (8.1) compared with FOS (5.5) and

alphacel (6.8) diets. Thus, an inverse correlation existed between the luminal pH of rats fed a diet rich in rapidly fermentable carbohydrate (lactulose or sorbitol) and proliferating colon epithelial cells. This demonstrates that rapidly fermentable carbohydrates lower the colonic pH and this can lead to an increase in epithelial cell proliferation (Lupton *et al.* 1985). These research findings supported our FOS pH data, but not cell viability data, because the FOS diet had no significant effect on cell viability. The lowering of intra-luminal pH is known to suppress the growth of pathogens in the colon (Blaut 2002) and assists with intestinal motility (Dass 2007). This result did not support our bacterial data related to the FOS diet because the FOS diet significantly promoted the growth pathogenic bacteria such as *Allobaculum*. The effect of pH may not be relevant to the development of colon cancer. For example, one study suggested that the pH-dependent cytotoxicity of fecal bile acids was not associated with the formation of neoplasm in HT-29 cells (de Kok *et al.* 1999). In support of this, rats consuming WB diet had both a reduction in colon tumor development and a higher fecal pH when compared to rats fed oat bran diet (Zoran *et al.* 1997), in agreement with our WB data. Therefore, we can suggest that pH might play an important role by itself or additive effect with other fecal metabolites to protect colon cancer development.

Fecal metabolites including secondary bile acids are another factor that may be associated with promoting colon cancer development. Diets low in fiber or high in animal fats were shown to increase lithocholic acids (LCA) levels in FW that may induce DNA strand breaks, form DNA adducts, and inhibit DNA repair enzymes *in vivo* (Chaplin 1998). It was also suggested that secondary bile acids (e.g. DCA and LCA) act as promoters for colon cancer development in carcinogen treated rats (among others; Reddy

et al. 1989; Reddy *et al.* 1992; Reddy 1995; Salmelson *et al.* 1985). Diet significantly controls secretion and excretion of secondary bile acids such as DCA and LCA (Thompson *et al.* 1985; Villalon *et al.* 1992). It has also been shown that diets supplemented with fiber (from wheat bran or rye) significantly reduced the concentration of fecal secondary bile acids and lowered the fecal mutagens (e.g. fecapentaenes) in humans compared to those that did not receive dietary fiber supplements (Reddy *et al.* 1987). In our experiment, FW from FOS and alphacel diets significantly induced DNA damage on HCT-116 cells after 48 hr incubation although the percentage of dietary fiber (7%) was the same for all three experimental diets: WB, FOS, and alphacel. Reddy *et al.* (1992) reported that the effect of dietary fiber on secondary bile acids and bacterial enzymes depends on the type of dietary fiber consumed. The fermentation of dietary fiber increased the production of short chain fatty acids (SCFA) that inhibits the conversion of primary to secondary bile acids. For example, a high intake of dietary wheat bran fiber was shown to be associated with a significantly lowered incidence of colon tumors in rats. The authors postulated that this was due to lower secondary bile acids and increased SCFA in FW (Reddy *et al.* 1981). It was also found that feeding rats WB diets reduced colon tumor development, and produced a higher concentration of SCFA and more bulky stools than rats fed an oat bran diet (Zoran *et al.* 1997). These results agree with our tumor data that showed that the WB diet had a significant effect on colon tumor development in the AOM-treated rats compared to FOS and alphacel diets (**Table 2.03**). Therefore, it is a very difficult to make a conclusion regarding bile acids without having experimental data. Bile acids might be in lower concentration in FW. But, these low bile acids might have additive or synergistic effects with other metabolites or by itself in the

colon of rats. We suspected that the WB diet has a higher binding capacity for fecal bile acids than the other diets (e.g. FOS, oat bran, and alpahcel) that can reduce the availability of these bile acids to interact with epithelial cells in the colon. The WB diet had higher fecal bulking (volume and total amount of fecal output) capacity that lowered the concentration of bile acids in lumen. Additionally, WB diet had higher dilution potential (more water content in the feces) that can reduce the exposure to carcinogens in the colon. Intake of calcium supplements can decrease the molar ratio of hydrophobic to hydrophilic bile acids that helps to precipitate hydrophobic dihydroxy bile acids. In addition, intake of calcium phosphate with diet decreased the cytolytic activity of FW from 47±9% to 27±8% ($n = 12$, $P < 0.05$) in the intestinal lumen of rats, which decreased epithelial cell proliferation (Lapre *et al.* 1993).

We suspected that SCFA may have a protective role in colon cancer development. It was found that rats consuming a WB diet had reduced colon tumor development, and excreted a higher amount of SCFA and a more bulky stool than rats fed oat bran diets, which are completely and more rapidly fermented (Zoran *et al.* 1997). Sung *et al.* (2006) showed that intake of FOS increased the concentration of lactate and SCFAs in fecal samples of rats, and decreased luminal pH. This was also observed in the present results. We predicted that fecal SCFA play an important role in cell parameters. For example, FOS diet rapidly fermented in the upper gut specifically in the cecum of rats and produced a huge amount of SCFA compared with WB diet; therefore, FW of FOS and alphacel diets had significant effects on apoptosis, cell cycle, and DNA damage whereas WB did not show any significant effect on HCT-116 cells after 48 hr treatment. WB might have protective effects for normal epithelial cells. The SCFA may be an important

factor related to the tumorgenesis because all three diets produced significantly different amounts of SCFA through the fermentation in the cecum of rats. The fermentation process may play a role in colon cancer development because different types of dietary fibers have different rates of fermentation and their metabolites, specifically butyrate, are absorbed differently throughout the gut.

Our studies found that diets alter the bacterial composition in the gut of AOM-treated rats (alphacel vs. FOS vs. WB). We suspected that bacteria may be associated with colon cancer development or may be protective against the development of colon cancer. The human colon holds a huge variety of bacteria and most of them are anaerobic. Colonic microflora help to digest a wide variety of carbohydrates using their hydrolyase enzymes. They also ferment carbohydrates and help to develop a barrier that protects the gastrointestinal tract from pathogenic bacteria (Salminen *et al.* 1998). It was noticed that WB diet had significant effect on *Lactobacilli* bacteria growth that might play an important role in inhibiting tumor growth in AOM-treated rats. The isolated important bacterial species are shown in **Table 2.08.** It was found that *Lactobacilli* actively degrade nitrosamines (such as diphenylnitrosamine and dimethylnitrosamine) into parent amine and nitrite ions, as well as certain unknown volatile metabolites (Rowland and Grasso 1975). In addition, *Lactobacilli* are capable of binding to mutagenic compounds (e.g. pyrolyzates) depending on the luminal pH. These pyrolyzates are potent mutagenic and carcinogenic heterocyclic amines that are formed during processing of foods. It was also shown that intestinal bacteria including *Lactobacillus casei* YIT 9018 and dietary fiber can bind with carcinogenic heterocyclic amines and inhibit their mutagenic effects (Morotomi and Mutal 1986).

Studies have found that administration of lactic acid bacteria (LAB) (e.g. *Bifidobacteria* and *Lactobacilli*) significantly lowered the number of ACF formations in AOM Sprague Dawley rats (Lee and Lee 2000). However, our study did not find an association with *Bifidobacteria* in colon cancer development whereas *Lactobacilli* might have a significant effect against colon cancer development in F344 rats. ACF represent the earliest detectable lesions of colon cancer development, and the number and multiplicity of ACF eventually predicts the tumors incidence (Pretlow *et al.* 1991). The administration of *Lactobacillus casei* strain Shirota (LcS) via intrapleural method in tumor-bearing mice effectively inhibit tumor growth and increased survival rate by increasing the production of several cytokines (e.g. IFN-γ, IL-1β, and TNF-α) in the thoracic cavity (Matsuzaki 1998). The tumor necrosis factor-α (TNF-α) plays important roles in immunity, apoptosis, and cell survival (Szlosarek and Balkwill 2003). Another study by Kato *et al.* (1994) found that oral administration of *Lactobacillus casei* YIT 9018 in tumor bearing mice increased T-cell function. T cells (lymphocytes) are white blood cells that play a significant role in cell-mediated immunity. Lactic acid bacteria also increased the activity of cytochrome P450 reductase (Pool-Zobel *et al.* 1996) and glutathione S-transferase enzymes (Challa *et al.* 1997) that play important roles in the metabolism of carcinogens in rats. A 6 week administration of *Lactobacillus acidophilus* with milk supplements resulted in a lowering of the concentration of soluble bile acids in the feces of colon cancer patients (Lidbeck *et al.* 1991). In another 3 month study, it was found that administration of *Lactobacillus acidophilus and Bifidobacterium bifidum* in patients with colon adenomas significantly decreased the fecal pH and increased the proliferative activity of epithelial cells in the upper colon (Venitt 1988). In addition,

Lactobacilli and *Bifidobacteria* reduced microbial enzymes (e.g. beta-glucuronidases) that inhibited the conversion of AOM to colon carcinogenic metabolites (e.g. methylazoxymenthanol) (Sekine *et al.* 1991). This could be an important factor that is associated with our WB results. Lee and Lee (2000) found that administration of *Lactobacillus* species significantly lowered the formation of total ACF (38.6%) compared with *Bifidobacterium* (30.3%), but the combined administration of both lowered the percentage of ACF even more (41.5%). Therefore, we can say that WB diet significantly promoted the growth of *Lactobacillus* species in the gut of rats and that this may have a significant association with the prevention of colon tumor development.

It is possible that luminal bacteria or their metabolites play a significant role in colon cancer development. In the present experiment, all the diets had a similar total percentage dietary fiber, and the FOS and WB diets had a similar amount of total fermentable material, but they had significantly different effects on colon tumors incidence. The rats fed FOS diet had a higher colon tumor incidence than the rats fed WB diet. We suspected that these diets are fermented differently and produced diverse metabolites in different colonic regions (e.g. cecum, distal colon, and proximal colon) of the gut. For example, rats fed the WB diet produced the lowest concentration of SCFA including butyrate in the distal colon compared with the cecum and proximal colon that play significant roles in the prevention of colon cancer; cecum is the main site of fermentation (Zoran *et al.* 1997) but tumor occurrence is highest in the distal colon/rectum (Holt *et al.* 1996). It was also noticed that the concentration of butyrate was higher in the distal colon of rats consuming WB diet than other fibers (such as guar and oat bran) (Lupton and Kurtz 1993; McIntyre *et al.* 1993). On the other hand, Lupton and

Turner (1999) found that after 16 weeks of feeding on an oat bran diet, rats had significantly higher butyric acid concentration throughout the colon as compared to WB, and significantly higher tumor incidence. A previous study in our lab found that FOS significantly produced higher amounts of SCFA in the proximal colon compared to WB and alphacel diets (Chen *et al.* 2010). The relative amount of SCFA is shown in **Table 4.02**. This is possibly due to the fact that the FOS diet is fermented rapidly and completely producing high amounts of butyrate in the upper gut (cecum and proximal region) that are rapidly absorbed to promote cell growth proximally, apoptosis, and DNA damage. This is positively correlated with our FW data on the FOS diet that significantly induced apoptosis, cell cycle arrest, and DNA damage in HCT-116 cells compared to WB diet after 48 hr treatment. But, there is no butyrate remaining to reach in the distal colon. We also suspect that cells were not capable of preserving balanced cell proliferation and elimination of damaged cells; and thereby, cells turned into colon cancer. The proposed possible mechanism related to the FOS diet in the development of colon cancer is shown in **Figure 4.01** and **Table 4.03.** Our data suggest that the FOS diet significantly promoted *Allobaculum* bacteria that might an important association in the development of colon tumors in AOM-injected rats. On the other hand, alphacel (control) diet had (0%) fermentable dietary fiber (DF) and this diet was poorly fermented.

The WB diet was slowly and incompletely fermented and we postulate that it produced butyrate throughout the lower gut. We believe that this may stimulate cell growth, apoptosis, and DNA damage to an equal extent. As a result, cells may achieve a balance between cell growth and apoptosis. The proposed mechanism of action of the WB diet against colon cancer development is shown in **Figure 4.01** and **Table 4.03**. In

addition, WB diet composition contains multiple dietary fiber sources, different degree of carbohydrate polymers, and degree of polymerization, characteristics that differentiate the WB diet from other diets such as FOS and alphacel. In addition, different types of dietary fiber fermented differently and produce different types of metabolites with different amount. We suggest that the WB diet can maintain a suitable ratio of *Lactobacillus* bacteria in the gut of rats. *Lactobacillus* and *Bifidobacteria* are thought to improve health resistance to gut infections by inhibiting the growth of harmful bacteria, to reduce cholesterol levels, to produce digestive enzymes, to improve the immune response, and to produce vitamins (Gibson 1998; Holzapfel *et al.* 1998; Vanderhoof and Young 1998; Ziemer and Gibson 1998).

The protective value of dietary fiber appears to be linked to the production of butyrate throughout the lower gut. Our results suggest that butyrate did not show a significant effect on cell growth, but significantly enhanced apoptosis especially in the late and dead stages, and significantly induced more DNA damage on HCT-116 cells than FOS and alphacel diets. The most significant effect of butyrate on apoptosis and DNA damage are shown in **Figures 3.12 and 3.16.** Studies suggest that butyrate plays protective effects against the growth of most human colon cancer by inhibiting cell proliferation and histone deacetylase, and enhancing apoptosis and cell differentiation (Hague *et al.* 1993; Heerdt *et al.* 1994) as well as causing colonic gene expression changes (Chen *et al.* 2010). Recently, it has been shown that butyrate induced glutathione-S-transferase π in cultured CaCo-2 cells that enhanced the detoxification capacity of intestinal cells against carcinogens (Stein *et al.* 1996; Treptow-van Lishaut *et al.* 1999), and inhibited the genotoxic activity of nitrosamides and H_2O_2 in human colon

cells (Wollowski *et al.* 2001). Butyrate appears to play a significant role in cell proliferation and differentiation, induces apoptosis, and inhibits angiogenesis (Davis and Milner, 2009). However, our studies found that butyrate had no significant effect on cell viability, but significantly induced apoptosis on HCT-116 cells after 48 hr incubation. The effect of butyrate on cell viability and apoptosis is shown in **Figure 3.09** and **Table 3.02**. Butyrate metabolism has prolonged effects on histone acetylation and induces differentiation in colon cancer (Wong *et al.* 2006; Hijova and Chmelarova 2007). Data also suggest that butyrate may act to promote tumor initiation and growth. For example, administration of butyrate in drinking water in rats actually enhanced tumor development in rats compared with controls (Freeman 1986), although it is unclear whether the butyrate actually reached the large intestine or not. Caderni *et al* (1998) showed that there was no protective effect of butyrate with respect to ACF formation after butyrate was supplied in the form of slow release pellets to the colon of rats. Based on our findings suggested that butyrate term is controversy and further studies are required to understand the action of butyrate.

We have proposed two metabolic pathways regarding dietary fiber. In first pathway, fermentation of dietary fiber lowers the luminal pH that may have promoting effects on beneficial microflora that may develop a barrier that prevents pathogenic bacteria from invading the GIT tract. On the other hand, DF increased butyrate production that may increase cell proliferation and apoptosis, and decreased DNA damage, and then play a role in colon cancer protection. However, WB did not show significant effects on any cell parameters (e.g. cell growth, apoptosis, DNA damage, and cell cycle arrest), but WB significantly maintained the suitable ratio of beneficial

microflora (e.g. *Lactobacillus,* and *Roseburia*) in the feces of AOM-treated rats that might play a significant role against colon cancer development. Butyrate production is not the only factor that is altered after feeding dietary fiber. DF has many effects in the colon, including an impact on the bacterial community resident in the ascending colon. These bacteria can interact with the gut associated lymphatic tissue (GALT) to potentially promote heightened immunity (Davis and Milner 2009; Wexler 2007) that may play important roles in tumor development.

Tumor Development:

Colon cancer development is a multistage process and is very complex (Foulds 1958). There are many factors (e.g. genetics, dietary factors, genotoxic agents, smoking, etc.) that play a role in cancer initiation and progression of tumors. Chemicals such as azoxymethane (AOM) causes mutations in the epithelial cell DNA that disrupts apoptosis and increases DNA damage. This event sometimes triggers epithelial cell growth and division. This alters the balance between growth and apoptosis when appropriate genes are targeted. Butyrate, which is known to enhance apoptosis, may stimulate growth at this stage by providing a useable source of energy. Thus, butyrate may not prevent or help destroy transoformed cells but help in the formation of aberrant crypt foci (ACF). Fecal bacteria may also play a role in cancer development by increasing the production of SCFA through fermentation. This also decreases the luminal pH.

Butyrate, therefore, can play more than one role in cancer development. It has been suggested that butyrate, at moderate levels appears to have anticancer effects, but at high levels may not. Other metabolites including bile acids, certain amines, and

nitrosocompounds can also enhance the promotion of colon cancer. Bacterial enzymes such as beta-glucuronidase enhance the transformation by deconjugating AOM metabolites to make them more active. *Lactobacilli* and *Bifidobacteria*, which have no β-glucuronidase, are thought to be more beneficial because they play no role in AOM activation whereas *E. coli* and some *Clostridium* species have high β-glucuronidase activity (Gadelle et al. 1985). The combined actions of these chemicals/compounds plays role in various pathways to develop tumors in the colon of rats.

In our experiments, FW from FOS and alphacel diets significantly increased apoptosis and DNA damage in the *in vitro* test system, which is contradictory to literature findings. It may be that higher DNA damage is associated with higher tumor incidence in rats, which would explain the relationship we observed. It appears that the most beneficial effect related to dietary fermentable substrates is a moderate rate of fermentation to produce moderate levels of butyrate throughout the gut.

Table 4.02: The modified table from the growth and cecal SCFA of rats fed WB- or FOS-based diets containing various fermented metabolites (Chen *et al.* 2011).

Cecal contents SCFA μmol/g weight	Alphacel (Control)	WB	FOS	p-value
Acetate	105 ±12	162 ±6	98 ±22	<0.0001
Butyrate	21 ±3	130 ±9	29 ±14	<0.0001
Total	162 ±18	331 ±13	156 ±42	<0.0001
Cecal contents total SCFA, μmol	107 ±13	198 ±12	219 ±19	<0.0001

NB. Values are means ± SEM, n = 10, P-values reported for the Kruskal-Wallis nonparametric test (one-Way ANOVA) and pooled collection from 6–8 rats.

Table 4.03: The proposed action of SCFA along the gut.

Parameters	Cecum		Distal Colon	
	Cell Growth	Apoptosis	Cell Growth	Apoptosis
Alphacel	—	+	+	+
FOS	++	+	—	—
WB	~	~	~	~

NB: Symbols: + represents effect, — represents no effects, and ~ represents equilibrium condition

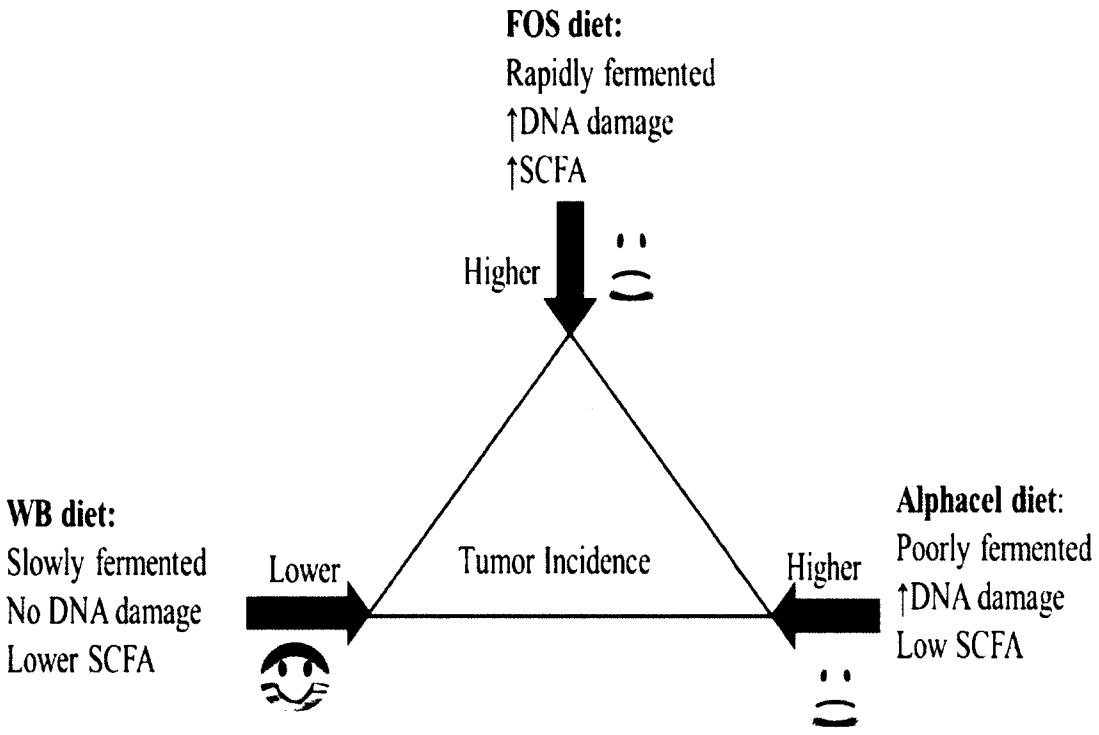

Figure 4.01: The proposed mechanism regarding the FOS, WB, and alphacel diets in the development of colon cancer.

CONCLUSION

Diets may be an important risk factor in the etiology and pathogenesis of colon cancer. Diet is a significant factor that affects the toxicity of FW against colon cancer cells *in vitro* and diets alter the bacterial composition in the gut of AOM-injected rats. Both effects are related to tumor appearance. Colonic microflora (e.g. *Allobaculum sp.*) are associated with increased colon cancer development in rats and *Lactobacillus sp.* and *Clostridium XI sp.* are associated with reduced colon cancer in AOM-injected rats. It is apparent that several factors must be involved in cancer development, even in simplified models such as the one used here. Determining their relative importance is difficult, given that diet can influence all of them.

FUTURE DIRECTIONS

A wide variety and diversity of research can be done on colon cancer. Studies can be done to determine whether bacteria or diets have protective activities against colon cancer upon feeding control diet with the administration of bacteria with drinking water. Further studies can be done on germ free and P450 genes deleted rats to identify significant roles of bacterial enzymes (e.g. β-glucuronidase) in metabolizing carcinogens (e.g. Azoxymethane (AOM) and 1,2-dimethyl hydrazine (DMH)) that are associated with the development of colon cancer. Furthermore, designing an experiment on agar plates to determine the interactions between carcinogenic compounds (e.g. AOM, DMH, etc.) and fecal bacteria under oxygen-free sterilized conditions is a possibility.

Calcium might have an additive or a synergistic effect with dietary fiber that may have a protective role in colon cancer development. Many studies on FW showed that calcium reduced the concentrations of secondary bile acids and was associated with a lowering of the FW toxicity agents on various colon cancer cells lines (such as CaCo-2, HT-29, and HCT-116). Therefore, a study can be designed to determine the effects of calcium on secondary bile acids in the colon of rats by feeding calcium supplemented diet.

APPENDICES

List of Publications

Altaf Mahmud[1,2,3], Kenneth Storey[1], Stephen P.J. Brooks[1,2], and Jayadev Raju[3].Fecal Water and Colon Carcinogenesis: Is fecal water an ideal biomarker for studying Colon Cancer?

[1]Department of Biology, Carleton University, Ottawa, ON; [2]Nutrition Research Division, and [3]Toxicology Research Division, Food Directorate; Health Canada, Ottawa, ON. (Ready to submit).

Altaf Mahmud[1,2,3], Kenneth Storey[1], Stephen P.J. Brooks[1,2], and Jayadev Raju[3]. Investigation on the relationship between dietary fiber, fecal bacterial composition, and colon cancer.

[1]Department of Biology, Carleton University, Ottawa, ON; [2]Nutrition Research Division, and [3]Toxicology Research Division, Food Directorate; Health Canada, Ottawa, ON. In preparation.

Communications at Scientific Conferences

Altaf Mahmud[1,2,3], Kenneth Storey[1], Stephen P.J. Brooks[1,2], and Jayadev Raju[3]. Investigation on the relationship between dietary fiber, fecal bacterial composition, and colon cancer.

[1]Department of Biology, Carleton University, Ottawa, ON; [2]Nutrition Research Division, and [3]Toxicology Research Division, Food Directorate; Health Canada, Ottawa, ON. In preparation. Poster Presentation in Ottawa-Carleton Institute of Biology Symposium. Ottawa, April 25-26, 2012.

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Appendix A: SUPPLEMENTAL DATA

Rats were fed one of 5 different diets according to the protocol outlined in the Materials and Methods section. The rats were sacrificed at 24 wks, the intestinal tracts removed and analysed for tumors by microscopy after staining with eosin and hematoxylin. Tumor statistics are shown in **Table A.01**.

Table A.01: Analysis of colon tumor incidence and size in rats fed diets containing different sources of DF and fermentable materials (modified from Raju *et al.*. unpublished data) .

Characteristics	Diets					
	Fructo-oligosaccharide	Wheat bran	Poly dextrose	Oat bran	Resistant starch	Control (Alphacel)
Number of rats (*n*)	24	23	24	24	24	24
Number of tumor-bearing rats	14	3	5	8	11	15
Tumor incidence (%)	58.33	13.04	20.83	33.33	45.83	62.50
Tumor multiplicity	1.50 ± 0.29	1.00 ± 0.00	1.40 ± 0.24	1.13 ± 0.12	1.36 ± 0.15	2.07 ± 0.27
Average tumor size (mm^2)/ tumor-bearing rat	30.66 ± 10.12	9.76 ± 2.43	26.41 ± 10.10	19.71 ± 6.74	21.70 ± 6.57	16.39 ± 4.35

Primary data that represent the composition of bacterial diversity in the feces of rats. Rats were fed one of 3 different diets and the isolation of bacterial population were described the Materials and Methods section. The bacterial species were classifed based on the difference in 16S rRNA gene sequences using statitical various softwares. The bacterial diversities are shown in **Table A.02.**

Table A.02: The percentage of bacterial populations (at the family level) in the gut of AOM-treated and Saline-treated rats fed three different diets including Alphacel, WB, and FOS. The results were analyzed using the RDPII program.

Bacteria Type	Phylum	Family	Alphacel AOM (%)	Alphacel Saline (%)	WB AOM (%)	WB Saline (%)	FOS AOM (%)	FOS Saline (%)
Gram Negative	Bacteroidetes	Porphyromonadaceae	0	2.1	2.1	0	2.4	0
		Bacteroidaceae	1.2	1.4	0.7	0	1.2	2.9
	Proteobacteria	Alcaligenaceae	0	0	0	0.6	0	0
Gram Positive	Firmicutes	Clostridiaceae	5.3	3.5	14.7	13.5	1.8	1.8
		Ruminococcaceae	54.2	46.7	38.5	38.3	31.3	44.5
		Lachnospiraceae	25.1	21.2	14.7	31.5	20.4	14.1
		Enterococcaceae	0	2.8	0	0	0	0
		Peptostreptococcaceae	1.2	2.8	4.9	3.4	0.6	0
		Peptococcaceae	0.6	0.7	0	0.6	0	0
		Veillonellaceae	0.6	0	0	0.6	0	0
		Thermoanaerobacteraceae	0.6	1.4	0	0.6	0.6	2.3
		Eubacteriaceae	0	0.7	0.7	0.6	0.6	0
		Erysipelotrichaceae	4.1	5	18.2	2.8	12.6	3.5
		Incertae Sedis XIV	4.7	6.4	1.4	3.4	21.7	27.5
	Actinobacteria	Bifidobacteriaceae	0	0	0.7	0	1.2	0
	Verrucomicrobia	Verrucomicrobiaceae	3.5	5	3.5	4.5	5.4	3.5

The comparison of bacterial libraries using 16S rRNA sequence abundance data were analyzed with LibShuff program is shwon in **Figure A.01**

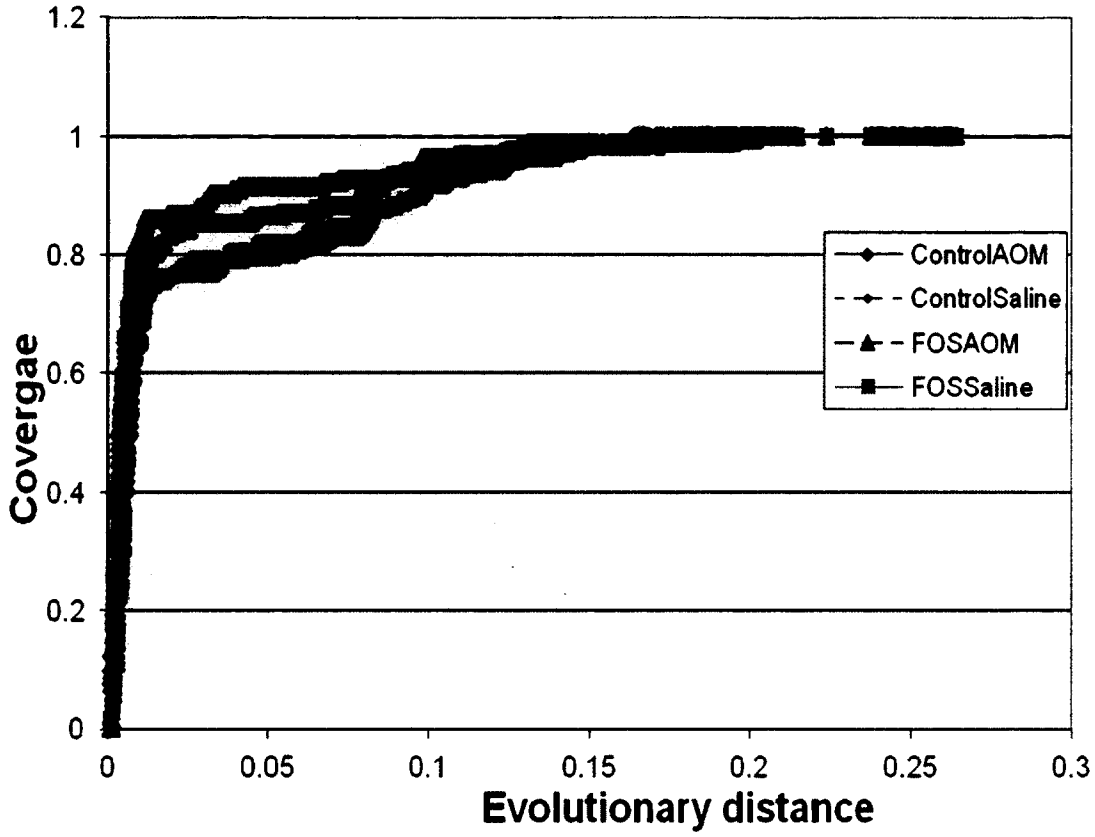

Figure A.01: The comparison of bacterial libraries based on 16S rRNA gene sequences.

Primary data that repesent the classification of bacteria into hierarchical taxa from phylum to genus rank along with a confidence value (95%) with p-value ≤ 0.05. The Library Comparison Tool (RDP II website) was used to taxonomically classify the libraries of 16S rRNA sequences using RDP naïve Bayeian classifier. This library comparison test helps us to identify the significant bacrial speices that might have a positive association in the development of colon cancer and bacterial species that might have a protective effect against colon cancer development. The bactetial comparison results are shown in **Table A.03** to **Table A:11.**

Table A.03: The bacterial comparison between Alphacel-AOM and Alphacel-Saline treatment.

Rank	Name	Treatment		Significance
		Alphacel-AOM	Alphacel-Saline	
class	Clostridia	119	158	3.16E-2
class	Bacilli	4	0	3.82E-2
family	Enterococcaceae	4	0	3.82E-2
genus	Enterococcus	4	0	3.82E-2
family	Porphyromonadaceae	3	0	8.43E-2

Table A.04: The bacterial comparison between FOS-AOM and FOS-Saline treatment

Rank	Name	Treatment		Significance
		FOS-AOM	FOS-Saline	
family	Erysipelotrichaceae	21	6	2E-3
genus	Turicibacter	6	0	1.41E-2
family	Ruminococcaceae	53	75	2.38E-2
genus	Clostridium IV	0	5	3.41E-2
genus	Coprococcus	10	3	5.05E-2
genus	Allobaculum	13	5	5.49E-2
genus	Parabacteroides	4	0	5.8E-2
genus	Blautia	13	24	6.88E-2

Table A.05: The bacterial comparison between WB-AOM and WB-Saline treatment.

Rank	Name	Treatment		Significance
		WB-AOM	WB-Saline	
family	Erysipelotrichaceae	26	6	1.08E-5
genus	Turicibacter	23	6	6E-5
family	Lachnospiraceae	17	54	1E-4
order	Clostridiales	103	158	3.6E-4
unclassified	Lachnospiraceae	15	47	NA
order	"Bacteroidales"	4	0	3.4E-2
genus	Ruminococcus	13	7	5.36E-2

Table A.06: The bacterial comparison between Alphacel-Saline and WB-Saline treatment.

Rank	Name	Treatment		Significance
		Alphacel-Saline	WB-Saline	
genus	Clostridium sensu stricto	7	24	1.88E-3
family	Ruminococcaceae	95	70	3.62E-3
genus	Lactobacillus	0	7	8.74E-3
genus	Clostridium XVIII	6	0	1.41E-2
genus	Ruminococcus	15	7	6.72E-2

Table A.07: The bacterial comparison between Alphacel-Saline and FOS-Saline treatment.

Rank	Name	Treatment		Significance
		Alphacel-Saline	FOS-Saline	
genus	Blautia	1	24	6.08E-7
genus	Clostridium IV	22	5	1.13E-3
family	Lachnospiraceae	45	69	3.74E-3
genus	Ruminococcus	15	4	1.36E-2
genus	Clostridium XVIII	6	0	1.69E-2
genus	Allobaculum	0	5	2.91E-2
family	Ruminococcaceae	95	75	5.24E-2

Table A.08: The bacterial comparison between WB-Saline and FOS-Saline treatment.

Rank	Name	Treatment		Significance
		WB-Saline	FOS-Saline	
genus	Blautia	0	24	3.11E-8
genus	Clostridium sensu stricto	24	3	4.81E-5
genus	Lactobacillus	7	0	9.57E-3
genus	Clostridium XI	6	0	1.87E-2
genus	Turicibacter	6	0	1.87E-2
genus	Clostridium IV	16	5	2.32E-2
genus	Bacteroides	0	5	2.67E-2
family	Lachnospiraceae	54	69	4.24E-2

Table A.09: Bacterial comparison between Alphacel-AOM and WB-AOM treatment.

Rank	Name	Treatment		Significance
		Alphacel-AOM	WB-AOM	
genus	Turicibacter	0	15	3.84E-7
family	Erysipelotrichaceae	8	17	1.08E-3
order	Clostridiales	119	60	1.02E-2
genus	Clostridium sensu stricto	6	11	1.88E-2
family	Ruminococcaceae	67	29	3.94E-2
genus	Lactobacillus	0	3	4.19E-2

Table A.10: Bacterial comparison between Alphacel-AOM and FOS-AOM treatment.

Rank	Name	Treatment		Significance
		Alphacel-AOM	FOS-AOM	
genus	Clostridium IV	11	0	2.11E-4
genus	Allobaculum	0	13	3.05E-4
family	Lachnospiraceae	35	70	8E-4
genus	Coprococcus	0	10	2E-3
genus	Blautia	1	13	2.29E-3
family	Ruminococcaceae	67	53	9.88E-3
genus	Ruminococcus	16	7	2.2E-2
genus	Turicibacter	0	6	2.47E-2
family	Erysipelotrichaceae	8	21	3.08E-2
genus	Enterococcus	4	0	4.41E-2

Table A.11: The bacterial comparison between WB-AOM and FOS-AOM treatment.

Rank	Name	Treatment		Significance
		WB-AOM	FOS-AOM	
family	Lachnospiraceae	17	70	3.65E-9
genus	Clostridium sensu stricto	21	3	3.48E-5
genus	Turicibacter	23	6	1.8E-4
genus	Allobaculum	0	13	3.34E-4
genus	Blautia	0	13	3.34E-4
genus	Coprococcus	0	10	2.15E-3
genus	Clostridium IV	6	0	9.09E-3
genus	Marvinbryantia	0	6	2.58E-2
genus	Lactobacillus	4	0	4.25E-2
genus	Ruminococcus	13	7	8.19E-2

Fecal water were prepared from the feces of rats and tested on human colon cancer cell line (e.g. HCT-116) to study the toxic effect of fecal water on cell growth which were outlined in the martials and methods section. Each experimental measure was repeated 6 times (technical repetitions) and these data were averaged to obtain a single measure of the tested effect (n=1). The individual measures were averaged to obtain a final mean and standard deviation. The primary data of cell viability after 48 hr treatement with various preparation of fecal water is shown in **Table A:12** and **Figures A.02** to **A.06.**

ViaLight Assay:

Table A.12: The number of HCT-116 living cells after 48 hr treatment with FW from three diet groups (alphacel, FOS and WB), as measured by a luminescence spectrometer.

Compound	Exp 1	Exp 2	Exp 2 (Repeated)	Exp 3	Exp 3 (Repeat)
Alpha1	5157.33	9746	1075.5	781.17	777.33
Alpha 3	6991.33	13757.3	1791	1566.17	1389
FOS1	5169.33	6377.5	547.67	553.5	622.67
FOS3	7410.73	13389.07	1562.8	1771.53	1261.43
WB1	6979.33	2211.5	988.5	1317	1045.17
WB3	8168.33	3070.83	1644.33	2006.5	1602.67
NaBut 10mM	6793.5	10155	674.5	658.83	382.5
NaBut 3mM	7769.83	2761	890.33	909.67	504.17
PBS(-)	12759.8	5415.17	3607	3460.83	2563.67
H_2O_2(+)	7.33	48.67	1	33.17	10.67
Compounds	SD 1	SD 2	SD 2 (Repeated)	SD 3	SD (Repeated)
Alpha1	155.35	191.35	120.99	116.43	84.62
Alpha 3	184.03	571.48	176.21	148.76	43.95
FOS1	110.77	307.73	39.98	92.06	62.48
FOS3	403.3	357.18	69.76	122.65	36.39
WB1	269.98	102.97	111.04	146.16	39.35
WB3	344.66	139.77	240.22	68.17	70.78
NaBut 10mM	317.6	466.36	75.87	33.85	42.36
NaBut 1mM	339.17	43.93	60.79	62.64	55.74
PBS(-)	285.65	468.04	145.66	182.7	114.69
H_2O_2(+)	33.26	26.3	4.73	17.28	7.63

NB: Exp 1- 3 represents experiments 1-3 that was performed with fecal water preparation 1-3 respectively. And repeat means the experiment was repeated due to inconsistency. Alpha, FOS, WB, and NaBut represents for Alphacel (Control), Fructo-oligosaccharide, Wheat bran, Sodium butyrate respectively. 1 and 3 stand for dilution factors where the original fecal water was diluted 1 and 3 times with PBS. SD means standard deviation.

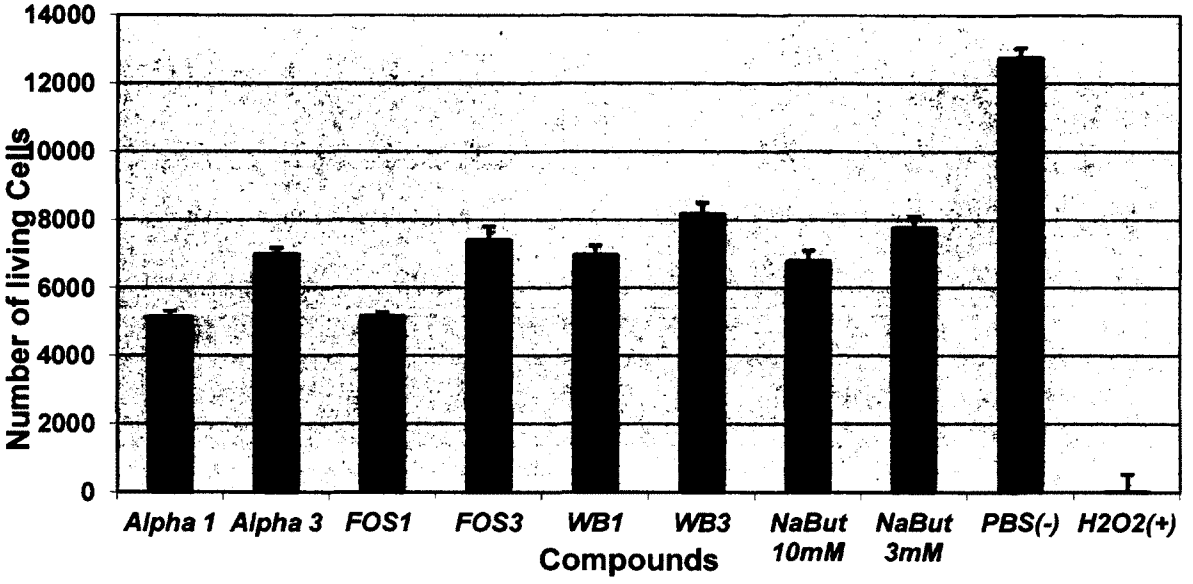

Figure A.02: The number of living HCT 116 cells after treatment with FW1 (fecal water preparation 1) of diets (e.g. alphacel, FOS, and WB) at 48 hr and detected by luminescence meter (ViaLight kit). In experiment 1, HCT 1116 cells were treated with NaBut 10mM and NaBut 3mM. The columns represent the mean (n=6) from 6 replicates and bars represent SD.

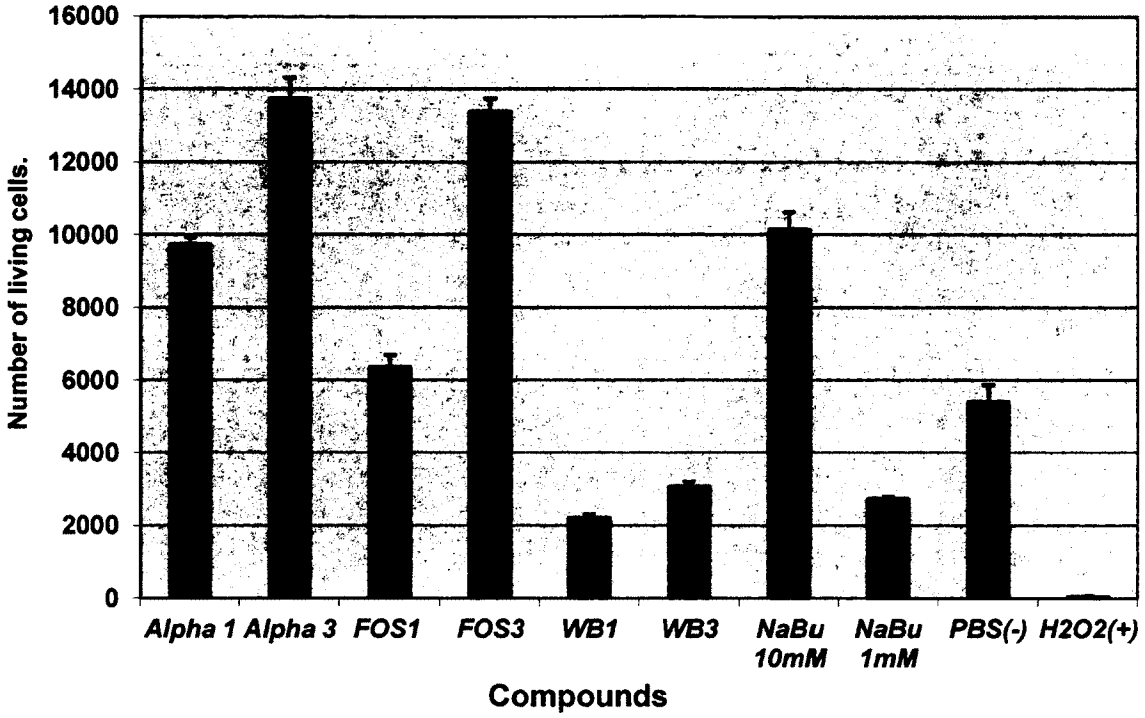

Figure A.03: The number of living HCT 116 cells after treatment with FW2 (fecal water preparation 2) of diets (e.g. alphacel, FOS, and WB) at 48 hr and detected by luminescence meter (ViaLight kit). The columns represent the mean (n=6) from 6 replicates and bars represent SD.

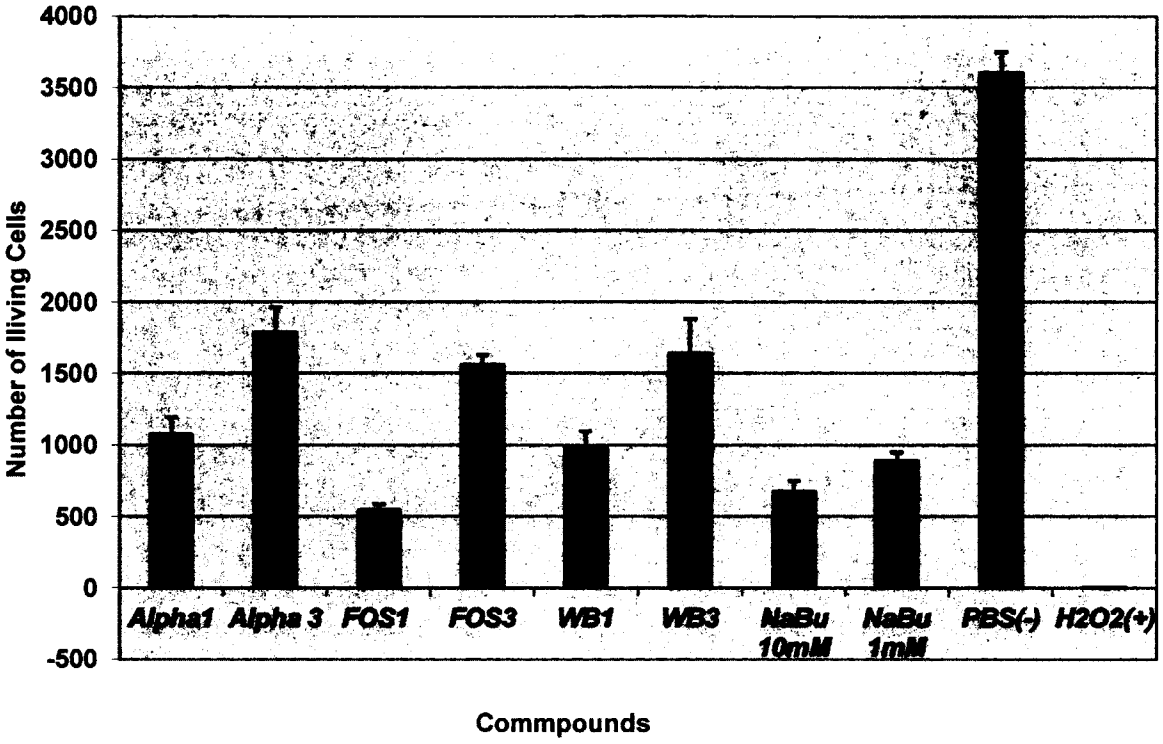

Figure A.04: The number of living HCT 116 cells after repeat treatment with FW2 (fecal water preparation 2) of diets (e.g. alphacel, FOS, and WB) at 48 hr and detected by luminescence meter (ViaLight kit). The columns represent the mean (n=6) from 6 replicates and bars represent SD.

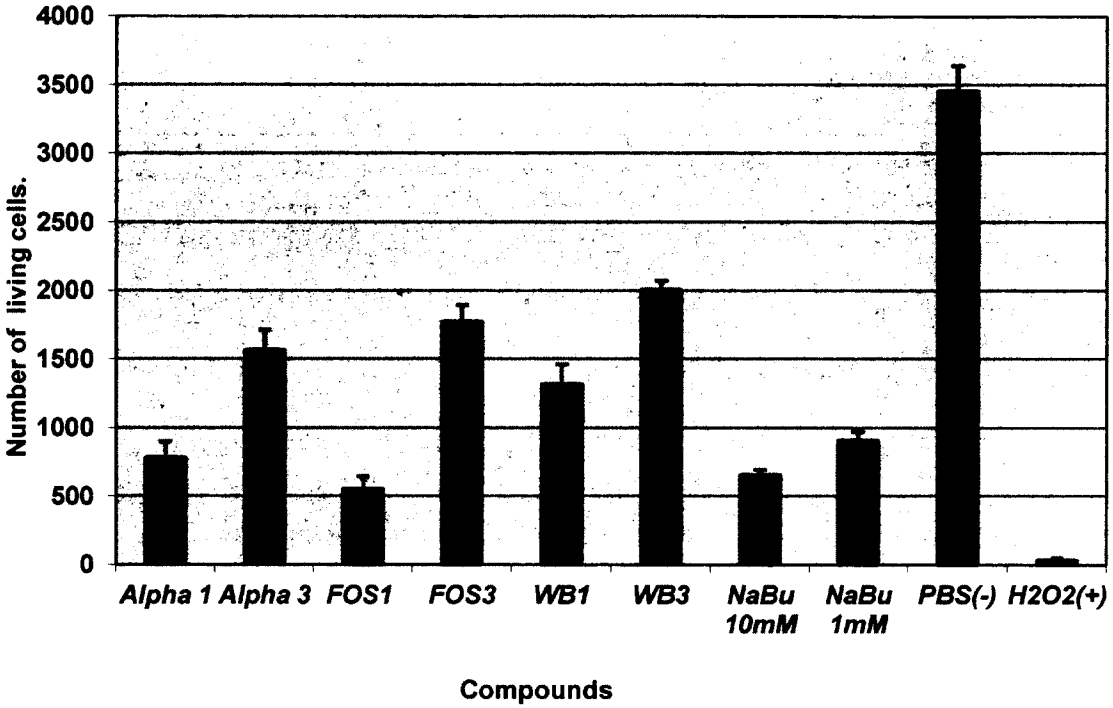

Figure A.05: The number of living HCT 116 cells after treatment with FW3 (fecal water preparation 3) of diets (e.g. alphacel, FOS, and WB) at 48 hr and detected by luminescence meter (ViaLight). The columns represent the mean (n=6) from 6 replicates and bars represent SD.

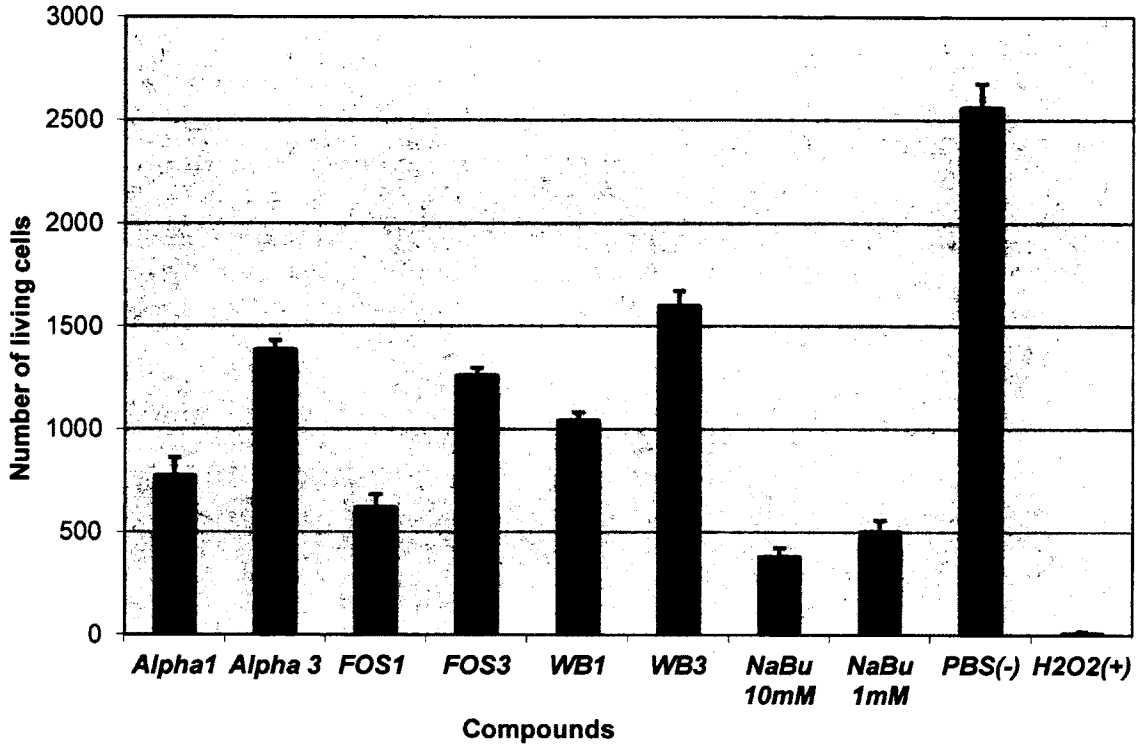

Figure A.06: The number of living HCT 116 cells after repeated treatment with FW3 (fecal water preparation 3) of diets (e.g. alphacel, FOS, and WB) at 48 hr and detected by luminescence meter (ViaLight kit). The columns represent the mean (n=6) from 6 replicates and bars represent SD.

Fecal water was prepared from the feces of rats and tested on human colon cancer cell line (e.g. HCT-116) to study the toxic effect of fecal water on apoptosis which were outlined in the Materials and Methods section. The percentage of apoptotic cells in different stages of apoptosis after 48 hr treatement with different preparation of fecal water. The primary results are shown in **Table A:13** and **Figures A.07 to A.10.**

AnnexinV and PI Assay:

Table A.13: The percentage of apoptotic HCT-116 cells in different stages of apoptosis after treatment with FW (e.g. alphacel (Control), WB, and FOS) at 48 hr, and the apoptotic cells were measured by flow cytometer (FITC Annexin V Apoptosis Detection Kit). Exp 1(R) represents experiment 1 (R) that was repeated with fecal water preparation 1.

Sample ID	Viable Cells (%)				Early Apoptotic Cells (%)				Late Apoptotic cells (%)				Dead cells (%)			
	Exp1	Exp1 (R)	Exp2	Exp3	Exp1	Exp1 (R)	Exp2	Exp3	Exp1	Exp1 (R)	Exp2	Exp3	Exp1	Exp1 (R)	Exp2	Exp3
PBS	81.7	79.37	64.28	82.44	1.56	3.23	5.35	3.32	16.22	17.21	29.38	13.97	0.53	0.19	0.98	0.28
Dead Cells	25.73	0.25	0.16	0.35	18.47	0.65	0.46	1.98	1.17	.029	1.19	0.31	54.63	98.8	98.19	97.36
NaBut 10mM	52.44	41.74	38.78	63.87	8.47	5.26	5.54	9.37	38.50	52.32	53.88	26.45	0.59	0.67	1.47	0.31
NaBut 1mM	47.08	35.44	44.17	76.15	8.74	11.69	5.52	6.67	43.73	52.55	47.70	17.84	0.45	0.32	2.61	0.35
Alpha 1	52.44	41.74	38.78	63.87	8.47	5.26	5.54	9.37	38.5	52.32	53.88	26.45	0.59	0.67	1.47	0.31
Alpha 3	47.08	35.44	44.17	76.15	8.74	11.69	5.52	5.67	43.73	52.55	47.7	17.84	0.45	0.32	2.61	0.35
FOS 1	35.3	57.81	58.91	65.66	16.17	15.7	8.42	15.66	47.35	21.57	30.88	18.37	1.17	4.91	1.79	0.3
FOS 3	68.26	39.1	62.57	74.25	7.38	18	4.97	5.64	23.41	41.84	30.05	19.63	0.95	1.06	2.53	0.47
WB 1	70.3	71.72	64.11	76.66	6.08	8.86	5.14	6.36	20.22	18.75	26.52	16.7	3.4	0.69	4.23	0.29
WB 3	77.3	77.88	59.84	68.83	3.57	6.95	4.48	7.36	18.28	14.95	32.84	23.15	0.86	0.22	2.84	0.66

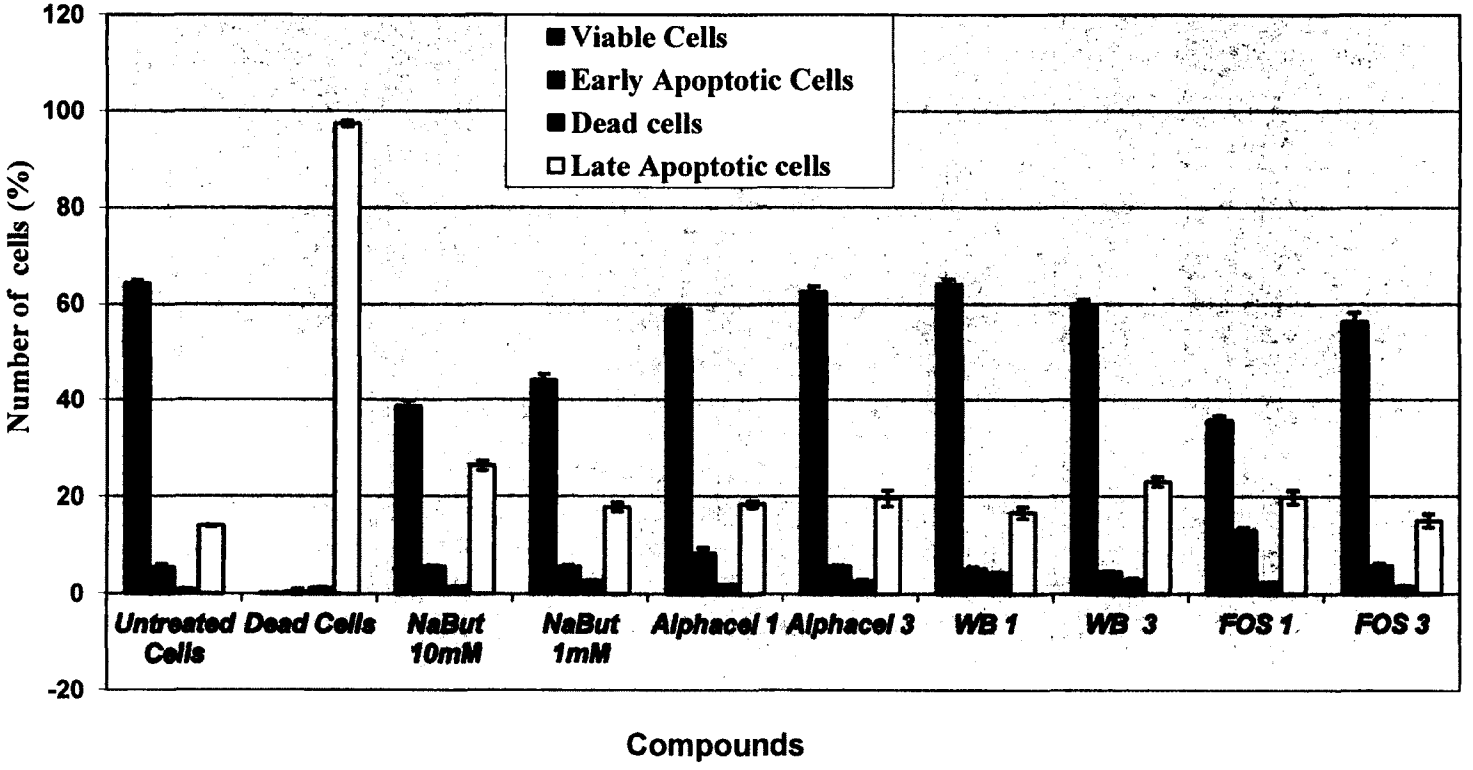

Figure A.07: The percentage of cells in different stages of apoptosis after treatment with FW1 (fecal water preparation 1) of diets (e.g. alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

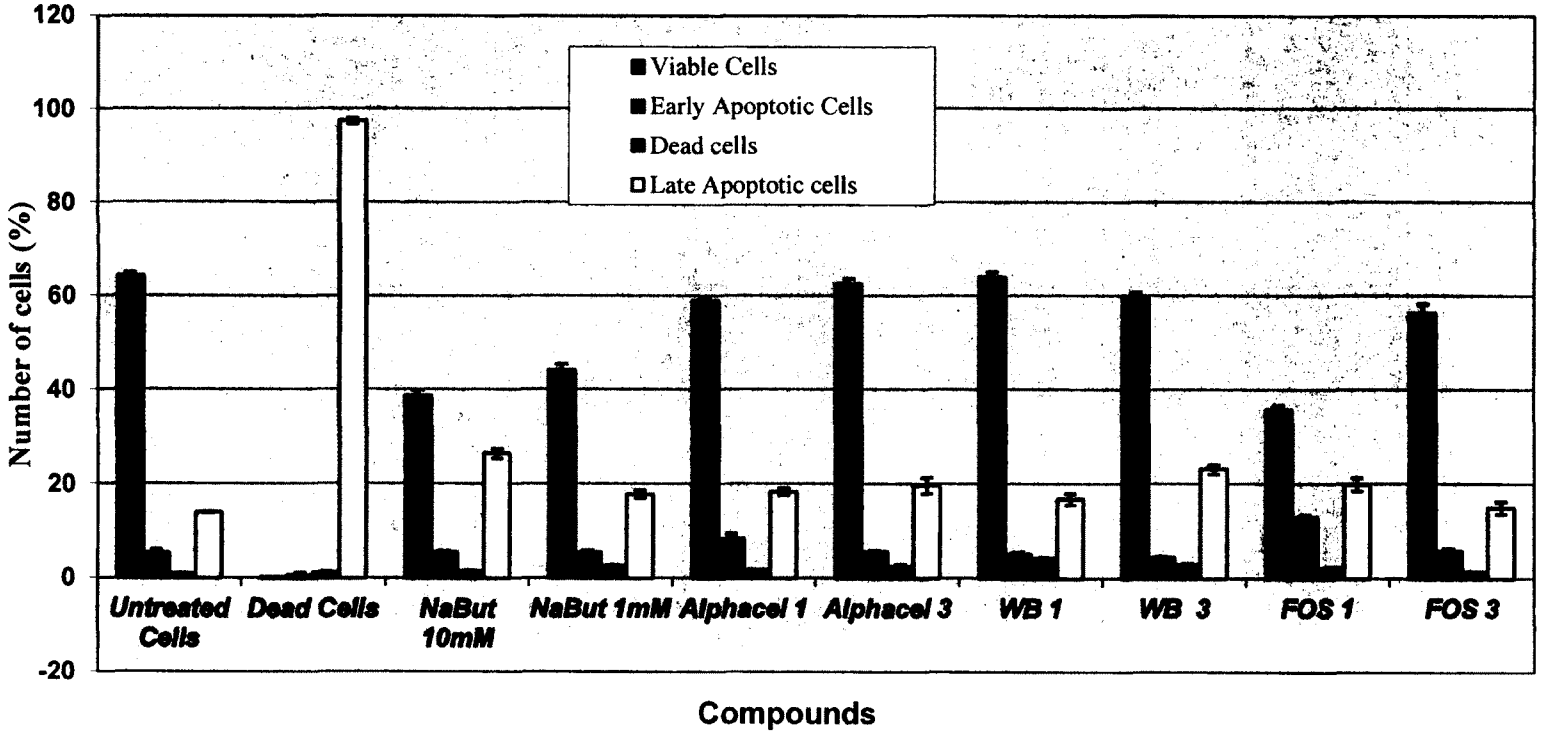

Figure A.08: The percentage of cells in different stages of apoptosis after repeated treatment with FW (fecal water preparation 1) of diets (e.g. alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

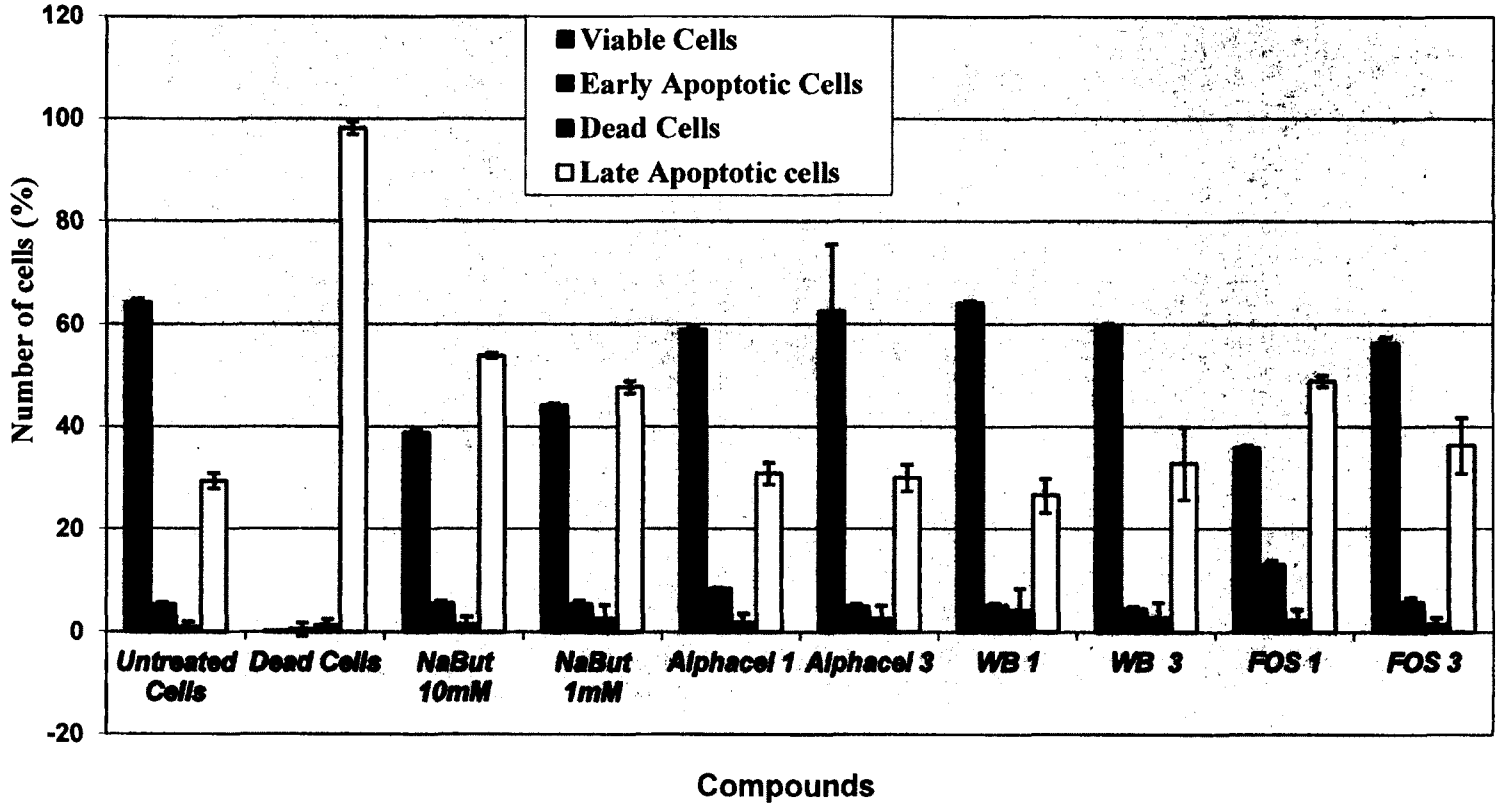

Figure A.09: The percentage of cells in different stages of apoptosis after treatment with FW2 (fecal water preparation 2) of diets (e.g. alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

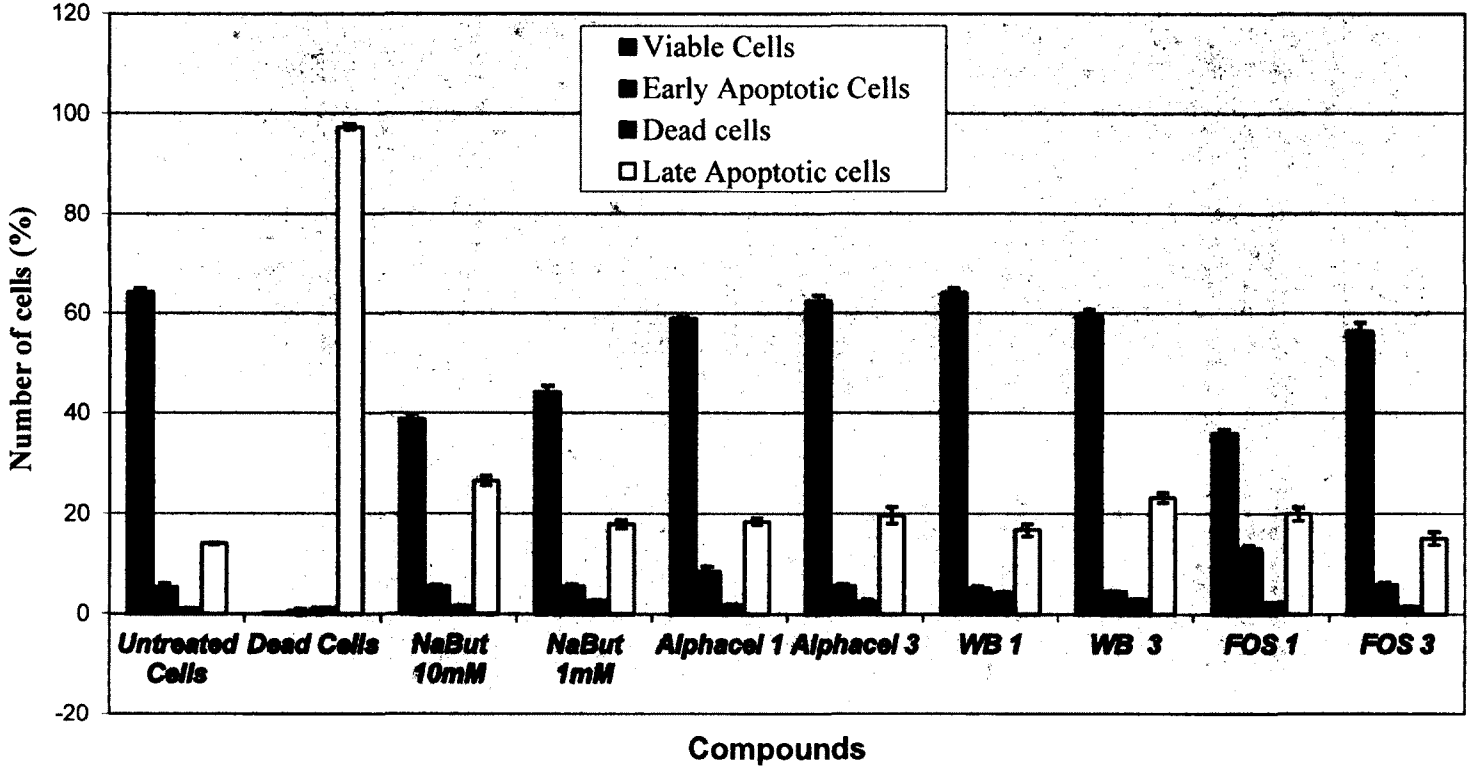

Figure A.10: The percentage of cells in different stages of apoptosis after treatment with FW 3 (fecal water preparation 3) of diets (e.g. alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

Fecal water ws prepared from the feces of rats and tested on a human colon cancer cell line (e.g. HCT-116) to study the toxic effect of fecal water on different phases of cell cycle which were described in the Materials and Methods section. The percentage of apoptotic cells in different phases of cell cycle after 48 hr treatement with different preparation of fecal water. The primary results of cell cycle arested are shown in **Table A:14** and **Figures A.11 to A.13.**

Cell Cycle Assay:

Table A.14: The percentage of apoptotic HCT-116 cells after 48 hr treatment with dietary fecal water in different phases of cell cycle.

Sample ID	G_0/G_1 (%)			G_2/M (%)			S Phase (%)		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
PBS (-)	88.52	85.94	86.44	4.09	1.61	1.79	7.39	12.45	11.76
SFP (+)	28.15	28.87	60.98	35.71	44.46	23.25	36.14	26.67	15.78
Alpha1	75.47	83.48	72.78	22.01	14.68	24.72	2.52	1.84	2.51
Alpha3	92.1	88.94	74.43	4.53	1.55	3.85	3.37	9.51	21.72
FOS 1	75.5	76.67	69.32	21.74	21.23	28.70	2.77	2.10	1.98
FOS 3	92.8	87.23	73.25	3.39	3.51	4.57	3.81	9.26	22.18
WB 1	82.42	84.94	89.93	15.49	13.73	2.49	2.09	1.33	7.59
WB 3	90.81	91.70	83.05	3.67	0.91	1.35	5.53	7.39	15.59
	SD 1	**SD 2**	**SD 3**	**SD 1**	**SD 2**	**SD 3**	**SD 1**	**SD 2**	**SD 3**
PBS (-)	0.41	0.45	0.41	0.42	0.45	0.79	0.2	0.24	0.39
SFP (+)	0.9	0.5	1.07	0.77	0.42	1.22	1.11	0.09	0.19
Alpha1	0.55	0.44	0.59	0.4	0.32	0.74	0.15	0.13	0.23
Alpha3	0.2	0.23	0.3	0.31	0.42	0.23	0.12	0.29	0.24
FOS 1	0.62	0.48	0.53	0.53	0.26	0.49	0.37	0.32	0.05
FOS 3	0.69	0.57	0.11	0.64	0.43	0.31	0.06	0.27	0.37
WB 1	0.57	0.34	0.34	0.56	0.27	0.2	0.02	0.2	0.37
WB 3	0.48	0.35	0.45	0.74	0.6	0.2	0.32	0.27	0.57

NB: SD represents the standard deviation of the experiment and SFP and PBS represent sulforaphane compound and phosphate buffer saline that was used as a positive and a negative control for cell cycle assay.

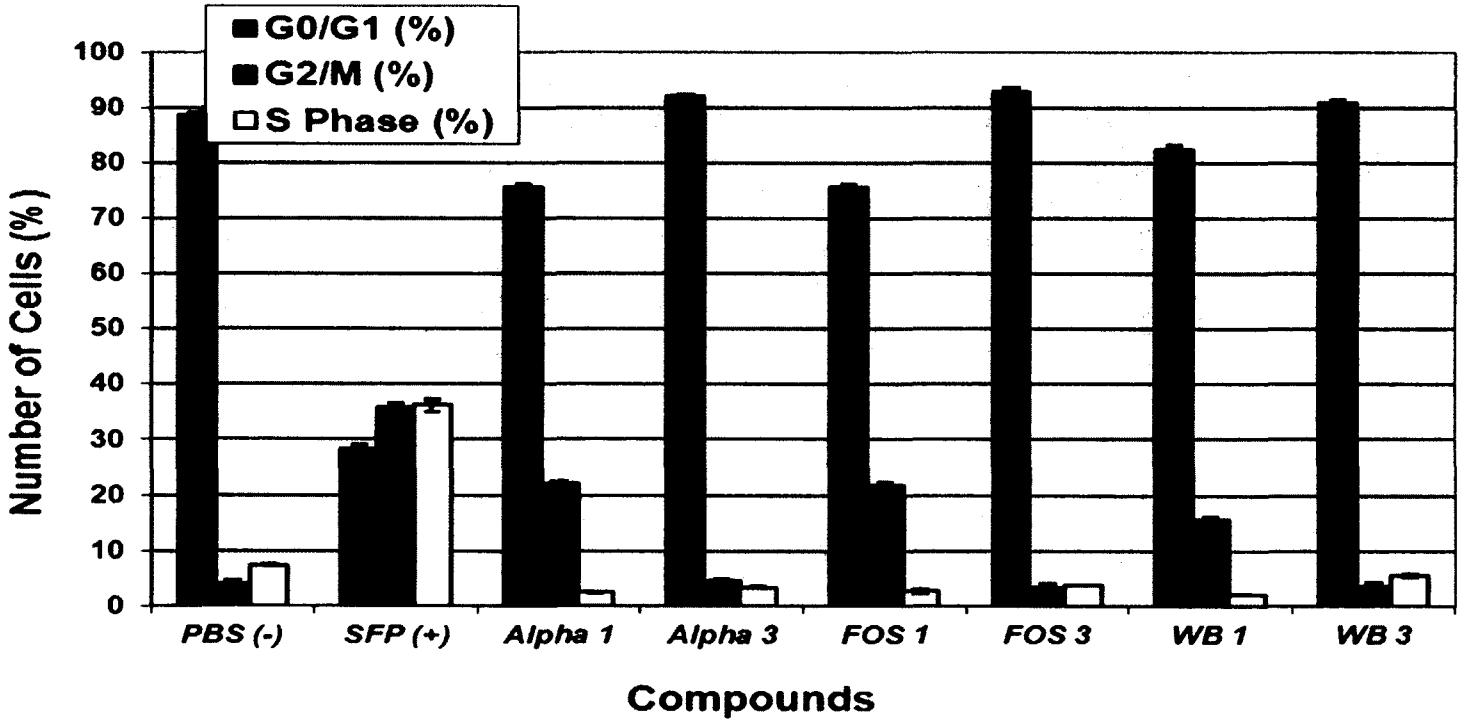

Figure A.11: The percentage of HCT-116 cells in different phases of cell cycle after treatment with FW1 (fecal water preparation 1) (e.g. Alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

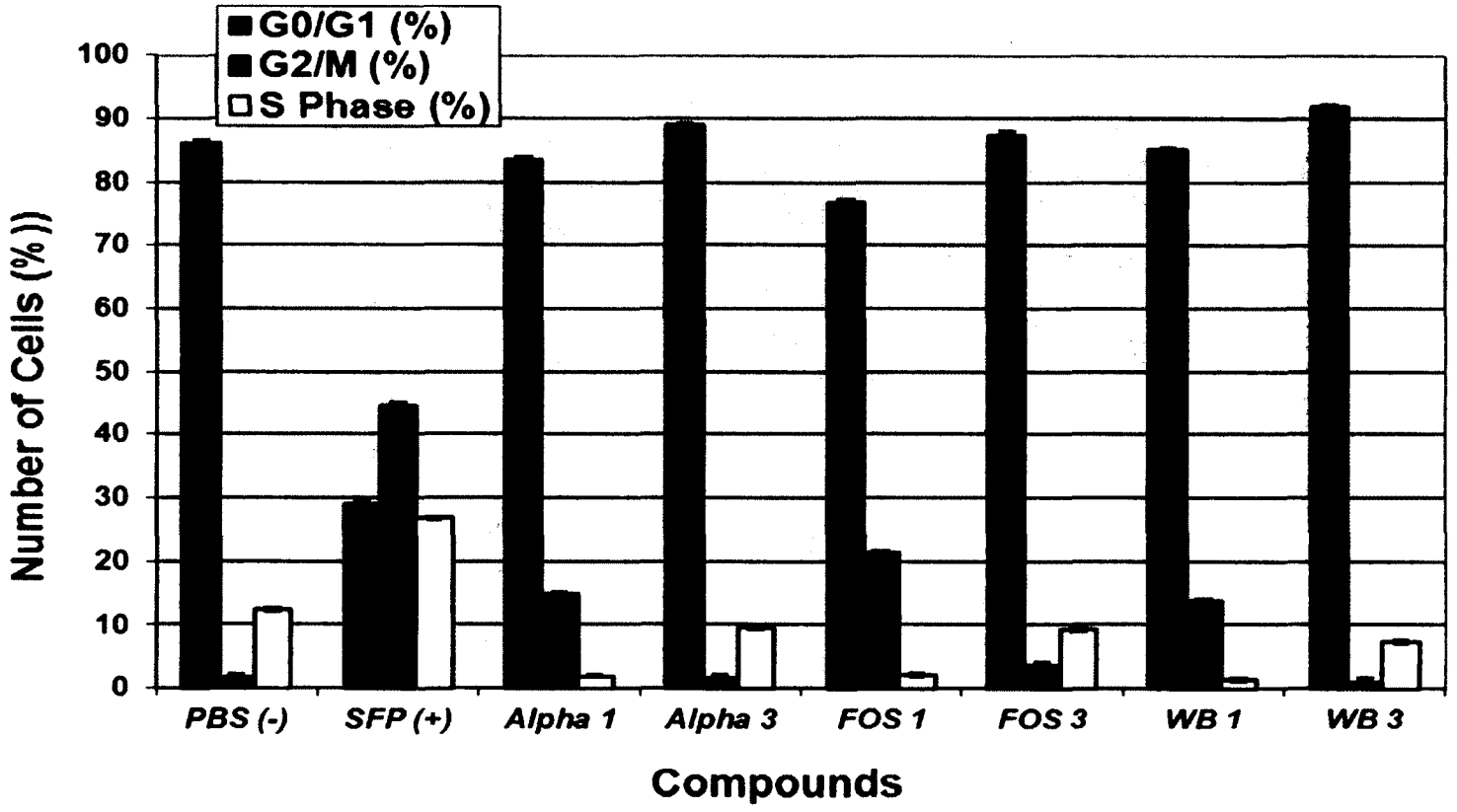

Figure A.12: The percentage of HCT-116 cells in different phases of cell cycle after treatment with FW2 (fecal water preparation 2) (e.g. Alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

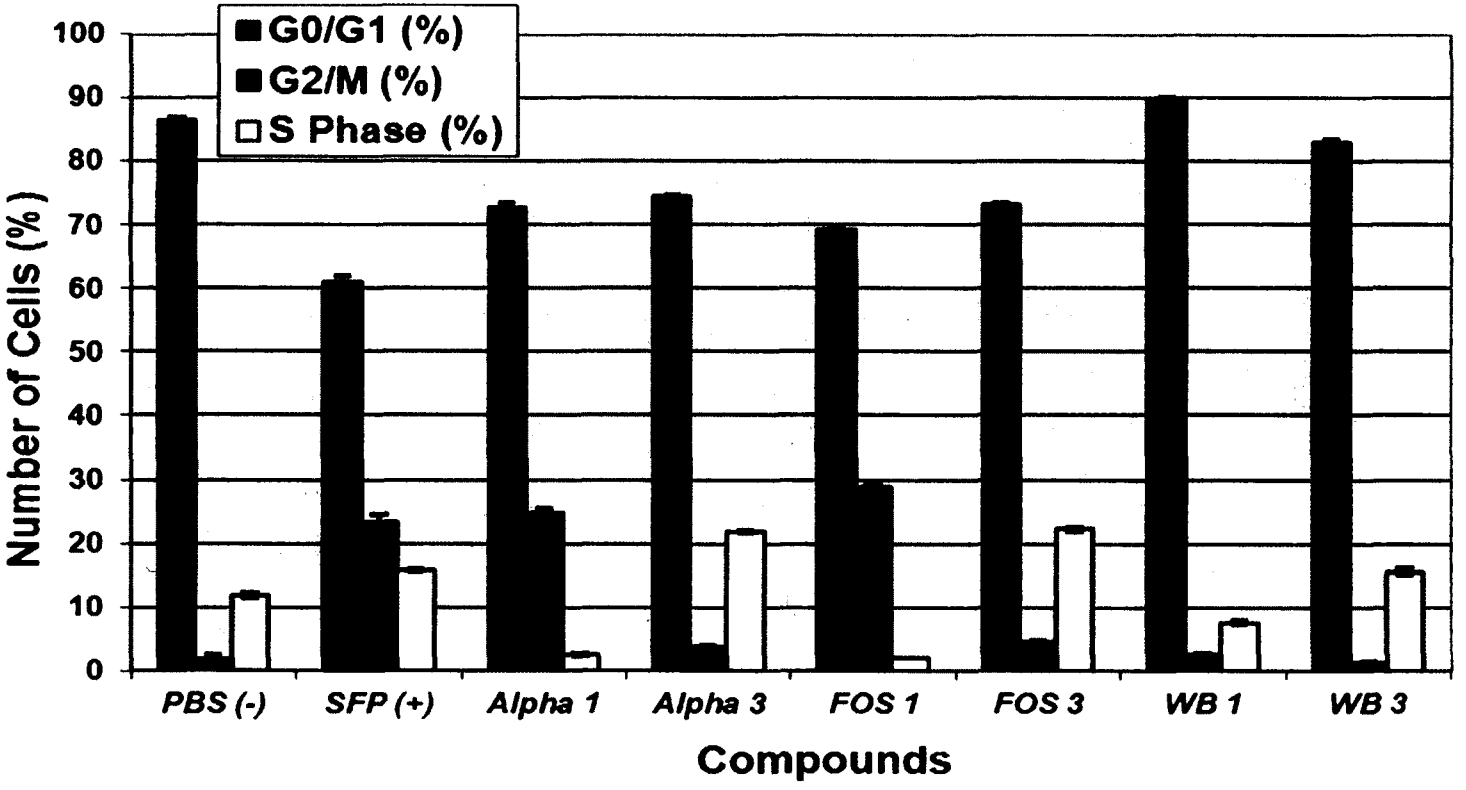

Figure A.13: The percentage of HCT-116 cells in different phases of cell cycle after treatment with FW3 (fecal water preparation 3) (e.g. Alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

Fecal water ws prepared from the feces of rats and tested on a human colon cancer cell line (e.g. HCT-116) to study the toxic effect of fecal water on DNA damage which was described in the Materials and methods section. The DNA damage was measured by flow cytometry after 48 hr treatement with different preparation of fecal water. The raw data of DNA damage are shown in **Table A:15** and **Figures A.14 to A.17.**

APO-BrdU Assay:

Table A.15: The percentage of apoptotic cells (DNA damage) of HCT-116 cells treated with FW from the alphacel, FOS, and WB after 48 hr treatments and apoptotic cells were detected by flow cytometer. Exp 3 (R) means experiment 3 was repeated with fecal water preparation 3.

Sample ID	Apoptotic Cells (%)			
	Exp 1	Exp 2	Exp 3	Exp 3 (R)
PBS	0.99	0.99	1.65	21.56
NaBut 10 mM	89.36	17.06	47.43	79.37
NaBut 1mM	98.87	30.40	39.87	67.56
Alpha 1	63.10	24.39	25.66	72.26
Alpha 3	38.30	6.50	6.56	28.89
WB 1	85.16	23.59	6.67	23.29
WB 3	46.64	5.93	6.18	12.84
FOS 1	50.60	29.86	32.70	60.44
FOS 3	14.19	7.84	11.36	32.16

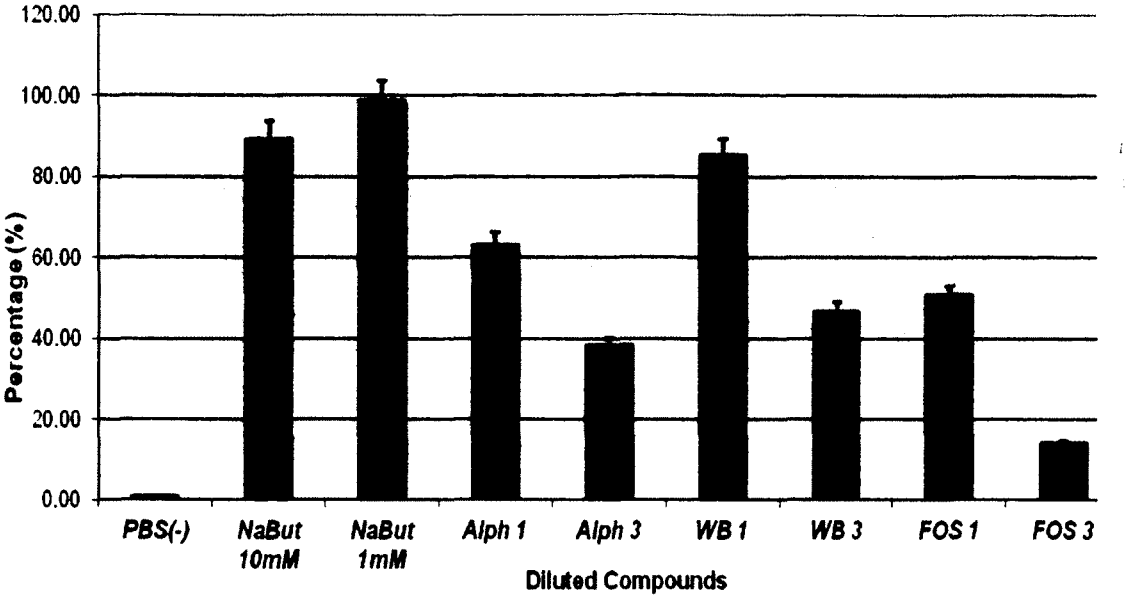

Figure A.14: The percentage of apoptotic HCT-116 cells that were treated with FW 1 (fecal water preparation 1) (e.g. Alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry.The columns represent the mean (n=3) from 3 replicates and bars represent SD.

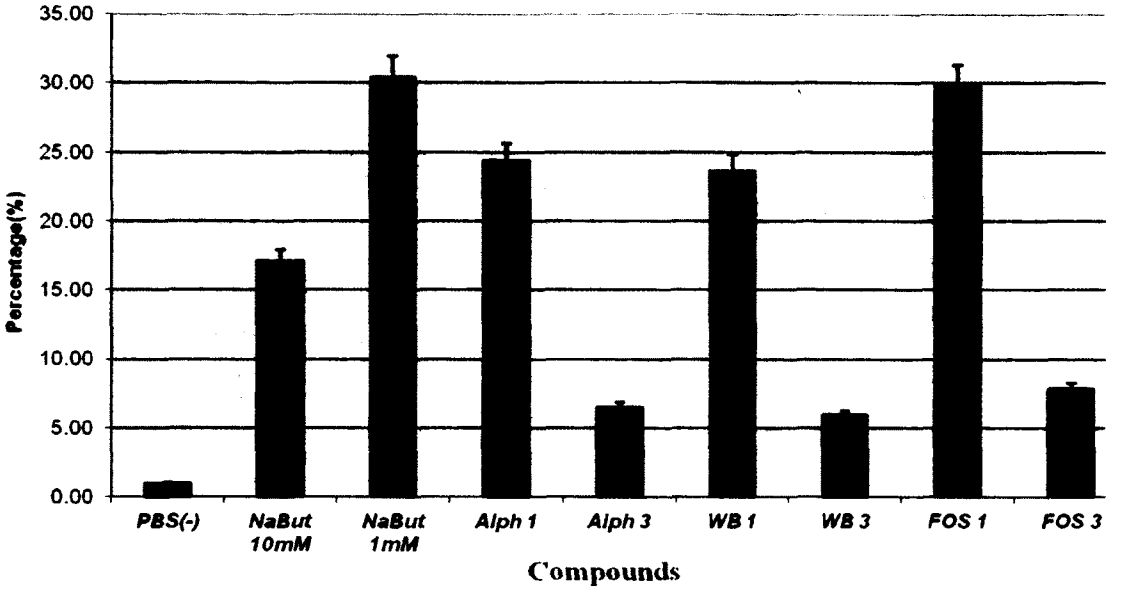

Figure A.15: The percentage of apoptotic HCT-116 cells that were treated with FW 2 (fecal water preparation 2) (e.g. Alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

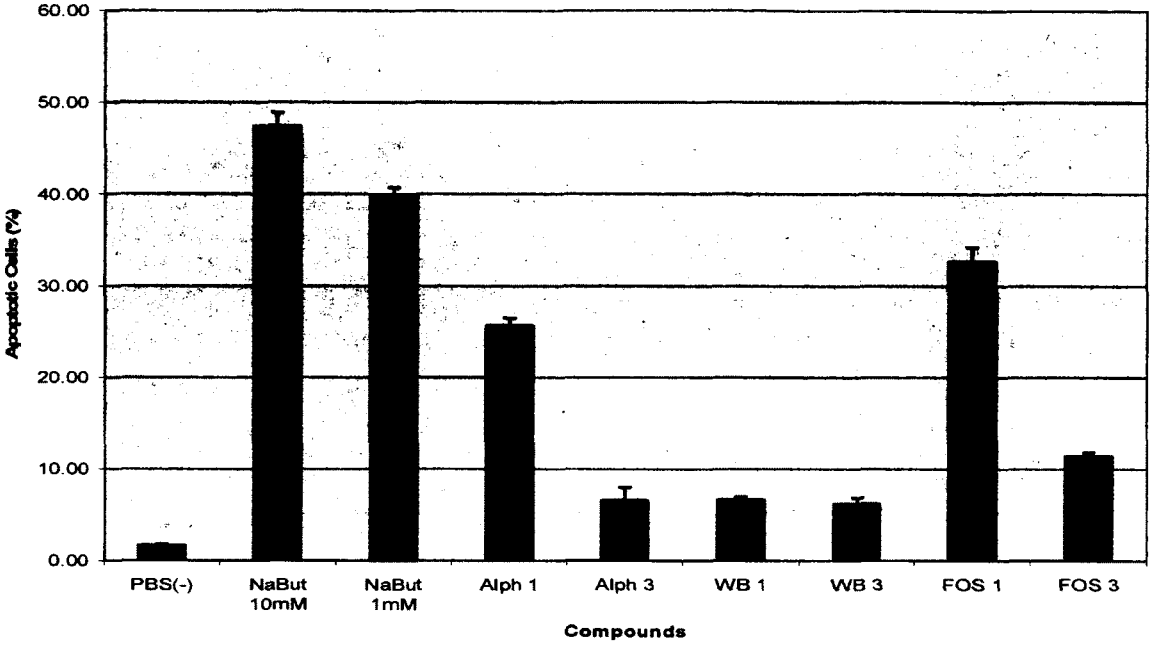

Figure A.16: The percentage of apoptotic HCT-116 cells that were treated with FW 3 (fecal water preparation 3) (e.g. Alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

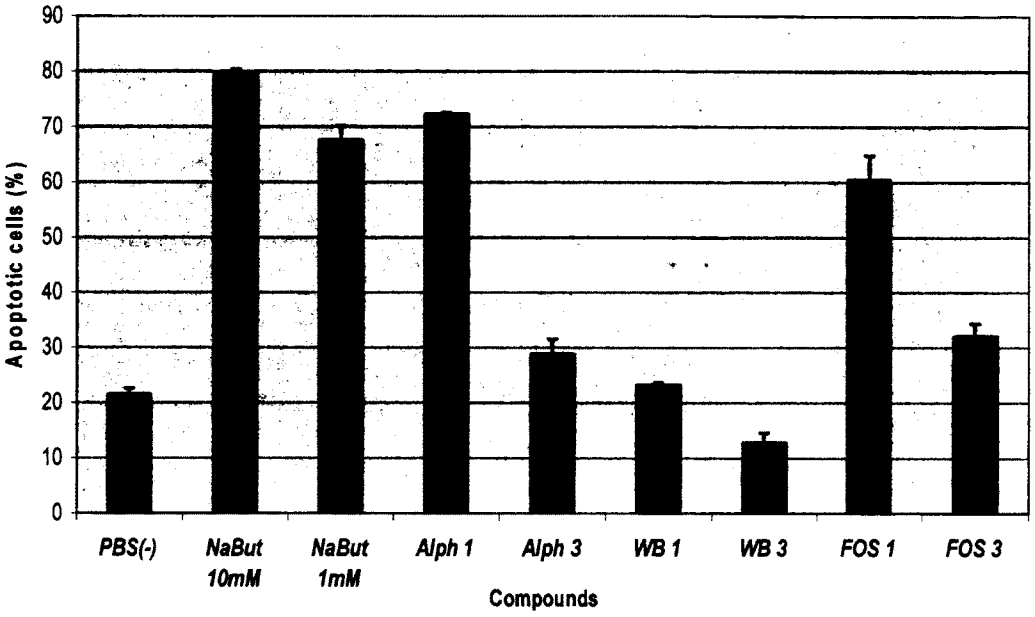

Figure A.17: The percentage of apoptotic HCT-116 cells that were repeated with FW 3 (fecal water preparation 3) (e.g. Alphacel, FOS, and WB) at 48 hr, as detected by flow cytometry. The columns represent the mean (n=3) from 3 replicates and bars represent SD.

SigmaPlot (version 12.0) statiscal program/software was used to analysze data from cell growth, apoptosis, cell cycle arrest, and DNA damage. A paired t-test was used to analyze the fecal water data. The reaults of paried t-test are shown in **Tables A.16 –A.44**

ViaLight Assay

Table A.16: The results of viability assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) difference between treatments (e.g. PBS versus WB) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Viable Cells Monday, March 05, 2012, 2:00:11 PM

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed ($P < 0.050$)

Test execution ended by user request, Signed Rank Test begun

Wilcoxon Signed Rank Test Monday, March 05, 2012, 2:00:11 PM

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
PBS(-)	4	0	8183.400	3497.372	21219.825
WB1	4	0	1764.250	1070.625	5787.372

W= -10.000 T+ = 0.000 T-= -10.000
Z-Statistic (based on positive ranks) = -1.826
P(est.)= 0.100 **P(exact)= 0.125, Therefore, Ratio = [Median PBS]/[Median WB] = 0.22**

The change that occurred with the treatment is not great enough to exclude the possibility that it is due to chance ($P = 0.125$).

Table A.17: The results of viability assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) difference between treatments (e.g. PBS versus NaBut) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Viable Cells Monday, March 05, 2012, 2:02:09 PM

Data source: Data 1 in Paried T-test Viablity Assay.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.127)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS(-) 4	0	10966.866	9740.385	4870.192	
NaBut 10mM	4	0	4570.458	4711.983	2355.991
Difference	4	0	6396.408	5201.921	2600.960

t = 2.459 with 3 degrees of freedom. (P = 0.091), [Median PBS]/[Median NaBut] = 0.42

95 percent confidence interval for difference of means: -1880.979 to 14673.795

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.091)

Power of performed test with alpha = 0.050: 0.344

The power of the performed test (0.344) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.18: The results of viability assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) difference between treatments (e.g. PBS versus Alphacel) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Viable Cells Monday, March 05, 2012, 2:03:13 PM

Data source: Data 1 in Paried T-test Viablity Assay.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.266)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS(-)	4	0	10966.866	9740.385	4870.192
Alphacel 1	4	0	4190.000	4208.131	2104.065
Difference	4	0	6776.866	5537.648	2768.824

t = 2.448 with 3 degrees of freedom. (P = 0.092), [Median PBS]/[Median Con] = 0.38

95 percent confidence interval for difference of means: -2034.737 to 15588.469

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.092)

Power of performed test with alpha = 0.050: 0.341

The power of the performed test (0.341) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.19: The results of viability assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) difference between treatments (e.g. PBS versus FOS) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Viable Cells Monday, March 05, 2012, 2:03:43 PM

Data source: Data 1 in Paried T-test Viablity Assay.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.157)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS(-)	4	0	10966.866	9740.385	4870.192
FOS1	4	0	3161.999	3055.477	1527.739
Difference	4	0	7804.867	6921.502	3460.751

t = 2.255 with 3 degrees of freedom. (P = 0.109), [Median PBS]/[Median FOS] = 0.28

95 percent confidence interval for difference of means: -3208.748 to 18818.482

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.109)

Power of performed test with alpha = 0.050: 0.291

The power of the performed test (0.291) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Apoptosis Assay:

Table A.20: The results of dead and late stages of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) difference between treatments (e.g. PBS versus NaBut) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Dead+Late Apoptosis Monday, March 05, 2012, 12:21:26 PM

Data source: Data 1 in Notebook2

Normality Test (Shapiro-Wilk) Passed (P = 0.910)

Treatment Name	N	Missing	Mean	Std Dev	SEM
NaBut 10mM	4	0	43.550	13.296	6.648
PBS	4	0	19.750	7.360	3.680
Difference	4	0	23.800	9.482	4.741

t = 5.020 with 3 degrees of freedom. (P = 0.015), [Median PBS]/[Median NaBut] = 0.45

95 percent confidence interval for difference of means: 8.712 to 38.888

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.015)

Power of performed test with alpha = 0.050: 0.894

Table A.21: The results of dead and late stages of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) difference between treatments (e.g. PBS versus WB) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Dead+Late Apoptosis Monday, March 05, 2012, 12:26:05 PM

Data source: Data 1 in Notebook2

Normality Test (Shapiro-Wilk) Passed (P = 0.600)

Treatment Name	N	Missing	Mean	Std Dev	SEM
WB 1	4	0	22.697	6.026	3.013
PBS	4	0	19.750	7.360	3.680
Difference	4	0	2.947	2.834	1.417

t = 2.080 with 3 degrees of freedom. (P = 0.129), [Median PBS]/[Median WB] = 1.75

95 percent confidence interval for difference of means: -1.562 to 7.457

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.129)

Power of performed test with alpha = 0.050: 0.248

The power of the performed test (0.248) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.22: The results of dead and late stages of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) difference between treatments (e.g. PBS versus Alphacel) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Dead+Late Apoptosis Monday, March 05, 2012, 12:24:51 PM

Data source: Data 1 in Notebook2

Normality Test (Shapiro-Wilk) Passed (P = 0.118)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	19.750	7.360	3.680
Alphacel 1	4	0	31.585	12.660	6.330
Difference	4	0	-11.835	13.606	6.803

t = -1.740 with 3 degrees of freedom. (P = 0.180), [Median PBS]/[Median Alpha] = 1.69

95 percent confidence interval for difference of means: -33.484 to 9.814

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.180)

Power of performed test with alpha = 0.050: 0.172

The power of the performed test (0.172) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.23: The results of dead and late stages of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and non-significant ($p \geq 0.05$) difference between treatments (e.g. PBS versus FOS) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Dead+ Late Apoptosis Monday, March 05, 2012, 1:03:04 PM

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.416)

Treatment Name	N	Missing	Mean	Std Dev	SEM
FOS 1	4	0	34.615	16.066	8.033
PBS	4	0	19.750	7.360	3.680
Difference	4	0	14.865	11.912	5.956

t = 2.496 with 3 degrees of freedom. (P = 0.088), [Median PBS]/[Median FOS] = 1.75

95 percent confidence interval for difference of means: -4.089 to 33.819

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.088)

Power of performed test with alpha = 0.050: 0.353

The power of the performed test (0.353) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.24: The results of viable stage of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus Alphacel) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Viable Cells Monday, March 05, 2012, 1:19:54 PM

Data source: Data 1 in Paried T-test (Viable Cells).JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.629)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	76.948	8.546	4.273
Alphacel 1	4	0	54.420	13.211	6.605
Difference	4	0	22.528	17.304	8.652

t = 2.604 with 3 degrees of freedom. (P = 0.080), [Median PBS]/[Median Alpha] = 0.71

95 percent confidence interval for difference of means: -5.006 to 50.061

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.080)

Power of performed test with alpha = 0.050: 0.382

The power of the performed test (0.382) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.25: The results of viable stage of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus FOS) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Viable Cells Monday, March 05, 2012, 1:20:21 PM

Data source: Data 1 in Paried T-test (Viable Cells).JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.844)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	76.948	8.546	4.273
FOS 1	4	0	51.200	15.634	7.817
Difference	4	0	25.748	12.538	6.269

t = 4.107 with 3 degrees of freedom. (P = 0.026), [Median PBS]/[Median FOS] = 0.57

95 percent confidence interval for difference of means: 5.796 to 45.699

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.026)

Table A.26: The results of viable stage of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus WB) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Viable Cells Monday, March 05, 2012, 1:20:58 PM

Data source: Data 1 in Paried T-test (Viable Cells).JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.912)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	76.948	8.546	4.273
WB 1	4	0	70.697	5.169	2.584
Difference	4	0	6.250	4.679	2.339

t = 2.672 with 3 degrees of freedom. (P = 0.076), [Median PBS]/[Median WB] = 0.92

95 percent confidence interval for difference of means: -1.195 to 13.695

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.076)

Power of performed test with alpha = 0.050: 0.400

The power of the performed test (0.400) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.27: The results of viable stage of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus NaBut) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Viable Cells Monday, March 05, 2012, 1:21:28 PM

Data source: Data 1 in Paried T-test (Viable Cells).JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.983)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	76.948	8.546	4.273
NaBut 10mM	4	0	49.207	11.401	5.700
Difference	4	0	27.740	7.942	3.971

t = 6.986 with 3 degrees of freedom. (P = 0.006), [Median PBS]/[Median NaBut] = 0.12

95 percent confidence interval for difference of means: 15.102 to 40.378

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.006)

Power of performed test with alpha = 0.050: 0.991

Table A.28: The results of early apoptosis stage of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus Alphacel) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Early Apoptosis Monday, March 05, 2012, 1:13:36 PM

Data source: Data 1 in Paried T-test (Early Apoptosis).JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.098)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	3.368	1.555	0.778
Alphacel 1	4	0	13.988	3.719	1.859
Difference	4	0	-10.620	5.146	2.573

t = -4.127 with 3 degrees of freedom. (P = 0.026), [Median PBS]/[Median Alpha] = 4.15

95 percent confidence interval for difference of means: -18.809 to -2.431

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.026)

Power of performed test with alpha = 0.050: 0.759

Table A.29: The results of early apoptosis stage of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus FOS) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Early Apoptosis Monday, March 05, 2012, 1:13:49 PM

Data source: Data 1 in Paried T-test (Early Apoptosis).JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.274)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	3.368	1.555	0.778
FOS 1	4	0	14.182	2.029	1.015
Difference	4	0	-10.815	2.916	1.458

t = -7.418 with 3 degrees of freedom. (P = 0.005), [Median PBS]/[Median FOS] = 4.21

95 percent confidence interval for difference of means: -15.455 to -6.175

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.005)

Power of performed test with alpha = 0.050: 0.995

Table A.30: The results of early apoptosis stage of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus WB) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Early Apoptosis Monday, March 05, 2012, 1:14:03 PM

Data source: Data 1 in Paried T-test (Early Apoptosis).JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.656)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	3.368	1.555	0.778
WB 1	4	0	6.610	1.588	0.794
Difference	4	0	-3.242	2.540	1.270

t = -2.553 with 3 degrees of freedom. (P = 0.084), [Median PBS]/[Median WB] = 1.96

95 percent confidence interval for difference of means: -7.285 to 0.800

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.084)

Power of performed test with alpha = 0.050: 0.368

The power of the performed test (0.368) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.31: The results of early apoptosis stage of apoptosis were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus NaBut) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Early Apoptosis Monday, March 05, 2012, 1:15:18 PM

Data source: Data 1 in Paried T-test (Early Apoptosis).JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.456)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	3.368	1.555	0.778
NaBut 10mM	4	0	7.160	2.068	1.034
Difference	4	0	-3.792	3.213	1.607

t = -2.361 with 3 degrees of freedom. (P = 0.099), [Median PBS]/[Median NaBut] = 2.13

95 percent confidence interval for difference of means: -8.905 to 1.320

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.099)

Power of performed test with alpha = 0.050: 0.318

The power of the performed test (0.318) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Cell Cycle Assay:

Table A.32: The results of G_0/G_1 phase of cell cycle assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus WB) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: G_0/G_1 phase Monday, March 05, 2012, 2:33:12 PM

Data source: Data 1 in Paried T-test Cell Cycle Assay G0-G1.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.930)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS (-)	3	0	86.967	1.368	0.790
WB 1	3	0	85.763	3.822	2.207
Difference	3	0	1.203	4.798	2.770

t = 0.434 with 2 degrees of freedom. (P = 0.706), [Median PBS]/[Median WB] = 0.99

95 percent confidence interval for difference of means: -10.716 to 13.123

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.706)

Power of performed test with alpha = 0.050: 0.058

The power of the performed test (0.058) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.33: The results of G_0/G_1 phase of cell cycle assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus FOS) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: G_0/G_1 phase Monday, March 05, 2012, 2:32:46 PM

Data source: Data 1 in Paried T-test Cell Cycle Assay G0-G1.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.951)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS (-)	3	0	86.967	1.368	0.790
FOS 1	3	0	73.830	3.949	2.280
Difference	3	0	13.137	3.926	2.267

t = 5.795 with 2 degrees of freedom. (P = 0.029), [Median PBS]/[Median FOS] = 0.85

95 percent confidence interval for difference of means: 3.383 to 22.890

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.029)

Power of performed test with alpha = 0.050: 0.808

Table A.34: The results of G_0/G_1 phase of cell cycle assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus Alphacel) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: G_0/G_1 phase Monday, March 05, 2012, 2:32:11 PM

Data source: Data 1 in Paried T-test Cell Cycle Assay G0-G1.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.093)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS (-)	3	0	86.967	1.368	0.790
Alphacel 1	3	0	77.243	5.566	3.214
Difference	3	0	9.723	6.298	3.636

t = 2.674 with 2 degrees of freedom. (P = 0.116), [Median PBS]/[Median Con] = 0.08

95 percent confidence interval for difference of means: -5.921 to 25.367

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.116)

Power of performed test with alpha = 0.050: 0.295

The power of the performed test (0.295) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.35: The results of G_2/M phase of cell cycle assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus WB) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: G_2/M Phase Monday, March 05, 2012, 2:39:10 PM

Data source: Data 1 in Paried T-test Cell Cycle Assay G2-M.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.108)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS (-)	3	0	2.497	1.383	0.798
WB 1	3	0	10.570	7.053	4.072
Difference	3	0	-8.073	6.396	3.693

t = -2.186 with 2 degrees of freedom. (P = 0.160), [Mean PBS]/[Mean WB] = 4.23

95 percent confidence interval for difference of means: -23.961 to 7.814

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.160)

Power of performed test with alpha = 0.050: 0.211

The power of the performed test (0.211) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.36: The results of G_2/M phase of cell cycle assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus FOS) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: G_2/M Phase Monday, March 05, 2012, 2:38:48 PM

Data source: Data 1 in Paried T-test Cell Cycle Assay G2-M.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.388)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS (-)	3	0	2.497	1.383	0.798
FOS 1	3	0	23.890	4.173	2.410
Difference	3	0	-21.393	4.878	2.816

t = -7.596 with 2 degrees of freedom. (P = 0.017), [Mean PBS]/[Mean FOS] = 9.57

95 percent confidence interval for difference of means: -33.511 to -9.276

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.017)

Power of performed test with alpha = 0.050: 0.942

Table A.37: The results of G2/M phase of cell cycle assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus Alphacel) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: G_2/M Phase Monday, March 05, 2012, 2:38:18 PM

Data source: Data 1 in Paried T-test Cell Cycle Assay G2-M.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.982)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS (-)	3	0	2.497	1.383	0.798
Alphacel 1	3	0	20.470	5.194	2.999
Difference	3	0	-17.973	4.930	2.846

t = -6.314 with 2 degrees of freedom. (P = 0.024), [Mean PBS]/[Mean WB] = 8.20

95 percent confidence interval for difference of means: -30.221 to -5.726

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.024)

Power of performed test with alpha = 0.050: 0.860

Table A.38: The results of S phase of cell cycle assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus WB) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: S Phase Monday, March 05, 2012, 2:43:50 PM

Data source: Data 1 in Paried T-test Cell Cycle Assay S Phase.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.290)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS (-)	3	0	10.533	2.744	1.584
WB 1	3	0	3.670	3.416	1.972
Difference	3	0	6.863	3.729	2.153

t = 3.188 with 2 degrees of freedom. (P = 0.086), [Mean PBS]/[Mean WB] = 0.35

95 percent confidence interval for difference of means: -2.401 to 16.128

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.086)

Power of performed test with alpha = 0.050: 0.390

The power of the performed test (0.390) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.39: The results of S phase of cell cycle assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus FOS) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: S Phase Monday, March 05, 2012, 2:43:23 PM

Data source: Data 1 in Paried T-test Cell Cycle Assay S Phase.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.173)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS (-)	3	0	10.533	2.744	1.584
FOS 1	3	0	2.283	0.426	0.246
Difference	3	0	8.250	3.157	1.822

t = 4.527 with 2 degrees of freedom. (P = 0.045), [Mean PBS]/[Mean FOS] = 0.22

95 percent confidence interval for difference of means: 0.409 to 16.091

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.045)

Power of performed test with alpha = 0.050: 0.632

Table A.40: The results of S phase of cell cycle assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus Alphacel) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: S Phase Monday, March 05, 2012, 2:43:03 PM

Data source: Data 1 in Paried T-test Cell Cycle Assay S Phase.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.437)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS (-)	3	0	10.533	2.744	1.584
Alphacel 1	3	0	2.290	0.390	0.225
Difference	3	0	8.243	2.999	1.732

t = 4.760 with 2 degrees of freedom. (P = 0.041), [Mean PBS]/[Mean Con] = 0.22

95 percent confidence interval for difference of means: 0.792 to 15.694

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.041)

Power of performed test with alpha = 0.050: 0.670

BrdU Assay (DNA damage)

Table A.41: The results of BrdU assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus NaBut) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Apoptotic Cells Monday, March 05, 2012, 2:12:36 PM

Data source: Data 1 in Paried T-test BrdU Assay.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.980)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	6.297	10.180	5.090
NaBut 10 mM	4	0	58.305	32.800	16.400
Difference	4	0	-52.008	29.923	14.962

t = -3.476 with 3 degrees of freedom. (P = 0.040), [Mean PBS]/[Mean NaBut] = 9.26

95 percent confidence interval for difference of means: -99.622 to -4.393

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.040)

Power of performed test with alpha = 0.050: 0.613

Table A.42: The results of BrdU assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus Alpahcel) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Apoptotic Cells Monday, March 05, 2012, 2:13:06 PM

Data source: Data 1 in Paried T-test BrdU Assay.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.224)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	6.297	10.180	5.090
Alphacel 1	4	0	46.353	24.915	12.457
Difference	4	0	-40.055	19.447	9.724

t = -4.119 with 3 degrees of freedom. (P = 0.026), [Mean PBS]/[Mean Alpha] = 7.36

95 percent confidence interval for difference of means: -71.000 to -9.110

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.026)

Power of performed test with alpha = 0.050: 0.758

Table A.43: The results of BrdU assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus WB) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Apoptotic Cells Monday, March 05, 2012, 2:13:42 PM

Data source: Data 1 in Paried T-test BrdU Assay.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.114)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	6.297	10.180	5.090
WB 1	4	0	34.677	34.571	17.286
Difference	4	0	-28.380	38.305	19.153

t = -1.482 with 3 degrees of freedom. (P = 0.235), [Mean PBS]/[Mean WB] = 5.51

95 percent confidence interval for difference of means: -89.332 to 32.572

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.235)

Power of performed test with alpha = 0.050: 0.123

The power of the performed test (0.123) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Table A.44: The results of BrdU assay were analyzed to assess a significant ($p \leq 0.05$) and a non-significant ($p \geq 0.05$) relationship between treatments (e.g. PBS versus FOS) using paired T-test (SigmaPlot 12.0 software).

Paired t-test: Apoptotic Cells Monday, March 05, 2012, 2:14:21 PM

Data source: Data 1 in Paried T-test BrdU Assay.JNB

Normality Test (Shapiro-Wilk) Passed (P = 0.518)

Treatment Name	N	Missing	Mean	Std Dev	SEM
PBS	4	0	6.297	10.180	5.090
FOS 1	4	0	43.400	14.606	7.303
Difference	4	0	-37.102	9.381	4.690

t = -7.910 with 3 degrees of freedom. (P = 0.004), [Mean PBS]/[Mean WB] = 6.89

95 percent confidence interval for difference of means: -52.030 to -22.175

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant change (P = 0.004)

Power of performed test with alpha = 0.050: 0.998