EFFECT OF ANTIOXIDANT-DIETARY FIBER MIXTURES ON CANCER GROWTH IN COLORECTAL CANCER-INDUCED RATS

By

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ABBREVIATIONS USED IN THIS THESIS

ACF	Aberrant Crypt Foci
AGRF	Australian Genome Research Facility
AI	Apoptotic Index
AOM	azoxymethane
AP	Alkaline phosphatase
APC	Adenomatous Polyposis Coli
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BARF	*B-type Raf kinase*
BLAST	Basic Local Alignment Search Tool
C	Cellulose
CFU	Colony Forming Units
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability Pathway
Cin	Cincau
CinL	Cincau Leave
COX-2	cyclooxygenase-2
CRC	Colorectal Cancer
CtBP1	C-terminal Binding Protein-1
DCC	Deleted in Colorectal Carcinoma
DE	Degree of Esterification
DF	Dietary fiber
DGGE	Denaturing gel gradient electrophoresis
DMEM	Dulbecco's Modified Eagle's Medium
DMH	dimethyhydrazine
DNA	Deoxyribonucleic acid
DP	degree of polymerization
DSS	Dextran Sulphate Sodium
EGCG	(-)-epigallocatechin-3-gallate
FAP	Familial Adenomatous Polyposis
FB	Faecal Blank
FOS	fructose oligosaccharide
FS	fermentation supernatant
HCA	Heterocyclic Aromatic Amines
HDAC	Histone deacetylase
I	Inulin
IAP	Intestinal Alkaline Phosphatase
IBD	Inflammatory Bowel Disease
iNOS	inducible nitric oxide synthases
LDH	Lactate dehydrogenase
LSD	Less significant difference
MDA	Malondialdehide
MDF	mucin-depleted foci
MSI	Microsatelite Instability
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

NAD	nicotinamide adenine dinucleotide
NEEA	non essential amino acid
NOx	Nitrogen oxide
NSAIDs	non steroidal anti-inflammatory drugs
P	pectin
PARP	Poly(ADP-ribose) Polymerase
PC	Pectin-Cellulose
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PGE2	prostaglandin E (2)
PGE3	prostaglandin E (3)
ROS	Reactive Oxygen Species
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
SCFA	short-chain fatty acids
SEM	Standard error of mean
SHIME	Simulator of the Human Intestinal Microbial Ecosystem bromide
UPGMA	Unweighted pair group with mathematical averages

ABSTRACT

Colorectal cancer (CRC) incidence is rising significantly in most Countries due to increasing prosperity. Epidemiological studies indicate that dietary fiber and antioxidants may protect against CRC. Dietary fiber is thought to suppress colorectal cancer growth via the production of short chain fatty acids (SCFA) in the colon, where specific compounds are produced via bacterial breakdown of the fiber. Colonic bacteria are also involved in antioxidant metabolism in the colon, and they can increase antioxidant bioavailability and activity. My research aimed to study the beneficial effect of different combinations of dietary fiber and antioxidant sources including dried green cincau extracts in the colon, these effects were examined in both *in vitro* and *in vivo* models of colon cancer. Green cincau (*Premna oblongifolia* Merr) is an Indonesia plant where the extract has high dietary fiber and antioxidant activity and was also tested in this thesis.

SCFA significantly inhibited proliferation while inducing differentiation of Caco-2 cells irrespective of the media pH. Caspase 3 and 7 (key mediators in the extrinsic and intrinsic apoptotic pathway) activities were affected by both pH and SCFA, but there was no interaction between them. Caco 2 cells were less proliferated in low media pH as this condition induced cell apoptosis. Butyrate induced cell death was observed through both caspase3/7-dependent and -independent pathways as indicated by increased caspase 3/7 activity.

Fermentation experiments using anaerobic batch cultures inoculated with human fecal slurries showed that soluble fiber (pectin and inulin)

resulted in significantly higher SCFA production than that observed with insoluble (cellulose) fiber. In Caco-2 cells, inhibition of cell growth was dependent on the amount of SCFA generated during fermentation in particular butyrate. However, the effect of fermentation supernatant (FS) on cell differentiation and apoptosis was not able to be explained by the butyrate content, as high butyrate in the FS did not always promote differentiation and the apoptotic process. Apoptotic, necrotic and autophagic pathways might all be involved in cell death in response to FS treatment. The ability of the supernatant to modulate parameters of cell growth, differentiation and apoptosis was dependent on butyrate concentration and, possibly, unidentified compounds.

Using the Azoxymethane (AOM)-induced rat model of CRC it was found that 0.1% epigallocatechin-3-gallate (EGCG) increased some individual SCFA concentrations (acetate and butyrate) in digesta when the dietary fiber source was cellulose (CE), and an opposite effect was observed when the dietary fiber source was pectin (PE). Pectin-EGCG combined induced cancer progression, characterized by an increase in total number of aberrant crypt foci (ACF), and also an increase in the proliferating cell nuclear antigen (PCNA) labelling index and PCNA positive cells. This effect was associated with increasing lipid peroxidation in the liver. The protective effect of antioxidant EGCG consumption against colon cancer development appears to be dependent on the type of the dietary fiber source in the diet and the mechanism particularly through the modification of antioxidant/prooxidant properties of the EGCG.

The beneficial effect of individual dietary fibers does not automatically synergize with the positive effects of potential antioxidants, and their combined effect will depend on how they interact with the colon microbiota of the individual. Natural mixtures of dietary fiber and antioxidant sources (as found in fruits, vegetables and plant extracts) may exhibit protective effect against CRC, and utilization of these sources should consider the processing method such as the drying process to protect their potency. In conclusion the work presented in this thesis suggests that the consumption of fresh dietary fiber antioxidants sources may pose the greatest protection against CRC.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree of diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Adelaide, 29 August 2012

Samsu Udayana Nurdin

PREFACE

This thesis is based on the three major research projects that I have conducted during my PhD. It has 7 chapters arranged chronologically including general introduction (Chapter 1), three journal articles (Chapter 2, 3 and 4), Conclusions and Future direction (Chapter 5), Appendix (Chapter 6) and References (Chapter 7). I present this thesis mainly as three journal articles prepared for submission, so there will be some repetition of concepts, abbreviations and definitions, but all references are listed in one chapter (Chapter 7). Although these papers will be eventually submitted to different journals for ease of reading they all conform to the same style in the thesis. Some data and information supporting the main thesis can also be found in Appendix (Chapter 6).

Samsu Udayana Nurdin

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PUBLSHED WORK

Part of the work in this thesis has been published or is in preparation for publishing.

In Preparation for Publication:

Nurdin, SU., Le Leu, RK., Young, GP., and Abbott, C. (2012). **SCFA MODULATION ON PROLIFERATION, DIFFERENTIATION AND APOPTOSIS DID NOT DEPEND ON THE pH.**

Nurdin, SU., Le Leu, RK., Young, GP., and Abbott, C. (2012). **EXAMINING THE EFFECTS OF COMBINATIONS OF NON-DIGESTIBLE CARBO-HYDRATE SOURCES ON SHORT CHAIN FATTY ACID PRODUCTION USING AN *in vitro* FAECAL FERMENTATION SYSTEM AND CACO-2 CELLS AS A MODEL OF THE HUMAN COLON.**

Nurdin, SU., Le Leu, RK., Young, GP. Aburto-Medina A, Ball AS, and Abbott, C. (2012). **PROTECTION EFFECT OF PHENOLIC ANTIOXIDANT ON COLORECTAL CANCER-INDUCED RATS IS DEPENDS ON DIETARY FIBER SOURCE IN THE DIET**

Conference Presentation

Nurdin, SU., Le Leu, RK., Young, GP., and Abbott, C. (2011). **EFFECT OF SHORT CHAIN FATTY ACID AND pH ON THE PROLIFERATION AND DIFFERENTIATION OF CACO-2 CELLS**, poster , at ASMR SA Scientific Meeting in Adelaide, 8th June 2011

Nurdin, SU., Le Leu, RK., Young, GP., and Abbott, C. (2011). **EXAMINING THE EFFECTS OF COMBINATIONS OF NON-DIGESTIBLE CARBO-HYDRATE SOURCES ON SHORT CHAIN FATTY ACID PRODUCTION USING AN in vitro FECAL FERMENTATION SYSTEM AND CACO-2 CELLS AS A MODEL OF THE HUMAN COLON**, poster, at Asean Food Conference in Bangkok Thailand, 15-18 June 2011

1. GENERAL INTRODUCTION

Colorectal cancer (CRC), most commonly known as bowel cancer, is cancer that starts in either the colon or the rectum. Most CRC cases are not genetically inherited but caused by external factors. External factors such as food derivatives can induce genetic alteration or induce cancer development in all stages of carcinogenesis (Berlau et al., 2004). Increased intake of red and processed meat for instance, is associated with significantly increased risk of CRC (Xu et al., 2012, Takachi et al., 2011, Fung et al., 2012) whereas diets high in protein, fiber, and carbohydrate may reduce the risk of the disease (Sun et al., 2012, Hauner et al., 2012, Arafa et al., 2011).

Fruit and vegetables are two dietary factors suggested to reduce CRC risk (Magalhaes et al., 2011a, van Duijnhoven et al., 2009, Flood et al., 2008, Magalhaes et al., 2012). Modifications of diet by increasing consumption of fruit and vegetables and reducing red and processed meat intake, as well as maintenance of physical activity and appropriate body mass can substantially reduce CRC incidence and mortality (Gingras and Beliveau, 2011). The efficacy of these foods might be due both to their dietary fiber and antioxidant content. Dietary fiber inhibits CRC through several mechanisms including increasing bile acid excretion, increasing short chain fatty acid production, increasing antioxidant levels, increasing vitamin and mineral absorption, decreasing caloric intake and by its carcinogen binding effects (Lattimer and Haub, 2010). Dietary antioxidants are associated with the modulation of signalling cascades, and expression of genes involved in the regulation of cell proliferation, differentiation, apoptosis, metastasis, and angiogenesis as

well as exhibiting anti-inflammatory and/or antioxidant effects (Araujo et al., 2011, Pan et al., 2011).

Nutrients as well as non-nutrient metabolism in the colon, by indigenous bacteria lead to the formation of a large number of compounds that may have beneficial or adverse effects on human health (Scott et al., 2011, Blaut and Clavel, 2007). The composition of bacteria in the colon depends on the type of undigested dietary intake reaching the colon(Louis et al., 2007). Diets have indirect effect on the gastrointestinal function of the host through their effects on the composition and activity of the human gut microbiota as well as the gut environment (Walker et al., 2011). Since bacterial species have different metabolic activities, specific diets have various consequences for health, dependent on the effect exerted on the bacterial population (Scott et al., 2011).

In the past, dietary fiber and antioxidants have received a lot of attention due to their ability to modulate CRC progression. Dietary fiber and antioxidants that are not digested in the small intestine will reach the colon and be metabolized by colon microbiota ((Manach et al., 2005, Saura-Calixto et al., 2010). At the same time, antioxidants will modulate the colon microbial population via their action as antibacterials or substrates, and metabolism of antioxidants will produce further simple metabolites with varied activity(Saura-Calixto et al., 2010, Bellion et al., 2008). Although the anticancer activities of dietary fiber and antioxidant have been well documented (Pool-Zobel, 2005, Lattimer and Haub, 2010, Araujo et al., 2011, Pan et al., 2011), little is known of their efficacy when they are combined. A

major aim of this thesis was to investigate the ability of dietary fiber and antioxidants to exert synergetic effect in *in vitro* and *in vivo* models. This introduction will review our current and past understanding of the CRC carcinogenesis process and the factors influencing its progress focussing mainly on diets. Firstly the biology of colon cancer and current animal models for colon carcinogenesis will be discussed. Secondly the effect of dietary fiber and antioxidant as well as specific dietary fiber such as inulin, pectin and cellulose on CRC will be reviewed. Finally green cincau (*Premna oblongifolia* L Mer.) an indonesian traditional dietary fiber will be introduced. The purpose of this review is to set the context in which the present study will be conducted.

1.1. Biology of Colorectal cancer

Cancer arises as a result of a series of genetic events that alter the normal characteristic of cells. Cancer cells have lost their ability to control molecular pathways organizing cell proliferation, differentiation, cell death, and migration. As a result, the cells undergo an irreversible change characterized by overgrowth and invasiveness. In humans carcinogenesis is a multistep process including initiation, promotion and progression (Fearon and Vogelstein, 1990), each of which will be described below.

In the initiation step, loss of function or mutations in protein-encoding genes that regulate cell growth occurs (Fearon and Vogelstein, 1990, Harrison and Benziger, 2011). The morphology of mutated cells during the initiation step is not different from the surrounding normal cells. Promotion is the second step in the transformation of a normal cell into a cancerous cell

and it is clinically and pathologically detectable(Fearon and Vogelstein, 1990). The progression is the phase in which the mutated cells develop into malignant cells. In this phase, the neoplasmic cells show progressively increased invasiveness and develop the ability to metastasize.

CRC develops through a multistep process that results from the progressive accumulation of mutations and epigenetic alterations in tumour suppressor genes and oncogenes (Migheli and Migliore, 2012). Fearon and Vogelstein (Fearon and Vogelstein, 1990) proposed the first genetic model for colorectal tumorigenesis (Fig. 1.1). In this model, genetic alteration in proto-oncogenes and tumour suppressor genes were suggested as the major cause of cancer development. Proto-oncogenes encode proteins that regulate cell division thus mutations in these genes enhance cellular growth, while tumour suppressor genes encode proteins that normally inhibit cell division or induce cell death and mutation in these genes can upregulate cell proliferation (Fearon and Vogelstein, 1990). Hyperproliferation is initiated when chromosome 5q loss occurs(Fearon and Vogelstein, 1990). This chromosome contains the Familial adenomatous polyposis (FAP) gene (a tumour suppressor gene) that controls the Wnt/β-catenin signalling pathway and mutations of this gene cause increased β-catenin nuclear localisation resulting in uncontrolled proliferation(Fearon and Vogelstein, 1990, Fodde, 2002) . Epigenetic events (DNA hypomethylation) are proposed to be involved preceding early adenoma formation. K-ras mutations (an oncogene) that occur during loss of chromosome 17q (containing p53 tumour suppressor gene) and 18p (containing DCC tumour suppressor gene) are

involved in the following steps and lead to an increase in malignancy (Fodde, 2002, Phelps et al., 2009).

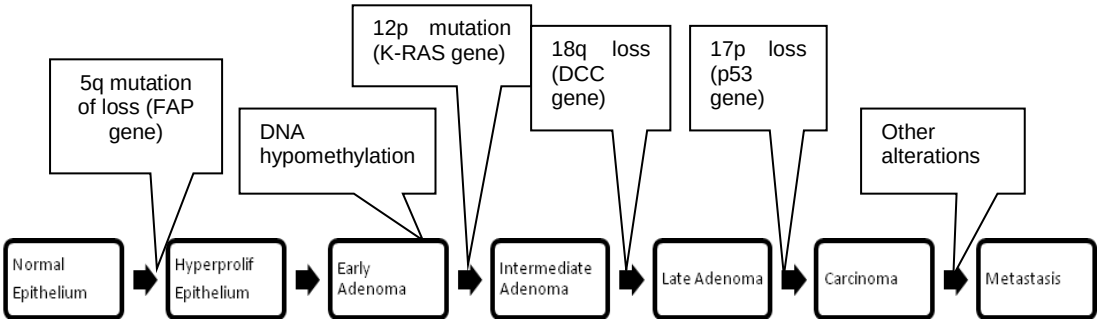

Figure 1-1. A genetic model for colorectal tumorigenesis (from Fearon and Vogelstein, 1990)

Recent research has improved our understanding of the molecular events involved in colorectal carcinogenesis. For example, Phelps et al., found that APC mutations are insufficient to cause tumor initiation (hyperproliferation), but the contribution of transcriptional corepressor C-terminal binding protein-1 (CtBP1) is needed to initiate a tumor (Phelps et al., 2009). In addition in order for tumorgenesis to progress, K-ras activation and β-catenin nuclear localization are required to promote adenoma progression to carcinoma. Based on more recent research, four molecular pathways in colorectal carcinogenesis have been identified including the chromosomal instability pathway (CIN), the CpG island methylator phenotype pathway (CIMP), the microsatellite instability pathway (MSI) and the serrated pathway (Fig 1.2) (Harrison and Benziger, 2011). In this new model, the initiation process is not only through APC mutation (adenoma formation) but also through B-type Raf kinase *(*BARF) mutation (hyperplastic polyp formation) (Harrison and Benziger, 2011) . BARF mutation is involved in the early steps

of the serrated adenocarcinoma pathway, a new distinct variant of CRC, that accounts for about 7.5% of CRC (Makinen, 2007).

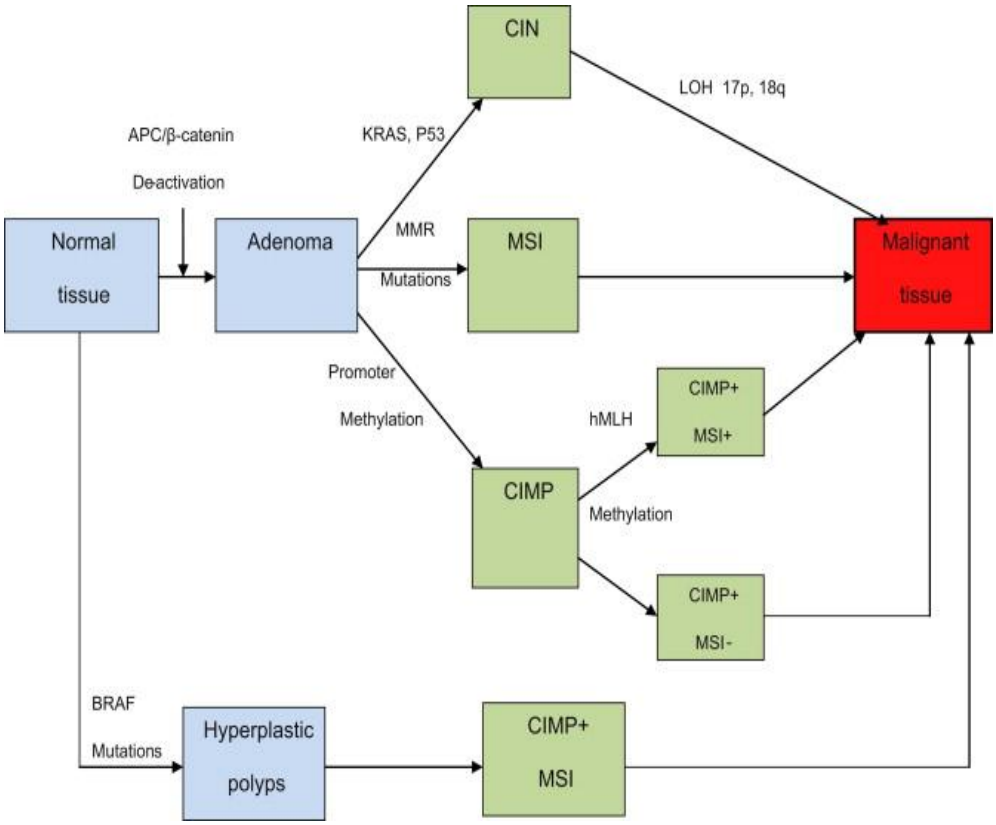

Figure 1-2. New model of adenoma-carcinoma(from Harrison and Benziger, 2011)

According to this model, the pathway that was first proposed by Fearon and Vogelstein is only one of four pathways underlying the molecular mechanism for CRC carcinogenesis. The Fearon and Vogelstein pathway is similar to the CIN pathway suggested in the new model, and this alteration occurs in 80-85% of CRC (Migheli and Migliore, 2012) and is thus the most common cause of CRC (Harrison and Benziger, 2011). DNA hypomethylation, which was proposed to precede K-ras mutation in the Fearon and Vogelstein pathway, has now been revealed to be a different pathway in the new model called CIMP, and this is the second most common

pathway (Harrison and Benziger, 2011). The events that occur during the initiation and progression process not only involve the accumulation of gene mutations but also the alteration of morphological and cellular events. One such event is the formation of ACF and this step is believed to be a early histological biomarker of colonic tumour development (Pan et al., 2011). In many animal models (discussed below) ACF are used as biomarkers to study the effects of new treatments or nutritional interventions for preventing CRC.

Epigenetic alteration modifies gene expression without altering DNA sequence. Epigenetic alterations have been detected in CRC carcinogenesis, such as DNA methylation, histone modification and microRNA dysregulation (Migheli and Migliore, 2012). Epigenetic modification is inducible by environmental factors including diets (Migheli and Migliore, 2012). Williams et al. found nutrients (folate, vitamins B6 and B12) and antioxidants (vitamin C and E, β-carotene) could affect DNA methylation and reduce CRC risk in white Americans (Williams et al., 2010).

Inflammation also plays an important role in colorectal carcinogenesis, especially CRC associated inflammatory bowel disease (IBD) and is likely to be involved in other forms of sporadic, as well as heritable, colon cancer (Terzic et al., 2010). The inflammatory process generates pro-inflammatory cytokines, reactive nitrogen species and reactive oxygen species, and the last two potentially affect the molecular pathway through their reactivity against DNA, RNA, lipids and proteins (Pan et al., 2011).

1.2. Rodent models for CRC prevention

Ideally, the efficacy of food, phytochemicals or other chemo-preventative agents for CRC prevention should be tested in humans. However, nutritional studies in human are time consuming and expensive, and may put the subjects under a health risk. Therefore, animal models are valuable in development of chemo-preventative agents and/or in understanding how nutritional factors can exhibit protection against CRC. In animal models, carcinogenesis can be induced by a chemical carcinogen or diet modification or if the models have been genetically engineered, carcinogenesis can arise spontaneously (Femia and Caderni, 2008). There are some animal models that have been used for studying the anticancer activity of natural and synthetic compounds. The most common and widely used as a carcinogen-induced animal models are the dimethyhydrazine (DMH) or azoxymethane (AOM) models. The genetic model, APC^{min} mice is also a promising model and common choice for CRC preventive studies (Femia and Caderni, 2008, Perse and Cerar, 2011, Corpet and Pierre, 2005).

Perse and Cerar have reviewed morphological and molecular alterations in the DMH and AOM-induced animal models (Perse and Cerar, 2011). These authors found that the DMH/AOM model has similarity with human sporadic CRC in term of their morphological and molecular characteristics. Tumours in this model develop through aberrant crypt foci (ACF), adenoma and carcinoma formation and the rats have APC and K-ras mutations, as well as β-catenin accumulation in the colon cell nuclei like in human tumours (Corpet and Pierre, 2005, Hu et al., 2009). DMH/AOM

induced ACF formation in the rat colon are like pre-neoplastic lesions in human colons (Bird, 1995, Bird, 1987). AOM has been used as a carcinogen in rat models for the evaluation of the chemo-preventative agents, and by using ACF as a biomarker of colon cancer, this method shows accurate results to predict the efficacy of the agents against colon cancer (Wargovich et al., 1996).

DMH and AOM are equally effective at inducing colon cancer, but DMH is more hazardous than AOM for the researchers (Femia and Caderni, 2008). Cancer in these rodent models is induced with 150 mg/kg DMH or 10-15 mg/kg AOM respectively subcutaneously injections twice for two consecutive week, and the rodents killed after 8-12 weeks of the second DMH/AOM injection (short-term study) or after more than 40 weeks of the second injection for long-term study. ACF are scored in the short-term study whilst the number of colonic tumours are assessed in the long-term study (Perse and Cerar, 2011). The AOM-induced ACF model is more useful for selection of potential chemo-preventative agents against CRC (Raju, 2008). Numerous studies have used dosages of 20 mg/kg body weight of DMH given for 15-27 consecutive weeks or 15-20 mg/kg body weight of AOM given for 2 consecutive weeks with ACF data as one of end point biomarkers (Aranganathan and Nalini, 2012, Nalini et al., 2012, Won et al., 2012, Sivagami et al., 2012, Lahouar et al., 2012, Zhao et al., 2011, Gourineni et al., 2011). The chemical carcinogen induced rat model is much more efficient than a dietary induction model as in a dietary model it can take 1.5 to 2.0 years of feeding on the high risk colon cancer diet to induce the CRC (Yang et al., 2008).

APC^{min} mice, have a mutation of APC gene which is similar to that found in patients with Familial Adenomatous Polyposis (FAP) and sporadic colon cancers. APC^{min} mice develop tumours in small intestine spontaneously, and have been used for studying effects of dietary fiber or phytochemicals on CRC prevention (Mutanen et al., 2000, Fini et al., 2011). Even though these mice have an APC mutation, they have no K-ras or p53 mutations whereas mutations in these genes are detected in human tumours (Corpet and Pierre, 2005). Moreover, as the effect of dietary fiber and phytochemicals against CRC in the colon is in response to the interaction between these agents and the colon microenvironment, evidence of inhibitory effects of chemo-preventative agents against tumour development in small intestine of APC^{min} mice cannot predict the efficacy of these agents in humans (Femia and Caderni, 2008).

1.3. Effect of Diet on Colorectal Cancer Risk

CRC carcinogenesis is a multistep process initiated by alteration of genes, classified into sporadic and hereditary case (Benito and Diaz-Rubio, 2006). More than 95% CRC is considered as sporadic and arising in patients without significant hereditary risk (Watson and Collins, 2011). Thus a large proportion of CRC are related to the environmental factors such as lifestyle and obesity, and are preventable (Gingras and Beliveau, 2011). The strongest contributing lifestyle risk factor for colorectal cancer is diet (Watson and Collins, 2011). The increased CRC incidence in developed countries is attributed to the western diet. This diet is characterised by increased quantities of red meat and processed meat, but reduced fruit and vegetables

intake compared to diets in developing countries. Adoption of this diet by recently developed countries, such as Japan, has increased the CRC incident in this country (Takachi et al., 2011).

Several mechanisms have been proposed underlying the association between red and processed meat and CRC including stimulation of insulin secretion, high content of saturated fat and heme iron, as well as carcinogenic heterocyclic amine precursors (Chan and Giovannucci, 2010). Recent reports support this hypothesis. Consumption of high amounts of meat increased levels of serum C-peptide, a biomarker for pancreatic insulin secretion, and this increase was associated with an increased CRC risk (Fung et al., 2012). Heme iron present in meat promotes CRC through its action on the endogenous formation of carcinogenic N-nitroso compounds and the formation of cytotoxic and genotoxic aldehydes by lipoperoxidation (Bastide et al., 2011). High temperature cooking of meat forms heterocyclic aromatic amines (HCA), and HCA intake was positively associated with CRC risk, regardless of phenotypes involved in the metabolizing process (Barbir et al., 2012). It is also possible that thermoresistant oncogenic bovine viruses can contaminate meat preparations, and concomitant or subsequent exposure to chemical carcinogens arising during cooking procedures results in an increased risk of CRC (zur Hausen, 2012).

In contrast with red meat, high intake of fruit and vegetables might help to reduce the risk of CRC. Recent data from a systematic review and meta-analysis of human diets indicate the risk of CRC was decreased when fruit and vegetables intake was high (Magalhaes et al., 2012). Consumption

of 100-200 g/day of fruits and vegetables will reduce CRC risk by 10% (Aune et al., 2011b). Several mechanisms by which fruit and vegetables reduce CRC risk have been proposed. Fruit and vegetables are good sources of fiber that may lead to diminished risk of CRC by increasing stool bulk, decreasing transit time in the colon, diluting potential carcinogens, and induction of SCFA production (Kumar et al., 2012). Fruit and vegetables contain a large number of potentially anticarcinogenic agents including carotenoids, vitamins C and E, selenium, as well as phenolic that have the ability to induce detoxification enzymes or form antineoplastic agents (drugs that prevent or inhibit the maturation and proliferation of neoplasms), and/or antioxidant effects (Steinmetz and Potter, 1991). Polyphenols are abundant in plant-based foods including fruits and vegetables and have a significant inverse association with CRC (Zamora-Ros et al., 2012). A case control study indicated CRC risk was inversely correlated with the daily number of apple servings (a rich source of flavonoids), with the most significant reductions observed when the intake was one or more apple servings daily (Jedrychowski and Maugeri, 2009). However, carotenoids (individual or total) that are frequently found in fruits and vegetables, or vitamin A, C and E and selenium have no significant associations with a decreased CRC risk (Park et al., 2009, Papaioannou et al., 2011). Therefore, phenolic compounds in fruits and vegetables are suggested to have a more prominent role in CRC prevention than vitamin antioxidants. Phenolic compounds protect against CRC through induction of cell differentiation, Wnt signalling inhibition, and reduction of oxidative DNA damage (Lea et al., 2010, Sharma et al., 2010, Miene et al., 2009, Lea et al., 2008).

Thousands of polyphenol compounds have been identified in higher plants including edible plants as a secondary metabolites and are generally involved in defence against ultraviolet radiation or aggression by pathogens (Manach et al., 2004). The polyphenol content of a large number of foods and beverages from plant origin have been assayed, and the richest sources were various spices and dried herbs, cocoa products, some darkly coloured berries, some seeds (flaxseed) and nuts (chestnut, hazelnut) and some vegetables, including olive and globe artichoke heads (Perez-Jimenez et al., 2010). In the beverage group, coffee was the richest with the main content being chlorogenic acids, followed by black and green tea which are rich in catechins, theaflavins and proanthocyanidins(Perez-Jimenez et al., 2010).

Tea is one of the most commonly consumed beverages in the world. Both green and black tea and their constituents, such as tea polyphenols, have been shown to have CRC-inhibitory properties in numerous *in vitro* and *in vivo* animal studies (Zhou et al., 2012, Shimizu et al., 2011, Khatiwada et al., 2011, Shimizu et al., 2008). However, the effect of these beverages on CRC risk in human is not conclusive, with some studies indicating an inverse association with CRC risk (Yang et al., 2011, Fujiki et al., 2012) and other studies not producing consistent results (Sinha et al., 2012, Zhang et al., 2010).

1.4. Dietary fiber and colorectal cancer

Dietary fiber is the edible part of plants, primarily derived from plant material and is composed of complex, nonstarch carbohydrates and lignin that are not digested and absorbed in the small intestine (Turner and Lupton,

2011). Dietary fiber reaches the colon and the beneficial effects of dietary fiber arise in this site after enzymatic, microbiological and physiological processes take place (Louis et al., 2007). Dietary fibers that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improving the health of the host are classified as prebiotics (Gibson and Roberfroid, 1995).

Dietary fiber in nature is often in a mixed form and can be classified as soluble and insoluble. Soluble dietary fiber dissolves in water forming viscous gels consisting of pectin, gums, inulin-type fructans and some hemicelluloses, and has fermentable properties (Lattimer and Haub, 2010). In contrast, insoluble dietary fiber is not soluble in water and is difficult to degrade by the colon microbial flora. Insoluble dietary fiber commonly comes from parts of the plant cell wall such as lignin, cellulose and some hemicelluloses (Lattimer and Haub, 2010).

There are some studies indicating an inverse relationship between dietary fiber intake and CRC risk in the population (Aune et al., 2011a, Bener, 2011, Arafa et al., 2011). Specific dietary fiber fractions isolated from some plants such as inulin, pectin and cellulose have received much attention as indicated by the number of studies in this area in the last 10 years. In the following section, efficacy of inulin, pectin and cellulose against CRC will be discussed.

1.4.1. Inulin

Inulin are polysaccharides having linear or branch chains consisting mainly of fructose monomers connected by β-2,1 linkage (Fig. 1.3). Inulin are

found in a number of cereals including wheat, and in fruits or vegetables such as, banana, onion, and garlic (Roberfroid and Delzenne, 1998). Thus inulin is widely distributed in nature as plant stored carbohydrate (Niness, 1999). For industrial needs, inulin is extracted from chicory (*Cichorium intybus)* (Roberfroid and Delzenne, 1998, Niness, 1999), Jerusalem artichoke (*Kluyveromyces marxianus*), or synthesized from sucrose molecules (Roberfroid and Delzenne, 1998).

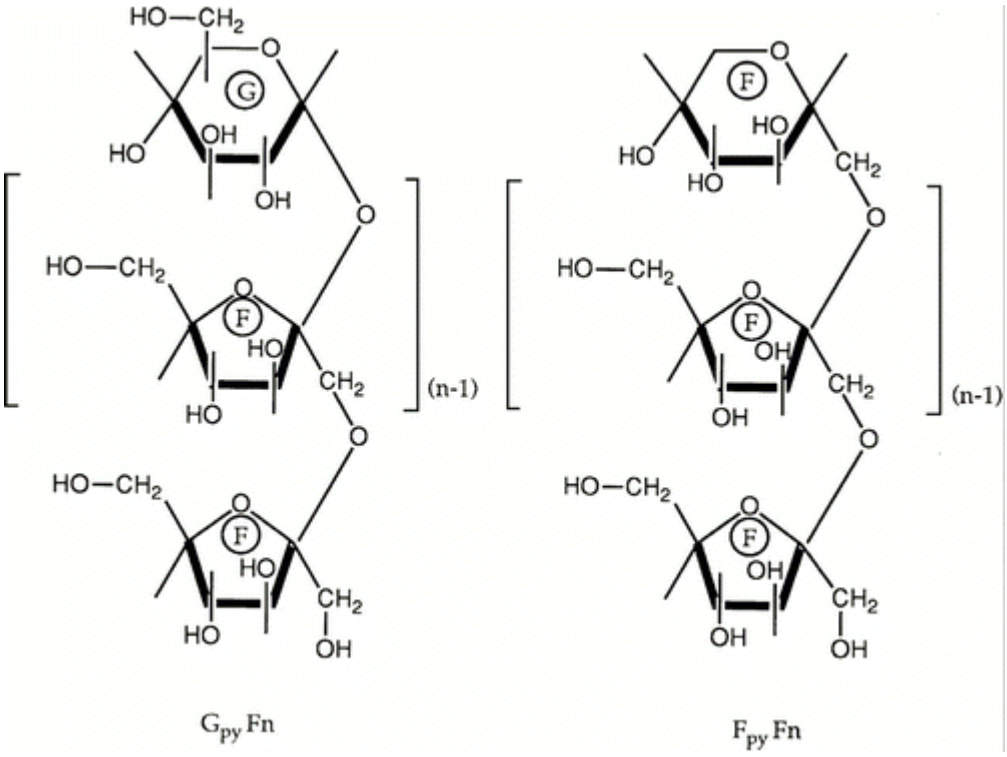

Figure 1-3. Chemical structure of inulin-type fructans. n is the degree of polymerization or the number of β-D-fructofuranose; G and F stand for glucose and fructose, respectively; GpyFn is α-D-glucopyranosyl-[β-D-fructofuranosyl](n-1)-D-fructofuranoside (Roberfroid and Delzenne, 1998)

The characteristics of inulin mainly depend on the degree of polymerization (DP). Natural inulin extracted from chicory contains fructan with a variety of DP and exists as mixed fructan such that chicory inulin

powder has an average DP of 10-12 (Roberfroid and Delzenne, 1998). High performance inulin which is a commercial inulin, mainly contains a high DP (the average DP is 25), produced through elimination of the low DP molecules through a separation process (Roberfroid and Delzenne, 1998, Niness, 1999). Inulin is also processed by the food industry to produce oligofructose with DP 2-10 as a result of partial enzymatic hydrolysis (Roberfroid and Delzenne, 1998).

Fermentation of inulin using human faecal inoculums had a profound effect on growth of the *bifidobacteria spp*. (Wang and Gibson, 1993, Pompei et al., 2008) and *lactobacilli spp*. (Bertkova et al., 2010) in a batch culture system. In more advanced culture systems mimicking the human intestinal microflora, similar results were found (de Wiele et al., 2004, Pompei et al., 2008). This system, termed as the simulator of the human intestinal microbial ecosystem (SHIME), consisted of five double-jacketed vessels simulating the stomach, small intestine, ascending colon, transverse colon and descending colon, respectively, and was run at a temperature of 37^{o}C, with a total retention time of 76 h (de Wiele et al., 2004, Pompei et al., 2008). Using this system, de Wiele et al combined molecular analytical techniques with conventional methods to study the colon associated bacteria, and found that inulin (fibrulin instant with DP ranging between 3 and 60) induced significant *bifidobacteria spp*. counts with more than 1 log CFU ml^{-1} from the proximal to distal colon (de Wiele et al., 2004, Pompei et al., 2008). In an animal model, mice fed diets with fructans (oligosaccharide or inulin) had 50% lower densities of *Candida ablican* in the small intestine compared with those fed

diets containing cellulose. In addition mice, administred inulin had less systemic infected mortality caused by *Listeria monocytogenes* or Salmonella *typhimurium* (Buddington et al., 2002).

To elucidate which species of bifidobacteria spp. is stimulated by inulin, Ramirez-Farias (Ramirez-Farias et al., 2009) carried out a study examining the effects of inulin on human gut microbiota. In this research, six healthy adult subjects consumed 10 g inulin per day for 21 days (treatment group) and six healthy adult subjects did not ingest the inulin for the same period. Faecal samples from both groups were collected in the 16^{th} day of the experiment. Using real-time PCR analysis, the four most prevalent *Bifidobacterium spp* were *B. longum, B. pseudocatenulatum, B. adolescentis and B. bifidum* and the two later showed significant response to inulin consumption in human volunteers with the proportion increasing from 0.89 to 3.9% to 0.22 to 0.63% of the total microbiota, respectively (Ramirez-Farias et al., 2009). Unfortunately, this favourable change is not consistent, especially in animal model, because feeding inulin also has the potential to decrease membrane intestinal resistance again mobilization of pathogenic bacteria such as *Salmonella* (Ten Bruggencate et al., 2004). Rats fed a restricted diet containing 60g/kg fructo-oligosacharide FOS or inulin orally subjected with gastric gavage with one ml of saline containing 30 g/l sodium bicarbonate and 2×10^9 colony forming units (CFU) of *Salmonella enterica serovar Enteritidis* had higher salmonella colonies than the rats fed control diet with the same level of Salmonella orally (Ten Bruggencate et al., 2004). In this research, both prebiotics also markedly stimulated salmonella translocation

to extraintestinal sites, as indicated by an increased urinary excretion of Nitrogen oxide metabolites. These authors also found that high salmonella colonisation and translocation caused severe infection and decreased animal growth. They suggested that inulin induced SCFA production in the colon but it also may lead to irritation of epithelial mucosa. As a consequence, the mucosa barrier might be disturbed and promote bacteria migration. Further work by this group (Rodenburg et al., 2008) found that the impaired mucosa barrier function of rat colon fed restricted diet containing FOS (a short chain inulin) was due to altered energy metabolism of colonic epithelial cells as indicated by induction of gene expression involved in oxidative phosphorylation and TCA cycle in colonic mucosa.

Inulin is a prebiotic dietary fiber polysaccharide that has been intensively studied as an anti CRC agent. Pool-Zobel concluded the mechanism by which inulin inhibited CRC growth was by reducing exposure to genotoxic carcinogens in the gut, or by reducing their genotoxic effects, inhibition of cancer cell growth, modulation of gene expression, and reduction of metastatic activities of the cancer cells (Pool-Zobel, 2005). It is suggested the compounds that inhibit cancer growth originate from inulin being fermented to produce SCFA, especially butyrate. However, further research (Beyer-Sehlmeyer et al., 2003, Sauer et al., 2007) also indicated that non-SCFA compounds contained in the inulin fermentation supernatant may also be involved in inhibiting colon cancer.

Recently Munjal et al conducted a study further supporting a role for SCFAs in inhibiting colon cancer growth (Munjal et al., 2009). They evaluated

the anticancer effects of an inulin fermentation supernatant fraction in human colon cancer cell lines, LT97 and HT29. Their research indicated that the supernatant, mainly via its SCFA content, was able to inhibit growth and induce apoptosis in the colon cancer cells. Previously, opposite results were found by Beyer-Sehlmeyer et al. (Beyer-Sehlmeyer et al., 2003). In the Beyer-Sehlemeyer et al study the fermentation supernatant from inulin and other fibers were more active than SCFA mixtures or butyrate in inhibiting cell cancer growth. The efficacy of the supernatant suggested that non-butyrate compounds present from the breakdown or fermentation of inulin also have the capacity to increase the anti-proliferative activity of butyrate and prevent chemoresistence in cancer cells. In addition, Sauer et al. (Sauer et al., 2007) found that inulin fermentation supernatant beneficially modulated expression of genes related to xenobiotic metabolism in primary human colon cells, and this efficacy was more pronounce than butyrate's alone.

Considering the data from the *in vivo* animal studies the effects of inulin against colon cancer growth are inconsistent. Pool-Zoobel et al. (Pool-Zobel et al., 2005) and Pool-Zoobel and Sauer (Pool-Zobel and Sauer, 2007) have reviewed the role of inulin on reduction of CRC risk, and they concluded inulin favorably modulates parameters of colon cancer risks in *in vivo* model. However, several other researchers have revealed opposite results in animal model (Mutanen et al., 2000, Pajari et al., 2000, Misikangas et al., 2005, Misikangas et al., 2008). These differences seem to be due to the different animal models used, chemically induced model vs mutated model.

The efficacy of inulin against colon cancer in chemically induced rats has been studied by Reddy et al. (Reddy et al., 1997), Hughes and Rowland (Hughes and Rowland, 2001), and Buddington et al. (Buddington et al., 2002). Inulin significantly reduced total ACF per colon in rats induced with azoxymethane (AOM) (Reddy et al., 1997). Inulin also induced apoptosis in the colon of rats feed dimethylhydrazine (DMH) (20 mg/kg) as indicated by reduction of apoptotic index (AI) in this compartment (Hughes and Rowland, 2001). Based on the AI, inulin was more effective in the distal compared to the proximal colon. To elucidate at which stage of colon cancer development inulin was most effective, Verghese et. al. (Verghese et al., 2002) fed three groups (initiation, promotion, initiation and promotion) of AOM-induced rats with diet containing 10 % inulin at three different periods. From this study, it might be concluded that inulin suppresses tumour formation especially at the promotion stage.

Chemical-induced rat models fed inulin do not always affect all biomarkers of tumorgenesis favourably. Inulin failed to down-regulate β-glucuronidase activity in the colon of AOM-induced rats (Hughes and Rowland, 2001). This enzyme plays a prominent role in genotoxicity of food-derived carcinogenic heterocyclic aromatic amines (Humblot et al., 2007). Even though inulin inhibits crypt multiplicity, it does not suppress the number of multicrypt cluster ≥ 4 of ACF per foci (Reddy et al., 1997). The number of crypts, four or more per foci has been suggested as a consistent predictor of colon tumor incidence (Pereira et al., 1994).

In contrast the ineffectiveness of inulin against ACF formation in the colon of AOM-induced rats has been published by Rao et al.(Rao et al., 1998). Diet containing 10% inulin did not significantly reduce the total ACF in the colon and crypt multiplicity but suppressed the number of ACF/cm^2. Compared with 0.2% piroxicam, a strong inhibitor of colon carcinogenesis in animal models, 10% coffee fiber or 10% pectin, inulin was found to be the least effective (Rao et al., 1998).

A mutagenic animal model of colon cancer (Apc^{min} mice) has been used to evaluate the antitumorigenic activity of inulin and these results contradict those observed using the chemical induced AOM animal model of colon cancer described above (Mutanen et al., 2000, Pajari et al., 2003, Misikangas et al., 2005). Mutanen et al. (Mutanen et al., 2000) found that 2.5 % inulin in the diet did not inhibit tumour formation in Apc^{min} mice. Tumor incidence in mice fed inulin reached 100%, while mice fed control diet or non-fiber diet had 88% and 71% tumour incidence, respectively. These authors also found the mice fed inulin had higher cytosolic β-catenin level than those fed non-fiber diet. β-catenin is involved in Wnt signalling and its activity induces cell proliferation (Akiyama, 2000). Therefore, high levels of cytosolic-β-catenin will upregulate cell proliferation and accelerate tumour growth. To further elucidate whether inulin induces intestinal tumour formation in Apc^{min} mice, Pajari et al. (Pajari et al., 2003) evaluated the β-catenin expression and cellular localization. A high fat diet containing 10% inulin and a high fat diet containing no fiber were fed to Apc^{min} mice from the age of 6 weeks to the age of 9, 12 or 15 weeks. The total adenoma number in the small intestine of

animals fed inulin was higher at week 12 and this gap was not significant at time point of 15 weeks. In both groups, adenoma size in the distal small intestine increased with time, and at weeks 12 and 15 the adenoma size in the inulin group was wider than the non-fiber group. Further analysis indicated that inducing effect of inulin on tumour formation and growth correlated with an increased β-catenin levels and nuclear localization during the tumorigenesis process (Pajari et al., 2003). These results have been confirmed by Misikangas et al using the same model (Misikangas et al., 2005). Inulin depressed β-catenin formation and induced its migration to the nucleus concomitant with cyc D1 accumulation in the membrane and cytosol. β-catenin activated cyc D1 to push the cells mainly into G1 to S phase. Accumulation of cyc D1 in the nucleus enhanced cell proliferation and lead to tumor formation. The level of cell signalling proteins present in Apc^{min} mice fed inulin diet were increased in accordance with the enlargement of the adenoma size (Misikangas et al., 2008).

Recently, inulin has been combined with non-steroidal anti-inflammatory drugs (NSAIDs) and probiotics to evaluate their short-term modulation effect on luminal or mucosal colon of patients with familial adenomatous polyposis (FAP) (Friederich et al., 2011). Probiotic is a viable microbe that has beneficial health affects to the host through its effects in the intestinal tract(Roberfroid, 2000). After four weeks intervention, the authors found that combinations of probiotic/inulin/NSAIDs did not enhance the chemoprevention effect of NSAIDs (sulindac) against adenomas in patients with FAP. Moreover, in a phase II experiment, 6-month intervention with

oligofructose-enriched inulin (as ORAFTI®Synergy1) did not reduce CRC risk on subjects 40 years or older cancer patients (Limburg et al., 2011).

1.4.2. Pectin

Pectin is a polysaccharide composed of D-galacturonic acid units linked by a (1-4) glycosodic bond (Fig. 1.4). Pectin makes up about one third of the cell wall dry substance of higher plants, and it is, mainly, found in the middle lamella of the cell wall, commercially, it is extracted using hot dilute acid at pH about 2 (Sriamornsak, 2003). The gelling characteristics of pectin are determined by the molecular size and degree of esterification (DE) (Thakur et al., 1997). Commonly, pectin from different sources has different properties due to variations in this parameter. In the food industry, pectin is used in jams, jellies, frozen foods, and more recently in low-calorie foods as a fat and/or sugar replacer(Thakur et al., 1997). In the pharmaceutical industry, it is used to reduce blood cholesterol levels and to treat gastrointestinal disorders (Thakur et al., 1997).

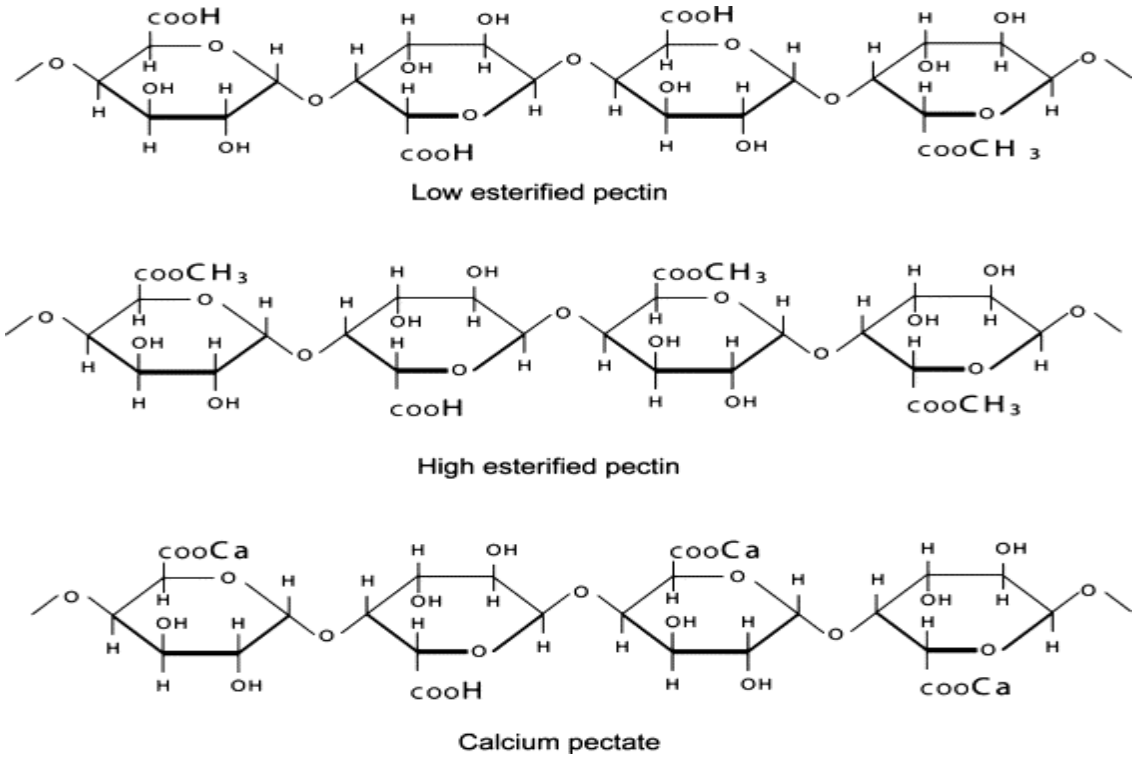

Figure 1-4. Chemical structure of pectin (Khotimchenko et al., 2007)

Several *in vitro* studies demonstrated the beneficial effect of pectin in CRC protection (Henningsson et al., 2002, Waldecker et al., 2008, Chen et al., 2010, Vanamala et al., 2008, Cao et al., 2011). Pectin is fermentable by human faecal bacteria and produces high proportion of acetic acid *in vitro* (Titgemeyer et al., 1991). In combination with guar gum, fermentation of this dietary fiber produced a higher proportion of butyric acid (Henningsson et al., 2002).

A recent study showed the structures of oligosaccharides fractionated from pectin had a significant impact on fermentation by human faecal bacteria, with greatest bifidogenic activity seen with the low-molecular-weight oligoarabinosides and oligogalactosides (Onumpai et al., 2011). Fermentation supernatant from incubation of human fecal slurry with apple pectin was rich in butyrate and it inhibited histonedeacetylase in nuclear

extracts from tumour cell lines (Waldecker et al., 2008). Moreover, faecal water prepared from rehydrated lyophilized faeces of mice fed diet containing pectin offered a better protection against DNA damage compared to faecal water prepared with faeces of mice fed diet containing cellulose (Chen et al., 2010).

Data from animal models does not conclusively support the efficacy of pectin against CRC development. Rats fed citrus pectin had larger caecal total concentrations of SCFA than those fed inulin and lactitol (Nilsson et al., 2006). Diet containing 7% apple pectin increased butyrate producing *Clostridiales* and genes encoding butyryl-coenzymes A Coa transferase, which is involved in butyrate production (Licht et al., 2010). Pectin significantly suppressed the formation of AOM-induced ACF as well as crypt multiplicity and number of ACF/cm^2 in rats fed low fat (5%) diet for 10 weeks (Rao et al., 1998). Diet containing 6% of pectin inhibited the increase in cell proliferation and crypt length in transmissible murine colonic hyperplasia induced by *Cytrobacter rodentium* through blocking the increase in cellular β-catenin, cyc D1 and c-myc levels (Umar et al., 2003). Combination of pectin and fish oil induced colonocyte apoptosis in AOM-alone and irradiated AOM rats through the depression of peroxisome proliferator-activated receptor δ expression and prostaglandin E (2) (PGE2) level, and elevation of PGE3 concentration (Vanamala et al., 2008). These authors also found that at the ACF stage, the zone of proliferation was reduced in fish oil-pectin group compared to the corn oil-cellulose group. Further research indicated that diet containing 20% pectin enhanced Smad3 expression and activation in the gut thus reducing the risk of CRC development (Cao et al., 2011). In contrast,

pectin did not inhibit tumor development in APC^{min} mice (Jacobasch et al., 2008).

Jacobasch et al. fed APC^{min} mice with diet containing 10% high or low methoxyl pectin(Jacobasch et al., 2008). After an 8 week feeding period, these authors found that both pectin induced SCFA production with a high proportion of acetate but low butyrate, induced bile acids secretion, upregulated colorectal development and reduced antioxidant capacity. Pectin also induced β- glucuronidase activities in the faeces of DMH induced rats (Freeman, 1986, Licht et al., 2010). Enzyme β-glucuronidase is a pivotal enzyme in the genotoxicity of a common food-borne carcinogens (Humblot et al., 2007) and a prime factor in the aetiology of colon cancer (Kim and Jin, 2001).

1.4.3. Cellulose

The cell walls of fruit and vegetables generally consist of middle lamella and primary wall layers rich in dietary fiber (Heredia et al., 1995). Middle lamella is composed predominantly by peptic substances whereas the primary wall mostly contains cellulose (Heredia et al., 1995, Heitman et al., 1989, Cameron et al., 1989, Sakamoto et al., 1996). Cellulose is a linear chain of β(1→4) linked glucose monomers (Fig. 1.5), water insoluble and inert to digestive enzymes in the small intestine but it can be fermented to a certain degree in the large intestine (Lattimer and Haub, 2010).

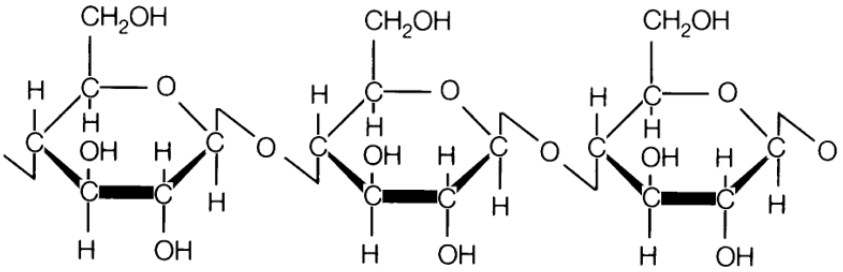

Figure 1-5. Chemical structure of cellulose (Colebrook 2012)

The effect of cellulose on CRC risk has been evaluated in both *in vitro* and *in vivo* models (Barry et al., 1995, Heitman et al., 1989, Cameron et al., 1989, Sakamoto et al., 1996). Some studies on dietary fiber used cellulose as the source of dietary fiber for control group or basal diet (Jacobasch et al., 2008, Cho et al., 2011, Le Leu et al., 2009); therefore less attention is addressed to its effects when discussing the overall experiment results. Generally, the cellulose diet (5.0-15.0%) demonstrates better effects against colon carcinogenesis when it was compared to no added fiber diet (Chen et al., 2010, Nakaji et al., 2004, Heitman et al., 1989, Cameron et al., 1989)

Using a simple *in vitro* batch system inoculated with fresh human faeces, 7.2% cellulose was fermented by faecal microbiota and showed no greater SCFA production than controls (no added dietary fiber) or soluble dietary fiber (Vincent et al., 1990; Barry et al., 1995). Faecal water of BALB/cJ mice fed diet containing cellulose had lower secondary bile acid concentrations and faecal β-glucuronidase activity than faecal water from mice on no dietary fiber diet and was able to inhibit faecal water-induced deoxyribonucleic acid (DNA) damage in Caco-2 cells (Chen et al., 2010).

Earlier studies using an animal model of colon carcinogenesis demonstrated that cellulose posed beneficial effects against cancer

development (Heitman et al., 1989, Cameron et al., 1989, Sakamoto et al., 1996). The effects of different levels of dietary cellulose on colonic crypt mitotic activity and colon carcinogenesis were studied by Heitman et al. (1989) and showed that addition of 5 or 15% cellulose suppressed DMH-enhanced mitotic activity in the colon crypts during the initiation and promotion stages of carcinogenesis as indicated by a significant reduction of colon adenocarcinomas in rats. Similar results were found by Cameron et al. (1989) where rats fed a diet containing cellulose (5 and 15%) were injected with DMH and had a significant suppression in the crypt mitotic activity compared to no added fiber. Other studies showed that total cancer volume per rat fed diet containing 10% cellulose was significantly lower than the rats fed basal diet or 10% resistant starch diet in DMH-induced colonic carcinogenesis rats model even though the resistant starch diet increased butyrate concentration (Sakamoto et al., 1996). Guar gum enhanced colon tumour formation in DMH-induced rats compared to control (no added dietary fiber) and this enhancement was suppressed when two-thirds of the guar gum in the diet was replaced with cellulose (Okazaki et al., 2002). Cellulose (15%) decreased tumour induction rates in DMH induced rats fed diet containing 5, 10, 15% lard suggesting the preventive effect of cellulose against large bowel tumorigenesis was greater than the tumour promoting effect of fat (Nakaji et al., 2004).

1.5. Antioxidants and Colorectal cancer development

Antioxidants are enzymes or other organic molecules that can protect from damage caused by free radical molecules. Numerous dietary nutrients

such as vitamin C (ascorbic acid), vitamin E, carotenoids and non-nutrients phytochemicals such as polyphenol compounds have antioxidant properties (Pan et al., 2011). Fruit and vegetables are rich in these compounds therefore it is believed that these compounds have prominent role in the efficacy of fruit and vegetables in inhibiting some diseases (Araujo et al., 2011, Millen et al., 2007, Hermsdorff et al., 2012). *In vitro* and *in vivo* research exists to support the preventive effect of antioxidant compounds against colon cancer but epidemiology studies in humans give inconsistent results (An et al., 2011, Ju et al., 2010, Nagendraprabhu and Sudhandiran, 2011, Wang et al., 2012, Roncucci et al., 1993).

1.5.1. Ascorbic acid

Ascorbic acid (1-3 mM) decreased RKO and SW480 colon cancer cell proliferation and induced apoptosis and necrosis, and this was accompanied by downregulation of specific protein factors (Pathi et al., 2011). Pathi et al also found ascorbic acid decreased expression of several Sp-regulated genes that are involved in cancer cell proliferation and survival, as well as the expression of epidermal growth factor receptor gene and cyc D1 gene, and had an effect on angiogenesis. In HT 16 cells, vitamin C significantly increased apoptosis through upregulation of p53 compared to untreated cells (An et al., 2011). Previously, DMH-induced rats fed 0.25 or 1% sodium ascorbate had lower incidence of colon cancer than control (Reddy et al., 1982).

1.5.2. Vitamin E

Vitamin E consists of tocopherol (α-, β-, γ-, and δ-tocopherols) and tocotrienol (α-, β-, γ-, and δ-tocotrienols) where tocotrienols have better anticancer properties than tocopherols (Ling et al., 2012, Smolarek and Suh, 2011). Within the tocopherol group, δ-tocopherol showed better prevention against the development of ACF in AOM-treated rats (Yang and Suh, 2012). Earlier research indicated that a δ-tocopherol-rich mixture of tocopherols effectively inhibited colon carcinogenesis in AOM-induced mice through apoptotic induction, anti-inflammatory effects, as well as antioxidative and reactive nitrogen species-trapping activities (Ju et al., 2010).

1.5.3. Carotenoids

Carotenoids are all compounds with the key structural elements of polyene chain connected to rings and functional groups that provide bright coloration in plants and animals (Sliwka and Partali, 2012). Various carotenoids have anti-cancer effects in some types of cancer *in vitro* but these effects are not supported by human clinical trials (Tanaka et al., 2012). Carotenoids, including α- and β-carotene and canthaxanthin, significantly suppressed cell viability, DNA synthesis and cell proliferation and thus showed growth-inhibitory effects on two human colon cancer cell lines (DLD-1 and Colo 320DM) (Onogi et al., 1998). Lycopene, a major component in tomato, exhibited potential anti-carcinogenic activity through suppression of protein kinase B (also known as Akt) activation and non-phosphorylated β-catenin protein levels in HT-29 cells (Tang et al., 2008). Akt activation and β-catenin regulate cell proliferation and survival, cell growth (size), glucose

metabolism, cell motility and angiogenesis (Testa and Tsichlis, 2005; Taurin et al., 2006). Quercetin (flavonoid) or β-carotene supplementation reduced the number of ACF in AOM-induced rats fed high-fat diet, the expression of inducible nitric oxide synthases (iNOS) was reduced by quercetin and cyclooxygenase-2 (COX-2) expression was slightly reduced by ß-carotene supplementation (Choi et al., 2006). Astaxanthin another carotenoid exhibited an anti-inflammatory and anti-cancer effects by inducing apoptosis in DMH-induced rat colon carcinogenesis (Nagendraprabhu and Sudhandiran, 2011).

1.5.4. Polyphenols

Polyphenols are a group of phytochemicals mostly found in plant-based foods (Tsao, 2010). Polyphenols have anticancer activity through many mechanisms including modulation of signal transduction pathways, inhibition of cell growth and transformation, induction of apoptosis, reduction in invasiveness and angiogenesis (Lambert et al., 2005). In colon cancer cell lines, dietary polyphenols inhibit cell proliferation, induce differentiation and apoptosis, inhibit angiogenesis and metastasis, and exhibit antioxidant properties (Araujo et al., 2011, Lea et al., 2008). Previous research showed that polyphenol-rich fractions from raspberry extract after the digestion process, have the ability to protect HT29 colon cancer cells from DNA damage induced by hydrogen peroxide, decrease the number HT29 cells entering the cell cycle, and inhibit HT115 colon cancer cell invasion (Coates et al., 2007). In the DMH-induced rat model, phenolic acid rich extract from Adlay (*Coix lachryma-jobi L. var. ma-yuen Stapf*) exhibited anti preneoplastic mucin-depleted foci (MDF) formation through suppressing chronic

inflammation (Chung et al., 2010). In the same rat model, adenoma colon formation was also inhibited when the rats were fed diet containing polyphenol extracts from red wine (Femia et al., 2005).

The potential protective role that antioxidants (vitamin antioxidants or polyphenols) may provide against CRC is not supported by all epidemiological evidence. The Fukuoka CRC Study found that the beneficial effects of carotenes, vitamin C, and vitamin E against CRC risk were not supported in men or women (Wang et al., 2012). A systematic review failed to demonstrate the protective effect of antioxidants because the data showed that vitamin A, C and E, selenium and β-carotene (as single or combination with other antioxidants) were not effective in the chemoprevention of colorectal neoplasia in the general population (Papaioannou et al., 2011). Moreover, other studies demonstrated that both vitamin antioxidants from food but also from supplements were not associated with a reduced CRC risk (Park et al., 2009). Even though polyphenols exhibited anticancer effects in a rat model (Femia et al., 2005), a prospective study indicated moderate consumption of red wine rich in polyphenols did not reduce CRC risk (Chao et al., 2010). Intake of a combination of all antioxidants measured as the total antioxidant capacity intake did not indicate any protection effect against CRC (Mekary et al., 2010). In contrast, several studies conducted in the 1990s showed beneficial effects of antioxidant compounds against colon cancer (Longnecker et al., 1992, Roncucci et al., 1993, Paganelli et al., 1992). It may be that recently dietary intake of high risk CRC diets such as the western diet have spread globally in comparison to diets of the 90s era. This unhealthy

diet cause a decreased overall antioxidant status of the community and a general increase in consumption of red meat and fast or more processed food rich in bad fats (Lesgards et al., 2002). As a consequence, consumption of antioxidants by the general community may be enough to induce beneficial health effects.

The beneficial effects of antioxidant compounds (vitamin and polyphenol compounds) in some *in vitro* and *in vivo* studies mentioned above is in agreement with the results of epidemiology studies on protective effect of fruit and vegetables against CRC (Magalhaes et al., 2012). Discrepancy between the antioxidant effect and the fruit and vegetables effect on CRC as mentioned above may suggest that only when antioxidant compounds are endogenously found in fruit and vegetables do they produce anti-cancer properties. Overall anti-colon cancer activity of fruit and vegetables may be a result of synergetic effect from antioxidant compounds and other compounds. Previously, it has been found that dietary fiber exhibited synergetic effects with antioxidant compounds especially phenolic compounds in disease protection (Pozuelo et al., 2012, Jimenez et al., 2008, Juskiewicz et al., 2011a, Palafox-Carlos et al., 2011). Therefore, it is possible that combinations of antioxidants and dietary fiber will exert pronounced protections against CRC.

1.6. Green cincau (*Premna oblongifolia* Merr.)

Green cincau (*Premna oblongifolia* Merr) is a tropical plant belonging to the Verbenaceae family (Fig. 1.6). The leaves of this plant are

commonly extracted with water to prepare a traditional drink containing polysaccharide forming gel. The extract has high pectin content (19.71%) and antioxidant activity as well as interesting characteristics (high water holding capacity and viscosity), suggesting possible uses in the development of fibre enriched foods (Nurdin et al., 2005).

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Figure 1-6. Green cincau plant (*Premna oblongifolia* Merr.)

There are limited publications on this plant, but preliminary research on the leaf extract indicates promising results(Nurdin et al., 2003). Hot water extract of the leaf (25-50 ppm) induced interferon-γ, interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) in mouse spleen cells, indicating the extract has the ability to induce cell-mediated immune responses *in vitro* (Nurdin et al., 2003). Diet containing green cincau extract produced more faeces in rats than diet containing inulin(Nurdin, 2007b). This author also found rats fed green cincau extract have faeces consistency softer compared to rat faeces of rats on diet containing cellulose but harder than with diet containing inulin, implying that the extract exhibits a laxative effect in the rats. The extract also

effectively induced the growth of lactic acid bacteria in the colon compared to cellulose (Nurdin, 2007b).

1.7. Study Hypothesis

SCFA in the colon can be absorbed via non-ionic diffusion or SCFA/anion exchange process and the rate of SCFA uptake is determined by luminal pH, whereas lowering luminal pH induces SCFA absorption. Combining dietary fibers will exert synergetic effects on SCFA pattern in fermentation supernatant (FS) prepared from incubation of dietary fibre combinations with human faecal flora and offer beneficial effects on proliferation, differentiation, and apoptosis of Caco-2 cell. Different dietary fiber sources will cause different effects on phenolic metabolism and bioactivities on AOM-induced aberrant crypt foci (ACF) formation in rat model of colorectal cancer.

1.8. Aim of my PhD

Epidemiological studies indicate dietary fiber and antioxidants may protect against CRC. However, not all dietary fiber or antioxidant sources show protection. Furthermore, there is limited evidence of the effects of combinations of specific dietary fibers and antioxidants on CRC. There is a possibility that combinations of different fibers and antioxidants will exert a synergistic effect. Dietary fiber is thought to suppress CRC growth via the production of SCFA, which are specific compounds produced in the colon via bacterial breakdown of the fiber. The major SCFA are acetate, propionate and butyrate, of which butyrate is thought to have the greatest effect in protecting against cancer. The efficacy of SCFA against CRC is affected by

intraluminal factors including colon pH, therefore, the first aim of the current study (Chapter 2) was to elucidate the effect of pH as a measure of intraluminal factors pH on modulation of Caco-2 cells proliferation, differentiation, and apoptosis induced by SCFA.

Different dietary fibers depending on how they are broken down (fermented) by the colonic bacteria will produce variable amounts of butyrate. In nature, dietary fiber exists in mixtures such as dietary fiber in fruit and vegetables. It is suggested that dietary fiber mixtures will exhibit more pronounced effect than single dietary fiber. Dried green cincau extract contains 52.0 % total dietary fiber and around 20.0 % of it is pectin. It would be possible that dietary fiber in cincau extract is a mixture of dietary fiber. Therefore, the second aim of my research (Chapter 3) was to study the effect of dietary fiber combinations, including dried cincau extracts, on SCFA pattern of fermentation supernatant prepared from incubation of dietary fiber combinations with human faecal flora and then, evaluate the effect of the fermentation supernatants on the proliferation, differentiation, and apoptosis of Caco-2 cell line.

There is some evidence that supports the beneficial effect of fruit and vegetables as protection against CRC. The combination of dietary fiber and antioxidant contained in fruit and vegetables may exert the protective effects. Green cincau extract contains both dietary fiber and antioxidant compounds. As *in vitro* research on the preventive effects of green cincau extracts against CRC indicated promising results (Chapter 3), therefore, in the third results chapter (Chapter 4), I aimed to evaluate the effect of combinations of dietary

fibre and antioxidant sources on AOM -induced ACF formation in a rat model of CRC. I fed different combinations of dietary fiber and antioxidant sources including dried cincau extracts so as to maximize the beneficial effect in the colon and examined the resultant effects in an animal model of colon cancer.

2. SCFA MODULATION OF PROLIFERATION, DIFFERENTIATION AND APOPTOSIS DID NOT DEPEND ON THE pH

2.1. Abstract

The main end products of dietary fiber breakdown are short-chain fatty acids (SCFA), mainly acetate, propionate, and butyrate. SCFA are thought to play a role in maintaining normal bowel function and may contribute to protection against gastrointestinal diseases. The SCFA profile and colon microenvironment may be an important factor in determining the degree of protection and is directly related to the amount of dietary fiber and/or undigested starch that is subjected to the microbial degradation in the large bowel. The objectives of this research were to investigate the effect of SCFA mixtures on the proliferation and differentiation of Caco-2 cells cultured in a range of pH levels. The SCFA were prepared as two different mixtures (Mixture 1 contained 18.8 mM SCFA at the ratio of 69:15:16; Mixture 2 contained 50.8 mM SCFA at the ratio 75:14:11; for acetate, propionate, and butyrate, respectively), these were compared with single SCFA butyrate (5, 10, and 20 mM), propionate (40 mM) and acetate (40 mM). SCFA significantly inhibited proliferation while inducing differentiation of Caco-2 cells irrespective of the media pH. Caspase 3 and 7 (key mediators apoptosis in the extrinsic and intrinsic apoptotic pathway) activities were affected by both pH and SCFA, but there was no interaction between them. Caspase 3 and 7 were decreased by increasing pH, but the effect of SCFA on these caspases was depended on the SCFA types. Butyrate induced apoptosis as indicated by increased caspase 3/7 activity resulting in growth inhibition

caused by mitochondrial dysfunction as well as induced cell death associated with membrane damage. In conclusion the effect of SCFA on cell proliferation, differentiation and apoptosis depends on the SCFA pattern. Butyrate induced cell death through both caspase3/7-dependent and -independent pathways.

2.2. Introduction

Colorectal cancer (CRC) is one of the most common cancers that occurs in the Western world. Although there is a genetic component to this disease, the majority of cases occur sporadically with external factors such as diet and nutrition playing key roles (Watson and Collins, 2011). Some food derivatives produced in gastrointestinal tract have been shown to induce or inhibit genetic damage or cancer development at different stages (Berlau et al., 2004). For example, metabolism of nutrients or non-nutritive compounds in the colon by indigenous bacteria leads to the formation of a large number of compounds that may have beneficial or adverse effects on human health (Blaut and Clavel, 2007) .

Compounds such as short chain fatty acids (SCFA), which are produced via bacterial fermentation of undigested dietary fibers are capable of inhibiting cancer (Pool-Zobel and Sauer, 2007, Sauer et al., 2007). Butyrate, one such SCFA, is the principal energy source for colonocytes and appears to offer the greatest protection against CRC (Pool-Zobel et al., 2005). Butyrate has been show to inhibit cell proliferation, induce cell differentiation as well as induce apoptosis in colorectal cancer cell lines

including Caco-2 (Orchel et al., 2005) and HT-29 cell lines (Barnard and Warwick, 1993, Domokos et al., 2010).

SCFA can be absorbed by colonocytes and acts via an unknown mechanism to inhibit histone deacetylase activity and consequently expression of genes associated with colorectal cancer (Kamitani et al., 2001, Marks et al., 2001). The rate of SCFA uptake determines how effective these compounds are in inhibiting growth of colorectal cancer cells (Kautenburger et al., 2005). Therefore, factors affecting SCFA absorption into the colon will help to determine their efficacy against CRC. SCFA in the colon can be absorbed via nonionic diffusion or SCFA/anion exchange process (Reynolds et al., 1993, Charney et al., 1998, Tyagi et al., 2002) but nonionic diffusion is the main transport mechanism (Charney et al., 1998). SCFA are in a dissociated form at physiological pH conditions and they are protonated when the pH is decreased (Cardone et al., 2005). As a consequence, lowering luminal pH induces SCFA absorption (Herrmann et al., 2011). However, the induction of absorption of SCFA are not observed with lower (< 5.5) pH (Stein et al., 2000). Butyrate absorption was significantly higher in an acidic environment (pH 5.5) compared to an alkaline environment (pH 7.5) (Reynolds et al., 1993).

Colon pH is variable and is affected by several factors including diet (Lupton and Kurtz, 1993) and disease (Nugent et al., 2001). In normal patients , colon pH also depends on the colon region, and it can range between 6.3 in right (proximal) colon to 7.4 in left (distal) colon (Evans et al., 1988). Moreover, cancer cell naturally tend to produce and secrete more acid as a consequence of rapid growth, resulting in a acidic extracellular

microenvironment (Gillies et al., 2002). This abnormality has been observed during the earliest step of cancer development (Cardone et al., 2005). Therefore, the objectives of the current study were (1) to elucidate the efficacy of SCFA against colorectal cancer over a range of pH, and (2) to investigate the effect of SCFA on the proliferation, differentiation, and apoptosis in the CRC cell line.

2.3. Material and Methods

2.3.1. Cell culture

Human colorectal carcinoma cells Caco-2 (passage 33-40) were obtained from the American Type Culture Collection (ATCC Number CCL-247). Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St Louis, MO) supplemented with 10% (v/v) foetal bovine serum (Bovogen, Victoria, Australia), 1% nonessential amino acid (Sigma-Aldrich), and 100 U/ml penicillin-streptomycin (Sigma-Aldrich) at 37^{o}C under 5% CO_2. For experiments, the pH of the supplemented media were adjusted using 0.1N HCl or 0.1N NaOH to obtain pH levels of 6.0, 6.5, 7.0, and 7.5 (before adjusting, pH media was approximately 7.00)

2.3.2. SCFA stock solutions in pH adjusted media

SCFA stock solutions were prepared using the method described by Kiefer et al., (2006). Na-Butyrate, Na-acetate, and Na-propionate (Sigma-Aldrich) were diluted in supplemented DMEM. SCFA mixtures (treatment solutions) were made as two types of mixtures whereas the molar ratios was based on the molar ratio of fermentation supernatant of dietary fiber with

fresh human faeces reported by Kiefer et al. (2006): mixture 1 (Mix 1) contains 18.8 mM SCFA in the ratio of 69:16:15 and mixture 2 (Mix 2) contains 50.8 mM SCFA in the ratio 75:11:14 for acetate, butyrate, and propionate, respectively. The single SCFA experiments utilised butyrate at levels of 5 mM (But5), 10 mM (But10), and 20 mM (But20), propionate (40 mM) and acetate (40 mM) (Table 2.1) prepared by diluting the SCFA stock solution (100-500 mM) in media. The stock solutions were stored until use at -20^{o}C.

Table 2-1. Final concentration of SCFA in media

Treatment	Final concentration (mM)			
	Acetate	Butyrate	Propionate	Total
Mix 1	12.97	3.01	2.82	18.80
Mix 2	38.10	5.59	7.11	50.80
But5		5.00		5.00
But10		10.00		10.00
But 20		20.00		20.00
Prop			40.00	40.00
Acet	40.00			40.00
Ctrl				0.00

2.3.3. Proliferation assay

Caco-2 cells were cultured in 96-well plates (Costar®, Corning incorporated, NY, USA)) at a density of 1.0 x 10^5 cells/mL in pH adjusted media, and then incubated for 24h to allow cell adherence prior to SCFA treatment. After SCFA treatment, the cells were incubated for 48h, the treatment media was discarded and 100 uL of 0.5 mg/mL 3-4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich) solution was added to each well. The cells were then incubated (37^{o}C, 5% CO_2) for 1h (so MTT could be metabolized). The supernatant was discarded and the formazan (MTT metabolic product) was resuspended in 80 µl 20% SDS in 0.02 M HCl and the plate was incubated in the dark for 1 h. The

optical density (OD) was read at 570 nm and background absorbance at 630 nm (FLUOstar omega, BMG labtech). Optical densities were converted to a total number of live cells using a linear regression plot. For each experiment a control plate was prepared containing 5000-80000 cells/well ranges and OD was measured and plots created (Young et al., 2005).Results were expressed as the number of live cells in wells containing treatment compared with the number of cells in control wells (medium alone).

2.3.4. Alkaline phosphatase (AP) activity assay

For AP assay, 3 mL of 1.0 x 10^5 cells/mL were cultured in the supplemented media for 24h. The media was removed and replaced with pH adjusted media (pH 6.0 and pH 7.5) containing SCFA Mix 2, 20 mM butyrate (B20), 40 mM propionate (P), 40 mM acetate (A), respectively. After 48h incubation, the cells were trypsinized and suspended in 50 mM Tris-HCl buffer, pH 10.0, and homogenized by sonication (Sonicator®, Ultrasonic Processor XL) for 2 x 10 second. To remove cell debris, homogenized cells were centrifuged at 3000 rpm for 10 min and the supernatant was used for the assay.

AP activity was measured by hydrolysis of p-nitro phenol phosphate (Sigma-Aldrich) (5 mM) and expressed in units (the number of µmol p-nitrophenol liberated in 1 min measured at 400 nm per mg protein). p-Nitrophenol (Sigma-Aldrich) (0-200 µM) was used to generate a standard curve (Beyer-Sehlmeyer et al., 2003).

2.3.5. Caspase 3-7 and lactate dehydrogenase (LDH) assay

Caco-2 cells (150 µL) were cultured in 96-well white plates (Costar®), at a density of 1 x 10^5 cells/mL in pH adjusted media (pH 6.0 and 7.5). The cells were incubated for 24h to allow the cell to adhere prior to treatment with 50 µL of the SCFA solution. After SCFA treatment, the cells were incubated for 48h.

To elucidate the role of caspase 3 and 7, key mediators apoptosis in the extrinsic and intrinsic apoptotic pathway(Lakhani et al., 2006), in cell death, the most effective SCFA treatment in induction of caspase 3/7 production was applied to the Caco 2 cells in combination with a caspase inhibitor. Caspase inhibitor was used to inhibit apoptotic process due to SCFA treatment and Staurosporine 5µM (Sigma-Aldrich) was used as a positive control to induce apoptosis. Briefly, Caco-2 cells (150 µL) were cultured as described above for 24h. Caspase-3/7 inhibitor (Ac-DEVD-CHO, Promega) (10µM) was added 1 h preceding SCFA treatment. Quantification of caspase-3 and -7 activities were carried out using the Caspase-GloR 3/7 assay kit (Promega, USA). In parallel, the CytoTox-ONE™ Homogeneous Membrane Integrity assay kit (Promega, USA) was employed to quantify the Lactate dehydrogenase (LDH) enzyme activity. These treatments were also applied to separate Caco 2 cells cultured in 96 wells plate for determination of cell proliferation using MTT assay.

2.3.6. Statistical analysis

All experiments were performed in triplicate with three replicates (r = 3). Results are expressed as the mean ±SEM. Statistical analysis was

carried out using the statistical program SPSS version 19. One way-Anova with LSD test was used. Results were considered significant if $p < 0.05$ (see Table 6.1.-6.13).

2.4. Results

2.4.1. Effect of SCFA and pH on Caco-2 cell proliferation

There was no interaction between pH and SCFA in inhibiting Caco-2 cell proliferation (Table 2.2, $p<0.05$). Caco-2 cells had more cell proliferation when the media pH was normal (pH 7.0) compared to when the media pH was lowered (pH 6.0 and 6.5) (Fig. 2.1A-D). SCFA significantly inhibited Caco-2 cell proliferation irrespective of the media pH (5.7×10^5 cells/mL control media vs. 3.1×10^5 cells/mL SCFA media)($p<0.05$). No significant differences in inhibition were observed when the Caco-2 cells were cultured in media containing SCFA Mix 2, 10 and 20 mM butyrate or 40 mM propionate, respectively, at all pH levels (Fig.2.1 A-D, $p<0.05$). Mix 2 that contained 5.59 mM and 7.11mM of butyrate and propionate, respectively (Table 2.1), exhibits anti-proliferation activity similar with But10, But20 and Pro those have higher level of butyrate or propionate. High concentration of SCFA Mix 2 or single (But10 and But20) were more effective than the lower levels, Mix 1 and But5 respectively. Cell proliferation in cells treated with Mix 1 and But 5 was not significantly different even though Mix 1 has lower butyrate concentration than But5 (3.01 mM and 5.00 mM, respectively).

Table 2-2. P* values of one way ANOVA test for effect of SCFA and pH on cell proliferation, differentiation and apoptosis.

	Treatments		
Parameters	SCFA	pH	SCFA x pH
Cell proliferation	0.00*	0.02*	0.25
Cell differentiation	0.03*	0.89	0.92
Apoptosis (Caspase3/7 activity)	0.00*	0.00*	0.83

*Data is considered as significant different when $p < 0.05$.

In the next part of the study only the high concentrations of SCFA Mix 2, But20, Prop and Acet were applied to the Caco 2 cells cultured in media at pH 6.0 and 7.5. This decision was made because the proliferation data indicated that the ability of SCFA (mixture or single) to inhibit cell growth was concentration dependent.

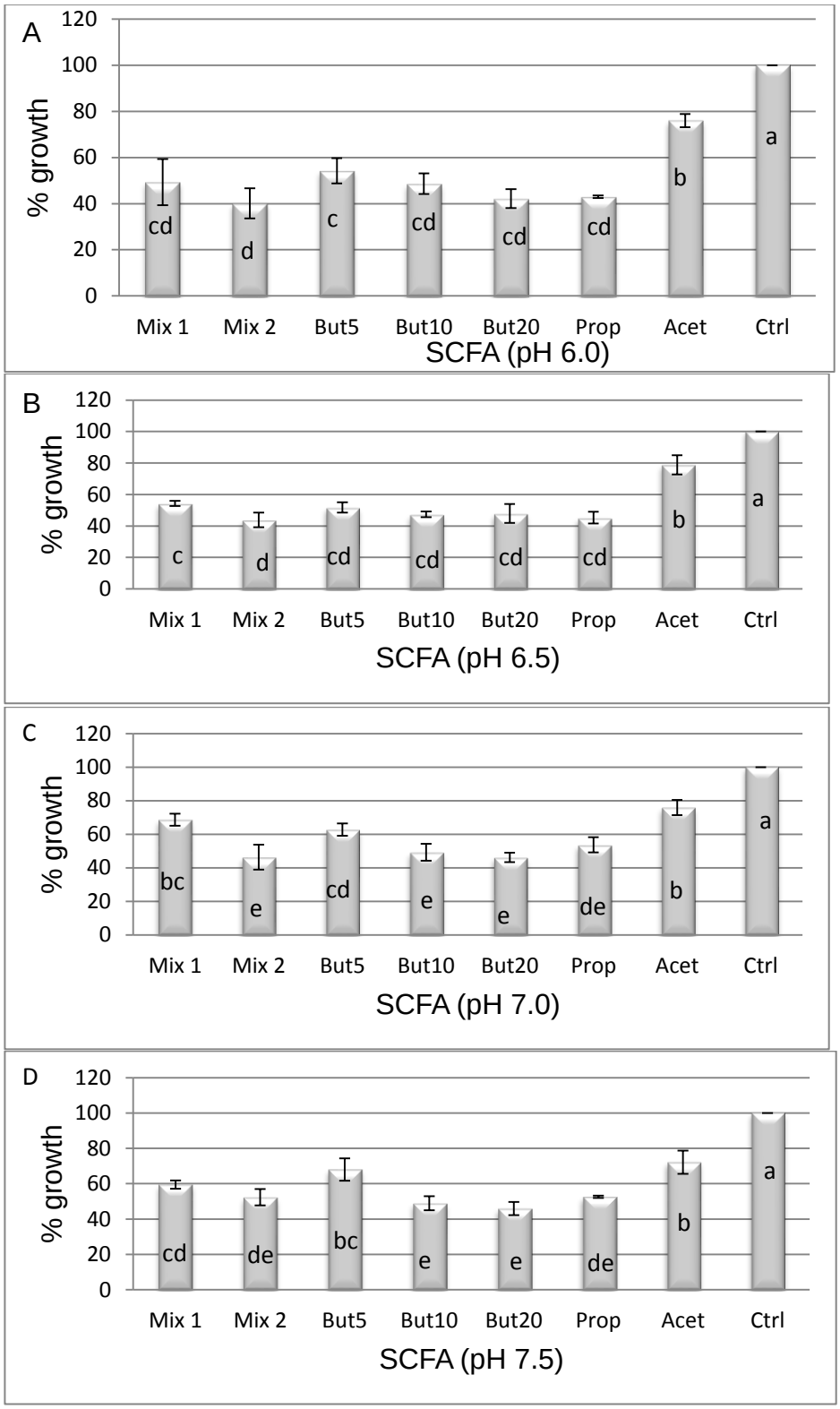

Figure 2-1. Effect of SCFA on Caco-2 cell proliferation in media (A) pH 6.0, (B) pH 6.5, (C) pH 7.0 and (D) pH 7.5.

Cells were seeded 1 day before the SCFA treatment (day 0), then incubated for 48 h in media containing SCFA in pH 6.0, 6.5, 7.0 or 7.5. Cell proliferation was measured using the MTT assay and expressed as % growth against control of each media. Mix 1 contained 18.8mM SCFA at the ratio of 69:15:16 and Mix 2 contained 50.8mM SCFA at the ratio 75:14:11; for acetate, propionate, and butyrate respectively But5, But10 and But20 were 5, 10, and 20 mM butyrate respectively; Prop was 40 mM propionate; Acet was 40 mM acetate; Ctrl was control (media without SCFA). The bars represent the mean, and the lines are SEM of three independent experiments each performed in triplicates. Data points denoted by different superscripts (letters on the bar) differ significantly with $p < 0.05$.

2.4.2. Effect of SCFA and pH on Caco-2 cell differentiation

Cell differentiation was measured based on alkaline phosphatase (AP) activities. Activity of this enzyme increases during cell differentiation (Matsumoto et al., 1990). AP activity was significantly stimulated by SCFA, the stimulation was not pH dependent and there was no interaction between them (Table 2.2, $p<0.05$). Mix 2, But20 and Prop exhibited a stronger effect than Acet, whilst the acetate and Ctrl achieved similar levels of stimulation (Fig. 2.2 A and B, $p<0.05$).

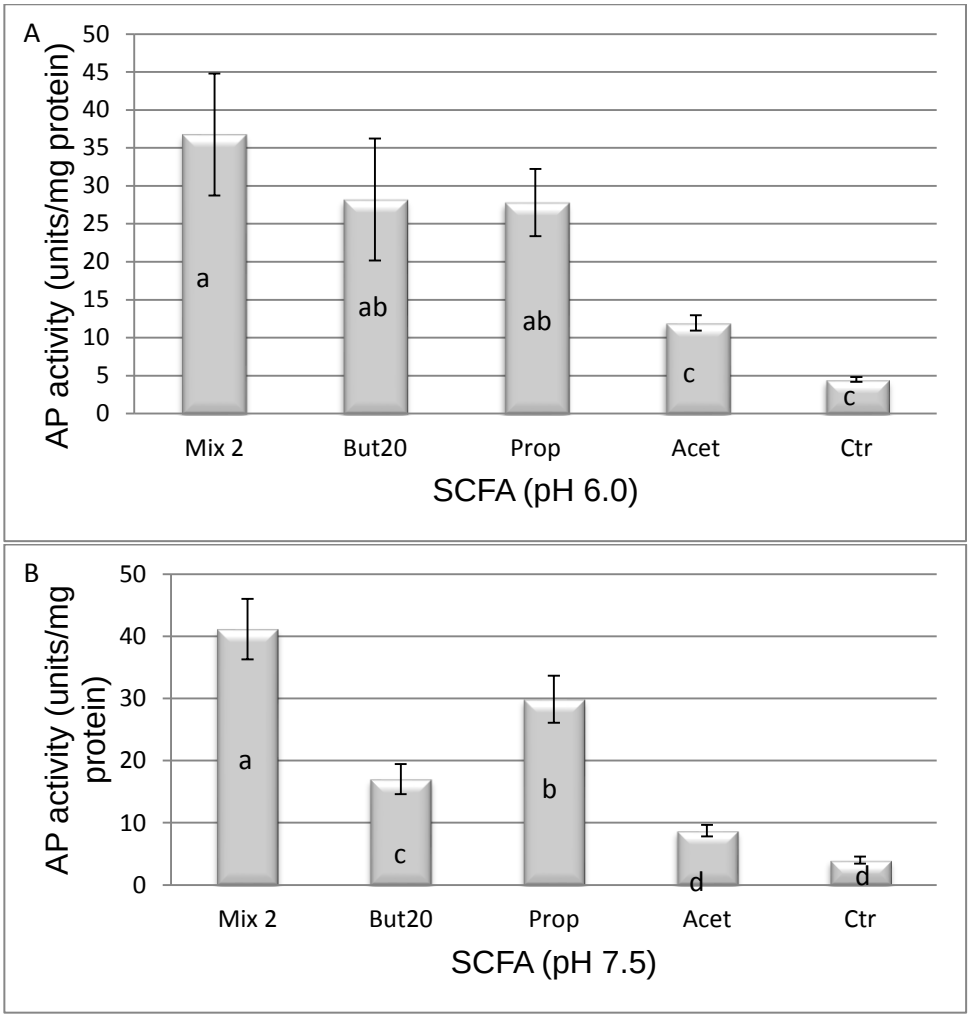

Figure 2-2. Effects of SCFA on alkaline phosphatase (AP) activity of Caco-2 cells cultured at pH 6.0 (A) and pH 7.5 (B) media.

Cells were seeded in 6 well plates for 24 h, then incubated for 48 h in media containing SCFA at different pH. AP activity was measured by hydrolysis of p-nitrophenolphosphate (5mM) and expressed in units (the number of µmol p-nitrophenol liberated in 1 min measured at 400 nm per mg protein); Mix 2 contained 50.8mM SCFA at the ratio 75:14:11; for acetate, propionate, and butyrate, respectively; But20 was 20 mM butyrate; Prop was 40 mM propionate; Acet was 40mM acetate; Ctrl was control (media without SCFA). The bars represent the mean, and the lines are SEM of three independent experiments each performed in triplicate. Data points denoted by different superscripts (letters on the bar) differ significantly with $p < 0.05$.

2.4.3. Effect of SCFA and pH on Caspase 3/7 activity

Caspase 3 and 7 mediate apoptosis in the extrinsic and intrinsic apoptotic pathways (Lakhani et al., 2006, Lamkanfi and Kanneganti, 2010). Activities of these caspases were affected by both pH and SCFA, but there is no interaction between them (Table 2.2, $p < 0.05$). Caspase 3-7 were more active in pH 6.0 compared to pH 7.5 (Fig. 2.3 A and B; $p<0.05$). Butyrate (20mM) increased caspase activity higher than the activity induced by B and Prop. Caspase 3/7 activity induction was not observed when the Caco 2 cells were treated with 40 mM acetate ($P<0.05$).

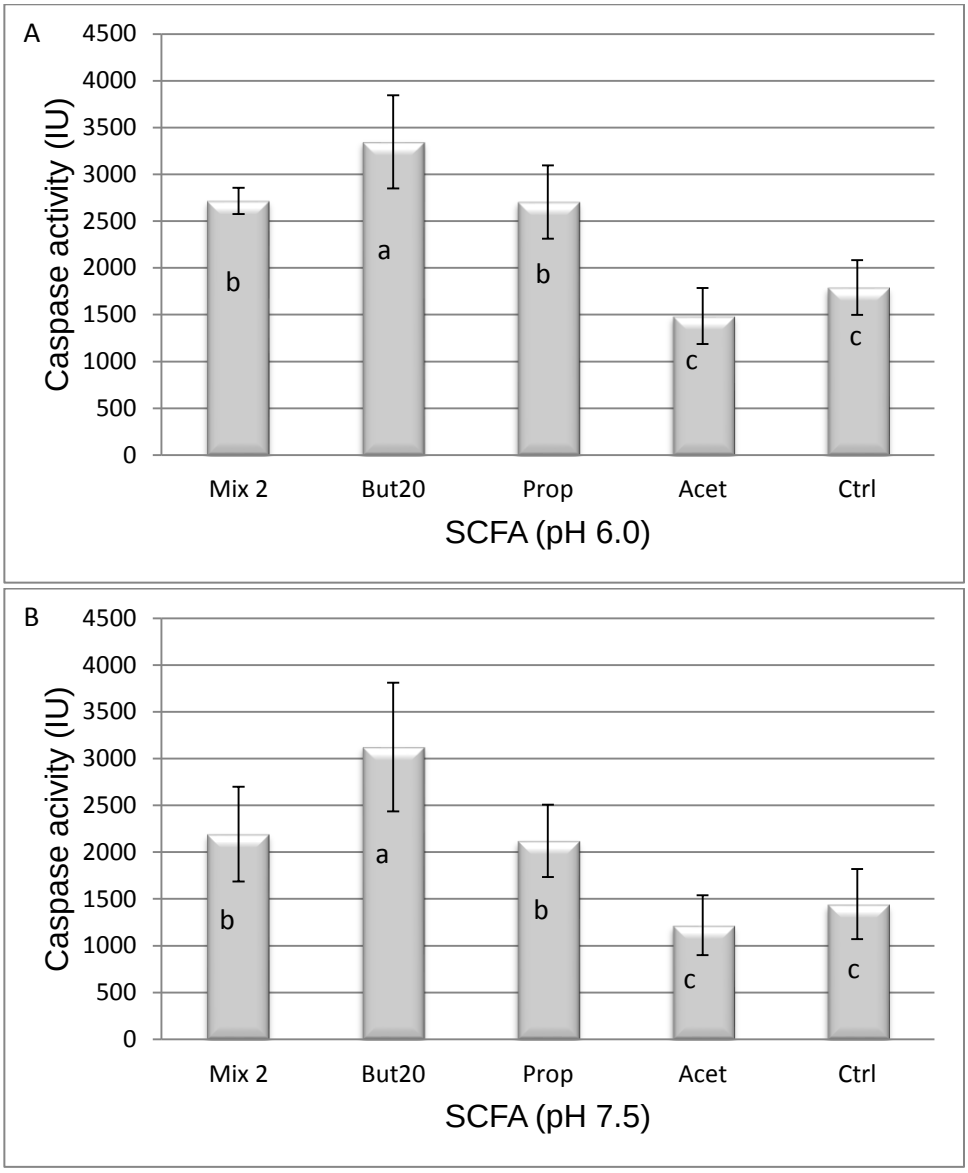

Figure 2-3. Effects of SCFA on caspase 3/7 activity of Caco-2 cells cultured in pH 6.0 (A) and pH 7.5 (B) media.

Cells were seeded 1 (24 h) day before the treatment with SCFA (day 0), then incubated for 48 h in media containing SCFA in pH 6.0 (A) or 7.5 (B). Caspase-3 and -7 activities were measured using the Caspase-GloR 3/7 assay kit (Promega, MI, USA). Mix 2 contained 50.8mM SCFA at the ratio 75:14:11; for acetate, propionate, and butyrate, But20 was 20 mM butyrate; Prop was 40 mM propionate; Acet was 40 mM acetate; Ctrl was control (media without SCFA). The bars represent the mean, and the lines are SEM of three independent experiments each performed in triplicates. Data points

denoted by different superscripts (letters on the bar) differ significantly with $p < 0.05$.

2.4.4. Role of apoptosis mechanism in cell death induced by SCFA.

I chose two assay methods to measure the level of cell death: lactate dehydrogenase (LDH) release and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) reduction assay were used to study the role of apoptosis mechanism in cell death induced by SCFA. As the effect of SCFA on caspase 3/7 activity was not depends on the media pH, the cell death mechanism was studied on at one pH level (pH 6.0). Caspase-3/7 acivity were more active in pH 6.0 than in pH 7.5 (Table 2.2 and 6.6). In butyrate treated cells, caspase 3/7 was significantly activated, but it decreased below the level of control sample when the caspase inhibitor was applied (Fig. 2.4A; $p<0.05$). However, the level of cell death assessed by the MTT method was not significantly different between these groups (Fig 2.4C, $p<0.05$) indicating cell death was occurring even though the apoptotic process was inhibited. Cell membrane damage in 20 mM butyrate (But20) tended to be higher than in caspase inhibitor+20 mM butyrate (IBut20) as indicated by their LDH release (Fig. 2.4B, $p<0.05$), suggested that cell death mechanism induced by But20 through apoptosis as well as necrosis pathway.

Staurosporine (5 µM) (Stau), an initiator of apoptosis in many different cell types, induced caspase 3/7 activity, and as But20, incubation with caspase inhibitor 1 hour preceding its addition (IStau) suppressed this effect. Percentage of cell growth or LDH release in the Stau and IStau treated cells confirmed that apoptosis was not the single pathway by which Staurosporine induced cell death (Fig. 2.4B and C, $p<0.05$). Cell membrane and mitochondrial damage occurred due to staurosporine treatment.

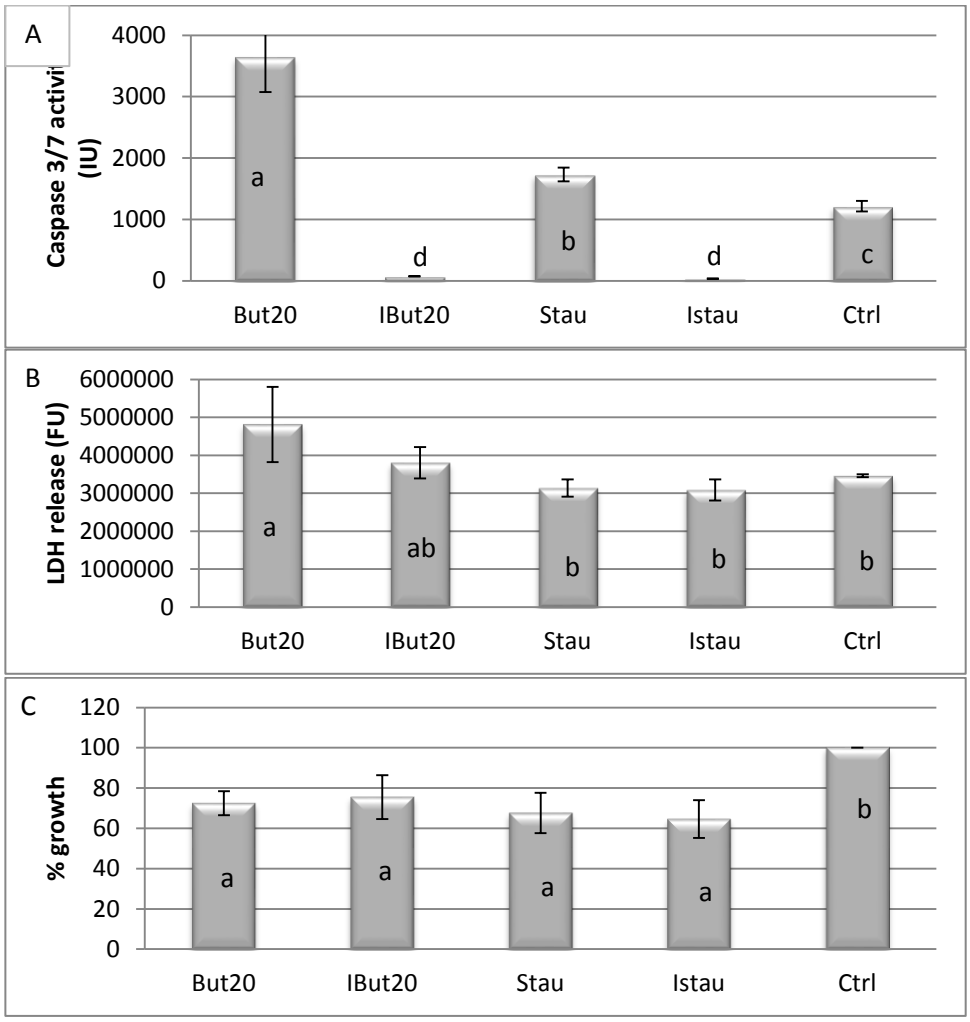

Figure 2-4. Effects of butyrate on the caspase 3/7 (A), LDH (B) activity and Caco 2 cell proliferation (C) with or without caspase inhibitor.

Cells were seeded 1 day before the SCFA treatment (day 0), then incubated for 48 h in media containing 20 mM butyrate in pH 6.0. Caspase inhibitor (10µM) (Ac-DEVD-CHO) was added 1 hour preceding SCFA treatment. Caspase 3-7 activity was measured using the Caspase-GloR 3/7 assay kit (Promega, USA), the LDH activity was quantified using the CytoTox-ONE™ Homogeneous Membrane Integrity assay kit (Promega, USA) and cell proliferation was measured using the MTT assay and expressed as % growth against control (media without SCFA). But20 was 20 mM butyrate; IBut20 was caspase inhibitor+20 mM butyrate; Stau was 5 µM staurosporin; IStau was caspase inhibitor+5 µM staurosporin; Ctrl was control (media without

SCFA). The bars represent the mean, and the lines are SEM of three independent experiments each performed in triplicates. Data points denoted by different superscripts (letters on the bar) differ significantly with $p < 0.05$.

2.5. Discussion

Inhibition of proliferation, induction of differentiation and apoptosis are the suggested mechanisms by which SCFA inhibits colorectal cancer growth (Fung et al., 2012). The type of SCFA and the level of their uptake may play an important role in their efficacy against colon cancer (Charney et al., 1998, Lan et al., 2007). SCFA are weak acids where their dissociation level is affected by the environmental pH. As the transport mechanism in the colon depends on ionic/nonionic state, it was suggested that the effect of SCFA against colon cancer development depends on the pH colon environment. However, my results indicated that there was no interaction between pH and SCFA in inhibition of the growth of cultured colon cancer cells, induction of differentiation or apoptosis. SCFA mixtures mimicking fermentation supernatant of dietary fiber with fresh human faeces (Mix 2), Butyrate 20 mM (But 20) and propionate 40 mM (Prop) significantly suppressed Caco 2 cell growth and differentiation irrespective of media pH. Effect of SCFA was determined by the SCFA type and concentration, but it was not affected by the pH of the media.

High pH (pH 7.0 and 7.5) was more suitable for Caco 2 cell growth than low pH (pH 6.0 and pH 6.5) (Fig. 2.1A-D; $p<0.05$). Previous results indicated that reducing pH (from 7.5 to 6.5) decreased cell proliferation in some mammalian cell lines (HeLa and Chang liver cells) (Mackenzie et al., 1961). When hypotetraploid spontaneously transformed cell lines were incubated at pH 6.4 instead of 7.2 after 6 hours only about 40% of these cells were still viable (Overgaard, 1976). In HT29 cells, low pH media (pH

5.0) resulted in reduced cell proliferation, which correlated with ultra-structural features suggestive of a more differentiated phenotype (Fitzgerald et al., 1997). Therefore, it is suggested that normal pH (pH 7.0) is the optimal pH for Caco 2 cell activities including cell proliferation even the presence of SCFA addition (Table 6.2).

Previous research indicated that SCFA have an anti-proliferative effect on colorectal cancer cell lines (Hinnebusch et al., 2002) and that each acid has slightly different effects (Basson et al., 1998, Hinnebusch et al., 2002). Acetate has a less potent anti-proliferative effect than butyrate and propionate as reported by Fu et al. where Caco-2 cells had shorter doubling time when they were cultured on media supplemented with 10 mM acetate compared with butyrate or propionate at the same level (Fu et al., 2004)l. My research supported this finding, that even at higher concentrations than previously examined (40 mM), acetate had less pronounced effects than butyrate (in concentration of 5, 10 and 20 mM) in inhibition of cell proliferation (Fig 2.1). Differences between SCFA Mix 1 and 40 mM of acetate were also observed, indicating that butyrate as a single source or within a mixed SCFA, is more favourable than acetate for cancer prevention.

The differentiation marker gene, intestinal alkaline phosphatase (IAP), was upregulated by 10 mM of butyrate and propionate through induction of histone hyperacetylation but not by 10 mM (Hinnebusch et al., 2002) or 40 mM acetate (Kiefer et al., 2006). In contrast, Whitehead et al. (Whitehead et al., 1986) found increased AP activity upon the addition of acetate (1.0 and 10.0 mM), propionate (1.0 mM) and most markedly by butyrate at a concentration of 1.0 mM. My results show that all SCFA increase alkaline

phosphatase activity with highest values for SCFA Mix 2, butyrate 20 mM and propionate and a much less for acetate. The inconsistency between my results and those of others may be due to differences in the cell line tested. In my research the Caco-2 cell line was used, while Hinnebusch *et al.* (Hinnebusch et al., 2002) and Kiefer *et al* (Kiefer et al., 2006)**.** used HT-29 cell lines, and Whitehead *et al.* (Whitehead et al., 1986) used a LIM1215 cell line.

The ability of SCFA (mixture B, butyrate 20 mM and propionate 40 mM) to induce apoptosis was confirmed by my findings demonstrating the induction effect of these compounds on caspase 3/7 activity (Fig. 3B; $P<0.05$). Matthews et al. have reported that butyrate on its own and in combination with propionate induced apoptosis and G2-M arrest to a greater extent than propionate alone in the Caco 2 cell line (Matthews et al., 2012). My results supported the efficacy of butyrate as an apoptosis inducer where this compound induced higher levels of caspase 3/7 activity than SCFA Mix 2, propionate or acetate. Previously, fluorescence-activated cell sorting (FACS) analysis also revealed that only butyrate increased apoptosis compared with untreated control cells, whereas other short chain fatty acids including acetate, propionate, valerate and caproate did not affect the level of apoptosis (Hinnebusch et al., 2002).

Synergetic effect between butyrate, propionate and acetate in cell proliferation inhibition was observed in my study where Mix 1 and Mix 2, which respectively have lower butyrate concentration than But5 and But10, demonstrated similar inhibition, demonstrated similar inhibition. This synergetic association was also detected between butyrate, propionate or

acetate as indicated by their effect on cell differentiation and apoptosis. It has been reported previously that butyrate synergistically modulated histone acetylation with propionate when these two SCFA were added into substrate of HT 29 cell lines (Kiefer et al., 2006).

To elucidate the role of caspase 3/7 in cell death induced by SCFA, caspase inhibitor was added 1h preceding the SCFA treatment. The caspase 3/7, LDH release and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) reduction were measured after 48h incubation. The LDH assay is an accurate method to assay cell death with membrane damage such as necrotic cell death, meanwhile MTT is suitable for cell death with mitochondrial dysfunction without membrane damage (Kim et al., 2009).

My results indicated that butyrate at 20 mM induced cell death through both caspase3/7-dependent and -independent pathways. 20 mM butyrate induced apoptosis as indicated by increasing of caspase 3/7 activity (Fig. 2.4A, $p<0.05$)) resulting in growth inhibition caused by mitochondrial dysfunction (Fig 2.4C, $p<0.05$). Moreover, 20 mM butyrate also induced cell death associated with membrane damage (Fig. 2.4B, $p<0.05$). Inhibition of Caspase 3/7 activity by a caspase inhibitor did not affect cell growth inhibition by 20 mM butyrate indicating cell death mechanism by which 20 mM butyrate inhibited cell growth was caspase-independent. Alternatively, the cell death mechanism induced by 20 mM butyrate appears to be dependent on the addition of a caspase inhibitor; cells died through an apoptotic pathway only when 20 mM butyrate was applied, but when caspase inhibitor was added, a switch in the cell death pathway occurred, it may be to a necrotic (Prabhakaran et al., 2004) or autophagy (Shao et al., 2004, Yu et al., 2006).

Considering that a non-significant reduction in LDH release occurred following pre-incubation of But20 with a caspase inhibitor (Fig 2.4B), it is possible that the inhibition of caspase 3/7 switched butyrate to operate via the autopaghic pathway. This suggestion is supported by staurosporin data. Staurosporine induced caspase 3/7 activity (Fig. 4A, $p<0.05$) and caused mitochondrial dysfunction (Fig. 2.4C, $p<0.05$). However, inhibition of caspase 3/7 activity induced by staurosporin did not increase the cell growth or upregulate LDH release demonstrating that a caspase inhibitor can switch apoptosis to an autophagic cell death mechanism. These results are supported by other work indicating that butyrate was able to induce cell death through apoptotic, necrotic, or apoptotic pathway (Shao et al., 2004, Domokos et al., 2010). Moreover it has been found that inhibition of the caspase cascade with zVAD-fmk switched the apoptotic response to necrotic cell death in cortical cell lines (Prabhakaran et al., 2004) and activation of caspase 3/7 can also upregulate autophagic cell death such as in HeLa cell lines (Rikiishi, 2011)

2.6. Conclusion

SCFA effect on inhibition of cell growth or induction of differentiation and apoptosis in Caco 2 cells was not depend on the pH media. Cell growth was better at the pH 7.0 than the lower pH level of pH 6.0 or 6.5. Butyrate has synergetic effect with propionate or acetate on inhibition of cell proliferation and induction of cell differentiation. Butyrate induced cell death through both caspase3/7-dependent and -independent pathways. Addition of a caspase inhibitor preceding butyrate treatment switched the cell death

mechanism, from apoptotic to autophagic cell death pathway. Dietary fibre that promote SCFA pattern may provide protection against colorectal cancer development.

3. EXAMINING THE EFFECTS OF COMBINATIONS OF NON-DIGESTIBLE CARBOHYDRATE SOURCES ON SHORT CHAIN FATTY ACID PRODUCTION USING AN *in vitro* FECAL FERMENTATION SYSTEM AND CACO-2 CELLS AS A MODEL OF THE HUMAN COLON

3.1. Abstract

Colorectal cancer (CRC) is a leading cause of cancer worldwide. Diet high in dietary fiber has been suggested as protective modifiers. Fiber is comprised principally of non-starch polysaccharides, which depending on the type of fiber, is subjected to varying degrees of fermentation by the resident bacterial ecosystem in the gastrointestinal tract. Short chain fatty acids (SCFA) are one of the compounds produced by fermentation of dietary fiber. Research has shown that mixtures of dietary fibre types may be more beneficial than a single dietary fiber type. The aim of this study was to investigate the effect of different dietary fiber types and their combination (pectin, inulin, cellulose and green cincau) on SCFA production using anaerobic batch cultures inoculated with human faecal slurries. Green cincau (*Premna oblongifolia* Merr) is an Indonesia plant where the extract has high dietary fiber content. Furthermore, fermentation supernatants (FS) were evaluated in Caco-2 cells (treated with FS for 48h) for effect on cell proliferation, differentiation, and apoptosis. SCFA production was dependent on the type of dietary fiber. Soluble fiber (pectin and inulin) resulted in significantly higher SCFA production than observed with insoluble (cellulose) fiber. Compared with inulin alone, the combination of inulin and cellulose reduced SCFA production including the amount of butyrate. Combinations of pectin and inulin resulted in the highest production of butyrate, although the proportion of butyrate was much lower than observed with inulin alone (14.4 and 41.4 %, respectively). In Caco-2 cells, inhibition of cell growth was

dependent on the amount of SCFA generated (in particular butyrate), with the inulin as the fiber source resulting in a 53% inhibition in cell growth. However, their effect on cell differentiation and apoptosis were not explainable by their butyrate content. Apoptotic, necrotic and autophagic pathways might all be involved in cell death in response of FS treatment. In conclusion, soluble dietary fibre and cincau extract had a great influence on SCFA production in the fermentation supernatant, and the ability of the supernatant to modulate parameters of cell growth, differentiation and apoptosis was dependent on butyrate concentration and unidentified compounds

3.2. Introduction

Compounds such as short chain fatty acids (SCFA), which are produced via bacterial fermentation of undigested dietary fibers are capable of inhibiting cancer(Pool-Zobel and Sauer, 2007, Gingras and Beliveau, 2011). Butyrate, one such SCFA is the principal energy source for colonocytes and appears to offer the greatest protection against colorectal cancer (CRC) as it promotes a normal colonocyte phenotype, enhances cell apoptosis and differentiation and inhibits proliferation in CRC cells *in vitro* (Munjal et al., 2009). The type of dietary fiber consumed influences the proportion and distribution of SCFA in the gastrointestinal tract (Juskiewicz and Zdunczyk, 2004, Pompei et al., 2008, Nilsson et al., 2006). Fast fermentable dietary fiber is fermented in the proximal colon and results in increased SCFA levels in this region of the gut, conversely, slow fermentable dietary fiber will reach the distal colon and modulate SCFA production at this site.(Juskiewicz and Zdunczyk, 2004, Pluske et al., 1998).

Anti CRC activity of inulin-type fructan has been intensively studied (Pool-Zobel, 2005, Pool-Zobel and Sauer, 2007). Inulin supplementation has been shown

to reduce number of tumours per animal and aberrant crypt foci (ACF) in AOM-induced F344 male rats (Reddy et al., 1997, Verghese et al., 2002). Dietary inulin appears to be protective in the early and late stages of carcinogenesis and tends to more effective in the distal colon than proximal colon (Buddington et al., 2002), suggesting that it reaches this part of the colon in order to modulate microbial activity (Hughes and Rowland, 2001). In combination with lycopene and probiotics, inulin induced apoptosis, inhibited cell proliferation and ACF formation in the colon of DMH-induced rats (Dias et al., 2010). Inulin intake reduced CRC levels in rats fed high lipid diet or chemical-induced CRC through decreasing faecal enzyme activity and bile acid concentration (Wu and Chen, 2011, Bertkova et al., 2010).

Pectin is a dietary fiber that makes up about 30% of the plant cell wall and is fermentable by human faecal bacteria (Rao et al., 1998, Sriamornsak, 2003). This fiber produces high proportions of acetic acid *in vitro* and *in vivo* (Rao et al., 1998). Rats fed pectin produced more SCFA than those fed inulin and lactitol (Nilsson et al., 2006). Moreover, pectin significantly suppressed the formation of AOM-induced ACF as well as crypt multiplicity and the number ACM/colon cm^2 in rats fed a low fat (5%) diet (Rao et al., 1998). Research has shown diets containing 6% pectin inhibit increases in cell proliferation and crypt length in transmissible murine colonic hyperplasia induced by *Cytrobacter rodentium* via blocking increases in cellular β-catenin, cyclin D1 and c-myc levels (Umar et al., 2003). Another study found the fermentation supernatant from incubation of human faecal slurry with apple pectin was rich in butyrate and inhibited histone deacetylase in nucleus extracted from tumour cell lines (Waldecker et al., 2008), therefore has the ability to induce apoptosis (Anh et al., 2012).

Many researchers have shown mixtures of two dietary fiber types are more beneficial than a single dietary fiber (Juskiewicz and Zdunczyk, 2004, Henningsson et al., 2002, Muir et al., 2004b, Khan and Edwards, 2005). Rats administered diets containing guar gum or pectin produced low proportions of butyrate in comparison to rats fed mixtures of both (Henningsson et al., 2002). Compared with control or wheat bran diets alone, diets containing a combination of wheat bran and resistant starch produced higher wet and dry faecal output, a lower faecal pH and faecal ammonia, as well as lower levels of faecal phenol (Muir et al., 2004a). Other studies have also shown that a combination of inulin and guar gum results in total SCFA higher than individual guar gum, but no significant difference was observed in total SCFA yielded by inulin alone (Khan and Edwards, 2005). These authors also found, after 24 h, the SCFA production rate from fermentation of individual inulin or guar gum decreases, but when these fibers were combined, the production rate kept increasing indicating that a combination of fiber sources may be more beneficial.

In the current study, I tested a traditional fiber source (Cincau) from Indonesia that has high pectin content for its ability to inhibit colon carcinogenesis. Extracts of the green leaves of the cincau (*Premna oblongifolia Merr.*) contains about 20% pectin (Nurdin et al., 2005). Traditionally, the leaves are extracted with hot water to prepare a refreshing drink, or are consumed as a traditional medicine for inflammation or fever. Research on the extract indicates that it has ability to induce cell-mediated immune responses *in vitro* (Nurdin et al., 2003). As a dietary fiber, the extract has a laxative effect and it effectively induced the growth of lactic acid bacteria in the colon (Nurdin, 2007a).

There appears to be an abundance of data suggesting combining dietary fibers will exert synergetic effects and offer promising effects for colon health. In

accordance, the aim of this research was to study the effect of dietary fiber combinations on SCFA pattern in fermentation supernatant (FS) prepared from incubation of dietary fibre combinations with human faecal flora and to evaluate the FS from the fiber on proliferation, differentiation, and apoptosis of Caco-2 cell.

3.3. Material and Methods

3.3.1. *In vitro* fermentation of dietary fiber.

Seven substrates as a single or a mixture of two dietary fibers (50:50) were tested: pectin (P), inulin (I), cellulose (C), pectin-cellulose mixture (PC), inulin-cellulose (IC), pectin-inulin (PI) and green cincau extract (Cin) (Table 3.1).

Table 3-1. Dietary fiber/dietary fiber mixture and their ratio

Dietary fiber	Abbreviation	Ratio (%)	Dietary fiber	Abbreviation	Ratio (%)
Pectin	P	100	Pectin+cellulose	PC	50:50
Inulin	I	100	Pectin+inulin	PI	50:50
Cellulose	C	100	Inulin+cellulose	IC	50:50
Cincau extract	Cinc	100	Fecal blank	FB	-

Briefly, 150 mg of dietary fiber was placed in a 15 ml capped tube, then 9 ml fermentation media was added (media contained 0.25% (w/v) Tryptone, 125 ppm (v/v) micro-mineral solution, 25 % (v/v) carbonate buffer solution, 25 % (v/v) macro-mineral solution, 0.1.25 ppm (w/v) of resazurine solution, and 3.35 % (v/v) reducing solution) (composition of the solution used in the dietary fiber fermentation can be seen Table 6.71-6.74). The pH of the media containing dietary fiber was adjusted to pH 7.0. For inoculums, fresh faecal slurry from healthy volunteers was pooled and

diluted in phosphate buffer to produce 10% (w/v) inoculums. Final concentration of inoculums was 1% (w/v) after mixing of 1 ml of 10% inoculums with 9 ml of media containing dietary fiber (DF). A negative control containing only fermentation media and faecal inoculum was prepared as a faecal blank (FB). All of the process was carried out in anaerobic chamber with rocking (SL Bacton IV anaerobic chamber) to maintain anaerobic conditions set at 37 ^{o}C for 24 h. Supernatants were sterilized by filtration (pore size 0.22 µm) (Minisart®, Sartorius) and stored at -80^{o} C until use.

3.3.2. Green Cincau Leave materials

Green cincau leaves (*Premna oblongifolia* Merr.) were collected from traditional farmers in Indonesia. The fresh leaves were dried in an oven at 50^{o}C (water content around 12%), ground into fine powder and imported into Australia using AQIS permit (IP07024278). Extracts were obtained by extracting of 5% dried cincau leaf powder using hot water (Nurdin et al., 2005) followed by freeze drying (Dynavac). Dried cincau extracts were ground. Table 3.2 shows the dried cincau extract composition as determined by CSIRO analytical test (Adelaide, South Australia).

Table 3-2. Composition of dried green cincau extract

	Concentration (%)
Moisture	4.4
Fat	4.4
Protein	13.3
Ash	12.2
Starch	1.8
Resistant starch	0.5
Soluble fiber	5.8
Insoluble fiber	46.3
Total fiber	52.1

3.3.3. Cell Culture

Human colorectal carcinoma cells Caco-2 were obtained from the American Type Culture Collection (ATCC Number CCL-247). Passages 76-85 of Caco 2 cells were used in this study. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Bovogen, Victoria, Australia), 100 U/ml penicillin-streptomycin (Sigma-Aldrich), and 1% nonessential amino acids (Sigma-Aldrich) in CO_2 incubator (37 ^{o}C and 5% CO2).

3.3.4. SCFA analysis

FS samples were homogenized in 3 volumes of internal standard solution (heptanoic acid, 1.68 mmol/L) (Sigma-Aldrich) and centrifuged at 3000 x *g* for 10 min. The supernatant was then distilled and 0.3 µL injected into a gas

chromatograph (HewlettPackard 5890 Series II A) equipped with a flame ionization detector and a capillary column (Zebron ZB-FFAP, 30 m x 0.53 mm i.d, 1-µm film, SGE). Helium was used as the carrier gas; the initial oven temperature was 120°C and was increased at 30°C/min to 190°C; the injector temperature was 210°C and the detector temperature was 210°C. A standard SCFA mixture containing acetate, propionate, and butyrate (Sigma-Aldrich) was used for calculations and the results are expressed as µmol/g of sample (Le Leu et al., 2005).

3.3.5. Proliferation assay.

Caco-2 cells were cultured in 96-well plates (Costar®, Corning incorporated, NY, USA) at a density of 1.0 x 10^5 cells/mL one day before treatment with FS (day 0), then incubated for 48 h in media containing 20% of FS. For standard curves, 1:2 serial dilutions were prepared to generate a standard curve of 5000-80000 cells per well, in final volume of 100 µl (Young et al., 2005).

After 48h treatment media was removed and 100 uL/well of medium containing 0.5 mg/mL 3-4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich) solution was added to each well and incubated (37°C, 5% CO 2) for 1h (to allow MTT to be metabolized).The formazan (MTT metabolic product) was resuspended in 80 µl 20% SDS (Amresco, Ohio, USA) in 0.02 M HCl (Sigma-Aldrich) and the plate was incubated in the dark for 1 h at room temperature. The optical density was read at 570 nm with back ground absorbance at 630 nm (FLUOstar omega, BMG labtech). Optical densities were converted to a total number of live cells using a linear regression plot. Results were expressed as the number of live cells in wells containing treatment compared with the number of cells in control wells (medium alone).

3.3.6. Alkaline Phosphatase (AP) activity assay

For the AP assay, 1.0 x 10^5 cells/mL were cultured in the supplemented media (DMEM) (Sigma-Aldrich) containing 10% FCS, 1% NEEA, and 100 U/mL penicillin-streptomicyn and 20 mM HEPES (Sigma-Aldrich)) for 24h. The medium was removed and replaced with media containing 20% of FS. After 48h incubation, the cells were trypsinized and resuspended in 50 mM Tris-HCl buffer, pH 10.0, and homogenized by sonification. The homogenized cells were centrifuged at 100,000 rpm for 30 min to remove cell debris.

AP activity was measured by hydrolysis of p-nitro phenol phosphate (5 mM) (Sigma-Aldrich) and expressed in units (the number of µmol p-nitrophenol liberated in 1 min measured at 400 nm per mg protein). p-nitrophenol (0-200 µM) was used to generate a standard curve (Beyer-Sehlmeyer et al., 2003).

3.3.7. Caspase 3-7 and LDH Assay

Caco-2 cells (150 µL) were cultured in 96-well white plates (Costar®) at a density of 1 x 10^5 cells/mL. The cells were Incubated 24 h to allow the cells to adhere prior to treatment with 20% FS. After FS treatment, the cells were incubated for 48h. Staurosporine 5µM (Sigma) was used as a positive control to induce apoptosis. Quantification of caspase-3/7 activities were carried out using the Caspase-GloR 3/7 assay kit (Promega, USA). In parallel, the CytoTox-ONE™ Homogeneous Membrane Integrity assay kit (Promega, USA) was employed to quantify the lactate dehydrogenase (LDH) enzyme activity, where 30 µl of samples was added with 70µ CytoTox-ONE™ Reagent and shake for 30 seconds, then incubated for 10 minutes. Add 35 µl of stop solution to each well, and record fluorescence with an excitation wavelength of 560nm and an emission wavelength of

590nm. The FS treatments were also applied to separate Caco 2 cells cultured in 96 wells plate for determination of cell proliferation using MTT assay.

To confirm the role of caspase 3/7 in the cell death mechanism, the inulin, Cincau extract, and faecal blank FS were applied to the Caco 2 cells in combination with a caspase inhibitor (10 µM). Cells were cultured in 96-well plates at as outlined above and caspase inhibitor (Ac-DEVD-CHO, Promega, WI, USA) was added 1 h preceding FS treatment.

3.3.8. Statistical analysis

All experiments were performed with three replicates in triplicate each time and the results are expressed as the mean ± standard error of mean (SEM). Statistical analysis was carried out with a statistical program SPSS version 19. One way-Anova with LSD test was used. Results were considered significant if $p < 0.05$ (see Table 6.14-6.31)

3.4. Results

3.4.1. SCFA content of dietary fiber fermentation supernatant

Fermentation of all dietary fibers individually or in combinations except for cellulose alone increased the yields of total SCFA in the FS in comparison to the FB ($p<0.05$) (Fig. 3.1A). The major products were acetate, propionate and butyrate, which were increased after incubation with dietary fibers (Fig 1C-D).

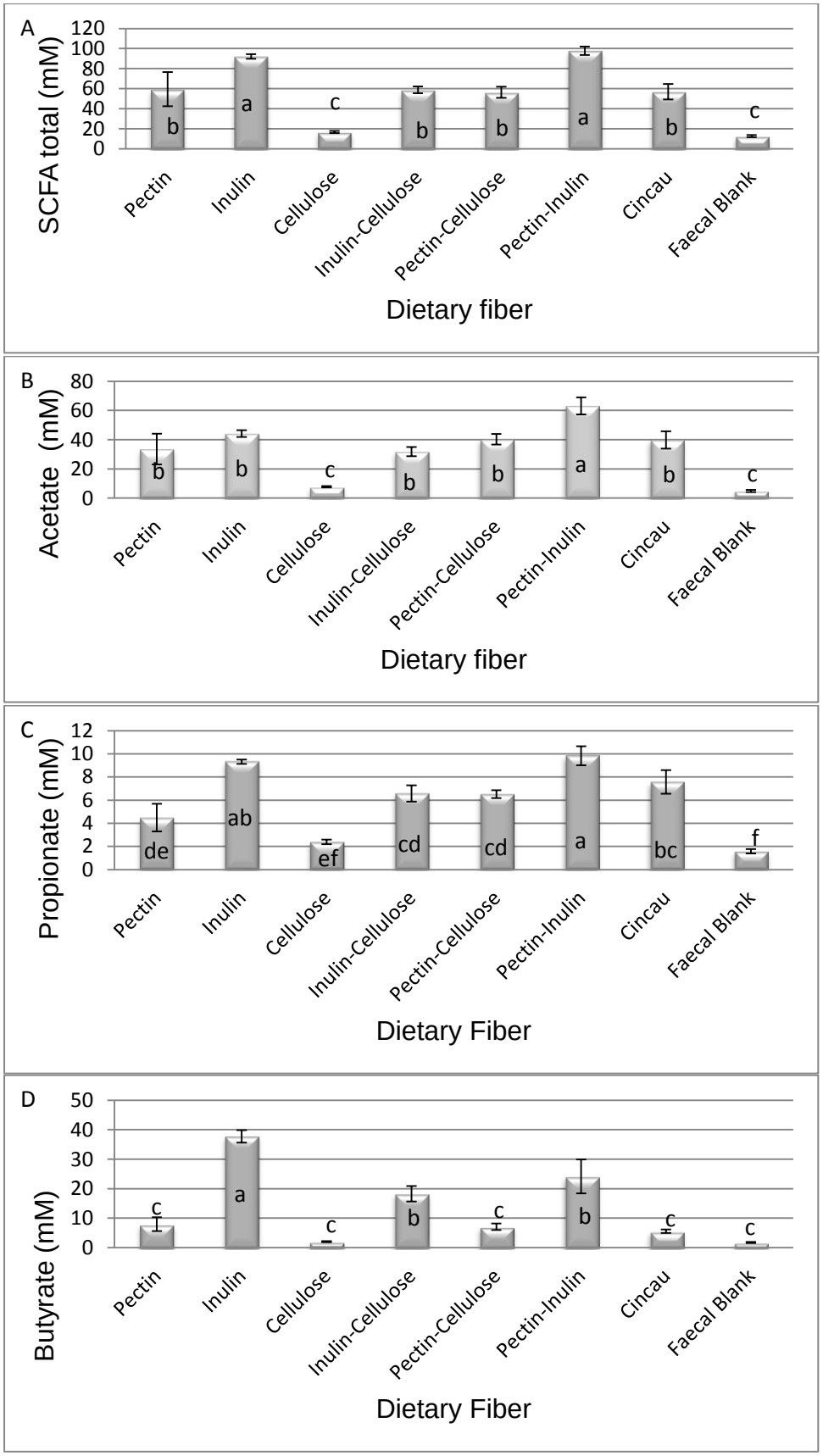

Figure 3-1. Effect of dietary fiber on the concentration of SCFA total (A), acetate (B), propionate (C), and butyrate of fermentation supernatants.

Dietary fibers (5%) were fermented with human faecal bacteria at 37^{o}C for 24 h in anaerobic conditions. The bars represent the mean, and the lines are SEM of four replicates. Data points denoted by different superscripts (letters on the bar) differ significantly when $p < 0.05$.

3.4.2. Effect of dietary fiber FS on Caco-2 cell proliferation.

Treatment of Caco 2 cells with FS affected cell proliferation. The cell number was significantly reduced after incubation of Caco 2 cells with 20% of FS compared to FB (Fig. 3.2, $p<0.05$). Incubation with inulin FS inhibited cell growth the most in compared with FB. Combination of inulin with pectin in the FS did not reduce the efficacy of inulin to inhibit cell growth.

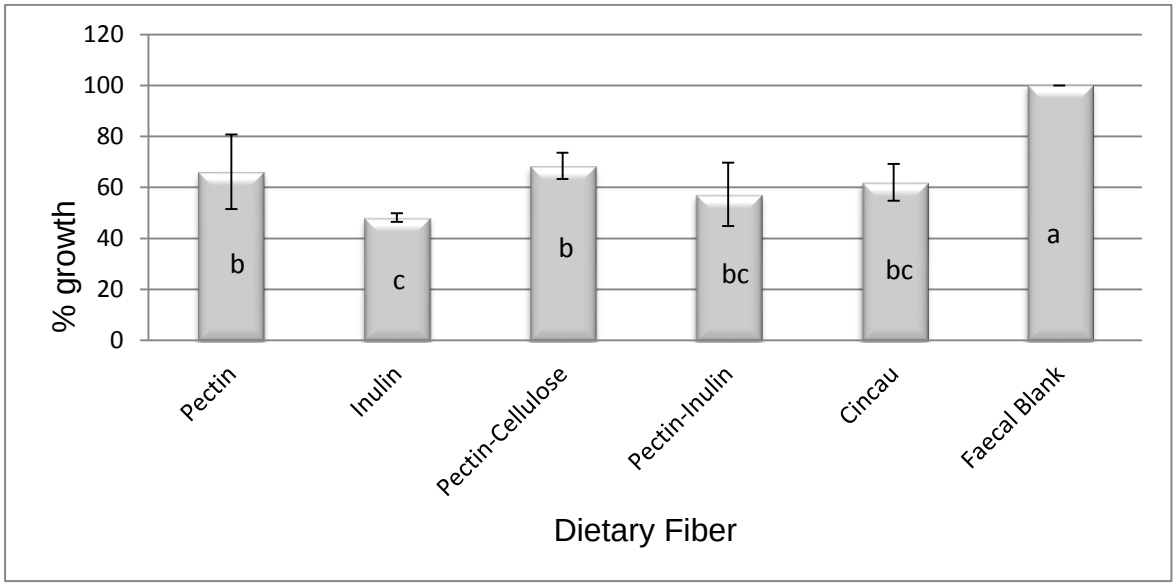

Figure 3-2. Effect of dietary fiber FS on the proliferation of Caco-2 cells.

Cells were seeded 1 day before the treatment with FS (day0), then incubated for 48 h in media containing 20% of FS. P, I, C, IC, PC, PI, Cin and FB were pectin, inulin, cellulose, pectin-cellulose mixture, pectin-inulin mixture, cincau extract and faecal blank, respectively. The bars represent the mean, and the lines are SEM of three independent experiments each performed in triplicates. Data points denoted by different superscripts (letters on the bar) differ significantly with $p < 0.05$.

3.4.3. Effect of dietary fiber FS on cell differentiation.

Cell differentiation was measured based on levels of the enzyme alkaline phosphatase (AP) present in the cells as the activity of AP increases during cell differentiation (Matsumoto et al., 1990). Stimulation of cell differentiation is one of the mechanisms by which SCFA slow cell cancer growth (Ding et al., 1998). All FS failed to increase AP enzyme levels with many caused significant decreases (Fig. 3.3, $p<0.05$). Cells that were incubated in FS containing Inulin and mixtures of pectin and inulin had significantly lower AP activity compared to FB, whereas incubated with FS containing pectin, mixture of pectin-cellulose and cincau displayed similar AP activities to FB ($p<0.05$).

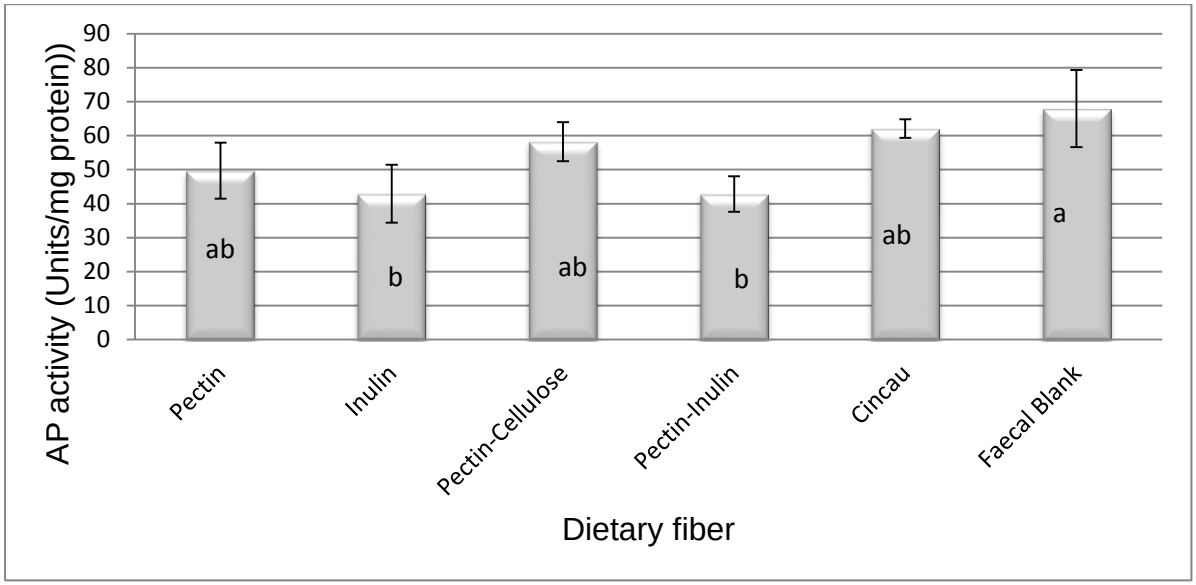

Figure 3-3. Effect of dietary fiber FS on AP enzyme levels.

Cells were seeded in 6 well plates until about 70% confluent, then incubated for 48 h in media containing 20% FS. AP enzyme activity was measured by hydrolysis of p-nitro phenol phosphate (5 mM) and expressed in units (the number of µmol p-nitrophenol liberated in 1 min measured at 400 nm per mg protein). The bars represent the mean, and the lines are SEM of three independent experiments each performed in triplicates. Data points denoted by different superscripts (letters on the bar) differ significantly with $p < 0.05$.

3.4.4. Effect of dietary fiber FS on caspase 3/7 activity.

Caspase 3 and 7 are key effectors of apoptosis and thus activities were measured using a fluorescent probe. Caspase 3/7 activity was affected by the type of dietary fiber fermented by faecal colon microbiota (Fig. 3.4, $p<0.05$). Pectin, individually or in combination with inulin, induce caspase 3/7 activity higher than no treatment (control). In contrast cincau extracts and faecal blank tended to suppress caspase 3/7 activity ($p<0.05$).

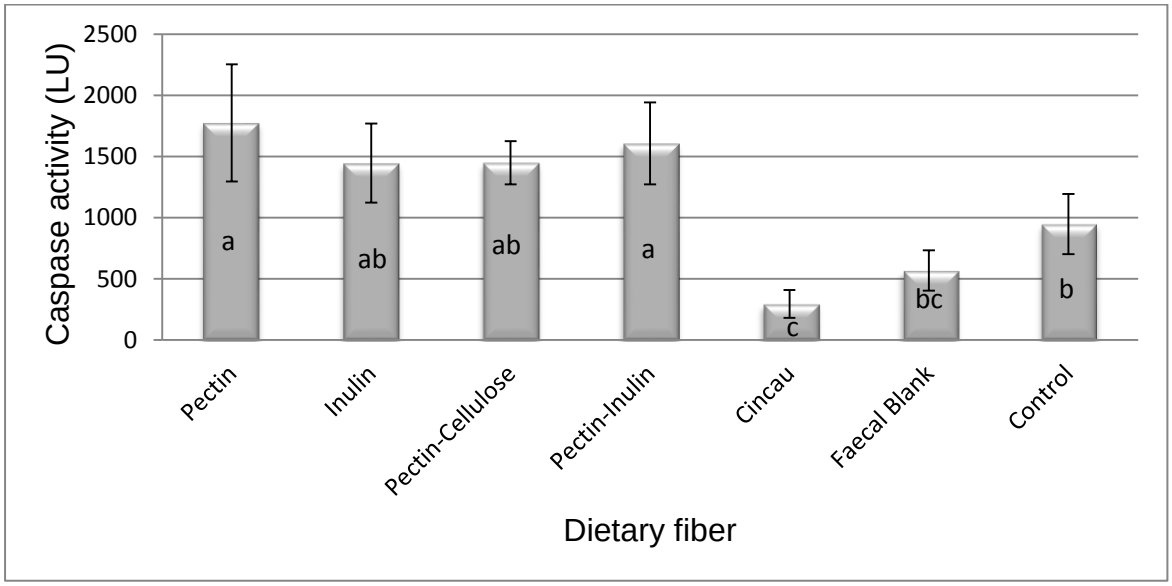

Figure 3-4. Effects of dietary fiber FS on caspase 3/7 activity.

Cells were seeded 1 day before the treatment with FS (day 0), then incubated for 48 h in media containing 20% FS. Caspase-3 and -7 activities were measured using the Caspase-GloR 3/7 assay kit (Promega, USA). Control is media without FS. The bars represent the mean, and the lines are SEM of three independent experiments each performed in triplicates. Data points denoted by different superscripts (letters on the bar) differ significantly with $p < 0.05$.

3.4.5. Mechanism of cell death induced by FS containing SCFA

Many methods are used for examining cell death. The extra cellular release of lactate dehydrogenase (LDH) is used to monitor cell damage and cell death. The 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) reduction assay is used to study viability as only live cells are able to metabolise MTT. To further examine the increase in caspase 3/7 activity triggered by FS from different dietary fiber, a caspase inhibitor was utilised. In inulin treated cells, caspase 3/7 activity was significantly higher than cincau and FB, but the difference was not detected when the caspase inhibitor was applied to the cells before the addition of the FS (Fig. 3.5 A; $p<0.05$). LDH release in cincau treated cells was lower compared to inulin and FB (Fig. 3.5B; $p<0.05$). LDH release was not affected by the addition of the caspase inhibitor. Both inulin and cincau FS inhibited cell growth and this inhibition was suppressed only partially by addition of the caspase inhibitor (Fig. 3.5C; $p<0.05$).

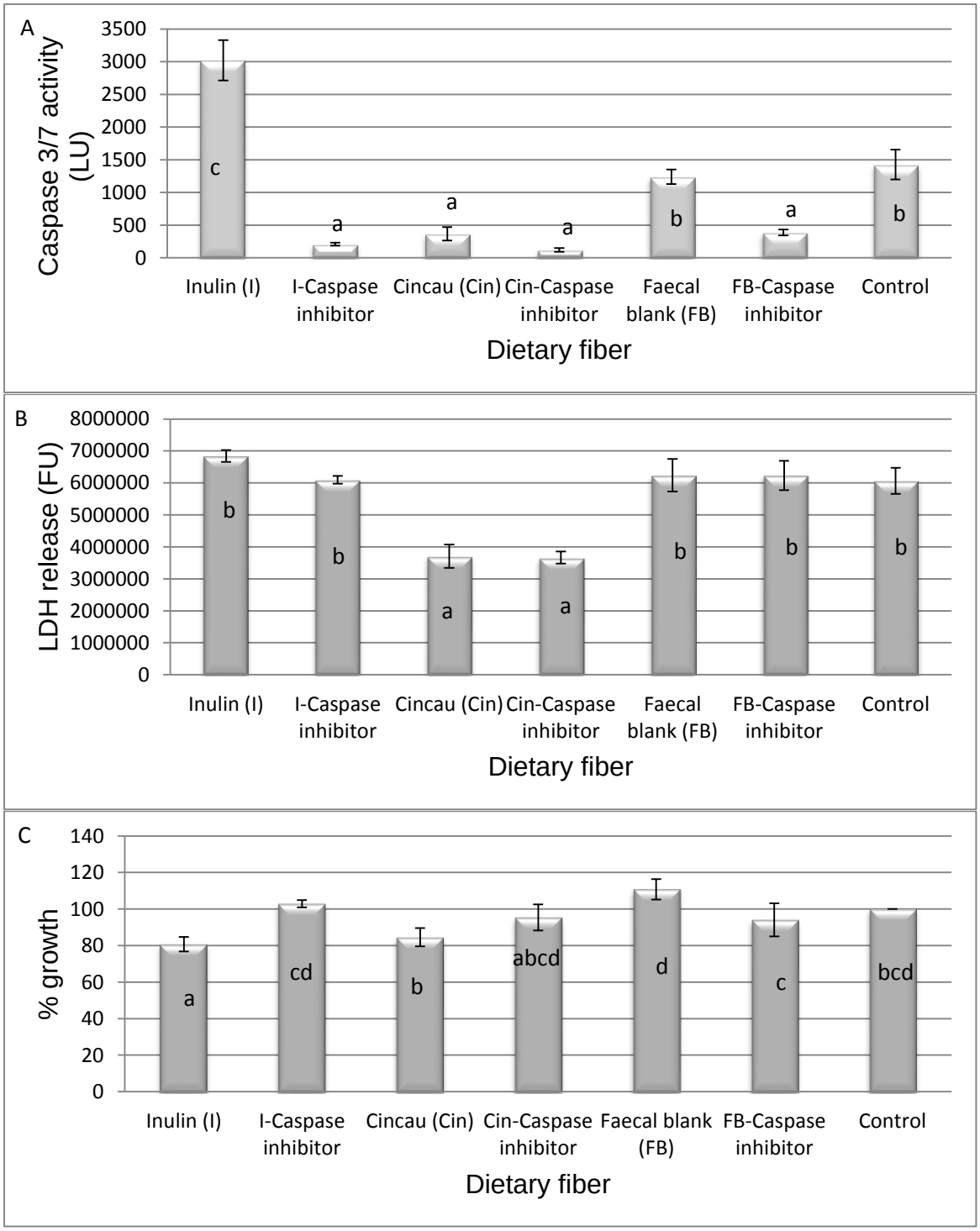

Figure 3-5. Effect of dietary fiber FS on caspase 3/7 activity (A), LDH release (B) and Caco 2 cell proliferation (C) with or without caspase inhibitor.

Cells were seeded 1 day before the treatment with FS (day 0), then incubated for 48 h in media containing 20% FS. Caspase inhibitor (10µM) was added 1 hour

preceding FS treatment. Caspase 3-7 activity was measured using the Caspase-GloR 3/7 assay kit (Promega, USA), the LDH activity was quantified using the CytoTox-ONE™ Homogeneous Membrane Integrity assay kit (Promega, USA) and cell proliferation was measured using the MTT assay and expressed as % growth against Control. Control is media without FS. The bars represent the mean, and the lines are SEM of three independent experiments performed in triplicates. Data points denoted by different superscripts (letters on the bar) differ significantly with $p < 0.05$.

3.5. Discussion

This study demonstrated that pectin and inulin alone, or in combination, had the greatest influence on individual and total SCFA production in the FS. This work demonstrates for the first time that cincau, a traditional food that is indigenous to Indonesia, can increase SCFA total concentrations. In particular that of acetate and propionate were increased. Inulin FS were able to inhibit the proliferation of Caco- 2 cells *in vitro* in a pattern that almost mirrored the amount of SCFA total present however, they had no effect on cell differentiation as measured by AP levels. Finally SCFA in the inulin FS were able to trigger apoptosis in Caco- 2 cells in a caspase 3/7 dependent pathway.

Inulin produced the highest concentration of butyrate among the dietary fibers tested. Butyrate levels in FS from cellulose and pectin increased significantly if these dietary fibers were mixed with inulin. Inulin is known to stimulate butyrate-producing bacteria (*Roseburia intestinalis*, *Eubacterium rectale*, *Anaerostipes caccae*), which in turn leads to higher concentrations and proportions of butyrate (Van den Abbeele et al., 2011). Fermentation of inulin with human faeces produced the highest concentration of butyrate compared to pectin, wheat, or corn starch (Paulsen et al., 1994) using a similar batch culture system. In a humanized rat model, inulin alone produced higher butyrate concentration than long chain arabinoxylans (Van den Abbeele et al., 2011), but in patients, the butyrogenic effect of inulin were not observed (Costabile et al., 2010).

My SCFA results suggest that FS from some dietary fiber will display different patterns when they were combined with others. Soluble dietary fibers will increase SCFA content from non-soluble dietary fiber, but the effect of the mixing on SCFA pattern (such as individual SCFA concentration) is not predictable.

Pectin has previously been shown to produce a high proportion of acetic acid *in vitro* (Titgemeyer et al., 1991) and *in vivo* (Rao et al., 1998). However, in the present study, the acetate content in pectin FS reached similar levels to inulin FS and inulin-cellulose FS, but lower than pectin-inulin FS. Moreover, even though previous research showed that rats fed pectin had larger caecal concentrations of SCFA than those fed inulin and lactitol (Nilsson et al., 2006), my results indicate that total SCFA from pectin FS were lower than inulin FS. Interestingly, when pectin was mixed with cellulose, the SCFA pattern in FS was similar with cincau. Cincau extract contains about 20% of pectin (Nurdin et al., 2005) and 52.1 % dietary fiber (Table 3.1), thus it is possible the remaining cincau dietary fiber is cellulose which can be expected as it is a plant product. Therefore, due to similarity of their fiber composition, FS from pectin-cellulose and Cincau contained similar SCFA composition.

Inulin increased butyrate concentration in cellulose and pectin FS (Fig. 3.1C, $p<0.05$), however the increase in butyrate in pectin-inulin FS did not lead to increased capacity to inhibit cell growth (Fig. 3.2, $p<0.05$). The butyrate content from pectin-inulin FS was nearly three-fold higher than pectin FS, while the propionate and acetate content from pectin-inulin FS was nearly double of pectin FS, however, per cent growth of the Caco-2 cells

cultured in media containing these fermentation supernatants was no different. This indicates that butyrate or combinations of butyrate with propionate or acetate are not the main factors in fermentation supernatant that affect cell growth, and that non-SCFA compounds also contained in the FS may be involved (Beyer-Sehlmeyer et al., 2003). Interestingly, cincau extract and pectin-cellulose FS which had lower concentrations of total or individual SCFA than pectin-inulin FS inhibited Caco- 2 cell growth to the same extent as pectin-inulin FS

Cell differentiation is one of the mechanisms by which SCFA act in order to slow cell cancer growth(Lanneau et al., 2007). This process requires cells to enter G1/G0 phase arrest and cell proliferation is then inhibited (Ding et al., 1998). Previous research indicates SCFA, mainly butyrate and propionate, induce cell differentiation (Basson et al., 1998). These two SCFA are suggested as the main compounds contributing to the anticancer activity of dietary fiber FS (Borowicki et al., 2010).

My results indicate FS from pectin, inulin, pectin-cellulose mixture, pectin-inulin mixture or cincau extract do not induce alkaline phosphatase a marker of cell differentiation (Fig. 3.3, $p<0.05$). Surprisingly, FS from fecal blank (FB) induced AP activity higher than pectin, inulin or pectin-inulin, even though all of these dietary fibre FS had high SCFA concentration. There are some possibilities to explain these phenomena. Firstly, the effect of butyrate on cell differentiation is dose dependent. It was previously butyrate induced cell differentiation of Caco-2 cells at a concentration of 0.1 mM, but when the butyrate level was increased to 5 mM, activity of this enzyme decreased (Orchel et al., 2005) . In the present study, inulin and pectin-inulin FS contain

37.7 and 24.1 mM of butyrate, respectively. When 20% of these FS were added to the media, the final concentration of butyrate in the media was 7.5 and 4.8 mM, respectively, whereas the final concentration of butyrate in media containing pectin, pectin-cellulose and cincau were only 1.6, 1.4 and 1.1mM, respectively. It is suggested that high level of butyrate in inulin and pectin-inulin may have led to the down regulation of AP activity. However, this explanation is not likely, as FB which contains no SCFA, elicited higher AP levels. Therefore, a second possibility needs to be considered to rationally explain the effects of FB. Previous research also found that FB had an effect that was unexplainable by SCFA content in FS (Beyer-Sehlmeyer et al., 2003, Sauer et al., 2007, Kiefer et al., 2006). For example, Sauer et al. found that metabolic activity of HT-29 cells was increased by 15% by FB supplementation, with levels increasing similar to those from inulin FS (Sauer et al., 2007). Moreover, these authors also found that FB enhanced gene expression of GSTA4, but inulin FS or SCFA mixture had no effects on this gene. GSTA4 is a gene encoding a glutathione S-transferase belonging to the alpha class 4 that has high catalytic efficiency with 4-hydroxyalkenals and other cytotoxic and mutagenic products of radical reactions and lipid peroxidation (Hubatsch et al., 1998). Taking this into account, data from this study indicate that effect of FS from dietary fiber on cell differentiation may depend on several factors including SCFA pattern and unidentified products formed during fermentation process or that originally exist in fresh faecal sample as a source of inoculums.

Our *in vitro* fermentation system used fresh faecal samples from volunteers. It should be noted that the diet consumed by volunteers

preceding collecting of faeces was not controlled or monitored. For example, Beyer_Sehlmeyer et al. (Beyer-Sehlmeyer et al., 2003) and Lux et al. (Lux et al., 2011) do not mention the nutritional and or health status of their faecal donors. Munjal et al. (Munjal et al., 2009) requested that healthy volunteers did not take supplements or antibiotics in the six months preceding the experiment. Faecal composition is determined by diet, not taking volunteer diet preceding the experiment into account will cause some bias. The present results indicate that the type of diet consumed by volunteers preceding collecting the faeces is highly likely to produces/contain unidentified compounds that have efficacy to induce differentiation and thus differentiation elicited by SCFA was not detectable. Therefore, in future, diet consumed preceding collecting the fresh faeces should be recorded or controlled, so its effect can be traced to the type of diet.

The ability of pectin, inulin, pectin-inulin, and pectin-cellulose FS to induce apoptosis was confirmed by their ability to increase caspase 3/7 activity compared to control (Fig. 3.4; $p<0.05$). In contrast, Cin and FB, when compared to control, decreased levels of caspase 3/7. Previous research has shown inulin induced apoptosis in HT-29 cells (Munjal et al., 2009) or in colon cancer rat model (Hughes and Rowland, 2001). Munjal et al. found there was a significant increase in poly(ADP-ribose) polymerase (PARP) cleavage in LT97 cells after incubation with the inulin FS, demonstrating its ability to induce apoptosis (Munjal et al., 2009). PARP cleavage is catalysed by caspase 3 and it has been proposed to prevent depletion of NAD (a PARP substrate) and ATP, which are required for apoptosis (Boulares et al., 1999). My results supports inulin or pectin-inulin FS is able to induce apoptosis

through caspase 3, as caspase 3/7 activity increased in Caco 2 cells incubated with these FS (Fig. 3.4, $p<0.05$).

Pectin or pectin-cellulose mixture also increased caspase 3/7 activity (Fig. 3.4, $p<0.05$), and this may support a role for pectin via its increase of SCFA as a dietary fiber that can affect the apoptosis process. Previously, induction of apoptosis through inactivation of PARP was found in AOM-induced rats fed diet containing fish oil and pectin (Vanamala et al., 2008). FS from pectin also inhibited histone deacetylase (HDAC) activity in nuclear extracts prepared from the colon tumour cell lines HT-29 and Caco-2 and in intact HeLa Mad 38 cells (Waldecker et al., 2008). Butyrate or other SCFA produced by the fermentation of pectin may be able to inhibit HDAC activity in order to induce gene transcription of caspase 3 and induce apoptosis (Anh et al., 2012, Hwang et al., 2009, Wallace et al., 2006),

Butyrate is the most potent SCFA for modulating colorectal cancer growth including induction of apoptosis (Matthews et al., 2012). However, my data indicate that modulation of apoptosis was not dependant on butyrate content. Compared with the pectin, inulin FS contained approximately five fold more butyrate and two fold more propionate (Fig. 3.1B and C), but their effect on caspase activity was not significantly different (Fig. 3.4, $p<0.05$). The effect of FS on caspase 3/7 activity was also unexplainable by total SCFA content of FS. For example, pectin-inulin contained about 1.7 fold SCFA total compared to pectin cellulose, but their caspase 3/7 activity was similar. Therefore, some factors other than SCFA might be involved in modulating caspase 3/7 activity (Munjal et al., 2009, Waldecker et al., 2008).

FS from inulin, cincau and FB were chosen to further elucidate the role of caspase 3/7 on cell death using the caspase inhibitor (Ac-DEVD-CHO) before the application of FS. LDH is an accurate method to assay cell death with membrane damage such as necrosis, while the MTT assay can measure differences in cell viability but it cannot tell whether cells are being killed via apoptosis or necrosis (Kim et al., 2009)

The FS from inulin induced cell death through a caspase3/7-dependent pathway and the release of caspase 3/7 could be inhibited by the addition of the caspase inhibitor and this led to an observed increase in Caco 2 cell viability. While the background level of LDH release in both control and all FS with and without caspase inhibitor are high the inhibitor did not affect LDH release. This finding suggests that the Caco 2 cells tested may have had a high percentage of necrotic cells present but this necrotic cell death is independent of FS and is not arising from cells in late apoptosis. This LDH release may be due to the fact that cells have been cultured for 48 h where the media was not changed.

Cincau extract FS suppressed Caco-2 cell growth compared to FB (Fig. 3.5C, $p<0.05$) but the mechanism appeared to be different to that of inulin. Compared to FB, cincau did not induce caspase 3/7 activity (Fig. 3.5A, $p<0.05$) and indeed less LDH was released from cells treated with either cincau or cincau and caspase inhibitor (Fig. 3.5B, $p<0.05$) suggesting cincau could protect cells from necrotic cell death. However these cells were less viable than the control cells. Previously, Huang et al. (Huang et al., 2010) found that *Solanum nigrum* Linn leaf extract, rich in polyphenols and anthocyanidin, caused cell death due to the induction of autophagy and

apoptosis. Acetone and ethyl acetate extracts from *Eupatorium odoratum* induced autophagic cell death in MCF-7 and Vero cell lines (Harun et al., 2012). Cincau was extracted from green cincau leaves (*Premna oblongifolia Merr.*). The extract contains alkaloids, saponins, phenol hydroquinones, molisch, benedict and tannins (Aryudhani, 2011). Therefore, it may be possible that the phytochemical compounds in the cincau induce autophaghic cell death which will not be measured by either the caspase or LDH assays.

3.6. Conclusion

Soluble dietary fibre and cincau extract had a great influence on SCFA production in the fermentation supernatant, and the ability of the supernatant to modulate parameters of cell growth, differentiation and apoptosis was dependent on butyrate concentration and unidentified compounds. In Caco-2 cells, inhibition of cell growth was dependent on the amount of SCFA generated in particular butyrate, with the inulin fiber source resulting in a 53% inhibition in cell growth. However, their effect on cell differentiation and apoptosis were not explainable by their butyrate content. Cincau FS appears to be able to kill cells by a method independent of apoptotic or necrotic pathways. Our research implies that beneficial effect of combination dietary fiber depend on how they interact with the colon microbial and suggests the important role of unidentified compound in modulation of the effect of dietary fiber on CRC carcinogenesis.

4. PROTECTIVE EFFECTS OF PHENOLIC ANTIOXIDANTS ON COLORECTAL CANCER-INDUCED RATS ARE DEPENDENT ON DIETARY FIBER SOURCE IN THE DIET

4.1. Abstract

Epidemiological studies indicate dietary fiber and antioxidants may protect against colon cancer. However, there is limited evidence of the effects of combinations of specific dietary fibers and antioxidants on colon cancer. The aim of my study was to evaluate the effect of combinations of dietary fiber and antioxidant sources on azoxymethane (AOM)-induced aberrant crypt foci (ACF) formation. Six week-old rats were fed an experimental diet containing 5% dietary fiber of cellulose (C) and pectin (P) or diet containing 5% cellulose or pectin and given water containing 0.1% epigallocatechin-3-gallate (EGCG) (CE or PE, respectively), green cincau extract (Cin) or green cincau leaves (CinL). After 4 weeks on experimental water and diets, each rat received subcutaneous injections of AOM (15 mg/kg body wt) once a week for 2 weeks, each animal was then maintained on their experimental diet until termination of the study at 8 weeks after the second AOM injection. Supplementation with 0.1% EGCG increased some individual SCFA concentration (acetate and butyrate) in digesta when the dietary fiber source was cellulose (CE), and an opposite effect was observed when the dietary fiber source was pectin (PE). PE induced cancer progression, characterized by an increase in total number of ACF and MDA level in compare with C. This effect was associated with increased lipid peroxidation in the liver. The protective effect of (-)-epigallocatechin-3-gallate

consumption against colon cancer development appears to be dependent on the type of the dietary fiber source in the diet.

4.2. Introduction

Colorectal cancer (CRC) incidence is rising significantly in most countries due to increasing prosperity (Long et al., 2012,Lee et al., 2012, Center et al., 2009b). The westernized lifestyle has been suggested as a main cause of the disease (Goh, 2007, Center et al., 2009a), mainly due to diet, obesity and inactivity (Gingras and Beliveau, 2011). The western diet is high in red and processed meat but low in fruits and vegetables; which significantly increase CRC risk. Therefore changes to the diet could lead to a decrease in CRC risk. Fruits and vegetables are two dietary factors suggested to reduce colorectal cancer risk (Magalhaes et al., 2011b,van Duijnhoven et al., 2009, Flood et al., 2008).

The cell wall of fruits and vegetables generally consists of middle lamella and a primary wall layer that is rich in dietary fiber, whereas middle lamella is composed predominantly by pectic substances whereas the primary wall mostly contains cellulose (Heredia et al., 1995). Cellulose is an inert substance and is classified as a non soluble dietary fiber with *in vitro* digestibility of around 7.2% (Barry et al., 1995). Previous *in vitro* research described in this thesis (Chapter 3) also demonstrated that SCFA concentration (total or individual) from cellulose fermentation supernatant was no different to the faecal blank (no added fiber).

Pectin is a soluble dietary fiber that makes up about 30% of the cell wall of higher plants (Sriamornsak, 2003) with high *in vitro* digestibility (Barry

et al., 1995, Titgemeyer et al., 1991, Rao et al., 1998). This fiber produces higher SCFA concentrations than cellulose *in vitro* (Vince et al., 1990) or inulin and lactitol *in vivo* (Nilsson et al., 2006); however the SCFA consisted of a high proportion of acetic acid (Titgemeyer et al., 1991, Rao et al., 1998). Research has shown fermentation supernatant from incubation of human faecal slurry with apple pectin is rich in butyrate and inhibits histone deacetylase in nuclear extracts from tumour cell lines (Waldecker et al., 2008). Moreover, pectin significantly suppresses the formation of AOM-induced aberrant crypt foci (ACF) as well as crypt multiplicity and number ACF/cm^2 in rats fed a low fat (5%) diet (Rao et al., 1998). Pectin also inhibited increases in cell proliferation and crypt length in transmissible murine colonic hyperplasia induced by *Cytrobacter rodentium* (Umar et al., 2003).

A wide variety of dietary antioxidants from fruit and vegetables, including vitamin antioxidants (Vitamin C and E, carotenoid), polyphenol compounds and dietary fibre, are metabolized in the gastrointestinal system. While some of these are absorbed in the small intestine, unabsorbed antioxidants reach the colon for further metabolism by microbiota (Manach et al., 2005). Previous research indicates dietary fiber induces colonic metabolism of the phenolic compounds linked/trapped by dietary fiber (Saura-Calixto et al., 2010). However, there is limited evidence on the effects of combinations of specific dietary fibers and antioxidants on colon cancer. There is a possibility that combinations of different fibers and antioxidants may exert a synergistic or antagonistic effect.

Diets have indirect effect on gastrointestinal function of the host through their effects on the composition and activity of the human gut microbiota as well as the gut environment (Gingras and Beliveau, 2011, Scott et al., 2011). Substrate availability for gut bacteria is the major determinant of microbial community complexity and metabolites in the intestine (Blaut and Clavel, 2007, Scott et al., 2011), therefore specific dietary interventions will change the type of bacterial groups dominating the colon (Scott et al., 2011, Walker et al., 2011). For example, daily consumption of inulin significantly increased *bifidobacteria spp.* and decreased Gram-positive cocci in stools (Gibson et al., 1995, Buddington et al., 1996, Kleessen et al., 2007, Kruse et al., 1999).

Microorganisms are involved intensively in phenolic metabolism in the gastrointestinal tract and their role determines the phenolic bioavailability (Dall'Asta et al., 2012). Phenolic compounds are degraded by colon microflora resulting in simpler derivative compounds, and the type of the metabolites are dependent on the bacterial types (Lee et al., 2006). Bioactivity of the microflora metabolites is varied, and some compounds are more active and some less active after fermentation (Bellion et al., 2008). Microflora fermentation with specific probiotic strains can also lead to a significant increase of free phenolic acids, therefore improving their bioavailability (Hole et al., 2012, Bellion et al., 2008).

Epigallocatechin-3-gallate (EGCG) is the major antioxidative polyphenolic compound of green tea (Takagaki et al., 2011). Studies in animal models of carcinogenesis have shown green tea and EGCG can inhibit tumorigenesis during the initiation, promotion and progression stages

(Shirakami et al., 2008). EGCG significantly suppressed the multiplicity and volume of colonic neoplasms compared to the AOM/dextran sulphate sodium (DSS) group, and resulted in a lesser degree of malignancy (Shirakami et al., 2008). Combinations of EGCG with curcumin (Xu et al., 2010) or sulindac (Ohishi et al., 2002) produced a synergistic effect on inhibition of colorectal cancer growth. Most trials examining the effects of EGCG on colorectal cancer have been carried out in rats fed diets containing cellulose or a standard diet (Caderni et al., 2000, Sengupta et al., 2003, Carter et al., 2007, Hao et al., 2007, Xiao et al., 2008). Therefore, it is interesting to assess whether substitution of the dietary fiber source will modulate the efficacy of EGCG or not.

By-products from citrus and apple juice production are the main sources of commercial pectin. Pectin concentration in these by-products is about 12.5 % and 25%, respectively (Sriamornsak, 2003). Pectin also can be extracted from an Indonesia (*Premna oblongifolia* Merr.) which the extract of the green leaf of the plant contains about 20% of pectin (Nurdin et al., 2005). Traditionally, the leaves are extracted with hot water to prepare a refreshing drink or consumed as a traditional medicine for inflammation or fever. Research on the extract indicates it has the ability to induce cell-mediated immune responses *in vitro* (Nurdin et al., 2003). As a dietary fiber, the extract also has a laxative properties and it effectively induces the growth of lactic acid bacteria in the colon (Nurdin, 2007a). As the extract from *Premna oblongifolia* Merr. leaves contain both high pectin levels in an unpurified form and phenolic compounds, it is worthwhile to compare the

efficacy of this traditional dietary fiber with other dietary fiber-antioxidant mixture in improving colon health.

The microflora population plays a significant role in metabolism of phenolic compounds in the colon (Lee et al., 2006, Bellion et al., 2008). As the diversity of colon microflora will be affected by dietary fiber, it is hypothesised that different dietary fiber sources will cause different effects on phenolic metabolism and bioactivities. The aim of this research was to evaluate the effect of a variety of combinations of dietary fibre and antioxidant sources on AOM-induced aberrant crypt foci (ACF) formation in rat model of colorectal cancer.

4.3. Material and Methods

4.3.1. Green Cincau Leave materials

Green cincau leafs (*Premna oblongifolia* Merr.) were collected from traditional farmers in Indonesia. The fresh leafs were dried in oven at 50oC to get dry leaves (water content around 12%), and ground to obtain dry cincau powder. Extracts were obtained from extraction of 5% dry cincau leaf powder using hot water followed by oven drying at 50^{o}C (Nurdin et al., 2005).

4.3.2. Animals and diet

All animal work was approved by the Flinders University Animal Welfare committee under ethics application 761/2010 and rats were obtained from the Animal Resource Centre, Perth, Western Australia. The animals, diets and the experimental procedure were prepared as described by Le Leu et al. (Le Leu et al., 2002). Dietary fibres were added into diets based on the

AIN-76A standard for purified diets for rats and mice (Report of the American Institute of Nutrition *ad hoc* Committee on Standards for Nutritional Studies, 1977). EGCG was given in tap water containing at 0.1% EGCG (Shimizu et al., 2008). Each group of rats was fed one of eight diets (Table 4.1). The first and the second groups (C and P) consumed the diet containing 5% cellulose (control diet) and pectin, respectively. The third and the fourth groups consumed the diet containing cellulose and pectin, respectively, and both groups were given tap water containing 0.1% EGCG (CE and PE groups). The fifth and sixth groups (Cin and CinL) were fed a diet containing 5% dried cincau extracts and dried cincau leaf, respectively (preparation of this material is described below). Animals were given free access to water and food.

Table 4-1. Composition of experimental diets (per 1 Kg)

	Experimental Groups					
	C	P	CE	PE	Cin	CinL
	(g)					
Casein	0.19	0.19	0.19	0.19	0.19	0.19
Corn Starch	0.43	0.43	0.43	0.43	0.43	0.43
Cellulose	0.05	-	0.05	-	-	0.05
Pectin	-	0.05	-	0.05	-	-
Cincau extract	-	-	-	-	0.05	-
Cincau leave powder	-	-	-	-	-	0.05
Corn oil	0.18	0.18	0.18	0.18	0.18	0.18
Sucrose	0.1095	0.1095	0.1095	0.1095	0.1095	0.1095
dl-Methionin	0.003	0.003	0.003	0.003	0.003	0.003
Choline	0.001	0.001	0.001	0.001	0.001	0.001
Mineral Mix	0.035	0.035	0.035	0.035	0.035	0.035
Vitamin Mix	0.01	0.01	0.01	0.01	0.01	0.01
EGCG in water			0.1%	0.1%		

Abbreviation: C, cellulose; P, pectin; CE, cellulose+0.1% EGCG; PE, pectin+0.1 EGCG; Cin, cincau extract;CinL, cincau leave; EGCG, epigallocatechin-3-gallate

At the beginning of Week 4 animals fed experimental diets were housed in metabolic cages for measuring of food intake and faecal output for 48 h. At the beginning of Week 5 each rat received a subcutaneous injection of AOM (15 mg/kg body wt; Sigma-Aldrich Chemical Co., St Louis, MO) once weekly for 2 weeks. After each subcutaneous injection, rats were monitored twice daily for any signs of an adverse reaction to AOM for the following three

weeks. Rats were maintained on their experimental diets for 8 weeks after the second AOM injection. Fresh faeces (faecal samples) were collected for SCFA analysis and pH measurement one week before termination. At the termination of the study (12 weeks) rats were anaesthetized and blood was collected via cardiac puncture. After this, laporotomy was performed and the large intestine was resected. The intestines were opened longitudinally and the contents were emptied. The colon was fixed flat for 24h and then were examined for colon tumours and the location and number of ACF (assessed with a dissection microscope) and recorded. Colon digesta (caecal samples) were collected for analysis of pH and SCFA concentrations. Caecal samples were obtained from each rat after termination through resection. Liver tissue was used for malondialdehide (MDA) measurements. Termination of rats was done for 5 consecutive days

4.3.3. Determination of SCFA composition and pH of fecal and cecal samples

Faecal and caecal (digesta) samples were homogenized in 3 volumes of internal standard solution (heptanoic acid, 1.68 mmol/L) and centrifuged at 3000 x *g* for 10 min. The supernatant was then distilled and 0.3 µL injected into a gas chromatograph (HewlettPackard 5890 Series II A) equipped with a flame ionization detector and a capillary column (Zebron ZB-FFAP, 30 m x 0.53 mm i.d, 1-µm film, SGE). Helium was used as the carrier gas; the initial oven temperature was 120°C and was increased at 30°C/min to 190°C; the injector temperature was 210°C and the detector temperature was 210°C. A standard SCFA mixture containing acetate, propionate, and butyrate was

used for calculation and the results are expressed as µmol/g of sample (Le Leu et al., 2005).

Faecal samples were collected from each rat by gently handling the rats until they produced a sample. Caecal samples were obtained from each rat after termination through resection. Samples (faecal or caecal) were collected from each rat and diluted in three volumes of 0.98% sodium chloride solution, vortexed briefly, and pH measured with a glass-embeded electrode (Eutech Instruments) (Winter et al., 2011)

4.3.4. Measuring ACF number and multiplicity

Colons were fixed flat in 10% buffered formalin solution containing 3.6% formaldehyde for 24 hours then transferred to 70% ethanol for histological processing. The mucosal surface of the colons was stained with methylene blue and the number of ACF was counted under a light microscope (Bird, 1987). Colons were divided into proximal, medium distal and distal sections. The proximal site was indicated by the herring bone region, and the distal was divided into medium distal and distal by halving the colon without proximal region. The sample was taken from slide box by blinding method and then the crypt was recorded randomly as ACF when its size was at least twice the normal..

4.3.5. Measuring proliferative activities

To assess the proliferative activity and the distribution of proliferating cells in the colonic crypts, immunostaining detecting the proliferating cell nuclear antigen (PCNA) was performed using standard immunohistochemical procedures (Le Leu et al., 2007). Briefly, deparaffinised sections were

rehydrated in a graded series of ethanol from 100% to 50% and then to distilled water. Antigen retrieval using pressure cooker in 0.01 M sodium citrate buffer was applied for 1h. The primary mouse monoclonal antibody (PC‑10, Santa Cruz, USA) was placed on the slides (1/500 dilution) and incubated overnight at room temperature. A Level 2 Ultra Streptavidin detection system (Signet Laboratories, Inc, USA) was used utilising biotinylated goat anti-mouse as the secondary antibody. The slides were counterstained for 1 min with haematoxylin. The cell number along the crypt and the number of PCNA positive cell were counted randomly under light microscopy (magnification 40x). The sample was taken from slide box by blinding method and then the cell number along the crypt and the number of PCNA positive cell were counted randomly under light microscopy (magnification 40x).

4.3.6. Measuring lipid peroxidation in rat liver

Lipid peroxidation in liver was evaluated by the fluorometric method based on the reaction of malondialdehide (MDA) and thiobarbituric acid. Results were expressed as μmol MDA per gram liver. The breakdown product of 1,1,3,3-tetraethoxypropane was used as standard (Ohkawa et al., 1979).

4.3.7. Measuring fecal bacterial community

Extraction of Bacterial DNA from Rat Faecal Samples

For DNA extraction, faecal samples were diluted 1:4 (w/vol) in 0.98% sodium chloride solution. DNA was extracted from about 500 mg diluted

samples using PowerSoil DNA Isolation Kit, a soil DNA extraction kit (Power Soil DNA Isolation kit, MO BIO Laboratories, Carlsbad, CA, USA) as per the manufacturer's protocol.

Bacterial 16S rDNA amplification

A mastermix containing 2 µl of purified DNA (145-371 ng/µl), 1 µl dNTPs 10 mM, 10 µl Go Taq Flexi buffer, 3 µl $MgCl_2$, 25 mM, 0.25 µl Go Taq Polymerase 5 U/L, 2 µl forward primer 63f 10 pmol/ µl, 2 µl reverse primer 1389r 10 pmol/ µl and 29.75 µl sterile water was prepared for PCR amplification. The thermocycling programme used consisted of 1 cycle 5 min at 95 °C; 30 cycles of 1 min at 95 °C, 1 min at 65 °C, 1.5 min at 72 °C; and a final extension at 72 °C for 10 min (Kadali et al., 2012).

Denaturing gel gradient electrophoresis (DGGE)

The colon bacteria in the digesta were monitored with DGGE following the method described by Pérez-Leblic et al. (Perez-Leblic et al., 2012). DGGE was performed with a D-code Universal Mutation Detection System (Bio Rad laboratories, Hercules, CA, USA). PCR products were loaded onto 6% polyacrylamide (Bio Rad, UK) gels containing a formamide-urea linear denaturing gradient of 25-65 %. Gels were run in 1 x TAE at a constant voltage of 60 V for 18 h at 60^{o}C. Bands were visualized by staining the gels with silver nitrate solution (12.5%). The gels were exposed to UV light to visualize the bands and digitalized in a Gel Doc 2000 (BioRad laboratories, Hercules, CA, USA). Digitized gel images were then analyzed with TotalLab analysis package (Non-linearDynamics, USA). Unweighted pair group with

mathematical averages (UPGMA) dendrograms were then generated with TotalLab and bacterial community diversity determined with Shannon–Weaver Diversity Index (H^o) (Girvan et al. 2003).

Excision, cloning and sequencing of selected bands from DGGE gels

Some bands were excised from DGGE gels with a sterile razor, placed in 40 µl sterile water, and incubated at 4°C for diffusion of DNA into the water. The bands were chose based on their appearance in DGGE, for instance the bands exist in groups fed pectin containing diets were cut and considered as bands appeared due to pectin intake. The DNA was cloned into pGEM®-T Easy Vector Cloning Kit (Promega, USA) according to the manufacturer's protocol. Competent JM109 *E. coli* cells were transformed and plated. White colonies were screened for inserts of the expected size by using primers T7 and SP6. Clones were purified with Wizard® *Plus* SV Minipreps (Promega, USA) and quantified with Nanodrop (Thermoscientific, USA). The samples were then sent for sequencing to AGRF (Australian Genome Research Facility) according to AGRF requirements (**www.agrf.org**.au). The sequences obtained were compared to available database sequences for bacteria using the Basic Local Alignment Search Tool (BLAST) (**http://blast.ncbi.nlm.nih.gov/**). Sequences with identity >95% were considered to represent the same taxonomic group

4.3.8. Statistical methods

Results are expressed as the mean ±SEM. Statistical analysis was carried out with a statistical program SPSS version 19. Two way-Anova with

LSD test was used. Results were considered significant if $p < 0.05$ (see Table 6.32-6.69)

4.4. Results

4.4.1. Body weight and daily intake

Throughout the experiment, all animals were healthy as indicated by a steady body weight gain. A slight body weight decrease was observed after the first and the second AOM injection, then weight steadily increased during the following weeks (Fig. 6.2). Body weight gains of rats fed diet containing pectin (P) and pectin+EGCG (PE) were lower than the rats in the cellulose group (C) or cellulose-EGCG group (CE) (Table 4.2). No difference was observed in the liver weight at the time of termination.

Data from rats housed in metabolic cages for 48 h indicated that daily water intake increased when the rats were fed a diet containing cincau (Cin or CinL) compared with those fed with cellulose diets (C or CE) or pectin alone (P). Water intake of CE and PE groups was not significantly different, so the EGCG intake of these groups was similar. Dietary fiber types and EGCG did not affect the rat's daily food intake (Table 4.2).

Table 4-2. The effects of dietary fiber sources and antioxidant on body weight gain, liver weight, water and food intake of azoxymethane (AOM)‑induced rats

	Experimental groups					
	C	P	CE	PE	Cin	CinL
Body weight gain (g)	368.7±18.6^{ab}	317.92±9.5^{cb}	371.2±14.8^{a}	319.2±11.6^{c}	365.5±16.7^{a}	345.9±13.2^{abc}
Liver weight (g)/body weight (g)	2.8±0.06	2.8±0.12	2.9±0.07	3.0±0.14	3.1±0.06	2.9±0.08
Water intake (ml/day/100 g bw)	5.81±0.39^{ab}	7.11±0.54^{ab}	5.62±0.23^{a}	7.52±0.62^{abc}	8.22±0.98^{c}	7.61±0.52^{bc}
Food intake (g/day/100 g bw)	5.79±0.33	5.22±0.14	5.28±0.43	5.58±0.28	6.04±0.51	5.87±0.28

Abbreviation: C, cellulose; P, pectin; CE, cellulose+0.1% EGCG; PE, pectin+0.1 EGCG; Cin, cincau extract;CinL, cincau leaf; EGCG, epigallocatechin-3-gallate. ^{a}Values are mean ± SEM (n=12). Means across rows with a different superscript are statistically significantly different at $p \leq 0.05$.

4.4.2. SCFA Concentration and pH

The colon digesta contained approximately twice the SCFA concentrations than faeces, especially in C, P and CE groups. The lowest SCFA concentrations were found in the cellulose diet (C) group. In general, my results indicate that 0.1% EGCG tended to increase SCFA concentration when the dietary fiber source was cellulose, and an opposite effect was observed when the dietary fiber source was pectin. Table 4.3 summarizes the SCFA concentrations in the colon digesta and faeces of rats administered the different experimental diets and drinking water.

The SCFA concentration of the colon digesta was elevated when cellulose in the control diet was substituted with pectin (P). Drinking water containing 0.1% EGCG decreased total SCFA of rats digesta fed with pectin diet (PE), 107 µmol/g in colon digesta of P group compared to 65.8 µmol/g in colon digesta of PE group (Table 4.3, $p<0.05$), but when the dietary fiber source was cellulose, this effect of EGCG was not seen. Ingestion of 0.1% EGCG increased acetate concentration in the digesta, from 18.2 µmol/g in C group to be 28.2 µmol/g in CE group (Table 4.3, $p<0.05$). Colon digesta of the pectin group contained higher acetate levels than C and CE groups, but the difference was not detected when rats in P group drank water containing 0.1% EGCG. Compared to cellulose, pectin induced propionate production in the digesta, but this effect was not significant in PE.

Water containing 0.1% EGCG increased the butyrate content of the colon digesta when the dietary fiber source was cellulose, but when the

dietary fiber source was pectin, the opposite effect was observed. Diet containing cinccau extract (Cin) or cincau leaf powder (CinL) produced acetate, propionate and butyrate levels in colon digesta in between those observed in the C and P group and but this did not reach statistical significant.

4.4.3. The number of aberrant crypt foci (ACF)

ACF were found in the colon of all rats injected with AOM. The number of ACF in the colon was higher in P and PE groups compared to rats fed cellulose diets (C) (Table 4.4). Although comparison between C and CE or P and PE showed no significant changes in the ACF parameters, we could observe that in general, the number of ACF in CE was lower than C and the number ACF in PE was slightly higher than P.

Table 4-3. The effects of dietary fiber sources and antioxidants on SCFA concentration in the digesta and faeces (μmol/g)a of AOM‑induced rats

	Experimental groups					
	C	P	CE	PE	Cin	CinL
			Digesta			
Total SCFA	45.9±8.4^{ab}	107.3±12.2^{c}	69.1±5.3^{b}	65.8±7.1^{b}	44.9±5.2^{a}	64.5±10.5^{ab}
Acetate	18.2±3.2^{a}	40.0±3.8^{c}	28.2±2.1^{b}	26.2±2.8^{ab}	19.1±2.0^{ab}	28.3±5.3^{b}
Propionate	5.0±1.0^{ab}	16.0±1.9^{d}	7.8±0.8^{bc}	9.2±1.0^{c}	4.0±0.5^{a}	5.7±1.1^{ab}
Butyrate	4.2±0.9^{a}	9.8±1.4^{c}	7.5±0.7^{bc}	6.9±1.7^{abc}	5.0±08^{ab}	6.5±1.0^{abc}
pH	6.9±0.06^{a}	6.7±0.08^{b}	6.7±0.05^{b}	6.7±0.07^{b}	6.7±0.05^{b}	6.7±0.05^{b}
			Faeces			
Total SCFA	14.8±2.0^{a}	44.4±8.3^{cd}	17.5±2.4^{ab}	51.9±9.0^{d}	30.8±3.9^{bc}	33.7±4.2^{c}
Acetate	5.8±0.6^{a}	14.5±3.0^{c}	7.2±0.9^{ab}	14.5±2.2^{c}	12.6±1.1^{bc}	13.7±1.2^{c}
Propionate	1.4±0.2^{a}	8.0±1.3^{b}	1.9±0.3^{a}	7.3±1.0^{b}	2.3±0.2^{a}	2.8±0.3^{a}
Butyrate	1.6±0.2^{a}	4.7±0.5^{b}	2.4±0.3^{a}	6.3±1.0^{b}	4.6±0.5^{b}	4.8±0.5^{b}
pH	6.7±0.06^{a}	6.4±0.05^{c}	6.4±0.04^{c}	6.5±0.05^{bc}	6.6±0.05^{b}	6.4±0.05^{c}
			SCFA absorption			
SCFA total	67.76%	58.62%	74.67%	21.12%	31.40%	47.75%
Butyrate	62.21%	52.28%	68.40%	8.70%	8.00%	26.15%

Abbreviation: C, cellulose; P, pectin; CE, cellulose+0.1% EGCG; PE, pectin+0.1 EGCG; Cin, cincau extract;CinL, cincau leaf.; EGCG, epigallocatechin-3-gallate.

[a] Values are mean ± SEM (n=12). Means across rows with a different superscript are statistically significantly different at $p \leq 0.05$.

Table 4-4. The effects of dietary fiber sources and antioxidant on AOM-induced ACF

	Experimental groups					
	C	P	CE	PE	Cin	CinL
ACF incidence	12/12	12/12	12/12	12/12	12/12	12/12
Number of ACF	73.2±12.1[a]	110.7±19.6[b]	64.7±8.5[a]	114.5±14.1[b]	79.7±10.7[ab]	88.8±10.7[ab]
1 crypt	32.9±5.2	53.6±10.9	31.5±4.5	52.3±5.6	43.4±4.3	44.5±5.0
2 crypts	26.8±4.7[a]	40.5±6.7[b]	23.7±[a]	41.3±5.9[b]	23.5±4.6[a]	30.2±4.5[ab]
3crypts	8.4±1.7	10.6±1.9	7.1±1.3	14.5±2.5	7.7±2.1	9.7±1.8
<4	68.2±11.2[a]	104.9±18.5[bc]	62.3±8.8[a]	108.2±13.0[c]	74.7±9.3[ab]	84.4±9.8[abc]
4≤	4.5±1.0	5.8±1.3	2.4±0.5	6.4±1.5	4.3±1.3	4.3±0.9
Proximal colon	1.6±0.6	2.0±0.8	0.6±0.2	3.6±2.7	3.2±1.9	2.4±1.4
Middle colon	38.8±6.4	64.3±14.3	43.0±7.2	63.0±11.3	44.1±9.7	49.8±8.1
Distal colon	32.8±6.7[ab]	44.4±6.5[bc]	21.2±2.9[a]	48.0±3.2[c]	32.4±4.4[ab]	36.4±6.8[bc]

Abbreviation: C, cellulose; P, pectin; CE, cellulose+0.1% EGCG; PE, pectin+0.1 EGCG; Cin, cincau extract;CinL, cincau leaf. [a] Values are mean ± SEM (n=12). Means across rows with a different superscript are statistically significantly different at $p \leq 0.05$.

The number of foci containing two aberrant crypts/focus was elevated significantly in P and PE groups compared to C, Cin or CinL group, but the elevation was not detected for foci containing 1 and 3 aberrant crypts/foci (Table 4.4). Substitution of cellulose (C) with Cin or CinL did not affect the number of foci containing <4 aberrant crypts/focus, but when rats were fed diet containing P, the number of foci containing <4 aberrant crypts/focus increased significantly compared to the C group (Table 4.4, $p<0.05$). Ingestion of 0.1% EGCG increased the number of foci containing <4 aberrant crypts/focus in P group. No significant difference was observed in the number of foci containing 4≤ aberrant crypts/focus irrespective of the diets or drink.

The total number of ACF was counted in the proximal, middle and distal region of the colon. In the proximal and middle colon, the total number of ACF was not affected by the diets or drink. However, in the distal part, P and PE group have total number of ACF higher than CE group.

4.4.4. Cell proliferation in distal colon

Cell proliferation was evaluated by assessing the PCNA staining in the distal colonic crypts, and Figure 4.1 shows the PCNA labelling index and the number of PCNA positive cells in the distal colon of the different groups. Rats fed diet containing pectin and drinking 0.1% EGCG (PE) have a higher PCNA labelling index and number of PCNA positive cells than P and CE groups ($P\leq 0.05$).

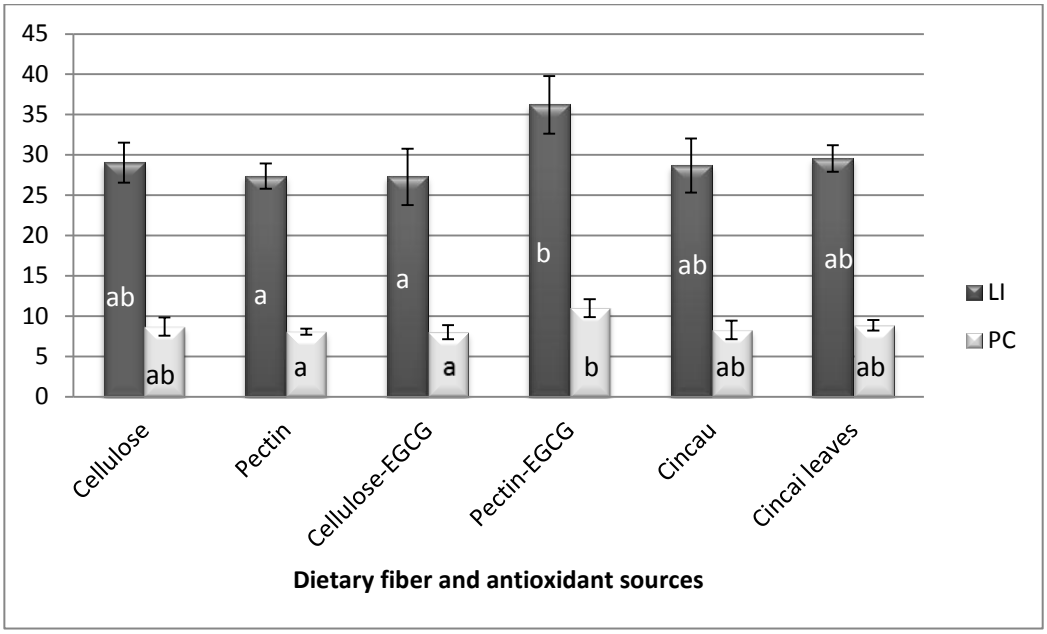

Figure 4-1. The effects of dietary fiber sources and antioxidant on PCNA labelling index (dark bar) and the number of PCNA positive cells (light bar) in the mucosa of distal colon of AOM‑treated rats.

Values are mean ± SEM of (n=12). Means with a different superscript (letters on the bar) are statistically significantly different at $p \leq 0.05$. Abbreviation: C, cellulose; P, pectin; CE, cellulose+0.1% EGCG; PE, pectin+0.1 EGCG; Cin, cincau extract;CinL, cincau leaf; LI, PCNA labelling index (%); PC, the number of PCNA positive cells.

4.4.5. Lipid peroxidation product

Liver MDA was significantly higher in PE and CinL than C, P, CE or Cin groups ($p \leq 0.05$) (Figure 4.2).

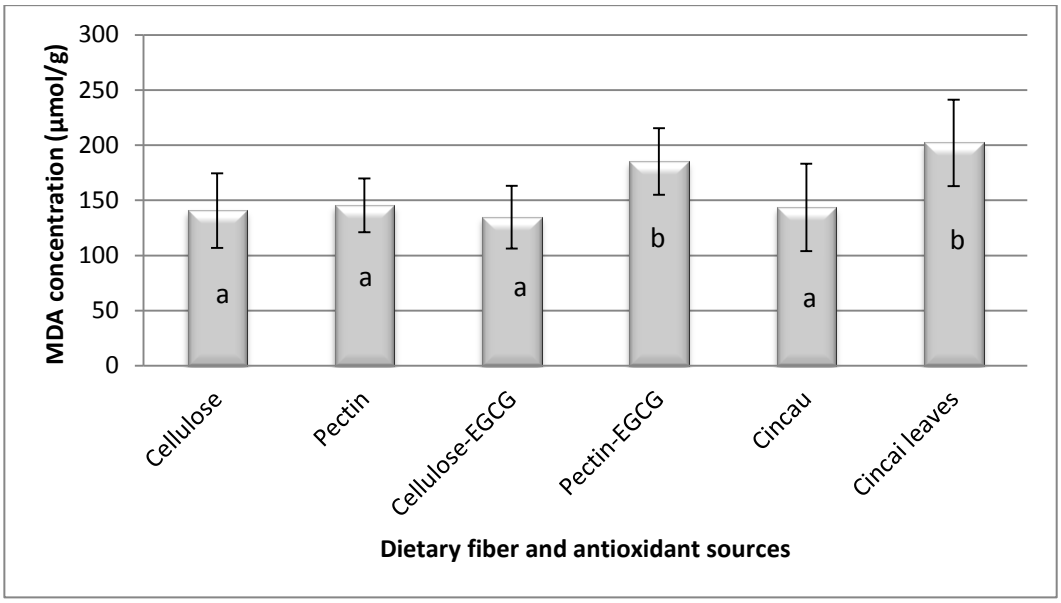

Figure 4-2. The effects of dietary fiber sources and antioxidant on MDA levels in the livers of AOM-treated rats.

Values are mean ± SEM of (n=10). Means with a different superscript (letters on the bar) are statistically significantly different at $p \leq 0.05$. Abbreviation: C, cellulose; P, pectin; CE, cellulose+0.1% EGCG; PE, pectin+0.1EGCG; Cin, cincau extract; CinL, cincau leaf; MDA, Malondialdehyde

4.4.6. Microbial profile of the colon digesta

Dietary fiber and EGCG induced different microbial profiles as indicated by different patterns on the acrylamide gel (Fig. 4.3 and 4.4). Considering the similarity index of those lanes with values higher than 0.4, Four distinct clusters (cluster one including lane 7, 8, 11, 12, 13 and 22; cluster two including lane 15, 3, 14, 16, and 19; cluster three including lane 1, 2, 9, 17, 18 and 20; cluster four including lane 6, 4, 5 and 21) (Fig. 4.2) were

seen. However, there was not a distinct cluster based on dietary fiber or antioxidant sources. Sequencing of selected bands confirmed the presence of species that commonly grow in the colon and produce SCFA (Fig. 4.3 and Table 4.4).

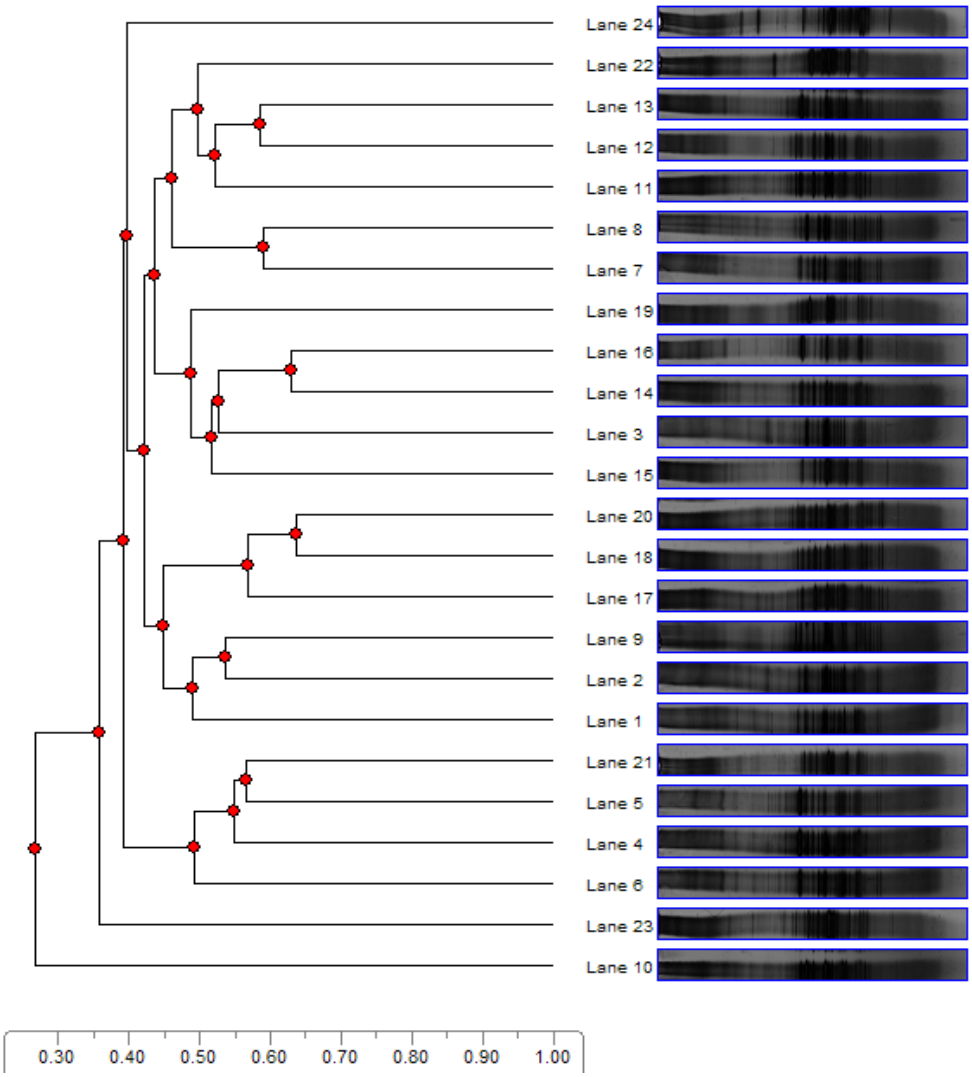

Figure 4-3. UPGMA dendrogram of 16S rDNA based DGGE profiles of digesta bacterial communities. Scale refers to similarity index

Lane 1-4 = CinL; Lane 5-8 = Cin; Lane 9-12 = PE; Lane 13-16 = CE; Lane 17-20 = P; Lane 21-24 = C.

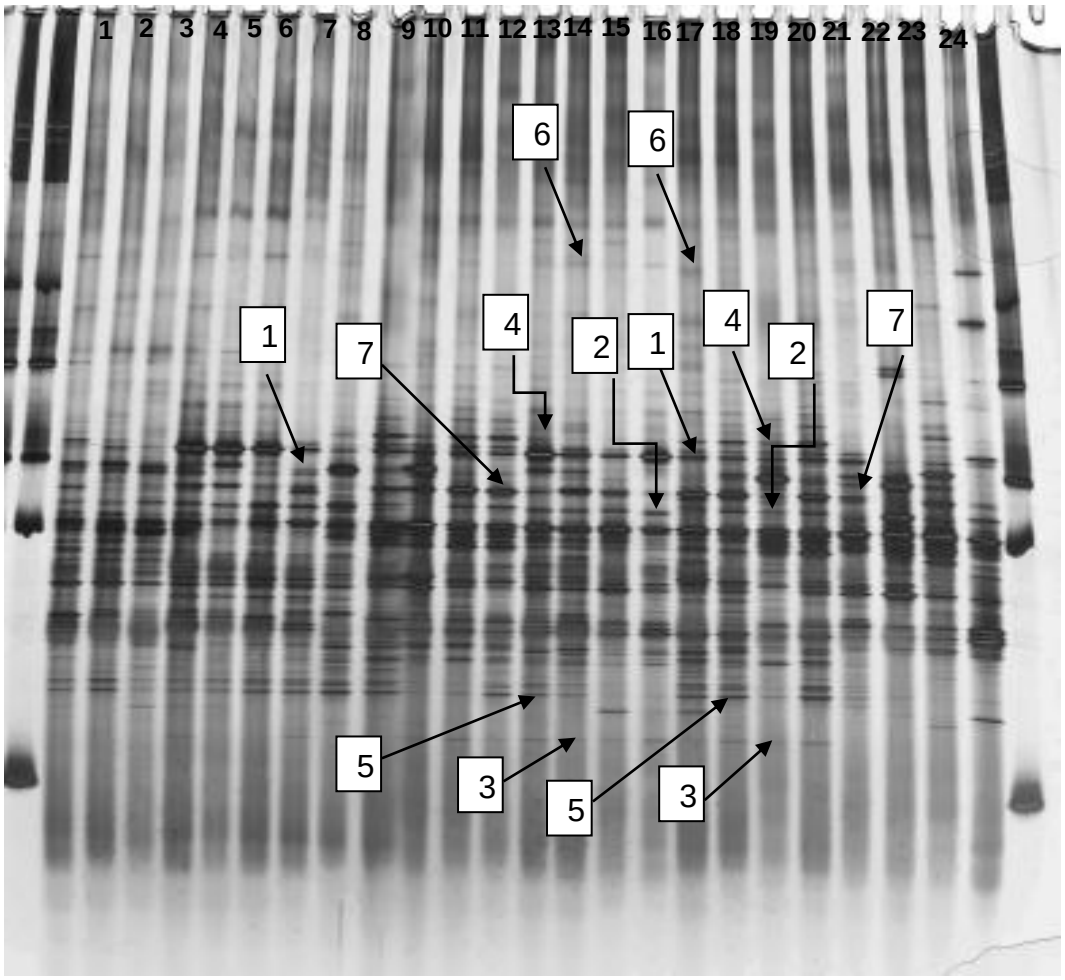

Figure 4-4. DGGE analysis of 16S rRNA gene fragment of total bacterial population from digesta of AOM-induced rats fed different dietary fiber and/or EGCG

Lane 1-4 = C; Lane 5-8 = P; Lane 9-12 = CE; Lane 13-16 = PE; Lane 17-20 = Cin; Lane 21-24 = CinL. Boxes numbered 1-7 were bands cut out for DNA extraction

Table 4-5. Closest relatives of band sequences excised from the acrylamide gel in Fig. 4.4. Samples are from digesta of AOM-induced rats fed different dietary fiber and/or EGCG.

Band	Fragment size/bp	Closest relative	Accession number	% Similarity	
1	196	*Lactobacillus johnsonii* DPC 6026	NC017477.1	100	Probiotic (Sgouras et al., 2005)
2	196	*Lactobacillus amylolyticus*	ADNY01000006.1	99	Lactid acid producer (Bohak et al., 1998)
3	173	*Oscillibacter valericigenes*	NC016048.1	97	Valerat producer (Iino et al., 2007)
4	170	*Clostridiales* sp. SM4/1	FP929060.1	96	Butyrate producer (genomesonline.org)
5	173	*Clostridium saccharolyticum*	NC014376.1	97	Acetic acid (Murray et al., 1982)
6	171	*Clostridiales* sp. SM4/1	FP929060.1	98	Butyrate producer (genomesonline.org)
7	177	*Lactobacillus amylolyticus*	ADNY01000006.1	100	Lactic acid producer (Bohak et al., 1998)

4.5. Discussion

This study has clearly shown that the efficacy of antioxidant (-)-epigallocatechin-3-gallate (EGCG) against AOM-induced colorectal cancer growth depends on the type of dietary fiber source added to the diet. Ingestion of EGCG to AOM-induced rats decreased colorectal cancer development when the rats were fed a diet containing cellulose. In contrast, when the dietary fiber source was pectin, EGCG promoted colon carcinogenesis. When compared with cellulose, dried cincau extracts and dried cincau leaves failed to demonstrate protection against CRC.

Pectin (P) enhanced the production of total SCFA including acetate, propionate and butyrate in digesta compared to cellulose (C). Pectin is a soluble dietary fiber and is more fermentable than cellulose (Barry et al., 1995, Titgemeyer et al., 1991). Surprisingly, ingestion of 0.1% EGCG by P group suppressed SCFA production in this group, from 107.3 µmol/g in P group to be 65.8 µmol/g in PE group (Table 4.3, $p < 0.05$), suggesting an inhibitory effect of EGCG or its metabolites on the fermentation process. This suggestion is supported by microbial analysis data of rats fed pectin and pectin+EGCG (Table 4.5). Reduction not only in total SCFA but also in individual acetate, propionate and butyrate levels indicates EGCG inhibits microbes involved in SCFA production. Butyrate producing bacteria (*Clostridiales sp. SM4/1*) and acetic acid producing bacteria (*Lactobacillus amylolyticus*) were detected in the digesta of these rats (Table 4.5), but the concentration of butyrate and acetic acid in PE group was lower than P group (Table 4.3). However, in the C group, inhibitory effects of EGCG or its

metabolites in SCFA production were not seen (Table 4.3, $p < 0.05$). These data suggest the effect of EGCG or its metabolites depend on the type of dietary fiber in the diet. Not all phenolic compounds suppress SCFA production in the colon; some are capable upregulating (Aprikian et al., 2003) and some of them have no effect (Juskiewicz et al., 2011b) . Therefore, in the CE group, EGCG or its metabolites has no effect on SCFA production.

Dried green cincau extract (Cin) and dried cincau leaf (CinL) did not exhibit any CRC protection effect when compared with cellulose (Table 4.4, Fig 4.1 and 4.2, $p<0.05$). It is possible that oven drying may result in a loss of phytochemical bioactive compounds in the dried cincau extracs (de Torres et al., 2010). There is a lack of data on polyphenolic content of green cincau leaf, however, like other tropical plants, they are thought to contain high concentrations of polyphenol compounds (Mustafa et al., 2010). It is possible that ingestion of cincau leaf extract may result in CRC prevention if phytochemical compounds in cincau are not lost during processing (Sikora et al., 2012). Moreover, cincau leaves contain chlorophyll and increasing chlorophyll intake, has previously been shown to reduce the risk of CRC in men (Balder et al., 2006), perhaps through inhibition of cytotoxic haem metabolite formation that has cytotoxic and hyperproliferative effects on colon cells (de Vogel et al., 2005). Chlorophyll was preserved better when dried using freeze-drying than oven-drying (Cosgrove and Guth, 1954). In the case of CinL, these dried leaves may contain lower anticancer compounds than the extracts.

The dominant microbial community in the colon depends on the type of the available substrate and the gut environment (Louis et al., 2007).

Cellulose and pectin induce a different microbial profile when they are administered to the rats (Fig. 4.3 and 4.4). As a consequence, when EGCG is given to rats fed cellulose (C) or pectin (P), it may be metabolized by a different microbial community in the rat colon depending on the diet. There are some bacterial groups that produce acetate, propionate and butyrate (Louis et al., 2007) but this will change in response to specific dietary interventions (Scott et al., 2011, Walker et al., 2011). Therefore, it is suggested that the microbes producing SCFA in C group and P group are different species, and thus they respond differently to EGCG or its metabolites.

Composition of human colon bacteria is individual (Walker et al., 2011), therefore, when subjects ingest a specific food, a specific metabolite profile differing in composition and level will be displayed by each subject (Gross et al., 2010). In this research, dietary fiber and mixed dietary fiber-EGCG intake did not exhibit distinct patterns in colon microbial profile (Fig. 4.3) suggesting an inter-individual variation of the initial composition of the gut microbiota in the rat (Walker et al., 2011). Some bands appear to be dominant in response to dietary fiber or antioxidant (Fig. 4.4). For instance, band 1 is detected as a distinct band in rats fed Cin (lane 5, 6 and 7) and CE (lane 13, 14, 15, and 16), but it also can be found with less intensity in PE (lane 9, 10, 11 and 12) or P (lane 17, 18, 19 and 20). This indicates that, even though rat colon microbial community has individual variation, dietary fiber and EGCG intake can produce marked changes in the gut microbiota (Walker et al., 2011).

There were differences in SCFA concentrations between digesta and faeces and in general SCFA concentration in the faeces was lower (Table 4.3). The high SCFA accumulation (smaller gap between SCFA faeces and digesta) refers to a smaller rate of SCFA absorption (Jacobasch et al., 2008). This study has shown the rate of SCFA absorption of PE group was lower than C, P and CE groups (Table 4.3) suggesting that SCFA transport in the colon may be down regulated. Moreover, butyrate absorption in the PE group was only 8.70 %, much lower than the C, P and CE groups (62.21%, 52.28% and 68.40%, respectively). Previously, Jacobasch et al. (Jacobasch et al., 2008) found that butyrate absorption was decreased as cancer progresses. Therefore, it may be that cancer of rats in PE group was the worse compared to other groups.

Normal colonic epithelial cells metabolize SCFA for energy production (Clausen and Mortensen, 1994). Transfer of these SCFA into colonocytes involves protein transporter sodium-coupled monocarboxylate transporter 1 (SMCT1) (Miyauchi et al., 2004) which is down regulated in colon cancer (Gupta et al., 2006) causing impaired SCFA absorption in the colon. This study has provided evidence to support the conclusion that cancer progression impaired SCFA absorption as the PE group that had the highest incident of ACF and cell proliferation absorbed less SCFA than C, P and CE groups. However, this data does not support a role for SCFA in colorectal cancer inhibition. The P group that produced the highest concentration of SCFA in their digesta had higher ACF than C and CE groups (Table 4.3 and 4.4).

The effect of feeding dietary pectin on colorectal cancer is not conclusive. In AOM-induced colon cancer rats model, pectin has previously been shown to significantly reduce tumour size and volume through upregulation of the proapoptotic caspase 3 and downregulation of proapoptotic bcl-2 family (Avivi-Green et al., 2000) as well as decreasing the multiplicity of colon tumours and the number of tumours (Ohkami et al., 1995, Watanabe et al., 1979) However, other experiments show that pectin induced activity of colon microbial enzymes that play a pivotal role in the genotoxicity of carcinogens (Freeman, 1986). In this experiment, pectin increased the number of ACF, possibly because of increasing bile acid secretion and COX-2 expression as shown previously (Jacobasch et al., 2008). Therefore, it is possible that efficacy of pectin in inhibition of CRC depend on some factors.

We demonstrate, for the first time, that a combination of pectin and EGCG induced cancer progression, characterized by an increase in total number of ACF, increase in the PCNA labelling index and PCNA positive cells and this effect was associated with increasing lipid peroxidation in the liver. Antioxidant and anticancer potential of EGCG did not prevent cancer initiation when this compound was administered together with pectin. Previously, 1% and 2% (w/v) tea infusion reduced the number of ACF as well as liver and colon lipid peroxidation significantly (Sengupta et al., 2003). Moreover a standardized green tea preparation consisting of 65% of (*2*)-epigallocatechin-3-gallate and 22% of other catechins inhibited cancer progression in AOM-induced rats (Xiao et al., 2008) and *Apc*$^{Min/+}$ *mice* model (Hao et al., 2007). The studies that show efficacy of EGCG against cancer initiation and progression were conducted using rat or mice model fed diet

containing non pectin dietary fiber. Ingestion of pectin as dietary fiber in our study most likely modified the colon environment resulting in circumstances that induced the pro-oxidant activity of EGCG.

The cancer preventative effect of EGCG antioxidant results through direct antioxidant or indigenous antioxidant induction activities (Lambert and Elias, 2010). However, there have been indications that EGCG caused oxidative damage in isolated and cellular DNA due to induction of H_2O_2 production (Furukawa et al., 2003). EGCG also induced pro-MMP-7 expression via O_2^- production in HT-29 and Caco-2 cell lines (Kim et al., 2007). Therefore, EGCG oxidation by superoxide is the possible mechanism by which EGGC exhibits pro-oxidant properties (Lambert and Elias, 2010).

The pro-oxidant ability of EGCG is one possible mechanism that could account for the pro-carcinogen effects observed from a diet combining pectin and EGCG. The addition of pectin highly increased the concentration of bile acids (deoxycholic acid and lithocholic acid) in comparison to a standard diet (Jacobasch et al., 2008). These acids, especially deoxycholic acid, cause lipid peroxidation by impairment of mitochondrial function, leading to the generation of free radicals (Hino et al., 2001) and induce lipid peroxidation in the liver (Delzenne et al., 1992). In our animal study, pectin administration may have increased the concentration of pro-oxidant bile acid in the colon resulting in the production of free radicals that are able to oxidize EGCG. As a result, EGCG poses pro-oxidant activity and induces more ACF formation.

4.6. Conclusion

Mixtures of dietary fiber and antioxidant *in vivo* do not always lead to positive synergistic effects in inhibiting CRC development. Combination of pectin and EGCG, a polyphenolic compound extracted from green tea, induced cancer progression that is characterized by increase in total number of aberrant crypt foci (ACF) and increase in cell proliferation, and this effect was associated with increasing lipid peroxidation in the liver. Furthermore, dried green cincau extract did not exhibit any CRC protective effects compared to cellulose and this may be caused by loss of its phytochemicals content during drying process. Overall, this research implies that beneficial effect of combination of dietary fiber and antioxidant does not automatically equate to their individual effect, but their combined effect depends on how they interact with the colon microbial community. Natural dietary fiber and antioxidant sources (as found in fruits, vegetables and plant extracts) may exhibit protective effect against CRC, and utilization of these source should consider the processing method such as drying process to protect their potency. Consumption of fresh dietary fiber antioxidants sources most likely to give the greatest protection

5. CONCLUSIONS AND FUTURE DIRECTIONS

CRC is a leading cause of cancer worldwide. Foods containing dietary fiber and antioxidants are suggested to be an important protective modifiers of CRC risk (Kumar et al., 2012,Yang and Suh, 2012, Pan et al., 2011). Dietary fiber is comprised principally of non-starch polysaccharides, which depending on the type of fiber, are subjected to varying degrees of fermentation by the resident bacterial ecosystem in the gastrointestinal tract producing the main end products of SCFA, mainly acetate, propionate, and butyrate (Turner and Lupton, 2011, Kumar et al., 2012). In this thesis the effect of different dietary fiber types (pectin, inulin, cellulose and cincau and their combination) on SCFA production using anaerobic batch cultures inoculated with human faecal slurries was studied. Soluble fiber (pectin and inulin) resulted in significantly higher SCFA production in fermentation supernatant (FS) than was observed with insoluble (cellulose) fiber. Also, the combination of inulin and cellulose reduced SCFA, including the amount of butyrate, compared with inulin alone while combinations of pectin and inulin resulted in the highest production of butyrate, although the proportion of butyrate was much lower than observed with inulin alone.

SCFA are thought to play a role in maintaining normal bowel function and may contribute to protection against gastrointestinal diseases (Chan and Giovannucci, 2010, Lattimer and Haub, 2010). To study the role of SCFA in CRC prevention, effect of pure SCFA (single or mixture) and FS containing SCFA on the proliferation, differentiation and apoptosis of Caco-2 cells were investigated. Pure SCFA (mixture or single) significantly inhibited proliferation

and induced differentiation and apoptosis of Caco-2 cells irrespective of the media pH. Even though the inhibition of cell growth by FS was dependent on the amount of SCFA generated (in particular butyrate) their effects on cell differentiation and apoptosis were not explainable by their butyrate content. Pure butyrate inhibited cell growth through induction of apoptosis as indicated by increased caspase 3/7 activity that could be inhibited with a caspase inhibitor. Interestingly, it may be possible that, besides SCFA, other phytochemical compounds in the cincau contribute to inhibition of cell growth through an autophaghic cell death mechanism which can not be measured by either the caspase or LDH assay (Chapter three). Cincau extracts contain dietary fiber and antioxidant, therefore it is suggested that the beneficial effect of the extracts was promoted by a combination of dietary fiber and antioxidant. Therefore, in a final study we evaluated the effect of dried cincau extract and combinations of dietary fiber and antioxidant sources on CRC carcinogenesis using AOM-induced rat model. Our results indicating that dried green cincau extract did not exhibit any CRC protective effects compared to cellulose might be due to loss of cincau phytochemical content during the drying process. Furthermore, we reveal that combination of dietary fiber (pectin) and antioxidant ((-)-epigallocatechin-3-gallate (EGCG) exhibit an adverse effect on CRC development.

Dietary fiber in nature does not exist as a single form but as a mixture. Pectin in apple for instance, exists as part of the cell wall of apple together with cellulose (Aprikian et al., 2003). Therefore, it is possible that the beneficial effects of fruit and vegetables are achieved by the combination of dietary fiber within different foods. Several *in vitro* and *in vivo* studies

(Juskiewicz and Zdunczyk, 2004, Henningsson et al., 2002, Muir et al., 2004a, Khan and Edwards, 2005) demonstrate the increased beneficial health effect when two specific dietary fibers are combined as indicated by some faecal parameters such as faecal butyrate content. My results demonstrate (Chapter three) that FS from some dietary fiber will display different patterns when they were combined with others. Soluble dietary fibers will increase SCFA content from non-soluble dietary fiber (Titgemeyer et al., 1991), but the effect of the mixing on the SCFA pattern (such as individual SCFA concentration) was not predictable.

Compounds such as SCFA, which are produced via bacterial fermentation of undigested dietary fibers are capable of inhibiting cancer (Scott et al., 2011). Butyrate, one such SCFA is the principal energy source for colonocytes and appears to offer the greatest protection against CRC (Hinnebusch et al., 2002, Whitehead et al., 1986). However my results showed that the ability of the FS to modulate parameters of cell growth, differentiation and apoptosis was not only dependent on the butyrate concentration but possibly also on other unidentified compounds. Butyrate contributed to the inhibition of growth when the dietary fiber was inulin, resulting in a 53% inhibition in cell growth, but the effects on cell differentiation and apoptosis were not explainable by its butyrate content alone. My data in accordance with others, indicate the existence of unidentified compound or compounds in FS that have the ability to modulate Caco-2 cell growth (Beyer-Sehlmeyer et al., 2003, Sauer et al., 2007, Kiefer et al., 2006). The compound/s may be a product of microbial fermentation or they may originally exist as a component of fresh fecal slurry. Future

research should consider the type of diets consumed by the volunteers as the source of colon microbial prior to collection of the fresh faeces, so the effects can be traced back to the type of diet. However this type of research may be difficult to perform as it would require volunteers to consume a restrictive diet for weeks at a time and would require wash out periods (Munjal et al., 2009, Kabeerdoss et al., 2012, Payne et al., 2012).

Previous research showed that inulin FS induced apoptotic cell death as measured by detection of poly(ADP-ribose) polymerase (PARP) cleavage using western blotting in the human colon LT97 but not HT29 cell lines after 24 h (Munjal et al., 2009). I results indicate that a non-apoptotic pathway might be involved in cell death in response to inulin FS treatment. I found that butyrate induced cell death through both a caspase 3/7 dependent and caspase 3/7 independent pathway (Chapter two). This suggests that butyrate in inulin FS may play an important role in colon cancer cells growth inhibition as both an inducer of apoptotic and non-apoptotic cell death. However, in cincau extract FS, butyrate is not the single bioactive compound, because even though it contains lower levels of butyrate than the inulin FS (5.5 mM and 37.7 M, respectively) the percentage of growth inhibition from the FS was no different. Interestingly, cincau did not induce caspase 3/7 activity, but it caused a release of lower level of LDH compared to faecal blank so its effects were likely not to be necrotic (Lockshin and Zakeri, 2004), and it was not affected by caspase inhibitor either. Thus there is the potential that cincau could trigger an autophagic cell death mechanism (Huang et al., 2010, Harun et al., 2012).

Green cincau (*Premna oblongifolia* Merr) is a tropical plant belonging to the *Verbenaceae* family. It is commonly extracted with water to prepare a traditional drink containing a polysaccharide forming gel. The extract had high pectin content (19.71%) and antioxidant activity as well as interesting characteristics (high water holding capacity and viscosity), suggesting possible uses in the development of fibre enriched foods (Nurdin et al., 2005). There are limited publications on this plant, but preliminary research on the leaf extract indicates promising results. Cincau extract contains some phytochemicals (Aryudhani, 2011) that, in combination with SCFA, may cause cell death (Lea et al., 2010). Solution of hot water extract of the cincau leaves (25-50 ppm (w/v)) induced interferon-γ, interleukin-2 and tumour necrosis factor-α in mouse spleen cells, indicating that the extract has the ability to induce cell-mediated immune responses *in vitro* (Nurdin et al., 2003). Cincau extract showed laxative effect and effectively induced the growth of lactic acid bacteria in the rat colon (Nurdin, 2007a). Revealing the efficacy of green cincau as a chemo-preventative agent for CRC would increase the economic value of this plant and would bring economic advantages for Indonesia. The work presented in this thesis suggests that further efficacy studies should be performed on this interesting plant based dietary fiber.

It has been mentioned above that it is the dietary fiber and antioxidants that are found in fruit and vegetables that lead to their beneficial effects against CRC. My results presented in Chapter Three suggested possible further promising effects when dietary fiber is naturally combined with phytochemical compounds (such as those found in cincau extracts).

Therefore the next project (Chapter four) was aimed to evaluate the effect of the green cincau extract and dietary fibre and phytochemicals (EGCG) mixture on CRC development using the AOM-induced ACF formation 13 wk rat model.

In the *in vitro* model (Chapter three), cincau extract FS inhibited cell growth significantly compared to faecal blank FS through induction of a caspase 3/7 independent cell death pathway. However, in the AOM-induced rat model, dried green cincau extract (Cin) and dried cincau leaves (CinL) did not exhibit any protective effects compared to cellulose. In the case of dried cincau extract, the discrepancy may be caused by the difference in the drying process applied between the two chapters. Cincau extract for *in vitro* experiment 2 (Chapter three) was dried using freeze drying, meanwhile cincau extract for *in vivo* experiment (Chapter four) was dried using oven drying at 50^{o}C. Freeze-drying was preserved chlorophyll better than oven-drying (Cosgrove and Guth, 1954). Due to financial and logistical reasons it was impractical to hot water extract and freeze dry the large amount of cincau required for a 12 wk feeding trial. As oven drying uses higher temperatures than freeze drying, loss of phytochemical bioactive compounds during the oven process will be higher than freeze drying process (de Torres et al., 2010) or novel compounds that are potentially toxic to cells may have formed.

Diet containing cincau extract (Cin) or cincau leaf powder (CinL) produced acetate, propionate and butyrate levels in colon digesta however these did not reach statistical difference compared to the cellulose group

(Chapter four). Previous work has indicated that the protective effect of consumption of dietary fiber against CRC development in AOM-induced rat appears to be related to SCFA production in the colon (Le Leu et al., 2007), therefore, the work presented here suggests that less protective effect of cincau extract is caused by less SCFA production. There is a lack of data on polyphenolic content of green cincau leaves, but like other tropical plants (Mustafa et al., 2010), cincau leaves are suggested to contain high levels of polyphenol compounds. Moreover, as cincau leaves contain chlorophyll and increasing chlorophyll intake was shown to reduce the CRC risk in men (Balder et al., 2006). It was proposed that chlorophyll works through the inhibition of cytotoxic heme metabolite formation that has cytotoxic and hyperproliferative effects on colon cells (de Vogel et al., 2005). Chlorophyll was preserved better when it was dried using the freeze-drying than oven-drying method (Cosgrove and Guth, 1954). More research is needed to establish the efficacy of green cincau leaves against CRC using material that is prepared using a more traditional method and freeze dried but this type of experiment may be costly.

Data in Chapter four also demonstrates that mixtures of dietary fiber and antioxidant *in vivo* do not always lead to positive synergetic effects in inhibiting CRC development. Combination of pectin and EGCG, a polyphenolic compound extracted from green tea, induced cancer progression that is characterized by increase in total number of aberrant crypt foci (ACF) and increase in cell proliferation, and this effect was associated with increasing lipid peroxidation in the liver. This adverse (cancer induction) effect was not observed when the pectin was substituted with the

cellulose. The protective effect of EGCG consumption against CRC development appears to be dependent on the type of the dietary fiber source in the diet and the mechanism may be via dietary fiber leading to modification of antioxidant/prooxidant properties. The addition of pectin may enhance concentration of pro-oxidant bile acid in the colon resulting in the harbouring of free radicals that are able to oxidize EGCG. As a result, EGCG poses pro-oxidant activity and induces more ACF formation and induce lipid peroxidation in the liver (Jacobasch et al., 2008, Hino et al., 2001, Delzenne et al., 1992).

Previous research showed that pectin increased butyrate producing *Clostridiales* and expression of genes involved in butyrate production (Licht et al., 2010). Pectin suppressed the formation of AOM-induced ACF as well as crypt multiplicity and number ACF/cm^2 in rats (Rao et al., 1998). It also inhibited the increase in cell proliferation and crypt length in transmissible murine colonic hyperplasia induced by *Cytrobacter rodentium* through blocking the increase in cellular β-catenin, cyclin D1 and c-myc levels (Umar et al., 2003). In combination with fish oil, pectin induced colonocyte apoptosis in AOM-induced rats through the depression of peroxisome proliferator-activated receptor δ expression and prostaglandin E(2) (PGE2) level, and elevation of PGE3 concentration (Vanamala et al., 2008) and enhanced Smad3 expression and activation in the gut thus reducing the risk of CRC development (Cao et al., 2011). Meanwhile, studies in animal models of carcinogenesis have shown that EGCG can inhibit tumorigenesis during the initiation, promotion and progression stages (Shirakami et al., 2008), suppress the multiplicity and volume of colonic neoplasms and result in a

lesser degree of malignancy (Shirakami et al., 2008). Additionally, EGCG in combinations with curcumin (Xu et al., 2010) or sulindac (Ohishi et al., 2002) produced a synergetic effect on inhibition of CRC growth. In my work when pectin and EGCG were combined (Chapter four), their beneficial effect on CRC prevention was not observed.

I also found possible inhibitory effects of EGCG or EGGC metabolites in SCFA production in the colon digesta when the source of dietary fiber was pectin (Chapter four). EGGC increased SCFA concentration when the dietary fiber source was cellulose, and an opposite effect was observed when the dietary fiber source was pectin. This may suggest that pectin and cellulose induce growth of different bacteria in the colon and the bacteria induced by pectin were suppressed by EGGC or EGGC metabolites. Considering that SCFA offer the greatest protection against CRC, this data reveal that the colon environment including bacteria composition plays a prominent role on efficacy of dietary fiber or antioxidant in colon cancer protection (Table 4.3, 4.4, and 4.5). Beneficial effects of mixtures of dietary fiber and antioxidant on CRC protection may not be not be possible to predict based on their single effect because the effect will be dependent on their interaction with the colon environment (Scott et al., 2011, Juskiewicz et al., 2011b).

My *in vivo* data demonstrate that SCFA concentration of colon digesta is not inversely associated with the ACF numbers. Rats fed diet containing pectin produced higher SCFA concentration in the colon than cellulose, and but they had total ACF higher than rats fed diets containing cellulose. This result suggests there are other compounds that may be involved in the carcinogenesis process in rats fed diet containing pectin. Concomitant with

the data in Chapter three, data in Chapter four suggest that SCFA concentration is not the single factor that modulates colon cancer progression. In future research, identification of compounds that may interact with SCFA to inhibit CRC development should be carried out.

CRC rates are increasing rapidly due to rapid economic development in many countries, and this increasing incidence can be attributed to lifestyle, hereditary and dietary factors (Bener, 2011). Globally, around 95% of CRC incidences are sporadic with the strongest risk factor being diet (Watson and Collins, 2011). Modifications of the diet, such as increasing consumption of foods from plant origin and reduction of red meat intake, could substantially reduce CRC incidence and mortality (Gingras and Beliveau, 2011). The beneficial effect of individual dietary fibers does not automatically synergize with the positive effects of potential antioxidants, and their combined effect will depend on how they interact with the colon microbiota of the individual (Juskiewicz et al., 2011b, Palafox-Carlos et al., 2011). Information derived from the function of different dietary fiber as well as their interactions among them or with phytochemicals and gut microbiota of the human host would improve our understanding of the effects of dietary fiber and antioxidants on CRC. It is also possible that increase in supplement intake in new developed countries contributes to augmentation of CRC incidence (Hart et al., 2012, Martinez et al., 2012). Public awareness campaigns on promoting prevention of modifiable risk factors (Bener, 2011) by promoting an increae in fresh fruit and vegetables intake and introduction of supplement guidelines on intake of combinations of vitamin and antioxidant supplements are urgently needed.

Natural mixtures of dietary fiber and antioxidant sources (as found in fruits, vegetables and plant extracts) may exhibit protective effects against CRC, and utilization of these sources should consider the processing method such as drying process to protect their potency (de Torres et al., 2010). Most phytochemicals that exhibit anticancer effects such as vitamins and phenolic compounds are heat sensitive, therefore, application of heat to process these compounds will increase their loss. Consumption of fresh dietary fiber antioxidants sources may result in the greatest protection against CRC(Hart et al., 2012).

6. APPENDIX

6.1. Analysis of Varian (Anova) and LSD Tables

Table 6-1. Anova of Effect of SCFA and pH on Caco-2 cell proliferation

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	96117.509[a]	31	3100.565	22.961	.000
Intercept	1039325.118	1	1039325.118	7696.745	.000
pH	2039.514	3	679.838	5.035	.002
SCFA	90674.437	7	12953.491	95.927	.000
pH * SCFA	3403.558	21	162.074	1.200	.251

Error	34568.799	256	135.034		
Total	1170011.425	288			
Corrected Total	130686.308	287			

a. R Squared = .735 (Adjusted R Squared = .703)

Table 6-2. LSD of Effect of pH on Caco-2 cell proliferation

(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6.00	6.50	-1.5674	1.93674	.419	-5.3814	2.2465
	7.00	-6.1889*	1.93674	.002	-10.0029	-2.3750
	7.50	-5.7731*	1.93674	.003	-9.5870	-1.9591
6.50	6.00	1.5674	1.93674	.419	-2.2465	5.3814
	7.00	-4.6215*	1.93674	.018	-8.4355	-.8075
	7.50	-4.2056*	1.93674	.031	-8.0196	-.3916
7.00	6.00	6.1889*	1.93674	.002	2.3750	10.0029
	6.50	4.6215*	1.93674	.018	.8075	8.4355
	7.50	.4159	1.93674	.830	-3.3981	4.2299
7.50	6.00	5.7731*	1.93674	.003	1.9591	9.5870
	6.50	4.2056*	1.93674	.031	.3916	8.0196
	7.00	-.4159	1.93674	.830	-4.2299	3.3981

Based on observed means. The error term is Mean Square(Error) = 135.034.

*. The mean difference is significant at the .05 level.

Table 6-3. LSD of Effect of SCFA on Caco-2 cell proliferation

(I) SCFA	(J) SCFA	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	Acet	-17.6300*	2.73896	.000	-23.0238	-12.2363
	B	12.4248*	2.73896	.000	7.0310	17.8185
	But10	9.6766*	2.73896	.000	4.2828	15.0704
	But20	12.0900*	2.73896	.000	6.6962	17.4837
	But5	-1.1483	2.73896	.675	-6.5420	4.2455
	Ctrl	-42.2153*	2.73896	.000	-47.6090	-36.8215
	Prop	8.4959*	2.73896	.002	3.1022	13.8897
Acet	A	17.6300*	2.73896	.000	12.2363	23.0238
	B	30.0548*	2.73896	.000	24.6610	35.4486
	But10	27.3066*	2.73896	.000	21.9129	32.7004
	But20	29.7200*	2.73896	.000	24.3262	35.1138
	But5	16.4818*	2.73896	.000	11.0880	21.8755
	Ctrl	-24.5852*	2.73896	.000	-29.9790	-19.1915
	Prop	26.1259*	2.73896	.000	20.7322	31.5197
B	A	-12.4248*	2.73896	.000	-17.8185	-7.0310
	Acet	-30.0548*	2.73896	.000	-35.4486	-24.6610
	But10	-2.7482	2.73896	.317	-8.1419	2.6456
	But20	-.3348	2.73896	.903	-5.7286	5.0590
	But5	-13.5730*	2.73896	.000	-18.9668	-8.1793
	Ctrl	-54.6400*	2.73896	.000	-60.0338	-49.2463
	Prop	-3.9288	2.73896	.153	-9.3226	1.4649
But10	A	-9.6766*	2.73896	.000	-15.0704	-4.2828
	Acet	-27.3066*	2.73896	.000	-32.7004	-21.9129
	B	2.7482	2.73896	.317	-2.6456	8.1419
	But20	2.4134	2.73896	.379	-2.9804	7.8071
	But5	-10.8249*	2.73896	.000	-16.2186	-5.4311
	Ctrl	-51.8919*	2.73896	.000	-57.2856	-46.4981
	Prop	-1.1807	2.73896	.667	-6.5744	4.2131

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But20	A	-12.0900*	2.73896	.000	-17.4837	-6.6962
	Acet	-29.7200*	2.73896	.000	-35.1138	-24.3262
	B	.3348	2.73896	.903	-5.0590	5.7286
	But10	-2.4134	2.73896	.379	-7.8071	2.9804
	But5	-13.2382*	2.73896	.000	-18.6320	-7.8445
	Ctrl	-54.3052*	2.73896	.000	-59.6990	-48.9115
	Prop	-3.5941	2.73896	.191	-8.9878	1.7997
But5	A	1.1483	2.73896	.675	-4.2455	6.5420
	Acet	-16.4818*	2.73896	.000	-21.8755	-11.0880
	B	13.5730*	2.73896	.000	8.1793	18.9668
	But10	10.8249*	2.73896	.000	5.4311	16.2186
	But20	13.2382*	2.73896	.000	7.8445	18.6320
	Ctrl	-41.0670*	2.73896	.000	-46.4608	-35.6732
	Prop	9.6442*	2.73896	.001	4.2504	15.0379
Ctrl	A	42.2153*	2.73896	.000	36.8215	47.6090
	Acet	24.5852*	2.73896	.000	19.1915	29.9790
	B	54.6400*	2.73896	.000	49.2463	60.0338
	But10	51.8919*	2.73896	.000	46.4981	57.2856
	But20	54.3052*	2.73896	.000	48.9115	59.6990
	But5	41.0670*	2.73896	.000	35.6732	46.4608
	Prop	50.7112*	2.73896	.000	45.3174	56.1050
Prop	A	-8.4959*	2.73896	.002	-13.8897	-3.1022
	Acet	-26.1259*	2.73896	.000	-31.5197	-20.7322
	B	3.9288	2.73896	.153	-1.4649	9.3226
	But10	1.1807	2.73896	.667	-4.2131	6.5744
	But20	3.5941	2.73896	.191	-1.7997	8.9878
	But5	-9.6442*	2.73896	.001	-15.0379	-4.2504
	Ctrl	-50.7112*	2.73896	.000	-56.1050	-45.3174

Based on observed means. The error term is Mean Square(Error) = 135.034.

*. The mean difference is significant at the .05 level.

Table 6-4. Anova of Effect of SCFA and pH on Caco-2 cell differentiation (Alkaline phosphatise activity)

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	4602.723[a]	9	511.414	1.567	.193
Intercept	12760.646	1	12760.646	39.089	.000
pH	6.601	1	6.601	.020	.888
SCFA	4294.739	4	1073.685	3.289	.032
pH * SCFA	301.384	4	75.346	.231	.918
Error	6528.982	20	326.449		
Total	23892.350	30			
Corrected Total	11131.705	29			

a. R Squared = .413 (Adjusted R Squared = .150)

Table 6-5. LSD of Effect of SCFA on Caco-2 cell differentiation (Alkaline phosphatise activity)

(I) SCFA	(J) SCFA	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Acet	B	-26.7327*	10.43151	.019	-48.4925	-4.9730
	But20	-12.2639	10.43151	.254	-34.0236	9.4959
	Ctrl	6.0927	10.43151	.566	-15.6671	27.8524
	Prop	-18.4909	10.43151	.092	-40.2507	3.2688
B	Acet	26.7327*	10.43151	.019	4.9730	48.4925
	But20	14.4689	10.43151	.181	-7.2909	36.2286
	Ctrl	32.8254*	10.43151	.005	11.0656	54.5851
	Prop	8.2418	10.43151	.439	-13.5180	30.0015
But20	Acet	12.2639	10.43151	.254	-9.4959	34.0236
	B	-14.4689	10.43151	.181	-36.2286	7.2909
	Ctrl	18.3565	10.43151	.094	-3.4032	40.1163
	Prop	-6.2271	10.43151	.557	-27.9868	15.5327
Ctrl	Acet	-6.0927	10.43151	.566	-27.8524	15.6671
	B	-32.8254*	10.43151	.005	-54.5851	-11.0656
	But20	-18.3565	10.43151	.094	-40.1163	3.4032
	Prop	-24.5836*	10.43151	.029	-46.3434	-2.8239
Prop	Acet	18.4909	10.43151	.092	-3.2688	40.2507
	B	-8.2418	10.43151	.439	-30.0015	13.5180
	But20	6.2271	10.43151	.557	-15.5327	27.9868
	Ctrl	24.5836*	10.43151	.029	2.8239	46.3434

Based on observed means. The error term is Mean Square(Error) = 326.449. *. The mean difference is significant at the .05 level.

Table 6-6. Anova of Effect of SCFA and pH on Caspase 3/7 activity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	24568864.65[a]	13	1889912.6	19.45	.000
Intercept	1.570E8	1	1.570E8	1616.49	.000
pH	1535000.96	1	1535000.9	15.80	.001
SCFA	13502280.41	5	2700456.1	27.801	.000
Block	8507987.04	2	4253993.5	43.795	.000
pH * SCFA	204627.55	5	40925.510	.421	.828
Error	1942672.07	20	97133.604		
Total	1.843E8	34			
Corrected Total	26511536.68	33			

a. R Squared = .927 (Adjusted R Squared = .879)

Table 6-7. LSD of Effect of SCFA on Caspase 3/7 activity

(I) SCFA	(J) SCFA	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Acet	B	-1102.2500*	179.938	.000	-1477.5952	-726.9048
	But20	-1882.7500*	179.9385	.000	-2258.0952	-1507.4048
	Ctrl	-265.1667	179.938	.156	-640.5118	110.1785
	Prop	-1061.0000*	179.938	.000	-1436.3452	-685.6548
	Stau	-345.6250	201.177	.101	-765.2736	74.0236
B	Acet	1102.2500*	179.938	.000	726.9048	1477.5952
	But20	-780.5000*	179.938	.000	-1155.8452	-405.1548
	Ctrl	837.0833*	179.938	.000	461.7382	1212.4285
	Prop	41.2500	179.938	.821	-334.0952	416.5952
	Stau	756.6250*	201.177	.001	336.9764	1176.2736
But20	Acet	1882.7500*	179.938	.000	1507.4048	2258.0952
	B	780.5000*	179.938	.000	405.1548	1155.8452
	Ctrl	1617.5833*	179.938	.000	1242.2382	1992.9285
	Prop	821.7500*	179.938	.000	446.4048	1197.0952
	Stau	1537.1250*	201.177	.000	1117.4764	1956.7736
Ctrl	Acet	265.1667	179.938	.156	-110.1785	640.5118
	B	-837.0833*	179.938	.000	-1212.4285	-461.7382
	But20	-1617.5833*	179.938	.000	-1992.9285	-1242.2382
	Prop	-795.8333*	179.938	.000	-1171.1785	-420.4882
	Stau	-80.4583	201.177	.693	-500.1070	339.1903

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Prop	Acet	1061.0000*	179.938	.000	685.6548	1436.3452
	B	-41.2500	179.938	.821	-416.5952	334.0952
	But20	-821.7500*	179.938	.000	-1197.0952	-446.4048
	Ctrl	795.8333*	179.938	.000	420.4882	1171.1785
	Stau	715.3750*	201.177	.002	295.7264	1135.0236
Stau	Acet	345.6250	201.177	.101	-74.0236	765.2736
	B	-756.6250*	201.177	.001	-1176.2736	-336.9764
	But20	-1537.1250*	201.177	.000	-1956.7736	-1117.4764
	Ctrl	80.4583	201.177	.693	-339.1903	500.1070
	Prop	-715.3750*	201.177	.002	-1135.0236	-295.7264

Based on observed means. The error term is Mean Square(Error) = 97133.604.

*. The mean difference is significant at the .05 level.

Table 6-8. Anova of Effect of SCFA and Caspase inhibitor on Caspase 3/7 activity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	24282667.267[a]	6	4047111.211	33.880	.000
Intercept	25317510.41	1	25317510.41	211.940	.000
SCFA	24280086.83	4	6070021.7	50.814	.000
Block	2580.433	2	1290.217	.011	.989
Error	955646.567	8	119455.8		
Total	50555824.25	15			
Corrected Total	25238313.83	14			

a. R Squared = .962 (Adjusted R Squared = .934)

Table 6-9. LSD of Effect of SCFA and Caspase inhibitor on Caspase 3/7 activity

(I) SCFA	(J) SCFA	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
B20	Ctrl	2328.5000*	282.201	.000	1677.7441	2979.2559
	IB20	3394.3333*	282.201	.000	2743.5774	4045.0892
	Istau	3431.1667*	282.201	.000	2780.4108	4081.9226
	Stau	1650.1667*	282.201	.000	999.4108	2300.9226
Ctrl	B20	-2328.5000*	282.201	.000	-2979.2559	-1677.7441
	IB20	1065.8333*	282.201	.005	415.0774	1716.5892
	Istau	1102.6667*	282.201	.004	451.9108	1753.4226
	Stau	-678.3333*	282.201	.043	-1329.0892	-27.5774
IB20	B20	-3394.3333*	282.201	.000	-4045.0892	-2743.5774
	Ctrl	-1065.8333*	282.201	.005	-1716.5892	-415.0774
	Istau	36.8333	282.201	.899	-613.9226	687.5892
	Stau	-1744.1667*	282.201	.000	-2394.9226	-1093.4108
Istau	B20	-3431.1667*	282.201	.000	-4081.9226	-2780.4108
	Ctrl	-1102.6667*	282.201	.004	-1753.4226	-451.9108
	IB20	-36.8333	282.201	.899	-687.5892	613.9226
	Stau	-1781.0000*	282.201	.000	-2431.7559	-1130.2441
Stau	B20	-1650.1667*	282.201	.000	-2300.9226	-999.4108
	Ctrl	678.3333*	282.201	.043	27.5774	1329.0892
	IB20	1744.1667*	282.201	.000	1093.4108	2394.9226
	Istau	1781.0000*	282.201	.000	1130.2441	2431.7559

Based on observed means. The error term is Mean Square(Error) = 119455.821.

*. The mean difference is significant at the .05 level.

Table 6-10. Anova of Effect of SCFA and Caspase inhibitor on LDH release

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.395E13	6	2.325E12	3.498	.053
Intercept	1.721E14	1	1.721E14	258.807	.000
SCFA	6.966E12	4	1.741E12	2.619	.115
Block	6.987E12	2	3.493E12	5.254	.035
Error	5.319E12	8	6.649E11		
Total	1.913E14	15			
Corrected Total	1.927E13	14			

a. R Squared = .724 (Adjusted R Squared = .517)

Table 6-11. LSD of Effect of SCFA and Caspase inhibitor on LDH release

(I) SCFA	(J) SCFA	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
B20	Ctrl	1861685.5*	665764.10	.023	326430.72	3396940.2
	IB20	937764.67	665764.10	.197	-597490.10	2473019.4
	Istau	1648073.8*	665764.10	.038	112819.06	3183328.6
	Stau	1618819.6*	665764.10	.041	83564.895	3154074.43
Ctrl	B20	-1861685.5*	665764.10	.023	-3396940.2	-326430.7
	IB20	-923920.83	665764.10	.203	-2459175.6	611333.938
	Istau	-213611.7	665764.10	.757	-1748866.4	1321643.10
	Stau	-242865.8	665764.10	.725	-1778120.6	1292388.93
IB20	B20	-937764.67	665764.10	.197	-2473019.4	597490.104
	Ctrl	923920.83	665764.10	.203	-611333.	2459175.60
	Istau	710309.16	665764.10	.317	-824945.60	2245563.93
	Stau	681055.00	665764.10	.336	-854199.7	2216309.77
Istau	B20	-1648073.8*	665764.10	.038	-3183328.6	-112819.06
	Ctrl	213611.67	665764.10	.757	-1321643.1	1748866.4
	IB20	-710309.17	665764.10	.317	-2245563.9	824945.60
	Stau	-29254.167	665764.10	.966	-1564508.9	1506000.6
Stau	B20	-1618819.7*	665764.10	.041	-3154074.4	-83564.89
	Ctrl	242865.8	665764.10	.725	-1292388.9	1778120.60
	IB20	-681055.00	665764.10	.336	-2216309.7	854199.77
	Istau	29254.17	665764.10	.966	-1506000.6	1564508.93

Based on observed means. The error term is Mean Square(Error) = 664862758447.471. *. The mean difference is significant at the .05 level

Table 6-12. Anova of Effect of SCFA and Caspase inhibitor on cell proliferation (% growth).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5169.959[a]	6	861.660	7.357	.006
Intercept	74398.727	1	74398.727	635.24	.000
SCFA	3410.118	4	852.530	7.279	.009
Block	1759.841	2	879.920	7.513	.015
Error	936.953	8	117.119		
Total	80505.639	15			
Corrected Total	6106.912	14			

a. R Squared = .847 (Adjusted R Squared = .732)

Table 6-13. LSD of Effect of SCFA and Caspase inhibitor on cell proliferation (% growth).

(I) SCFA	(J) SCFA	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
B20	Ctrl	-41.4173*	8.83626	.002	-61.7938	-21.0409
	IB20	-5.7618	8.83626	.533	-26.1383	14.6146
	Istau	-3.1030	8.83626	.735	-23.4794	17.2735
	Stau	-8.9378	8.83626	.341	-29.3143	11.4386
Ctrl	B20	41.4173*	8.83626	.002	21.0409	61.7938
	IB20	35.6555*	8.83626	.004	15.2791	56.0319
	Istau	38.3144*	8.83626	.002	17.9379	58.6908
	Stau	32.4795*	8.83626	.006	12.1031	52.8559
IB20	B20	5.7618	8.83626	.533	-14.6146	26.1383
	Ctrl	-35.6555*	8.83626	.004	-56.0319	-15.2791
	Istau	2.6589	8.83626	.771	-17.7176	23.0353
	Stau	-3.1760	8.83626	.729	-23.5524	17.2004
Istau	B20	3.1030	8.83626	.735	-17.2735	23.4794
	Ctrl	-38.3144*	8.83626	.002	-58.6908	-17.9379
	IB20	-2.6589	8.83626	.771	-23.0353	17.7176
	Stau	-5.8349	8.83626	.528	-26.2113	14.5416
Stau	B20	8.9378	8.83626	.341	-11.4386	29.3143
	Ctrl	-32.4795*	8.83626	.006	-52.8559	-12.1031
	IB20	3.1760	8.83626	.729	-17.2004	23.5524
	Istau	5.8349	8.83626	.528	-14.5416	26.2113

Based on observed means. The error term is Mean Square(Error) = 117.119.

*. The mean difference is significant at the .05 level.

Table 6-14. Anova effect of dietary fiber on Acetate content of fermentation supernatant

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10328.407[a]	10	1032.841	8.967	.000
Intercept	35184.750	1	35184.750	305.468	.000
Block	104.010	3	34.670	.301	.824
DF	10224.397	7	1460.628	12.681	.000
Error	2418.843	21	115.183		
Total	47932.000	32			
Corrected Total	12747.250	31			

a. R Squared = .810 (Adjusted R Squared = .720)

Table 6-15. Anova effect of dietary fiber on Acetate content of fermentation supernatant

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	Cin	-32.1400*	7.58891	.000	-47.9220	-16.3580
	FB	2.7575	7.58891	.720	-13.0245	18.5395
	I	-36.4125*	7.58891	.000	-52.1945	-20.6305
	IC	-24.0925*	7.58891	.005	-39.8745	-8.3105
	P	-25.9075*	7.58891	.003	-41.6895	-10.1255
	PC	-32.5700*	7.58891	.000	-48.3520	-16.7880
	PI	-55.3675*	7.58891	.000	-71.1495	-39.5855
Cin	C	32.1400*	7.58891	.000	16.3580	47.9220
	FB	34.8975*	7.58891	.000	19.1155	50.6795
	I	-4.2725	7.58891	.579	-20.0545	11.5095
	IC	8.0475	7.58891	.301	-7.7345	23.8295
	P	6.2325	7.58891	.421	-9.5495	22.0145
	PC	-.4300	7.58891	.955	-16.2120	15.3520
	PI	-23.2275*	7.58891	.006	-39.0095	-7.4455
FB	C	-2.7575	7.58891	.720	-18.5395	13.0245
	Cin	-34.8975*	7.58891	.000	-50.6795	-19.1155
	I	-39.1700*	7.58891	.000	-54.9520	-23.3880
	IC	-26.8500*	7.58891	.002	-42.6320	-11.0680
	P	-28.6650*	7.58891	.001	-44.4470	-12.8830
	PC	-35.3275*	7.58891	.000	-51.1095	-19.5455
	PI	-58.1250*	7.58891	.000	-73.9070	-42.3430
I	C	36.4125*	7.58891	.000	20.6305	52.1945
	Cin	4.2725	7.58891	.579	-11.5095	20.0545
	FB	39.1700*	7.58891	.000	23.3880	54.9520
	IC	12.3200	7.58891	.119	-3.4620	28.1020
	P	10.5050	7.58891	.181	-5.2770	26.2870
	PC	3.8425	7.58891	.618	-11.9395	19.6245
	PI	-18.9550*	7.58891	.021	-34.7370	-3.1730

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IC	C	24.0925*	7.58891	.005	8.3105	39.8745
	Cin	-8.0475	7.58891	.301	-23.8295	7.7345
	FB	26.8500*	7.58891	.002	11.0680	42.6320
	I	-12.3200	7.58891	.119	-28.1020	3.4620
	P	-1.8150	7.58891	.813	-17.5970	13.9670
	PC	-8.4775	7.58891	.277	-24.2595	7.3045
	PI	-31.2750*	7.58891	.000	-47.0570	-15.4930
P	C	25.9075*	7.58891	.003	10.1255	41.6895
	Cin	-6.2325	7.58891	.421	-22.0145	9.5495
	FB	28.6650*	7.58891	.001	12.8830	44.4470
	I	-10.5050	7.58891	.181	-26.2870	5.2770
	IC	1.8150	7.58891	.813	-13.9670	17.5970
	PC	-6.6625	7.58891	.390	-22.4445	9.1195
	PI	-29.4600*	7.58891	.001	-45.2420	-13.6780
PC	C	32.5700*	7.58891	.000	16.7880	48.3520
	Cin	.4300	7.58891	.955	-15.3520	16.2120
	FB	35.3275*	7.58891	.000	19.5455	51.1095
	I	-3.8425	7.58891	.618	-19.6245	11.9395
	IC	8.4775	7.58891	.277	-7.3045	24.2595
	P	6.6625	7.58891	.390	-9.1195	22.4445
	PI	-22.7975*	7.58891	.007	-38.5795	-7.0155
PI	C	55.3675*	7.58891	.000	39.5855	71.1495
	Cin	23.2275*	7.58891	.006	7.4455	39.0095
	FB	58.1250*	7.58891	.000	42.3430	73.9070
	I	18.9550*	7.58891	.021	3.1730	34.7370
	IC	31.2750*	7.58891	.000	15.4930	47.0570
	P	29.4600*	7.58891	.001	13.6780	45.2420
	PC	22.7975*	7.58891	.007	7.0155	38.5795

Based on observed means. The error term is Mean Square (Error) = 115.183.

*. The mean difference is significant at the .05 level.

Table 6-16. Anova of dietary fiber on Propionate content of fermentation supernatant

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	256.185[a]	10	25.619	12.213	.000
Intercept	1165.238	1	1165.238	555.516	.000
Block	2.401	3	.800	.382	.767
DF	253.784	7	36.255	17.284	.000
Error	44.049	21	2.098		
Total	1465.472	32			
Corrected Total	300.234	31			

a. R Squared = .853 (Adjusted R Squared = .783)

Table 6-17. LSD of dietary fiber on Propionate content of fermentation supernatant

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	Cin	-5.1750*	1.02410	.000	-7.3047	-3.0453
	FB	.8025	1.02410	.442	-1.3272	2.9322
	I	-6.9150*	1.02410	.000	-9.0447	-4.7853
	IC	-4.1800*	1.02410	.001	-6.3097	-2.0503
	P	-2.0900	1.02410	.054	-4.2197	.0397
	PC	-4.1150*	1.02410	.001	-6.2447	-1.9853
	PI	-7.4425*	1.02410	.000	-9.5722	-5.3128
Cin	C	5.1750*	1.02410	.000	3.0453	7.3047
	FB	5.9775*	1.02410	.000	3.8478	8.1072
	I	-1.7400	1.02410	.104	-3.8697	.3897
	IC	.9950	1.02410	.342	-1.1347	3.1247
	P	3.0850*	1.02410	.007	.9553	5.2147
	PC	1.0600	1.02410	.312	-1.0697	3.1897
	PI	-2.2675*	1.02410	.038	-4.3972	-.1378
FB	C	-.8025	1.02410	.442	-2.9322	1.3272
	Cin	-5.9775*	1.02410	.000	-8.1072	-3.8478
	I	-7.7175*	1.02410	.000	-9.8472	-5.5878
	IC	-4.9825*	1.02410	.000	-7.1122	-2.8528
	P	-2.8925*	1.02410	.010	-5.0222	-.7628
	PC	-4.9175*	1.02410	.000	-7.0472	-2.7878
	PI	-8.2450*	1.02410	.000	-10.3747	-6.1153
I	C	6.9150*	1.02410	.000	4.7853	9.0447
	Cin	1.7400	1.02410	.104	-.3897	3.8697
	FB	7.7175*	1.02410	.000	5.5878	9.8472
	IC	2.7350*	1.02410	.014	.6053	4.8647
	P	4.8250*	1.02410	.000	2.6953	6.9547
	PC	2.8000*	1.02410	.012	.6703	4.9297
	PI	-.5275	1.02410	.612	-2.6572	1.6022

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IC	C	4.1800*	1.02410	.001	2.0503	6.3097
	Cin	-.9950	1.02410	.342	-3.1247	1.1347
	FB	4.9825*	1.02410	.000	2.8528	7.1122
	I	-2.7350*	1.02410	.014	-4.8647	-.6053
	P	2.0900	1.02410	.054	-.0397	4.2197
	PC	.0650	1.02410	.950	-2.0647	2.1947
	PI	-3.2625*	1.02410	.004	-5.3922	-1.1328
P	C	2.0900	1.02410	.054	-.0397	4.2197
	Cin	-3.0850*	1.02410	.007	-5.2147	-.9553
	FB	2.8925*	1.02410	.010	.7628	5.0222
	I	-4.8250*	1.02410	.000	-6.9547	-2.6953
	IC	-2.0900	1.02410	.054	-4.2197	.0397
	PC	-2.0250	1.02410	.061	-4.1547	.1047
	PI	-5.3525*	1.02410	.000	-7.4822	-3.2228
PC	C	4.1150*	1.02410	.001	1.9853	6.2447
	Cin	-1.0600	1.02410	.312	-3.1897	1.0697
	FB	4.9175*	1.02410	.000	2.7878	7.0472
	I	-2.8000*	1.02410	.012	-4.9297	-.6703
	IC	-.0650	1.02410	.950	-2.1947	2.0647
	P	2.0250	1.02410	.061	-.1047	4.1547
	PI	-3.3275*	1.02410	.004	-5.4572	-1.1978
PI	C	7.4425*	1.02410	.000	5.3128	9.5722
	Cin	2.2675*	1.02410	.038	.1378	4.3972
	FB	8.2450*	1.02410	.000	6.1153	10.3747
	I	.5275	1.02410	.612	-1.6022	2.6572
	IC	3.2625*	1.02410	.004	1.1328	5.3922
	P	5.3525*	1.02410	.000	3.2228	7.4822
	PC	3.3275*	1.02410	.004	1.1978	5.4572

Based on observed means. The error term is Mean Square (Error) = 2.098.

*. The mean difference is significant at the .05 level.

Table 6-18. Anova of dietary fiber on Butyrate content of fermentation supernatant

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4651.358[a]	10	465.136	20.694	.000
Intercept	5443.679	1	5443.679	242.185	.000
Block	144.304	3	48.101	2.140	.126
DF	4507.054	7	643.865	28.645	.000
Error	472.024	21	22.477		
Total	10567.061	32			
Corrected Total	5123.382	31			

a. R Squared = .908 (Adjusted R Squared = .864)

Table 6-19. LSD of dietary fiber on Butyrate content of fermentation supernatant

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	Cin	-3.4925	3.35241	.309	-10.4642	3.4792
	FB	.2925	3.35241	.931	-6.6792	7.2642
	I	-35.6925*	3.35241	.000	-42.6642	-28.7208
	IC	-16.2350*	3.35241	.000	-23.2067	-9.2633
	P	-5.8850	3.35241	.094	-12.8567	1.0867
	PC	-5.0375	3.35241	.148	-12.0092	1.9342
	PI	-22.0925*	3.35241	.000	-29.0642	-15.1208
Cin	C	3.4925	3.35241	.309	-3.4792	10.4642
	FB	3.7850	3.35241	.272	-3.1867	10.7567
	I	-32.2000*	3.35241	.000	-39.1717	-25.2283
	IC	-12.7425*	3.35241	.001	-19.7142	-5.7708
	P	-2.3925	3.35241	.483	-9.3642	4.5792
	PC	-1.5450	3.35241	.650	-8.5167	5.4267
	PI	-18.6000*	3.35241	.000	-25.5717	-11.6283
FB	C	-.2925	3.35241	.931	-7.2642	6.6792
	Cin	-3.7850	3.35241	.272	-10.7567	3.1867
	I	-35.9850*	3.35241	.000	-42.9567	-29.0133
	IC	-16.5275*	3.35241	.000	-23.4992	-9.5558
	P	-6.1775	3.35241	.080	-13.1492	.7942
	PC	-5.3300	3.35241	.127	-12.3017	1.6417
	PI	-22.3850*	3.35241	.000	-29.3567	-15.4133
I	C	35.6925*	3.35241	.000	28.7208	42.6642
	Cin	32.2000*	3.35241	.000	25.2283	39.1717
	FB	35.9850*	3.35241	.000	29.0133	42.9567
	IC	19.4575*	3.35241	.000	12.4858	26.4292
	P	29.8075*	3.35241	.000	22.8358	36.7792
	PC	30.6550*	3.35241	.000	23.6833	37.6267
	PI	13.6000*	3.35241	.001	6.6283	20.5717

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IC	C	16.2350*	3.35241	.000	9.2633	23.2067
	Cin	12.7425*	3.35241	.001	5.7708	19.7142
	FB	16.5275*	3.35241	.000	9.5558	23.4992
	I	-19.4575*	3.35241	.000	-26.4292	-12.4858
	P	10.3500*	3.35241	.006	3.3783	17.3217
	PC	11.1975*	3.35241	.003	4.2258	18.1692
	PI	-5.8575	3.35241	.095	-12.8292	1.1142
P	C	5.8850	3.35241	.094	-1.0867	12.8567
	Cin	2.3925	3.35241	.483	-4.5792	9.3642
	FB	6.1775	3.35241	.080	-.7942	13.1492
	I	-29.8075*	3.35241	.000	-36.7792	-22.8358
	IC	-10.3500*	3.35241	.006	-17.3217	-3.3783
	PC	.8475	3.35241	.803	-6.1242	7.8192
	PI	-16.2075*	3.35241	.000	-23.1792	-9.2358
PC	C	5.0375	3.35241	.148	-1.9342	12.0092
	Cin	1.5450	3.35241	.650	-5.4267	8.5167
	FB	5.3300	3.35241	.127	-1.6417	12.3017
	I	-30.6550*	3.35241	.000	-37.6267	-23.6833
	IC	-11.1975*	3.35241	.003	-18.1692	-4.2258
	P	-.8475	3.35241	.803	-7.8192	6.1242
	PI	-17.0550*	3.35241	.000	-24.0267	-10.0833
PI	C	22.0925*	3.35241	.000	15.1208	29.0642
	Cin	18.6000*	3.35241	.000	11.6283	25.5717
	FB	22.3850*	3.35241	.000	15.4133	29.3567
	I	-13.6000*	3.35241	.001	-20.5717	-6.6283
	IC	5.8575	3.35241	.095	-1.1142	12.8292
	P	16.2075*	3.35241	.000	9.2358	23.1792
	PC	17.0550*	3.35241	.000	10.0833	24.0267

Based on observed means. The error term is Mean Square(Error) = 22.477.

*. The mean difference is significant at the .05 level.

Table 6-20. Anova of dietary fiber on SCFA total of fermentation supernatant

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	26785.872[a]	10	2678.587	13.283	.000
Intercept	101802.000	1	101802.000	504.831	.000
Block	790.756	3	263.585	1.307	.298
DF	25995.116	7	3713.588	18.416	.000
Error	4234.765	21	201.655		
Total	132822.637	32			
Corrected Total	31020.637	31			

a. R Squared = .863 (Adjusted R Squared = .798)

Table 6-21. Anova of dietary fiber on SCFA total of fermentation supernatant

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	Cin	-40.1800*	10.04	.001	-61.0620	-19.2980
	FB	4.1225	10.04	.686	-16.7595	25.0045
	I	-75.4350*	10.04	.000	-96.3170	-54.5530
	IC	-42.0250*	10.04	.000	-62.9070	-21.1430
	P	-42.7425*	10.04	.000	-63.6245	-21.8605
	PC	-39.6075*	10.04	.001	-60.4895	-18.7255
	PI	-81.0575*	10.04	.000	-101.9395	-60.1755
Cin	C	40.1800*	10.04	.001	19.2980	61.0620
	FB	44.3025*	10.04	.000	23.4205	65.1845
	I	-35.2550*	10.04	.002	-56.1370	-14.3730
	IC	-1.8450	10.04	.856	-22.7270	19.0370
	P	-2.5625	10.04	.801	-23.4445	18.3195
	PC	.5725	10.04	.955	-20.3095	21.4545
	PI	-40.8775*	10.04	.001	-61.7595	-19.9955
FB	C	-4.1225	10.04	.686	-25.0045	16.7595
	Cin	-44.3025*	10.04	.000	-65.1845	-23.4205
	I	-79.5575*	10.04	.000	-100.4395	-58.6755
	IC	-46.1475*	10.04	.000	-67.0295	-25.2655
	P	-46.8650*	10.04	.000	-67.7470	-25.9830
	PC	-43.7300*	10.04	.000	-64.6120	-22.8480
	PI	-85.1800*	10.04	.000	-106.0620	-64.2980
I	C	75.4350*	10.04	.000	54.5530	96.3170
	Cin	35.2550*	10.04	.002	14.3730	56.1370
	FB	79.5575*	10.04	.000	58.6755	100.4395
	IC	33.4100*	10.04	.003	12.5280	54.2920
	P	32.6925*	10.04	.004	11.8105	53.5745
	PC	35.8275*	10.04	.002	14.9455	56.7095
	PI	-5.6225	10.04	.581	-26.5045	15.2595

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IC	C	42.0250*	10.04	.000	21.1430	62.9070
	Cin	1.8450	10.04	.856	-19.0370	22.7270
	FB	46.1475*	10.04	.000	25.2655	67.0295
	I	-33.4100*	10.04	.003	-54.2920	-12.5280
	P	-.7175	10.04	.944	-21.5995	20.1645
	PC	2.4175	10.04	.812	-18.4645	23.2995
	PI	-39.0325*	10.04	.001	-59.9145	-18.1505
P	C	42.7425*	10.04	.000	21.8605	63.6245
	Cin	2.5625	10.04	.801	-18.3195	23.4445
	FB	46.8650*	10.04	.000	25.9830	67.7470
	I	-32.6925*	10.04	.004	-53.5745	-11.8105
	IC	.7175	10.04	.944	-20.1645	21.5995
	PC	3.1350	10.04	.758	-17.7470	24.0170
	PI	-38.3150*	10.04	.001	-59.1970	-17.4330
PC	C	39.6075*	10.04	.001	18.7255	60.4895
	Cin	-.5725	10.04	.955	-21.4545	20.3095
	FB	43.7300*	10.04	.000	22.8480	64.6120
	I	-35.8275*	10.04	.002	-56.7095	-14.9455
	IC	-2.4175	10.04	.812	-23.2995	18.4645
	P	-3.1350	10.04	.758	-24.0170	17.7470
	PI	-41.4500*	10.04	.000	-62.3320	-20.5680
PI	C	81.0575*	10.04	.000	60.1755	101.9395
	Cin	40.8775*	10.04	.001	19.9955	61.7595
	FB	85.1800*	10.04	.000	64.2980	106.0620
	I	5.6225	10.04	.581	-15.2595	26.5045
	IC	39.0325*	10.04	.001	18.1505	59.9145
	P	38.3150*	10.04	.001	17.4330	59.1970
	PC	41.4500*	10.04	.000	20.5680	62.3320

Based on observed means. The error term is Mean Square (Error) = 201.655.

*. The mean difference is significant at the .05 level.

Table 6-22. Anova of Effect of FS on Caco-2 cell proliferation (% growth)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5297.115[a]	7	756.731	4.758	.014
Intercept	80846.483	1	80846.483	508.367	.000
DF	5131.199	5	1026.240	6.453	.006
Block	165.916	2	82.958	.522	.609
Error	1590.318	10	159.032		
Total	87733.916	18			
Corrected Total	6887.433	17			

a. R Squared = .769 (Adjusted R Squared = .607)

Table 6-23. Anova of Effect of FS on Caco-2 cell proliferation (% growth)

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cin	FB	-35.3561*	10.29666	.006	-58.2985	-12.4137
	I	19.8260	10.29666	.083	-3.1164	42.7684
	P	-6.2180	10.29666	.559	-29.1604	16.7244
	PC	-.3914	10.29666	.970	-23.3338	22.5510
	PI	7.8924	10.29666	.461	-15.0500	30.8348
FB	Cin	35.3561*	10.29666	.006	12.4137	58.2985
	I	55.1821*	10.29666	.000	32.2397	78.1245
	P	29.1380*	10.29666	.018	6.1957	52.0804
	PC	34.9647*	10.29666	.007	12.0223	57.9071
	PI	43.2485*	10.29666	.002	20.3061	66.1909
I	Cin	-19.8260	10.29666	.083	-42.7684	3.1164
	FB	-55.1821*	10.29666	.000	-78.1245	-32.2397
	P	-26.0440*	10.29666	.030	-48.9864	-3.1016
	PC	-20.2174	10.29666	.078	-43.1598	2.7250
	PI	-11.9336	10.29666	.273	-34.8760	11.0088
P	Cin	6.2180	10.29666	.559	-16.7244	29.1604
	FB	-29.1380*	10.29666	.018	-52.0804	-6.1957
	I	26.0440*	10.29666	.030	3.1016	48.9864
	PC	5.8266	10.29666	.584	-17.1158	28.7690
	PI	14.1104	10.29666	.201	-8.8319	37.0528
PC	Cin	.3914	10.29666	.970	-22.5510	23.3338
	FB	-34.9647*	10.29666	.007	-57.9071	-12.0223
	I	20.2174	10.29666	.078	-2.7250	43.1598
	P	-5.8266	10.29666	.584	-28.7690	17.1158
	PI	8.2838	10.29666	.440	-14.6586	31.2262
PI	Cin	-7.8924	10.29666	.461	-30.8348	15.0500
	FB	-43.2485*	10.29666	.002	-66.1909	-20.3061
	I	11.9336	10.29666	.273	-11.0088	34.8760
	P	-14.1104	10.29666	.201	-37.0528	8.8319
	PC	-8.2838	10.29666	.440	-31.2262	14.6586

Based on observed means. The error term is Mean Square (Error) = 159.032.

Table 6-24. Anova of Effect of FS on Caco-2 cell differentiation (Alkaline phosphatise activity)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1766.973[a]	7	252.425	1.341	.325
Intercept	52415.279	1	52415.279	278.512	.000
Block	140.544	2	70.272	.373	.698
DF	1626.429	5	325.286	1.728	.216
Error	1881.973	10	188.197		
Total	56064.225	18			
Corrected Total	3648.946	17			

a. R Squared = .484 (Adjusted R Squared = .123)

Table 6-25. LSD of Effect of FS on Caco-2 cell differentiation (Alkaline phosphatise activity)

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cin	FB	-5.8772	11.201	.611	-30.8348	19.0805
	I	19.1210	11.201	.119	-5.8367	44.0786
	P	12.3637	11.201	.296	-12.5939	37.3214
	PC	3.8275	11.201	.740	-21.1301	28.7851
	PI	19.2030	11.201	.117	-5.7546	44.1606
FB	Cin	5.8772	11.201	.611	-19.0805	30.8348
	I	24.9981*	11.201	.050	.0405	49.9558
	P	18.2409	11.201	.134	-6.7167	43.1985
	PC	9.7047	11.201	.407	-15.2530	34.6623
	PI	25.0802*	11.201	.049	.1225	50.0378
I	Cin	-19.1210	11.201	.119	-44.0786	5.8367
	FB	-24.9981*	11.201	.050	-49.9558	-.0405
	P	-6.7572	11.201	.560	-31.7149	18.2004
	PC	-15.2935	11.201	.202	-40.2511	9.6642
	PI	.0821	11.201	.994	-24.8756	25.0397
P	Cin	-12.3637	11.201	.296	-37.3214	12.5939
	FB	-18.2409	11.201	.134	-43.1985	6.7167
	I	6.7572	11.201	.560	-18.2004	31.7149
	PC	-8.5362	11.201	.464	-33.4939	16.4214
	PI	6.8393	11.201	.555	-18.1183	31.7969
PC	Cin	-3.8275	11.201	.740	-28.7851	21.1301
	FB	-9.7047	11.201	.407	-34.6623	15.2530
	I	15.2935	11.201	.202	-9.6642	40.2511
	P	8.5362	11.201	.464	-16.4214	33.4939
	PI	15.3755	11.201	.200	-9.5821	40.3331
PI	Cin	-19.2030	11.201	.117	-44.1606	5.7546
	FB	-25.0802*	11.201	.049	-50.0378	-.1225
	I	-.0821	11.201	.994	-25.0397	24.8756
	P	-6.8393	11.201	.555	-31.7969	18.1183
	PC	-15.3755	11.201	.200	-40.3331	9.5821

Based on observed means. The error term is Mean Square (Error) = 188.197.

*. The mean difference is significant at the .05 level.

Table 6-26. Anova of Effect of FS on Caco-2 cell apoptosis (Caspase 3/7 activity)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7489584.5[a]	8	936198.0	5.905	.002
Intercept	23004852.0	1	23004852.0	145.100	.000
DF	5720880.5	7	817268.6	5.155	.004
Block	1768704.0	1	1768704.1	11.156	.004
Error	2378168.2	15	158544.6		
Total	40753381.0	24			
Corrected Total	9867752.8	23			

a. R Squared = .759 (Adjusted R Squared = .630)

Table 6-27. LSD of Effect of FS on Caco-2 cell apoptosis (Caspase 3/7 activity)

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CIN	CTR	-653.3333	325.109	.063	-1346.2884	39.6218
	FB	-272.3333	325.109	.415	-965.2884	420.6218
	I	-1150.5000*	325.109	.003	-1843.4551	-457.5449
	P	-1478.0000*	325.109	.000	-2170.9551	-785.0449
	PC	-1153.5000*	325.109	.003	-1846.4551	-460.5449
	PI	-1310.5000*	325.109	.001	-2003.4551	-617.5449
	STA	-694.5000*	325.109	.050	-1387.4551	-1.5449
CTR	CIN	653.3333	325.109	.063	-39.6218	1346.2884
	FB	381.0000	325.109	.260	-311.9551	1073.9551
	I	-497.1667	325.109	.147	-1190.1218	195.7884
	P	-824.6667*	325.109	.023	-1517.6218	-131.7116
	PC	-500.1667	325.109	.145	-1193.1218	192.7884
	PI	-657.1667	325.109	.061	-1350.1218	35.7884
	STA	-41.1667	325.109	.901	-734.1218	651.7884
FB	CIN	272.3333	325.109	.415	-420.6218	965.2884
	CTR	-381.0000	325.109	.260	-1073.9551	311.9551
	I	-878.1667*	325.109	.016	-1571.1218	-185.2116
	P	-1205.6667*	325.109	.002	-1898.6218	-512.7116
	PC	-881.1667*	325.109	.016	-1574.1218	-188.2116
	PI	-1038.1667*	325.109	.006	-1731.1218	-345.2116
	STA	-422.1667	325.109	.214	-1115.1218	270.7884
I	CIN	1150.5000*	325.109	.003	457.5449	1843.4551
	CTR	497.1667	325.109	.147	-195.7884	1190.1218
	FB	878.1667*	325.109	.016	185.2116	1571.1218
	P	-327.5000	325.109	.330	-1020.4551	365.4551
	PC	-3.0000	325.109	.993	-695.9551	689.9551
	PI	-160.0000	325.109	.630	-852.9551	532.9551
	STA	456.0000	325.109	.181	-236.9551	1148.9551

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P	CIN	1478.0000*	325.109	.000	785.0449	2170.9551
	CTR	824.6667*	325.109	.023	131.7116	1517.6218
	FB	1205.6667*	325.109	.002	512.7116	1898.6218
	I	327.5000	325.109	.330	-365.4551	1020.4551
	PC	324.5000	325.109	.334	-368.4551	1017.4551
	PI	167.5000	325.109	.614	-525.4551	860.4551
	STA	783.5000*	325.109	.029	90.5449	1476.4551
PC	CIN	1153.5000*	325.109	.003	460.5449	1846.4551
	CTR	500.1667	325.109	.145	-192.7884	1193.1218
	FB	881.1667*	325.109	.016	188.2116	1574.1218
	I	3.0000	325.109	.993	-689.9551	695.9551
	P	-324.5000	325.109	.334	-1017.4551	368.4551
	PI	-157.0000	325.109	.636	-849.9551	535.9551
	STA	459.0000	325.109	.178	-233.9551	1151.9551
PI	CIN	1310.5000*	325.109	.001	617.5449	2003.4551
	CTR	657.1667	325.109	.061	-35.7884	1350.1218
	FB	1038.1667*	325.109	.006	345.2116	1731.1218
	I	160.0000	325.109	.630	-532.9551	852.9551
	P	-167.5000	325.109	.614	-860.4551	525.4551
	PC	157.0000	325.109	.636	-535.9551	849.9551
	STA	616.0000	325.109	.078	-76.9551	1308.9551
STA	CIN	694.5000*	325.109	.050	1.5449	1387.4551
	CTR	41.1667	325.109	.901	-651.7884	734.1218
	FB	422.1667	325.109	.214	-270.7884	1115.1218
	I	-456.0000	325.109	.181	-1148.9551	236.9551
	P	-783.5000*	325.109	.029	-1476.4551	-90.5449
	PC	-459.0000	325.109	.178	-1151.9551	233.9551
	PI	-616.0000	325.109	.078	-1308.9551	76.9551

Based on observed means. The error term is Mean Square (Error) = 158544.550.

*. The mean difference is significant at the 0.05 level.

Table 6-28. Anova of Effect of FS and caspase inhibitor on Caco-2 cell apoptosis (Caspase 3/7 activity)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	20005421.310[a]	8	2500677.664	57.035	.000
Intercept	19647507.440	1	19647507.440	448.115	.000
Block	512498.167	2	256249.083	5.844	.017
DF	19492923.143	6	3248820.524	74.098	.000
Error	526137.500	12	43844.792		
Total	40179066.250	21			
Corrected Total	20531558.810	20			

a. R Squared = .974 (Adjusted R Squared = .957)

Table 6-29. Anova of Effect of FS and caspase inhibitor on Caco-2 cell apoptosis (Caspase 3/7 activity)

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cin	Ctrl	-1059.8333*	170.967	.000	-1432.3394	-687.3273
	FB	-872.1667*	170.967	.000	-1244.6727	-499.6606
	I	-2656.0000*	170.967	.000	-3028.5060	-2283.4940
	Icin	245.5000	170.967	.177	-127.0060	618.0060
	IFB	-21.1667	170.967	.904	-393.6727	351.3394
	II	158.3333	170.967	.373	-214.1727	530.8394
Ctrl	Cin	1059.8333*	170.967	.000	687.3273	1432.3394
	FB	187.6667	170.967	.294	-184.8394	560.1727
	I	-1596.1667*	170.967	.000	-1968.6727	-1223.6606
	Icin	1305.3333*	170.967	.000	932.8273	1677.8394
	IFB	1038.6667*	170.967	.000	666.1606	1411.1727
	II	1218.1667*	170.967	.000	845.6606	1590.6727
FB	Cin	872.1667*	170.967	.000	499.6606	1244.6727
	Ctrl	-187.6667	170.967	.294	-560.1727	184.8394
	I	-1783.8333*	170.967	.000	-2156.3394	-1411.3273
	Icin	1117.6667*	170.967	.000	745.1606	1490.1727
	IFB	851.0000*	170.967	.000	478.4940	1223.5060
	II	1030.5000*	170.967	.000	657.9940	1403.0060
I	Cin	2656.0000*	170.967	.000	2283.4940	3028.5060
	Ctrl	1596.1667*	170.967	.000	1223.6606	1968.6727
	FB	1783.8333*	170.967	.000	1411.3273	2156.3394
	Icin	2901.5000*	170.967	.000	2528.9940	3274.0060
	IFB	2634.8333*	170.967	.000	2262.3273	3007.3394
	II	2814.3333*	170.967	.000	2441.8273	3186.8394
Icin	Cin	-245.5000	170.967	.177	-618.0060	127.0060
	Ctrl	-1305.3333*	170.967	.000	-1677.8394	-932.8273
	FB	-1117.6667*	170.967	.000	-1490.1727	-745.1606
	I	-2901.5000*	170.967	.000	-3274.0060	-2528.9940
	IFB	-266.6667	170.967	.145	-639.1727	105.8394
	II	-87.1667	170.967	.619	-459.6727	285.3394

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IFB	Cin	21.1667	170.967	.904	-351.3394	393.6727
	Ctrl	-1038.6667*	170.967	.000	-1411.1727	-666.1606
	FB	-851.0000*	170.967	.000	-1223.5060	-478.4940
	I	-2634.8333*	170.967	.000	-3007.3394	-2262.3273
	Icin	266.6667	170.967	.145	-105.8394	639.1727
	II	179.5000	170.967	.314	-193.0060	552.0060
II	Cin	-158.3333	170.967	.373	-530.8394	214.1727
	Ctrl	-1218.1667*	170.967	.000	-1590.6727	-845.6606
	FB	-1030.5000*	170.967	.000	-1403.0060	-657.9940
	I	-2814.3333*	170.967	.000	-3186.8394	-2441.8273
	Icin	87.1667	170.967	.619	-285.3394	459.6727
	IFB	-179.5000	170.967	.314	-552.0060	193.0060

Based on observed means. The error term is Mean Square (Error) = 43844.792.

*. The mean difference is significant at the .05 level.

Table 6-30. Anova of Effect of FS and caspase inhibitor on Caco-2 cell proliferation (% growth)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3756.755[a]	8	469.594	8.180	.001
Intercept	177763.512	1	177763.512	3096.341	.000
Block	137.461	2	68.730	1.197	.336
DF	3619.294	6	603.216	10.507	.000
Error	688.930	12	57.411		
Total	182209.196	21			
Corrected Total	4445.685	20			

a. R Squared = .845 (Adjusted R Squared = .742)

Table 6-31. LSD of Effect of FS and caspase inhibitor on Caco-2 cell proliferation (% growth)

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cin	Ctrl	-25.4050*	6.18659	.001	-38.8844	-11.9256
	FB	-37.5552*	6.18659	.000	-51.0346	-24.0758
	I	-10.6775	6.18659	.110	-24.1569	2.8020
	Icin	-7.3638	6.18659	.257	-20.8432	6.1157
	IFB	-8.8365	6.18659	.179	-22.3159	4.6429
	II	-32.0324*	6.18659	.000	-45.5118	-18.5530
Ctrl	Cin	25.4050*	6.18659	.001	11.9256	38.8844
	FB	-12.1502	6.18659	.073	-25.6296	1.3292
	I	14.7275*	6.18659	.035	1.2481	28.2070
	Icin	18.0412*	6.18659	.013	4.5618	31.5207
	IFB	16.5685*	6.18659	.020	3.0891	30.0479
	II	-6.6274	6.18659	.305	-20.1068	6.8520
FB	Cin	37.5552*	6.18659	.000	24.0758	51.0346
	Ctrl	12.1502	6.18659	.073	-1.3292	25.6296
	I	26.8778*	6.18659	.001	13.3983	40.3572
	Icin	30.1915*	6.18659	.000	16.7120	43.6709
	IFB	28.7187*	6.18659	.001	15.2393	42.1981
	II	5.5228	6.18659	.390	-7.9566	19.0022
I	Cin	10.6775	6.18659	.110	-2.8020	24.1569
	Ctrl	-14.7275*	6.18659	.035	-28.2070	-1.2481
	FB	-26.8778*	6.18659	.001	-40.3572	-13.3983
	Icin	3.3137	6.18659	.602	-10.1657	16.7931
	IFB	1.8409	6.18659	.771	-11.6385	15.3204
	II	-21.3549*	6.18659	.005	-34.8344	-7.8755
Icin	Cin	7.3638	6.18659	.257	-6.1157	20.8432
	Ctrl	-18.0412*	6.18659	.013	-31.5207	-4.5618
	FB	-30.1915*	6.18659	.000	-43.6709	-16.7120
	I	-3.3137	6.18659	.602	-16.7931	10.1657
	IFB	-1.4728	6.18659	.816	-14.9522	12.0067
	II	-24.6686*	6.18659	.002	-38.1481	-11.1892

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IFB	Cin	8.8365	6.18659	.179	-4.6429	22.3159
	Ctrl	-16.5685*	6.18659	.020	-30.0479	-3.0891
	FB	-28.7187*	6.18659	.001	-42.1981	-15.2393
	I	-1.8409	6.18659	.771	-15.3204	11.6385
	Icin	1.4728	6.18659	.816	-12.0067	14.9522
	II	-23.1959*	6.18659	.003	-36.6753	-9.7165
II	Cin	32.0324*	6.18659	.000	18.5530	45.5118
	Ctrl	6.6274	6.18659	.305	-6.8520	20.1068
	FB	-5.5228	6.18659	.390	-19.0022	7.9566
	I	21.3549*	6.18659	.005	7.8755	34.8344
	Icin	24.6686*	6.18659	.002	11.1892	38.1481
	IFB	23.1959*	6.18659	.003	9.7165	36.6753

Based on observed means. The error term is Mean Square (Error) = 57.411.

*. The mean difference is significant at the .05 level.

Table 6-32. Anova of Effect of dietary fiber sources and antioxidant on body weight gain

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	41753.265[a]	5	8350.653	2.787	.024
Intercept	10843591.651	1	10843591.651	3618.562	.000
DF	41753.265	5	8350.653	2.787	.024
Error	197779.434	66	2996.658		
Total	11083124.350	72			
Corrected Total	239532.699	71			

a. R Squared = .174 (Adjusted R Squared = .112)

Table 6-33. Anova of Effect of dietary fiber sources and antioxidant on body weight gain

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
C	CE	-13.0917	22.348	.560	-72.3679	46.1846
	Cin	-12.3333	22.348	.583	-71.6096	46.9429
	CinL	9.3333	22.348	.678	-49.9429	68.6096
	P	41.2250	22.348	.070	-18.0513	100.501
	PE	47.3917	22.348	.038	-11.8846	106.667
CE	C	13.0917	22.348	.560	-46.1846	72.3679
	Cin	.7583	22.348	.973	-58.5179	60.0346
	CinL	22.4250	22.348	.319	-36.8513	81.7013
	P	54.3167	22.348	.018	-4.9596	113.592
	PE	60.4833*	22.348	.009	1.2071	119.759
Cin	C	12.3333	22.348	.583	-46.9429	71.609
	CE	-.7583	22.348	.973	-60.0346	58.5179
	CinL	21.6667	22.348	.336	-37.6096	80.9429
	P	53.5583	22.348	.019	-5.7179	112.834
	PE	59.7250*	22.348	.009	.4487	119.001
CinL	C	-9.3333	22.348	.678	-68.6096	49.9429
	CE	-22.4250	22.348	.319	-81.7013	36.8513
	Cin	-21.6667	22.348	.336	-80.9429	37.6096
	P	31.8917	22.348	.158	-27.3846	91.1679
	PE	38.0583	22.348	.093	-21.2179	97.3346
P	C	-41.2250	22.348	.070	-100.5013	18.0513
	CE	-54.3167	22.348	.018	-113.5929	4.9596
	Cin	-53.5583	22.348	.019	-112.8346	5.7179
	CinL	-31.8917	22.348	.158	-91.1679	27.3846
	PE	6.1667	22.348	.783	-53.1096	65.4429
PE	C	-47.3917	22.348	.038	-106.6679	11.8846
	CE	-60.4833*	22.348	.009	-119.7596	-1.2071
	Cin	-59.7250*	22.348	.009	-119.0013	-.4487
	CinL	-38.0583	22.348	.093	-97.3346	21.2179
	P	-6.1667	22.348	.783	-65.4429	53.1096

Based on observed means. The error term is Mean Square (Error) = 2996.658.

*. The mean difference is significant at the .05 level.

Table 6-34. Anova of Effect of dietary fiber sources and antioxidant on liver weight

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.698[a]	5	.340	1.567	.182
Intercept	595.183	1	595.183	2746.220	.000
DF	1.698	5	.340	1.567	.182
Error	14.304	66	.217		
Total	611.184	72			
Corrected Total	16.002	71			

a. R Squared = .106 (Adjusted R Squared = .038)

Table 6-35. Anova of Effect of dietary fiber sources and antioxidant on acetate concentration of AOM-induced rats digesta.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4606.738[a]	16	287.921	2.181	.018
Intercept	47097.178	1	47097.178	356.795	.000
Rat	860.070	11	78.188	.592	.826
DF	3727.809	5	745.562	5.648	.000
Error	6864.031	52	132.001		
Total	60409.311	69			
Corrected Total	11470.768	68			

a. R Squared = .402 (Adjusted R Squared = .217)

Table 6-36. LSD of Effect of dietary fiber sources and antioxidant on acetate concentration of AOM-induced rats digesta.

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	-2.8077	1.58369	.082	-7.0420	1.4267
	Cin	1.0113	1.58369	.526	-3.2230	5.2457
	CinL	-.6768	1.66099	.685	-5.1178	3.7643
	P	-11.0081*	1.58369	.000	-15.2425	-6.7737
	PE	-4.1992	1.61928	.012	-8.5287	.1304
CE	C	2.8077	1.58369	.082	-1.4267	7.0420
	Cin	3.8190	1.58369	.019	-.4153	8.0534
	CinL	2.1309	1.66099	.205	-2.3101	6.5719
	P	-8.2004*	1.58369	.000	-12.4348	-3.9661
	PE	-1.3915	1.61928	.394	-5.7210	2.9381
Cin	C	-1.0113	1.58369	.526	-5.2457	3.2230
	CE	-3.8190	1.58369	.019	-8.0534	.4153
	CinL	-1.6881	1.66099	.314	-6.1292	2.7529
	P	-12.0195*	1.58369	.000	-16.2538	-7.7851
	PE	-5.2105*	1.61928	.002	-9.5400	-.8810
CinL	C	.6768	1.66099	.685	-3.7643	5.1178
	CE	-2.1309	1.66099	.205	-6.5719	2.3101
	Cin	1.6881	1.66099	.314	-2.7529	6.1292
	P	-10.3313*	1.66099	.000	-14.7724	-5.8903
	PE	-3.5224	1.69496	.043	-8.0542	1.0095
P	C	11.0081*	1.58369	.000	6.7737	15.2425
	CE	8.2004*	1.58369	.000	3.9661	12.4348
	Cin	12.0195*	1.58369	.000	7.7851	16.2538
	CinL	10.3313*	1.66099	.000	5.8903	14.7724
	PE	6.8090*	1.61928	.000	2.4794	11.1385
PE	C	4.1992	1.61928	.012	-.1304	8.5287
	CE	1.3915	1.61928	.394	-2.9381	5.7210
	Cin	5.2105*	1.61928	.002	.8810	9.5400
	CinL	3.5224	1.69496	.043	-1.0095	8.0542
	P	-6.8090*	1.61928	.000	-11.1385	-2.4794

Based on observed means. The error term is Mean Square (Error) = 132.001.

*. The mean difference is significant at the .05 level.

Table 6-37. Anova of Effect of dietary fiber sources and antioxidant on propionate concentration of AOM-induced rats digesta.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1302.261[a]	16	81.391	5.409	.000
Intercept	4134.285	1	4134.285	274.731	.000
Rat	161.379	11	14.671	.975	.480
DF	1158.690	5	231.738	15.399	.000
Error	782.520	52	15.048		
Total	6511.727	69			
Corrected Total	2084.781	68			

a. R Squared = .625 (Adjusted R Squared = .509)

Table 6-38. LSD of Effect of dietary fiber sources and antioxidant on propionate concentration of AOM-induced rats digesta.

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	-2.8077	1.58369	.082	-7.0420	1.4267
	Cin	1.0113	1.58369	.526	-3.2230	5.2457
	CinL	-.6768	1.66099	.685	-5.1178	3.7643
	P	-11.0081*	1.58369	.000	-15.2425	-6.7737
	PE	-4.1992	1.61928	.012	-8.5287	.1304
CE	C	2.8077	1.58369	.082	-1.4267	7.0420
	Cin	3.8190	1.58369	.019	-.4153	8.0534
	CinL	2.1309	1.66099	.205	-2.3101	6.5719
	P	-8.2004*	1.58369	.000	-12.4348	-3.9661
	PE	-1.3915	1.61928	.394	-5.7210	2.9381
Cin	C	-1.0113	1.58369	.526	-5.2457	3.2230
	CE	-3.8190	1.58369	.019	-8.0534	.4153
	CinL	-1.6881	1.66099	.314	-6.1292	2.7529
	P	-12.0195*	1.58369	.000	-16.2538	-7.7851
	PE	-5.2105*	1.61928	.002	-9.5400	-.8810
CinL	C	.6768	1.66099	.685	-3.7643	5.1178
	CE	-2.1309	1.66099	.205	-6.5719	2.3101
	Cin	1.6881	1.66099	.314	-2.7529	6.1292
	P	-10.3313*	1.66099	.000	-14.7724	-5.8903
	PE	-3.5224	1.69496	.043	-8.0542	1.0095
P	C	11.0081*	1.58369	.000	6.7737	15.2425
	CE	8.2004*	1.58369	.000	3.9661	12.4348
	Cin	12.0195*	1.58369	.000	7.7851	16.2538
	CinL	10.3313*	1.66099	.000	5.8903	14.7724
	PE	6.8090*	1.61928	.000	2.4794	11.1385
PE	C	4.1992	1.61928	.012	-.1304	8.5287
	CE	1.3915	1.61928	.394	-2.9381	5.7210
	Cin	5.2105*	1.61928	.002	.8810	9.5400
	CinL	3.5224	1.69496	.043	-1.0095	8.0542
	P	-6.8090*	1.61928	.000	-11.1385	-2.4794

Based on observed means. The error term is Mean Square (Error) = 15.048.

*. The mean difference is significant at the .05 level.

Table 6-39. Anova of Effect of dietary fiber sources and antioxidant on butyrate concentration of AOM-induced rats digesta.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	356.246[a]	16	22.265	1.539	.121
Intercept	2916.199	1	2916.199	201.618	.000
Rat	127.896	11	11.627	.804	.636
DF	228.313	5	45.663	3.157	.015
Error	752.128	52	14.464		
Total	4160.256	69			
Corrected Total	1108.374	68			

a. R Squared = .321 (Adjusted R Squared = .113)

Table 6-40. LSD of Effect of dietary fiber sources and antioxidant on butyrate concentration of AOM-induced rats digesta.

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	-3.2139	1.55263	.043	-7.3652	.9374
	Cin	-.7864	1.55263	.615	-4.9377	3.3649
	CinL	-2.3066	1.62841	.163	-6.6606	2.0473
	P	-5.5545*	1.55263	.001	-9.7058	-1.4032
	PE	-2.6239	1.58753	.104	-6.8686	1.6207
CE	C	3.2139	1.55263	.043	-.9374	7.3652
	Cin	2.4275	1.55263	.124	-1.7238	6.5788
	CinL	.9072	1.62841	.580	-3.4467	5.2612
	P	-2.3406	1.55263	.138	-6.4920	1.8107
	PE	.5899	1.58753	.712	-3.6547	4.8346
Cin	C	.7864	1.55263	.615	-3.3649	4.9377
	CE	-2.4275	1.55263	.124	-6.5788	1.7238
	CinL	-1.5202	1.62841	.355	-5.8742	2.8337
	P	-4.7681*	1.55263	.003	-8.9194	-.6168
	PE	-1.8375	1.58753	.252	-6.0822	2.4071
CinL	C	2.3066	1.62841	.163	-2.0473	6.6606
	CE	-.9072	1.62841	.580	-5.2612	3.4467
	Cin	1.5202	1.62841	.355	-2.8337	5.8742
	P	-3.2479	1.62841	.051	-7.6018	1.1061
	PE	-.3173	1.66172	.849	-4.7603	4.1257
P	C	5.5545*	1.55263	.001	1.4032	9.7058
	CE	2.3406	1.55263	.138	-1.8107	6.4920
	Cin	4.7681*	1.55263	.003	.6168	8.9194
	CinL	3.2479	1.62841	.051	-1.1061	7.6018
	PE	2.9306	1.58753	.071	-1.3140	7.1752
PE	C	2.6239	1.58753	.104	-1.6207	6.8686
	CE	-.5899	1.58753	.712	-4.8346	3.6547
	Cin	1.8375	1.58753	.252	-2.4071	6.0822
	CinL	.3173	1.66172	.849	-4.1257	4.7603
	P	-2.9306	1.58753	.071	-7.1752	1.3140

Based on observed means. The error term is Mean Square Error) = 14.464.

*. The mean difference is significant at the .05 level.

Table 6-41. Anova of Effect of dietary fiber sources and antioxidant on SCFA total of AOM-induced rats digesta.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	40972.812[a]	16	2560.801	2.986	.001
Intercept	290597.808	1	290597.808	338.822	.000
Rat	7032.226	11	639.293	.745	.691
DF	34096.728	5	6819.346	7.951	.000
Error	45456.565	53	857.671		
Total	385704.126	70			
Corrected Total	86429.377	69			

a. R Squared = .474 (Adjusted R Squared = .315)

Table 6-42. LSD of effect of dietary fiber sources and antioxidant on SCFA total concentration of AOM-induced rats digesta

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	-23.2144	11.95597	.058	-55.1587	8.7298
	Cin	6.5118	11.95597	.588	-25.4324	38.4560
	CinL	-18.6757	12.53953	.142	-52.1791	14.8277
	P	-61.4113*	11.95597	.000	-93.3556	-29.4671
	PE	-20.2755	11.95597	.096	-52.2197	11.6688
CE	C	23.2144	11.95597	.058	-8.7298	55.1587
	Cin	29.7262	11.95597	.016	-2.2180	61.6705
	CinL	4.5388	12.53953	.719	-28.9646	38.0422
	P	-38.1969*	11.95597	.002	-70.1411	-6.2527
	PE	2.9390	11.95597	.807	-29.0052	34.8832
Cin	C	-6.5118	11.95597	.588	-38.4560	25.4324
	CE	-29.7262	11.95597	.016	-61.6705	2.2180
	CinL	-25.1875	12.53953	.050	-58.6908	8.3159
	P	-67.9231*	11.95597	.000	-99.8674	-35.9789
	PE	-26.7872	11.95597	.029	-58.7315	5.1570
CinL	C	18.6757	12.53953	.142	-14.8277	52.1791
	CE	-4.5388	12.53953	.719	-38.0422	28.9646
	Cin	25.1875	12.53953	.050	-8.3159	58.6908
	P	-42.7357*	12.53953	.001	-76.2391	-9.2323
	PE	-1.5998	12.53953	.899	-35.1032	31.9036
P	C	61.4113*	11.95597	.000	29.4671	93.3556
	CE	38.1969*	11.95597	.002	6.2527	70.1411
	Cin	67.9231*	11.95597	.000	35.9789	99.8674
	CinL	42.7357*	12.53953	.001	9.2323	76.2391
	PE	41.1359*	11.95597	.001	9.1917	73.0801
PE	C	20.2755	11.95597	.096	-11.6688	52.2197
	CE	-2.9390	11.95597	.807	-34.8832	29.0052
	Cin	26.7872	11.95597	.029	-5.1570	58.7315
	CinL	1.5998	12.53953	.899	-31.9036	35.1032
	P	-41.1359*	11.95597	.001	-73.0801	-9.1917

Based on observed means. The error term is Mean Square (Error) = 857.671.

*. The mean difference is significant at the .05 level.

Table 6-43. Anova of effect of dietary fiber sources and antioxidant on pH digesta of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.730[a]	16	.046	1.139	.353
Intercept	2392.672	1	2392.672	59734.717	.000
Block	.250	11	.023	.568	.843
DF	.440	5	.088	2.198	.072
Error	1.722	43	.040		
Total	2725.009	60			
Corrected Total	2.452	59			

a. R Squared = .298 (Adjusted R Squared = .036)

Table 6-44. LSD of effect of dietary fiber sources and antioxidant on pH digesta of AOM-induced rats

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	.2318*	.08534	.009	.0597	.4039
	Cin	.2493*	.09300	.010	.0618	.4369
	CinL	.2448*	.08745	.008	.0685	.4212
	P	.2102*	.08354	.016	.0417	.3786
	PE	.2031*	.09300	.034	.0155	.3906
CE	C	-.2318*	.08534	.009	-.4039	-.0597
	Cin	.0175	.09300	.852	-.1700	.2050
	CinL	.0130	.08745	.883	-.1634	.1894
	P	-.0217	.08354	.797	-.1901	.1468
	PE	-.0287	.09300	.759	-.2163	.1588
Cin	C	-.2493*	.09300	.010	-.4369	-.0618
	CE	-.0175	.09300	.852	-.2050	.1700
	CinL	-.0045	.09493	.962	-.1960	.1870
	P	-.0392	.09135	.670	-.2234	.1451
	PE	-.0463	.10007	.646	-.2481	.1556
CinL	C	-.2448*	.08745	.008	-.4212	-.0685
	CE	-.0130	.08745	.883	-.1894	.1634
	Cin	.0045	.09493	.962	-.1870	.1960
	P	-.0347	.08569	.688	-.2075	.1382
	PE	-.0418	.09493	.662	-.2332	.1497
P	C	-.2102*	.08354	.016	-.3786	-.0417
	CE	.0217	.08354	.797	-.1468	.1901
	Cin	.0392	.09135	.670	-.1451	.2234
	CinL	.0347	.08569	.688	-.1382	.2075
	PE	-.0071	.09135	.939	-.1913	.1771
PE	C	-.2031*	.09300	.034	-.3906	-.0155
	CE	.0287	.09300	.759	-.1588	.2163
	Cin	.0463	.10007	.646	-.1556	.2481
	CinL	.0418	.09493	.662	-.1497	.2332
	P	.0071	.09135	.939	-.1771	.1913

Based on observed means. The error term is Mean Square (Error) = .040.

*. The mean difference is significant at the .05 level.

Table 6-45. Anova of effect of dietary fiber sources and antioxidant on acetate concentration of faeces of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1501.625[a]	16	93.852	1.746	.065
Intercept	9265.301	1	9265.301	172.346	.000
Block	600.706	11	54.610	1.016	.445
DF	900.919	5	180.184	3.352	.010
Error	2956.802	55	53.760		
Total	13723.727	72			
Corrected Total	4458.427	71			

a. R Squared = .337 (Adjusted R Squared = .144)

Table 6-46. LSD of effect of dietary fiber sources and antioxidant on acetate concentration of faeces of AOM-induced rats

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	-1.4162	2.99333	.638	-9.4030	6.5707
	Cin	-6.8501	2.99333	.026	-14.8370	1.1367
	CinL	-7.9302	2.99333	.011	-15.9171	.0566
	P	-8.4084*	2.99333	.007	-16.3953	-.4216
	PE	-8.9663*	2.99333	.004	-16.9532	-.9795
CE	C	1.4162	2.99333	.638	-6.5707	9.4030
	Cin	-5.4340	2.99333	.075	-13.4208	2.5529
	CinL	-6.5141	2.99333	.034	-14.5009	1.4728
	P	-6.9923	2.99333	.023	-14.9791	.9946
	PE	-7.5502	2.99333	.015	-15.5370	.4367
Cin	C	6.8501	2.99333	.026	-1.1367	14.8370
	CE	5.4340	2.99333	.075	-2.5529	13.4208
	CinL	-1.0801	2.99333	.720	-9.0669	6.9067
	P	-1.5583	2.99333	.605	-9.5451	6.4285
	PE	-2.1162	2.99333	.483	-10.1030	5.8707
CinL	C	7.9302	2.99333	.011	-.0566	15.9171
	CE	6.5141	2.99333	.034	-1.4728	14.5009
	Cin	1.0801	2.99333	.720	-6.9067	9.0669
	P	-.4782	2.99333	.874	-8.4650	7.5086
	PE	-1.0361	2.99333	.731	-9.0229	6.9507
P	C	8.4084*	2.99333	.007	.4216	16.3953
	CE	6.9923	2.99333	.023	-.9946	14.9791
	Cin	1.5583	2.99333	.605	-6.4285	9.5451
	CinL	.4782	2.99333	.874	-7.5086	8.4650
	PE	-.5579	2.99333	.853	-8.5447	7.4290
PE	C	8.9663*	2.99333	.004	.9795	16.9532
	CE	7.5502	2.99333	.015	-.4367	15.5370
	Cin	2.1162	2.99333	.483	-5.8707	10.1030
	CinL	1.0361	2.99333	.731	-6.9507	9.0229
	P	.5579	2.99333	.853	-7.4290	8.5447

Based on observed means. The error term is Mean Square(Error) = 53.760.

*. The mean difference is significant at the .05 level.

Table 6-47. Anova of effect of dietary fiber sources and antioxidant on propionate concentration of faeces of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	611.078[a]	16	38.192	4.284	.000
Intercept	1132.925	1	1132.925	127.091	.000
block	97.395	11	8.854	.993	.464
df	513.683	5	102.737	11.525	.000
Error	490.286	55	8.914		
Total	2234.289	72			
Corrected Total	1101.364	71			

a. R Squared = .555 (Adjusted R Squared = .425)

Table 6-48. LSD of effect of dietary fiber sources and antioxidant on propionate concentration of faeces of AOM-induced rats

(I) df	(J) df	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	-.5462	1.21890	.656	-3.7985	2.7061
	Cin	-.9092	1.21890	.459	-4.1615	2.3431
	CinL	-1.3973	1.21890	.257	-4.6495	1.8550
	P	-6.4259*	1.21890	.000	-9.6782	-3.1736
	PE	-6.1901*	1.21890	.000	-9.4424	-2.9378
CE	C	.5462	1.21890	.656	-2.7061	3.7985
	Cin	-.3630	1.21890	.767	-3.6153	2.8893
	CinL	-.8511	1.21890	.488	-4.1033	2.4012
	P	-5.8797*	1.21890	.000	-9.1320	-2.6274
	PE	-5.6439*	1.21890	.000	-8.8962	-2.3916
Cin	C	.9092	1.21890	.459	-2.3431	4.1615
	CE	.3630	1.21890	.767	-2.8893	3.6153
	CinL	-.4881	1.21890	.690	-3.7404	2.7642
	P	-5.5167*	1.21890	.000	-8.7690	-2.2644
	PE	-5.2809*	1.21890	.000	-8.5332	-2.0286
CinL	C	1.3973	1.21890	.257	-1.8550	4.6495
	CE	.8511	1.21890	.488	-2.4012	4.1033
	Cin	.4881	1.21890	.690	-2.7642	3.7404
	P	-5.0286*	1.21890	.000	-8.2809	-1.7763
	PE	-4.7928*	1.21890	.000	-8.0451	-1.5406
P	C	6.4259*	1.21890	.000	3.1736	9.6782
	CE	5.8797*	1.21890	.000	2.6274	9.1320
	Cin	5.5167*	1.21890	.000	2.2644	8.7690
	CinL	5.0286*	1.21890	.000	1.7763	8.2809
	PE	.2358	1.21890	.847	-3.0165	3.4881
PE	C	6.1901*	1.21890	.000	2.9378	9.4424
	CE	5.6439*	1.21890	.000	2.3916	8.8962
	Cin	5.2809*	1.21890	.000	2.0286	8.5332
	CinL	4.7928*	1.21890	.000	1.5406	8.0451
	P	-.2358	1.21890	.847	-3.4881	3.0165

Based on observed means. The error term is Mean Square (Error) = 8.914.

*. The mean difference is significant at the .05 level.

Table 6-49. Anova of effect of dietary fiber sources and antioxidant on butyrate concentration of feces of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	255.615[a]	16	15.976	2.743	.003
Intercept	1195.753	1	1195.753	205.330	.000
block	63.071	11	5.734	.985	.471
df	192.544	5	38.509	6.613	.000
Error	320.296	55	5.824		
Total	1771.665	72			
Corrected Total	575.912	71			

a. R Squared = .444 (Adjusted R Squared = .282)

Table 6-50. LSD of effect of dietary fiber sources and antioxidant on butyrate concentration of faeces of AOM-induced rats

(I) df	(J) df	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	-.7534	.98519	.448	-3.3821	1.8753
	Cin	-2.9508*	.98519	.004	-5.5795	-.3221
	CinL	-3.2329*	.98519	.002	-5.8616	-.6042
	P	-3.0125*	.98519	.003	-5.6412	-.3838
	PE	-4.8970*	.98519	.000	-7.5257	-2.2683
CE	C	.7534	.98519	.448	-1.8753	3.3821
	Cin	-2.1974	.98519	.030	-4.8261	.4313
	CinL	-2.4795	.98519	.015	-5.1082	.1492
	P	-2.2591	.98519	.026	-4.8878	.3696
	PE	-4.1436*	.98519	.000	-6.7723	-1.5149
Cin	C	2.9508*	.98519	.004	.3221	5.5795
	CE	2.1974	.98519	.030	-.4313	4.8261
	CinL	-.2821	.98519	.776	-2.9108	2.3466
	P	-.0617	.98519	.950	-2.6904	2.5670
	PE	-1.9463	.98519	.053	-4.5749	.6824
CinL	C	3.2329*	.98519	.002	.6042	5.8616
	CE	2.4795	.98519	.015	-.1492	5.1082
	Cin	.2821	.98519	.776	-2.3466	2.9108
	P	.2204	.98519	.824	-2.4083	2.8491
	PE	-1.6641	.98519	.097	-4.2928	.9645
P	C	3.0125*	.98519	.003	.3838	5.6412
	CE	2.2591	.98519	.026	-.3696	4.8878
	Cin	.0617	.98519	.950	-2.5670	2.6904
	CinL	-.2204	.98519	.824	-2.8491	2.4083
	PE	-1.8845	.98519	.061	-4.5132	.7441
PE	C	4.8970*	.98519	.000	2.2683	7.5257
	CE	4.1436*	.98519	.000	1.5149	6.7723
	Cin	1.9463	.98519	.053	-.6824	4.5749
	CinL	1.6641	.98519	.097	-.9645	4.2928
	P	1.8845	.98519	.061	-.7441	4.5132

Based on observed means. The error term is Mean Square(Error) = 5.824.

*. The mean difference is significant at the .05 level.

Table 6-51. Anova of effect of dietary fiber sources and antioxidant on SCFA total of feces of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	17256.672[a]	16	1078.542	2.849	.002
Intercept	74571.932	1	74571.932	197.002	.000
block	4533.245	11	412.113	1.089	.387
df	12723.427	5	2544.685	6.722	.000
Error	20819.352	55	378.534		
Total	112647.956	72			
Corrected Total	38076.024	71			

a. R Squared = .453 (Adjusted R Squared = .294)

Table 6-52. LSD of effect of dietary fiber sources and antioxidant on SCFA total of feces of AOM-induced rats

(I) df	(J) df	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	-2.7471	7.94285	.731	-23.9404	18.4461
	Cin	-16.0663	7.94285	.048	-37.2595	5.1270
	CinL	-18.9555	7.94285	.020	-40.1487	2.2378
	P	-29.6518*	7.94285	.000	-50.8450	-8.4585
	PE	-37.1109*	7.94285	.000	-58.3041	-15.9176
CE	C	2.7471	7.94285	.731	-18.4461	23.9404
	Cin	-13.3191	7.94285	.099	-34.5124	7.8741
	CinL	-16.2083	7.94285	.046	-37.4016	4.9849
	P	-26.9046*	7.94285	.001	-48.0979	-5.7114
	PE	-34.3637*	7.94285	.000	-55.5570	-13.1705
Cin	C	16.0663	7.94285	.048	-5.1270	37.2595
	CE	13.3191	7.94285	.099	-7.8741	34.5124
	CinL	-2.8892	7.94285	.717	-24.0825	18.3040
	P	-13.5855	7.94285	.093	-34.7788	7.6077
	PE	-21.0446	7.94285	.011	-42.2378	.1487
CinL	C	18.9555	7.94285	.020	-2.2378	40.1487
	CE	16.2083	7.94285	.046	-4.9849	37.4016
	Cin	2.8892	7.94285	.717	-18.3040	24.0825
	P	-10.6963	7.94285	.184	-31.8895	10.4970
	PE	-18.1554	7.94285	.026	-39.3486	3.0379
P	C	29.6518*	7.94285	.000	8.4585	50.8450
	CE	26.9046*	7.94285	.001	5.7114	48.0979
	Cin	13.5855	7.94285	.093	-7.6077	34.7788
	CinL	10.6963	7.94285	.184	-10.4970	31.8895
	PE	-7.4591	7.94285	.352	-28.6523	13.7342
PE	C	37.1109*	7.94285	.000	15.9176	58.3041
	CE	34.3637*	7.94285	.000	13.1705	55.5570
	Cin	21.0446	7.94285	.011	-.1487	42.2378
	CinL	18.1554	7.94285	.026	-3.0379	39.3486
	P	7.4591	7.94285	.352	-13.7342	28.6523

Based on observed means. The error term is Mean Square (Error) = 378.534.

*. The mean difference is significant at the .05 level

Table 6-53. Anova of effect of dietary fiber sources and antioxidant on total ACF in the colon of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	57615.667[a]	16	3600.979	1.923	.038
Intercept	565516.125	1	565516.125	302.053	.000
Block	33033.375	11	3003.034	1.604	.123
DF	24582.292	5	4916.458	2.626	.034
Error	102973.208	55	1872.240		
Total	726105.000	72			
Corrected Total	160588.875	71			

a. R Squared = .359 (Adjusted R Squared = .172)

Table 6-54. LSD of effect of dietary fiber sources and antioxidant on total ACF in the colon of AOM-induced rats

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
C	CE	8.5000	17.66465	.632	-38.6331	55.6331
	Cin	-6.5000	17.66465	.714	-53.6331	40.6331
	CinL	-15.4167	17.66465	.387	-62.5498	31.7164
	P	-37.5000	17.66465	.038	-84.6331	9.6331
	PE	-41.3333	17.66465	.023	-88.4664	5.7998
CE	C	-8.5000	17.66465	.632	-55.6331	38.6331
	Cin	-15.0000	17.66465	.399	-62.1331	32.1331
	CinL	-23.9167	17.66465	.181	-71.0498	23.2164
	P	-46.0000	17.66465	.012	-93.1331	1.1331
	PE	-49.8333*	17.66465	.007	-96.9664	-2.7002
Cin	C	6.5000	17.66465	.714	-40.6331	53.6331
	CE	15.0000	17.66465	.399	-32.1331	62.1331
	CinL	-8.9167	17.66465	.616	-56.0498	38.2164
	P	-31.0000	17.66465	.085	-78.1331	16.1331
	PE	-34.8333	17.66465	.054	-81.9664	12.2998
CinL	C	15.4167	17.66465	.387	-31.7164	62.5498
	CE	23.9167	17.66465	.181	-23.2164	71.0498
	Cin	8.9167	17.66465	.616	-38.2164	56.0498
	P	-22.0833	17.66465	.217	-69.2164	25.0498
	PE	-25.9167	17.66465	.148	-73.0498	21.2164
P	C	37.5000	17.66465	.038	-9.6331	84.6331
	CE	46.0000	17.66465	.012	-1.1331	93.1331
	Cin	31.0000	17.66465	.085	-16.1331	78.1331
	CinL	22.0833	17.66465	.217	-25.0498	69.2164
	PE	-3.8333	17.66465	.829	-50.9664	43.2998
PE	C	41.3333	17.66465	.023	-5.7998	88.4664
	CE	49.8333*	17.66465	.007	2.7002	96.9664
	Cin	34.8333	17.66465	.054	-12.2998	81.9664
	CinL	25.9167	17.66465	.148	-21.2164	73.0498
	P	3.8333	17.66465	.829	-43.2998	50.9664

Based on observed means. The error term is Mean Square (Error) = 1872.240.

*. The mean difference is significant at the .01 level.

Table 6-55. Anova of effect of dietary fiber sources and antioxidant on single crypt per focus in the colon of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10922.389[a]	16	682.649	1.433	.161
Intercept	133472.222	1	133472.222	280.132	.000
DF	5246.611	5	1049.322	2.202	.067
gROUP	5675.778	11	515.980	1.083	.392
Error	26205.389	55	476.462		
Total	170600.000	72			
Corrected Total	37127.778	71			

a. R Squared = .294 (Adjusted R Squared = .089)

Table 6-56. Anova of effect of dietary fiber sources and antioxidant on two crypts per focus in the colon of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9639.056[a]	16	602.441	2.271	.013
Intercept	69316.056	1	69316.056	261.285	.000
GROUP	5733.278	11	521.207	1.965	.050
DF	3905.778	5	781.156	2.945	.020
Error	14590.889	55	265.289		
Total	93546.000	72			
Corrected Total	24229.944	71			

a. R Squared = .398 (Adjusted R Squared = .223)

Table 6-57. LSD of effect of dietary fiber sources and antioxidant on two crypts per focus in the colon of AOM-induced rats

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	3.0833	6.64942	.645	-10.2424	16.4091
	Cin	3.3333	6.64942	.618	-9.9924	16.6591
	CinL	-3.3333	6.64942	.618	-16.6591	9.9924
	P	-13.7500*	6.64942	.043	-27.0757	-.4243
	PE	-14.5000*	6.64942	.034	-27.8257	-1.1743
CE	C	-3.0833	6.64942	.645	-16.4091	10.2424
	Cin	.2500	6.64942	.970	-13.0757	13.5757
	CinL	-6.4167	6.64942	.339	-19.7424	6.9091
	P	-16.8333*	6.64942	.014	-30.1591	-3.5076
	PE	-17.5833*	6.64942	.011	-30.9091	-4.2576
Cin	C	-3.3333	6.64942	.618	-16.6591	9.9924
	CE	-.2500	6.64942	.970	-13.5757	13.0757
	CinL	-6.6667	6.64942	.320	-19.9924	6.6591
	P	-17.0833*	6.64942	.013	-30.4091	-3.7576
	PE	-17.8333*	6.64942	.010	-31.1591	-4.5076
CinL	C	3.3333	6.64942	.618	-9.9924	16.6591
	CE	6.4167	6.64942	.339	-6.9091	19.7424
	Cin	6.6667	6.64942	.320	-6.6591	19.9924
	P	-10.4167	6.64942	.123	-23.7424	2.9091
	PE	-11.1667	6.64942	.099	-24.4924	2.1591
P	C	13.7500*	6.64942	.043	.4243	27.0757
	CE	16.8333*	6.64942	.014	3.5076	30.1591
	Cin	17.0833*	6.64942	.013	3.7576	30.4091
	CinL	10.4167	6.64942	.123	-2.9091	23.7424
	PE	-.7500	6.64942	.911	-14.0757	12.5757
PE	C	14.5000*	6.64942	.034	1.1743	27.8257
	CE	17.5833*	6.64942	.011	4.2576	30.9091
	Cin	17.8333*	6.64942	.010	4.5076	31.1591
	CinL	11.1667	6.64942	.099	-2.1591	24.4924
	P	.7500	6.64942	.911	-12.5757	14.0757

Based on observed means. The error term is Mean Square (Error) = 265.289.

*. The mean difference is significant at the .05 level.

Table 6-58. Anova of effect of dietary fiber sources and antioxidant on three crypts per focus in the colon of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1189.889[a]	16	74.368	1.894	.041
Intercept	6766.722	1	6766.722	172.350	.000
Group	754.611	11	68.601	1.747	.087
DF	435.278	5	87.056	2.217	.065
Error	2159.389	55	39.262		
Total	10116.000	72			
Corrected Total	3349.278	71			

a. R Squared = .355 (Adjusted R Squared = .168)

Table 6-59. Anova of effect of dietary fiber sources and antioxidant on the crypts less than four crypts per focus in the colon of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	49079.389[a]	16	3067.462	1.868	.045
Intercept	505347.556	1	505347.556	307.719	.000
DF	21943.944	5	4388.789	2.672	.031
Block	27135.444	11	2466.859	1.502	.157
Error	90323.056	55	1642.237		
Total	644750.000	72			
Corrected Total	139402.444	71			

a. R Squared = .352 (Adjusted R Squared = .164)

Table 6-60. LSD of effect of dietary fiber sources and antioxidant on the crypts less than four crypts per focus in the colon of AOM-induced rats

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	5.8333	16.54407	.726	-38.3098	49.9765
	Cin	-6.5000	16.54407	.696	-50.6432	37.6432
	CinL	-16.2500	16.54407	.330	-60.3932	27.8932
	P	-36.7500	16.54407	.030	-80.8932	7.3932
	PE	-40.0000	16.54407	.019	-84.1432	4.1432
CE	C	-5.8333	16.54407	.726	-49.9765	38.3098
	Cin	-12.3333	16.54407	.459	-56.4765	31.8098
	CinL	-22.0833	16.54407	.187	-66.2265	22.0598
	P	-42.5833	16.54407	.013	-86.7265	1.5598
	PE	-45.8333*	16.54407	.008	-89.9765	-1.6902
Cin	C	6.5000	16.54407	.696	-37.6432	50.6432
	CE	12.3333	16.54407	.459	-31.8098	56.4765
	CinL	-9.7500	16.54407	.558	-53.8932	34.3932
	P	-30.2500	16.54407	.073	-74.3932	13.8932
	PE	-33.5000	16.54407	.048	-77.6432	10.6432
CinL	C	16.2500	16.54407	.330	-27.8932	60.3932
	CE	22.0833	16.54407	.187	-22.0598	66.2265
	Cin	9.7500	16.54407	.558	-34.3932	53.8932
	P	-20.5000	16.54407	.221	-64.6432	23.6432
	PE	-23.7500	16.54407	.157	-67.8932	20.3932
P	C	36.7500	16.54407	.030	-7.3932	80.8932
	CE	42.5833	16.54407	.013	-1.5598	86.7265
	Cin	30.2500	16.54407	.073	-13.8932	74.3932
	CinL	20.5000	16.54407	.221	-23.6432	64.6432
	PE	-3.2500	16.54407	.845	-47.3932	40.8932
PE	C	40.0000	16.54407	.019	-4.1432	84.1432
	CE	45.8333*	16.54407	.008	1.6902	89.9765
	Cin	33.5000	16.54407	.048	-10.6432	77.6432
	CinL	23.7500	16.54407	.157	-20.3932	67.8932
	P	3.2500	16.54407	.845	-40.8932	47.3932

Based on observed means. The error term is Mean Square (Error) = 1642.237.

*. The mean difference is significant at the .01 level

Table 6-61. Anova of effect of dietary fiber sources and antioxidant on the crypts more than four crypts per focus in the colon of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	416.333[a]	16	26.021	2.014	.028
Intercept	1540.125	1	1540.125	119.215	.000
DF	117.958	5	23.592	1.826	.123
bLOCK	298.375	11	27.125	2.100	.036
Error	710.542	55	12.919		
Total	2667.000	72			
Corrected Total	1126.875	71			

a. R Squared = .369 (Adjusted R Squared = .186)

Table 6-62. Anova of effect of dietary fiber sources and antioxidant on the ACF total in the proximal colon of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	393.889[a]	16	24.618	.873	.602
Intercept	360.014	1	360.014	12.766	.001
Group	320.819	11	29.165	1.034	.430
DF	73.069	5	14.614	.518	.761
Error	1551.097	55	28.202		
Total	2305.000	72			
Corrected Total	1944.986	71			

a. R Squared = .203 (Adjusted R Squared = -.029)

Table 6-63. Anova of effect of dietary fiber sources and antioxidant on the ACF total in the medium distal colon of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	23213.889[a]	16	1450.868	1.310	.224
Intercept	183719.014	1	183719.014	165.931	.000
Group	16234.819	11	1475.893	1.333	.232
DF	6979.069	5	1395.814	1.261	.294
Error	60896.097	55	1107.202		
Total	267829.000	72			
Corrected Total	84109.986	71			

a. R Squared = .276 (Adjusted R Squared = .065)

Table 6-64. Anova of effect of dietary fiber sources and antioxidant on the ACF total in the distal colon of AOM-induced rats

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10005.500[a]	16	625.344	1.895	.041
Intercept	92665.125	1	92665.125	280.798	.000
DF	5493.792	5	1098.758	3.330	.011
Group	4511.708	11	410.155	1.243	.282
Error	18150.375	55	330.007		
Total	120821.000	72			
Corrected Total	28155.875	71			

a. R Squared = .355 (Adjusted R Squared = .168)

Table 6-65. LSD of effect of dietary fiber sources and antioxidant on the ACF total in the distal colon of AOM-induced rats

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	11.6667	7.41628	.121	-3.1959	26.5292
	Cin	.4167	7.41628	.955	-14.4459	15.2792
	CinL	-3.5833	7.41628	.631	-18.4459	11.2792
	P	-11.5833	7.41628	.124	-26.4459	3.2792
	PE	-15.1667*	7.41628	.046	-30.0292	-.3041
CE	C	-11.6667	7.41628	.121	-26.5292	3.1959
	Cin	-11.2500	7.41628	.135	-26.1125	3.6125
	CinL	-15.2500*	7.41628	.045	-30.1125	-.3875
	P	-23.2500*	7.41628	.003	-38.1125	-8.3875
	PE	-26.8333*	7.41628	.001	-41.6959	-11.9708
Cin	C	-.4167	7.41628	.955	-15.2792	14.4459
	CE	11.2500	7.41628	.135	-3.6125	26.1125
	CinL	-4.0000	7.41628	.592	-18.8625	10.8625
	P	-12.0000	7.41628	.111	-26.8625	2.8625
	PE	-15.5833*	7.41628	.040	-30.4459	-.7208
CinL	C	3.5833	7.41628	.631	-11.2792	18.4459
	CE	15.2500*	7.41628	.045	.3875	30.1125
	Cin	4.0000	7.41628	.592	-10.8625	18.8625
	P	-8.0000	7.41628	.285	-22.8625	6.8625
	PE	-11.5833	7.41628	.124	-26.4459	3.2792
P	C	11.5833	7.41628	.124	-3.2792	26.4459
	CE	23.2500*	7.41628	.003	8.3875	38.1125
	Cin	12.0000	7.41628	.111	-2.8625	26.8625
	CinL	8.0000	7.41628	.285	-6.8625	22.8625
	PE	-3.5833	7.41628	.631	-18.4459	11.2792
PE	C	15.1667*	7.41628	.046	.3041	30.0292
	CE	26.8333*	7.41628	.001	11.9708	41.6959
	Cin	15.5833*	7.41628	.040	.7208	30.4459
	CinL	11.5833	7.41628	.124	-3.2792	26.4459
	P	3.5833	7.41628	.631	-11.2792	18.4459

Based on observed means. The error term is Mean Square (Error) = 330.007.

*. The mean difference is significant at the .05 level.

Table 6-66. Anova of effect of dietary fiber sources and antioxidant on the PCNA labelling indexes in the mucosa of distal colon of AOM-tinduced rats.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1534.290[a]	16	95.893	1.032	.440
Intercept	60280.133	1	60280.133	648.978	.000
Block	792.985	11	72.090	.776	.662
DF	743.513	5	148.703	1.601	.176
Error	4922.891	53	92.885		
Total	67873.262	70			
Corrected Total	6457.181	69			

a. R Squared = .238 (Adjusted R Squared = .007)

Table 6-67. LSD of effect of dietary fiber sources and antioxidant on PCNA labelling indexes in the mucosa of distal colon of AOM-induced rats.

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	2.9334	4.12661	.480	-5.3435	11.2104
	Cin	.3235	3.93456	.935	-7.5683	8.2152
	CinL	-.5097	3.93456	.897	-8.4014	7.3821
	P	1.6598	3.93456	.675	-6.2319	9.5516
	PE	-7.3116	3.93456	.069	-15.2033	.5802
CE	C	-2.9334	4.12661	.480	-11.2104	5.3435
	Cin	-2.6100	4.12661	.530	-10.8869	5.6669
	CinL	-3.4431	4.12661	.408	-11.7200	4.8338
	P	-1.2736	4.12661	.759	-9.5505	7.0033
	PE	-10.2450*	4.12661	.016	-18.5219	-1.9681
Cin	C	-.3235	3.93456	.935	-8.2152	7.5683
	CE	2.6100	4.12661	.530	-5.6669	10.8869
	CinL	-.8331	3.93456	.833	-8.7249	7.0586
	P	1.3364	3.93456	.735	-6.5554	9.2281
	PE	-7.6350	3.93456	.058	-15.5268	.2567
CinL	C	.5097	3.93456	.897	-7.3821	8.4014
	CE	3.4431	4.12661	.408	-4.8338	11.7200
	Cin	.8331	3.93456	.833	-7.0586	8.7249
	P	2.1695	3.93456	.584	-5.7222	10.0612
	PE	-6.8019	3.93456	.090	-14.6936	1.0898
P	C	-1.6598	3.93456	.675	-9.5516	6.2319
	CE	1.2736	4.12661	.759	-7.0033	9.5505
	Cin	-1.3364	3.93456	.735	-9.2281	6.5554
	CinL	-2.1695	3.93456	.584	-10.0612	5.7222
	PE	-8.9714*	3.93456	.027	-16.8631	-1.0797
PE	C	7.3116	3.93456	.069	-.5802	15.2033
	CE	10.2450*	4.12661	.016	1.9681	18.5219
	Cin	7.6350	3.93456	.058	-.2567	15.5268
	CinL	6.8019	3.93456	.090	-1.0898	14.6936
	P	8.9714*	3.93456	.027	1.0797	16.8631

Based on observed means. The error term is Mean Square (Error) = 92.885.

*. The mean difference is significant at the .05 level.

Table 6-68. Anova of effect of dietary fiber sources and antioxidant on the the MDA concentration of rat liver of of AOM‑induced rats.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	544429.905[a]	14	38887.850	20.193	.000
Intercept	1509055.087	1	1509055.087	783.594	.000
Rat	505570.856	9	56174.540	29.169	.000
DF	38859.049	5	7771.810	4.036	.004
Error	86661.595	45	1925.813		
Total	2140146.587	60			
Corrected Total	631091.500	59			

a. R Squared = .863 (Adjusted R Squared = .820)

Table 6-69. LSD of effect of dietary fiber sources and antioxidant on the the MDA concentration of rat liver of of AOM‑induced rats.

(I) DF	(J) DF	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C	CE	5.8758	19.62556	.766	-33.6521	45.4037
	Cin	-2.8351	19.62556	.886	-42.3630	36.6928
	CinL	-61.3705*	19.62556	.003	-100.8984	-21.8426
	P	-4.7240	19.62556	.811	-44.2519	34.8039
	PE	-44.5453*	19.62556	.028	-84.0732	-5.0174
CE	C	-5.8758	19.62556	.766	-45.4037	33.6521
	Cin	-8.7109	19.62556	.659	-48.2388	30.8170
	CinL	-67.2463*	19.62556	.001	-106.7742	-27.7184
	P	-10.5998	19.62556	.592	-50.1277	28.9281
	PE	-50.4211*	19.62556	.014	-89.9490	-10.8932
Cin	C	2.8351	19.62556	.886	-36.6928	42.3630
	CE	8.7109	19.62556	.659	-30.8170	48.2388
	CinL	-58.5354*	19.62556	.005	-98.0633	-19.0075
	P	-1.8889	19.62556	.924	-41.4168	37.6390
	PE	-41.7102*	19.62556	.039	-81.2381	-2.1823
CinL	C	61.3705*	19.62556	.003	21.8426	100.8984
	CE	67.2463*	19.62556	.001	27.7184	106.7742
	Cin	58.5354*	19.62556	.005	19.0075	98.0633
	P	56.6465*	19.62556	.006	17.1186	96.1744
	PE	16.8252	19.62556	.396	-22.7027	56.3531
P	C	4.7240	19.62556	.811	-34.8039	44.2519
	CE	10.5998	19.62556	.592	-28.9281	50.1277
	Cin	1.8889	19.62556	.924	-37.6390	41.4168
	CinL	-56.6465*	19.62556	.006	-96.1744	-17.1186
	PE	-39.8213*	19.62556	.048	-79.3492	-.2934
PE	C	44.5453*	19.62556	.028	5.0174	84.0732
	CE	50.4211*	19.62556	.014	10.8932	89.9490
	Cin	41.7102*	19.62556	.039	2.1823	81.2381
	CinL	-16.8252	19.62556	.396	-56.3531	22.7027
	P	39.8213*	19.62556	.048	.2934	79.3492

Based on observed means. The error term is Mean Square(Error) = 1925.813.

6.2. Dipeptidyl peptidase IV (DPIV) activity

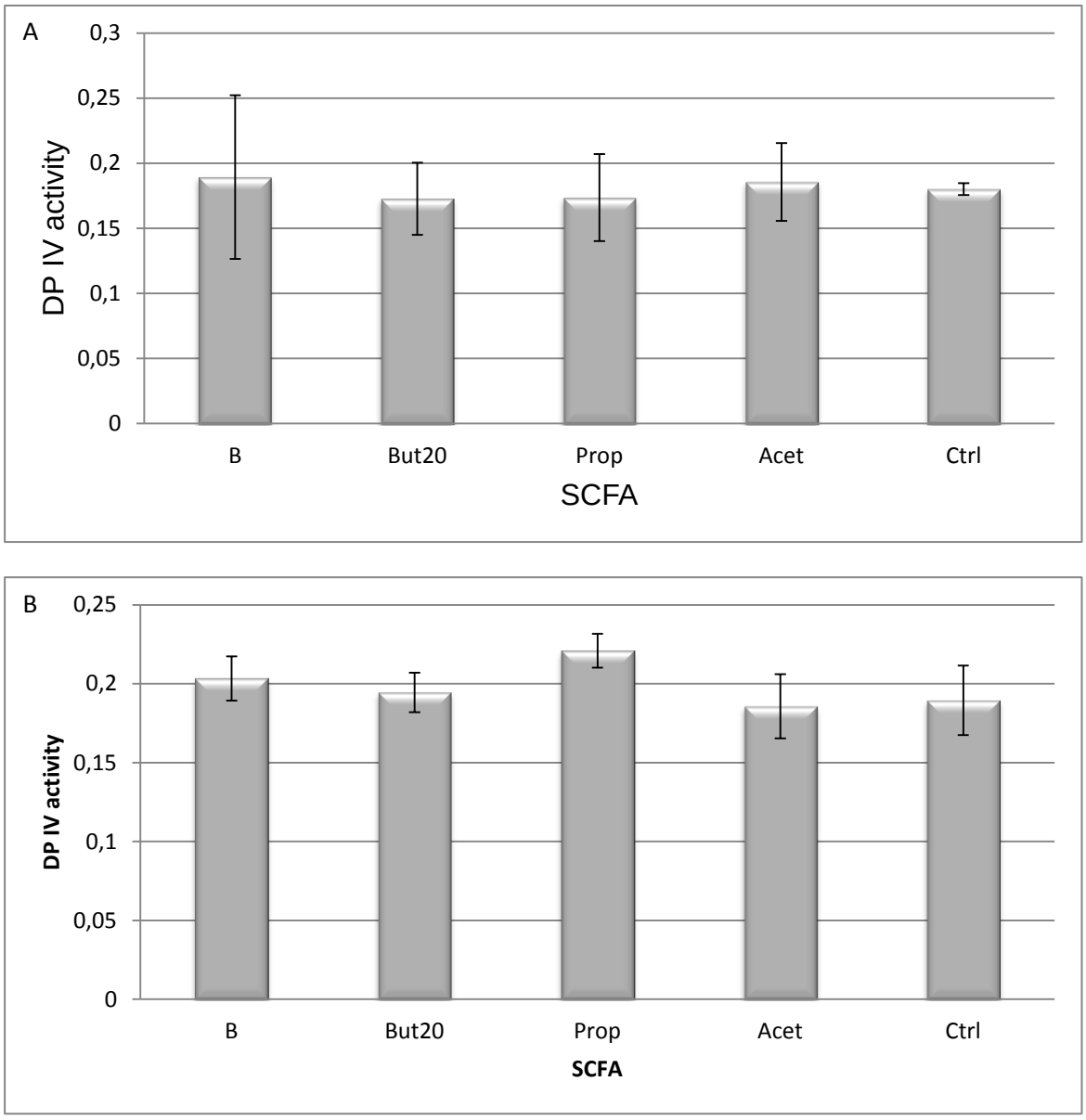

Figure 6-1. Effect of SCFA on the Dipeptidyl peptidase IV (DP IV) activity in (A) pH 6.0 and (B) pH 7.5 media.

Cells were seeded in 6 wells plate until about 70% confluent, then incubated for 48 hrs in media containing SCFA. DPPIV enzyme activity was measured spectrophotometrically using Gly-Pro-*p*NA as a DPPIV substrate and expressed as amount of enzyme which cleavage 1 mmol substrate per min per mg prot. The bars represent the mean, and the lines, SEM of three replicates.

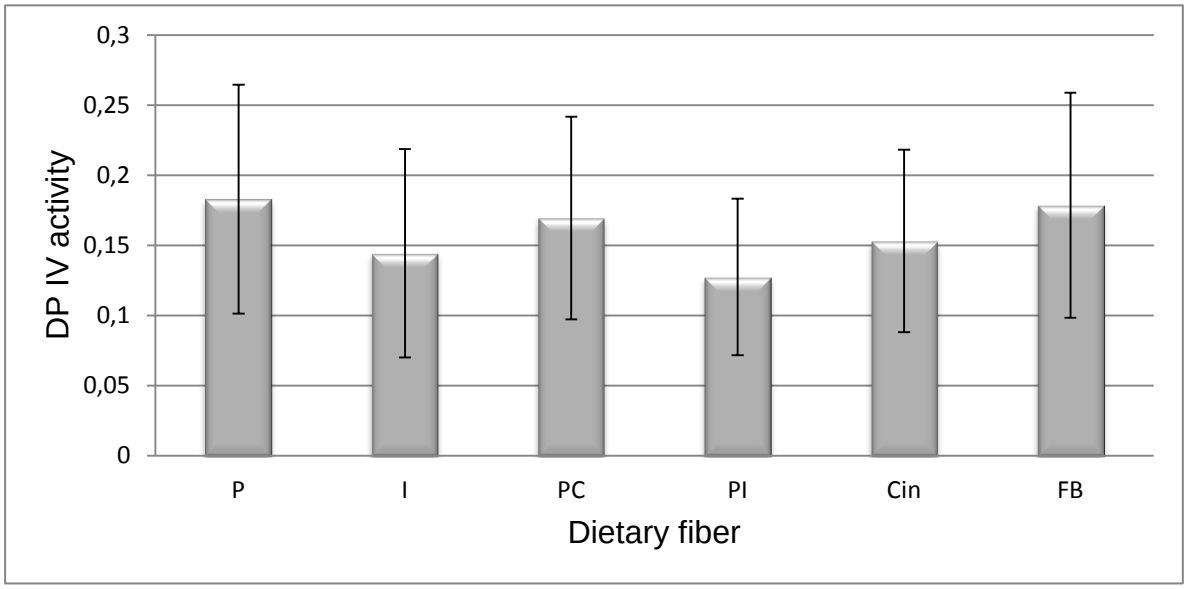

Figure 6-2.Effect of dietary fiber FS on the Dipeptidyl peptidase IV (DP IV) activity.

Cells were seeded in 6 wells plate until about 70% confluent, then incubated for 48 hrs in media containing FS. DPPIV enzyme activity was measured spectrophotometrically using Gly-Pro-*p*NA as a DPPIV substrate and expressed as amount of enzyme which cleavage 1 mmol substrate per min per mg protein. The bars represent the mean, and the lines, SEM of three replicates.

6.3. The effects of Dietary fiber sources and antioxidant on average weights of rats during 13-week experiment

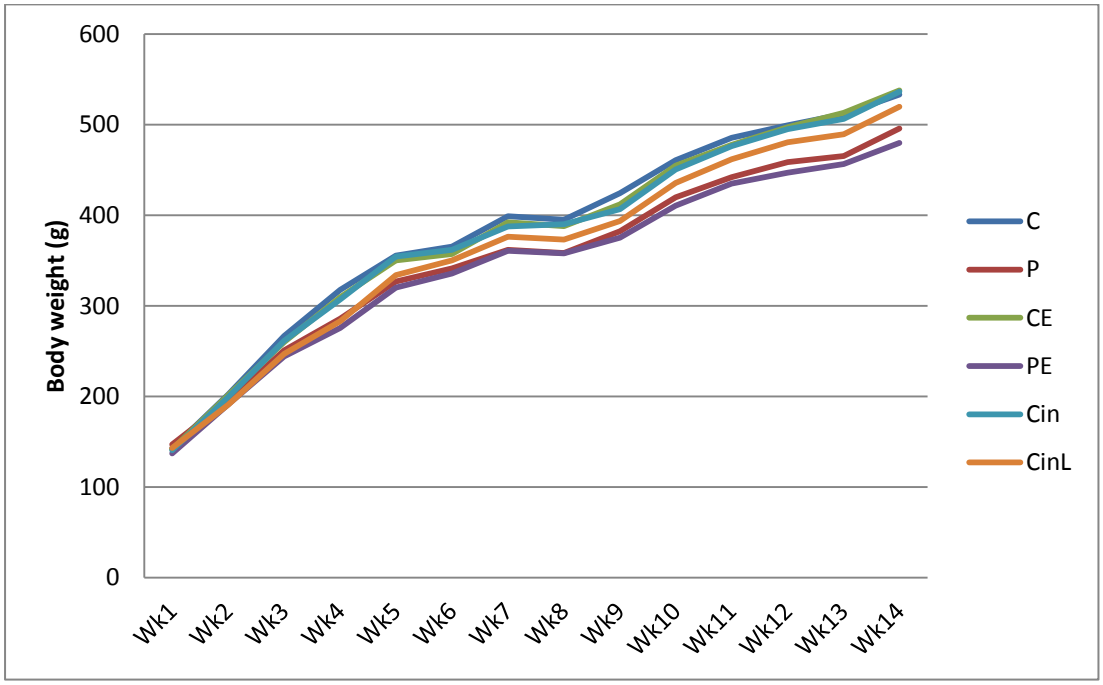

Figure 6-3. The effects of Dietary fiber sources and antioxidant on average weights of rats during 13-week experiment

Values are mean of 12 rats. Abbreviation: C, cellulose; P, pectin; CE, cellulose+0.1% EGCG; PE, pectin+0.1 EGCG; Cin, cincau extract;CinL, cincau leave; Wkn, week n^{th}.

6.4. Composition of chemicals used in dietary fiber fermentation

Table 6-70. Micromineral solution (per 1 L solution)

Compounds	Weigh (g)	Company
$CaCl_2.2H_2O$	132.00	Sigma-Aldrich
$MnCl_{2.}4H_2O$	100.00	Sigma-Aldrich
$CoCl_2.6H_2O$	10.00	Sigma-Aldrich
$FeCl_3.6H_2O$	80.00	Sigma-Aldrich

Table 6-71. Buffer solution (per 1 L solution)

Compounds	Weigh (g)	Company
$(NH_4)HCO_3$	4.00	Sigma-Aldrich
$NaHCO_3$	35.00	Sigma-Aldrich

Table 6-72. Macromineral Solution (per 1 L solution)

Compounds	Weigh (g)	Company
Na_2HPO_4	5.70	Sigma-Aldrich
KH_2PO_4	6.20	Sigma-Aldrich
$MgSO_4.7H_2O$	0.60	Sigma-Aldrich

Table 6-73. Reducing solution (per 1 L solution)

Reducing solution (per 1 L solution)		
Compounds	Weigh/Volume	Company
Cysteine hydrochloride	6.25 g	Sigma-Aldrich
$Na_2S.9H_2O$	6.25 g	Sigma-Aldrich
NaOH 1 M	40 ml	Sigma-Aldrich

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